

NOTE TO USERS

This reproduction is the best copy available.

UMI[®]

Characterization of novel genes involved in learning and memory in rodent models

Jonathan Brouillette

Department of Neurology and Neurosurgery
McGill University
Montréal, Québec, Canada

November 2007

A thesis submitted to McGill University in partial fulfilment of
the requirements of the degree of Doctor of Philosophy

© Jonathan Brouillette, 2007



Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 978-0-494-50782-7

Our file Notre référence

ISBN: 978-0-494-50782-7

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Acknowledgements

I would like to gratefully acknowledge all who contributed importantly and in various ways to my thesis.

First of all, I would like to thank my supervisor and mentor Dr. Rémi Quirion for giving me the opportunity to work in his laboratory. I am extremely grateful for the support he gave me throughout the duration of my Ph.D. in spite of his very busy schedule. I learned a tremendous amount working with him during all these years. His huge experience in science, his impressive knowledge in various fields of research, his capacity to communicate passion of research was truly inspirational. He taught me all aspects of the work going from the design of an experiment to the writing of manuscripts. I feel privileged to work with someone who will always be a source of inspiration and a great mentor. Thanks a lot for the confidence you placed in me.

I would also like to express my gratitude to the members of my advisory committee, Dr. Josephine Nalbantoglu and Dr. Édith Hamel, for their support, helpful discussions and constructive feedback throughout my training. During my Ph.D. I had the chance to work with both of them and appreciate their professionalism and great experience in science.

I would like to thank all members of my lab who contributed in various ways to this thesis... Mira, Jean-Guy, Yvan, Wen-Hua, Ara, Charles-Étienne, Naguib, Stéphane. Special thanks have to be dedicated to Mira Thakur who corrected practically everything that I have done and written in English (which means a lot of work!). Thank you also for organizing the various events in the lab and being there every time I needed you. Thank you also to Jean-Guy for his help in the last miles of my Ph.D. with the immunohistochemistry. Your amazing expertise on microscopy analysis techniques was incredibly helpful and your kindness makes it easy to work with you.

I really enjoyed working at the Douglas Hospital (oops the Douglas Mental Health University Institute!) which is really changing over the last few years to make it a better place to work. But one thing that has not changed is the

enthusiasm and generosity of people at the Douglas who bring this little something that makes you love it. Thanks to the people who worked or still working here among which I would like to mention a few such as the “French team”: Stéphanie, Hugues and Marion... see you soon; the member of the Douglas Hospital Organization for Students: Joe, Ian, Vincent, Mehereen, Geeta, Alexandra, Marc; the girls team at the communication: Caroline, Stéphanie, Marie-Gabrielle, Catherine; and so many others: Ramia, Ray, Rocio, Manon, Sarah, Danielle, Doris and Nicole. Special thanks to Serge who help me throughout all my Ph.D and particularly at the end. Also, I would like to thank the Luheshi lab for adopting me and for the good times... it's always a party with you guys and you are making such good coffee!

I would also like to take this occasion to thank my friends who are fed up listening to me about my thesis! It's always a pleasure François to go at your place to relax and watch a good hockey match and discuss with Julie. Thank you also to Bastien who is always willing for a game of pool or tennis as well as Manue for all the good times spent in canoe-camping, playing belote or queen scallop (we are already set for retirement!) and discussion around a bottle of wine (when I'm not sleeping!). A particular thank you to my childhood friend Gaétan that now lives in Uruguay but still have a special place in my heart. How to forget the Sachot family and good moments spent together in Montreal during last Christmas and during my trip to France... see you very soon.

To my mom and dad, I would like to say how much I love them and thank them for their unconditional love, understanding, generosity and for being there at each single step to support and encourage me. Thank you also to my sisters Annie and Julie... it will not be easy to be so far away from all of you for the next few years.

Finally, my Ph.D. and Douglas experiences would not have been the same at all without my sunshine, my one and only one little Cricri. Your energy, intensity and sensibility makes every moment pass with you unique... Je t'aime.

This thesis is dedicated to my mom and dad,
Thank you for everything

Table of Contents

Abstract	x
Résumé.....	xii
Contribution of the authors on co-authored papers.....	xiv
Original contributions to the body of knowledge	xvi
List of figures	xviii
List of tables.....	xix
Abbreviations	xx
Chapter I: Introduction.....	1
Chapter II: Review of the literature.....	5
1. Molecular and cellular mechanisms of memory formation	6
2. The hippocampus: a brain area critical for memory	9
2.1. Anatomy and neurotransmitter systems of the hippocampus	10
3. The Morris water maze task.....	13
4. Identification of novel memory genes using a microarray approach..	14
5. Aged memory-impaired rats	16
6. Signalling pathways involved in age-related memory deficits	16
7. Implication of the retinoid pathways in memory loss during aging ...	17
7.1. Retinoid transport.....	18
7.2. The retinol carrier transthyretin (TTR).....	19
7.3. Metabolism of retinoids	20
7.4. Retinoid receptors and their ligands.....	22
7.5. Distribution of retinoid receptors in the hippocampus.....	24
7.6. Role of retinoic acid (RA) in learning and memory	25
8. The environmental and food pollutant dioxin empties retinol stock ..	31
8.1. The pleiotropic action of dioxin is gender-specific	32
8.2. Source and noxious effects associated with exposure to dioxin	33
9. Role of retinoic acid signalling pathways in AD	35

9.1. Phenotypes and hallmarks of AD.....	35
9.2. The amyloid hypothesis of AD	38
9.3. Disruption of retinoic acid homeostasis in AD.....	40
9.4. Role of transthyretin in AD.....	41
9.5. Possible role of the pollutant dioxin in AD.....	42
9.6. Current treatments for AD	43
10. The scopolamine-induced amnesia model	44
10.1. Influence of nicotinic receptors on cholinergic, glutamatergic and GABAergic neurotransmission during memory processes	46
10.2. Influence of muscarinic receptors on cholinergic, glutamatergic and GABAergic neurotransmission during memory processes	47
11. Summary and aims of this thesis	54
Chapter III: Transthyretin: a key gene involved in the maintenance of memory capacities during aging.....	55
Preface.....	56
Abstract	58
Introduction.....	59
Materials and Methods.....	61
Results.....	65
Figures.....	71
Tables.....	77
Discussion	83
Acknowledgements.....	88
References	89
Chapter IV: The common environmental pollutant dioxin induced memory deficits by altering estrogen pathways and a major route of retinol transport involving transthyretin	96

Preface.....	97
Abstract	99
Introduction.....	100
Materials and Methods.....	103
Results.....	106
Figures.....	109
Discussion	116
Acknowledgements.....	120
References.....	121
Chapter V: Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABA_B receptors in scopolamine-induced amnesia.....	129
Preface.....	130
Abstract	132
Introduction.....	133
Material and Methods	135
Results.....	140
Figures.....	145
Tables	152
Discussion	159
Acnowledgements.....	164
References.....	165
Chapter VI: General Discussion.....	173
1. The aim of this general discussion.....	174

2. Hippocampal gene expression profile of aged memory-impaired rats	174
3. Hippocampal gene expression profiles of young and aged memory-unimpaired animals	177
4. Role of transthyretin during aging and in AD	179
4.1. Transthyretin as a biomarker of aging	179
4.2. Dual role of transthyretin in AD	181
4.3. Role of transthyretin in oxidative damage and calcium homeostasis	184
4.4. Link between neuroinflammation and transthyretin in AD	184
4.5. Susceptibility factors related to the development of AD and their relation to TTR.....	185
4.6. Relationship between apolipoprotein E and transthyretin in AD	187
4.7. Hypothyroidism as a risk factor for AD.....	188
4.8. Plasma transthyretin level is reduced in diabetes mellitus.....	189
4.9. Other susceptibility factors for AD and their relation to transthyretin	190
4.10. Injuries to the choroid plexus as a major source of altered transthyretin levels during aging.....	191
4.11. Possible future experiments to validate the key role of transthyretin in AD	195
5. Scopolamine-induced amnesia model.....	196
5.1. Age memory-impaired rats versus the scopolamine-induced amnesia models	199
6. Some future studies.....	201
7. Conclusion	204
References: Review of the literature and General Discussion	205
Appendix I: Neuroprotective effects of natural products: interaction with intracellular kinases, amyloid peptides and a possible role for transthyretin	

Appendix II: Hippocampal gene expression profiles in memory-impaired and memory-unimpaired aged rats

Appendix III: Gene expression profiling following chronic NMDA receptor blockade-induced learning deficits in rats

Appendix IV: Ethics approval certificates

Appendix V: Manuscript waivers

Abstract

Aging is often associated with the decline of numerous cognitive functions, especially learning and memory. Stable forms of long-term plasticity require the induction of a cascade of genes to produce and maintain structural changes associated with memory formation in the hippocampus, a brain area essential for spatial memory. Using a microarray approach, global alterations in gene expression were investigated in two animal models of memory deficits; namely Long-Evans rats with memory impairments following systemic administration of scopolamine (a muscarinic acetylcholine receptor antagonist), and a cohort of aged Long-Evans rats which naturally develop memory impairments during aging.

Among the various hippocampal genes differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) rats following spatial memory stimulation in the Morris water maze (MWM) task, decreased levels of transthyretin (TTR) gene expression were associated with aged memory deficits. Furthermore, we observed that TTR knock-out ($TTR^{-/-}$) mice develop memory deficits during aging. The mechanism involved in the effects of TTR in memory formation is apparently related to its role as retinol transporter since a treatment with its active metabolite retinoic acid (RA) reversed cognitive deficits in $TTR^{-/-}$ mice and aged rats.

To further investigate the role of RA in memory deficits, we used the common environmental pollutant dioxin (also known as TCDD; 2,3,7,8-tetrachlorodibenzo-p-dioxin) to empty retinol storage. We observed that treatment with TCDD induced memory impairments in the MWM task in adult female but not male mice. This gender-specific effect of dioxin on cognitive abilities is reversed by a treatment with RA combined or not with estrogen. In addition, TCDD-induced cognitive impairments are decreased in $TTR^{-/-}$ mice.

Scopolamine-treated rats are commonly used as a psychopharmacological model of memory dysfunctions similar to those seen in aging. We identified several novel candidate genes possibly involved in cognition that were altered in

scopolamine-treated adult rats following memory stimulation. For example, we found that the sustained overexpression of the glutamatergic receptor component Homer1a or 1c in the hippocampus abolished memory improvements induced by the GABA_B receptor antagonist, SGS742, in scopolamine-treated rats. Taken together, the various genes altered in the two animal models used in this thesis suggest potential novel targets to develop more effective therapeutic strategies for the treatment of age-related cognitive impairments.

Résumé

Les troubles de mémoire et d'apprentissage sont parmi les symptômes les plus fréquemment associés au vieillissement du cerveau. Notre capacité à mémoriser une information sur une longue période de temps nécessite la formation de nouvelles connections synaptiques dans certaines régions du cerveau tel l'hippocampe. Afin d'identifier les gènes impliqués dans la mémoire dont l'expression est modifiée lors du vieillissement, deux modèles animaux ayant des déficits de mémoire sont utilisés dans notre projet. Chez l'un de ces modèles, un antagoniste des récepteurs muscariniques, la scopolamine, est utilisé pour induire des déficits de mémoire. Pour le deuxième modèle, nous avons utilisé des animaux âgés, car tout comme l'humain, certains rats peuvent naturellement développer des troubles de mémoire avec l'âge. La capacité de mémorisation des rats est évaluée en utilisant un test d'apprentissage connu comme la piscine de Morris. Des micro-puces à ADN ont été employées afin d'analyser simultanément l'ensemble des gènes potentiellement impliqués dans la mémoire.

Nous avons entre autres observé que l'expression génique de la transthyrétine (TTR) est altérée dans l'hippocampe des rats âgés ayant des déficits de mémoire. Nous avons aussi observé des déficits de mémoire avec l'âge chez les souris dont le gène de la TTR a été invalidé par manipulation génétique. Le rôle de la TTR dans la formation de la mémoire semble être relié à sa fonction de transporteur de la vitamine A, car un traitement avec l'acide rétinique (AR) permet de prévenir les déficits de mémoire observés chez les souris n'ayant pas de TTR ainsi que chez les rats âgés.

Afin de déterminer plus précisément le rôle de l'AR dans les troubles de mémoire, nous avons utilisé la dioxine (aussi appelé TCDD; 2,3,7,8-tetrachlorodibenzo-p-dioxin), un polluant environnemental qui diminue les réserves en rétinol. Nous avons observé que le TCDD produit des déficits de mémoire sévères chez les souris femelles; ceux-ci étant prévenus par un traitement à l'AR combiné ou non à l'administration d'estrogènes. De plus, nous

avons démontré que les pertes de mémoire induites par le TCDD étaient diminuées chez les souris déficientes en TTR.

Les rats traités avec la scopolamine sont souvent utilisés comme modèle animal de déficits cognitifs. En utilisant ce modèle, nous avons identifié plusieurs gènes candidats pouvant expliquer les troubles cognitifs induits par la scopolamine. De plus, nous avons montré que la surexpression du gène Homer1a ou 1c (qui est associé au système glutamatergique) dans l'hippocampe de rats adultes est capable de renverser les effets bénéfiques de l'antagoniste des récepteurs GABA_B, le SGS742, sur les capacités cognitives des rats traités à la scopolamine. En conclusion, les gènes dont l'expression est altérée dans les deux modèles animaux utilisés dans cette thèse représentent de nouvelles cibles thérapeutiques pour le traitement des déficits de mémoires observés dans le vieillissement normal et pathologique.

Contribution of the authors on co-authored papers

In compliance with the regulations outlined by the Faculty of Graduate Studies and Research regarding paper-based thesis submission, outlined below are the published manuscripts, and the relative contributions of all the authors included in my thesis. Please note that as my Ph.D. supervisor, Prof. Rémi Quirion is a co-author on all manuscripts and he provided invaluable help throughout the course of this thesis project and made helpful comments on all the manuscripts generated from this work.

Chapter III: Brouillette J & Quirion R (2007) Transthyretin: a key gene involved in the maintenance of memory capacities during aging. *Neurobiol Aging*, Epub ahead of print.

As being a first author on this paper, I was responsible for all work, including experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Prof. Quirion.

Chapter IV: Brouillette J & Quirion R. The common environmental pollutant dioxin induced memory deficits by altering estrogen pathways and a major route of retinol transport involving transthyretin. *NeuroToxicology*, Submitted.

As a first author on this paper, I was responsible for all the work, including experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Prof. Quirion.

Chapter V: Brouillette J, Young D, During MJ & Quirion R (2007) Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABA_B receptors in scopolamine-induced amnesia. *J Neurochem*, 102, 1978-1989.

As a first author on this paper, I was responsible for the majority of the experimental design, data collection and analysis, as well as manuscript preparation under the guidance of my supervisor Prof. Quirion. Dr. Deborah Young and Dr. Matthew J. During (University of Auckland, New Zealand) generated recombinant adeno-associated virus containing Homer1a and 1c and revised drafts of the manuscript.

Original contributions to the body of knowledge

1) Transthyretin: a key gene involved in the maintenance of memory capacities during aging. Jonathan Brouillette & Rémi Quirion (2007) *Neurobiol Aging*, Epub ahead of print.

A. Decreased expression of TTR following a spatial memory challenge was associated with memory deficits in aged rats.

B. Complete genetic deletion of TTR in mice produces memory deficits during aging.

C. Time-dependent correlation suggest that decreased expression of the late response gene TTR was, at least partly, attributed to decreased protein level of C/EBP α and β .

D. Treatment with retinoic acid (RA), which bypasses TTR transport unlike retinol, can reverse cognitive deficits in TTR^{-/-} mice and aged rats.

Taken together, these results provided the first genetic, behavioural and molecular evidence that TTR is involved in the maintenance of normal cognitive processes during aging by acting on the fine regulation of brain retinoid availability and signalling pathways.

2) The common environmental pollutant dioxin induced memory deficits by altering estrogen pathways and a major route of retinol transport involving transthyretin. Jonathan Brouillette & Rémi Quirion. *NeuroToxicology*, Submitted.

A. The environmental and food pollutant dioxin induced robust memory deficits in adult female but not male mice.

B. Treatment with retinoic acid and/or estrogen reversed TCDD-induced memory impairments.

C. Cognitive deficits observed following exposure to dioxin are reduced in TTR^{-/-} mice.

Taken together, these results suggest significant cross-talk between retinoic acid and estrogen signalling pathways to the effects of TCDD on cognition. Our study also suggests that dioxin can alter memory function by disturbing the main route of retinol delivery to the brain.

3) Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABA_B receptors in scopolamine-induced amnesia. Jonathan Brouillette, Deborah Young, Matthew J During & Rémi Quirion R (2007) *J Neurochem*, 102, 1978-1989.

A. Scopolamine treatment affected the expression of genes associated with various neurotransmitter systems besides the cholinergic one in the dorsal hippocampus.

B. First evidence that muscarinic receptors may be implicated in the regulation of the glutamatergic receptor component Homer1a. Indeed, we observed that Homer1a gene expression is not transiently induced following spatial memory stimulation in scopolamine-treated rats.

C. A sustained up-regulation of either Homer1a or 1c in the hippocampus reversed memory enhancements induced by the GABA_B receptor antagonist SGS742 in scopolamine-treated rats.

Overall, this report revealed novel genes and mechanisms involved in scopolamine-induced amnesia, and demonstrated the involvement of both GABA and glutamate neurotransmission in this animal model of cognitive dysfunctions.

List of figures

Chapter II

Review of the literature

Fig. II.1. Molecular events that underlie the early and late phases of LTP.....	7
Fig. II.2. A three-dimensional organisation of the hippocampus in the rat brain...11	
Fig. II.3. The trisynaptic loop of the hippocampus.....	12
Fig. II.4. The Morris water maze task.....	13
Fig. II.5. The retinoid cascade.....	18
Fig. II.6. Retinol metabolism.....	21
Fig. II.7. RA-mediated gene expression.....	22
Fig. II.8. The amyloid cascade hypothesis of AD.....	39
Fig. II.9. Computer graphic model of A β binding to TTR.....	41
Fig. II.10. A model cholinergic synapse	45
Fig. II.11. Homer1 regulates glutamatergic post-synaptic signalling.....	52

Chapter VI

General Discussion

Fig. VI.1. The TTR cascade hypothesis of AD.....	181
Fig. VI.2. Administration of RA failed to reverse scopolamine-induced amnesia in 7-months old rats.....	200

List of tables

Chapter II

Review of the literature

Table II.1. Distribution of RAR α , β and γ and RXR α , β , and γ receptor proteins in the mouse CNS.....	24
Table II.2. Retinoid-regulated neuronal genes in the adult rodent brain.....	27
Table II.3. Localization of muscarinic ACh receptor subtypes in the brain.....	48

Chapter VI

General Discussion

Table VI.1. Susceptibility factors related to the development of AD.....	186

Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
A β	Amyloid- β peptide
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AhR	Aryl hydrocarbon receptor
AI	Aged memory-impaired
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activating protein 1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATRA	All- <i>trans</i> -retinoic acid
AU	Aged memory-unimpaired
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
C/EBP	CCAAT enhancer binding protein
ChAT	Choline acetyltransferase
CNS	Central nervous system
CP	Choroid plexus
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DS	Down Syndrome
EPSP	Excitatory post-synaptic potential
FAD	Familial Alzheimer's disease
FDA	Food and drug administration
GABA	γ -aminobutyric acid

GAD	Glutamic acid decarboxylase
GAP43	Growth-associated protein 43
HAH	Halogenated aromatic hydrocarbon
HDAC	Histone deacetylase complexe
H ₂ O ₂	Hydrogen peroxide
IEG	Immediate-early genes
IGF-1	Insulin-like growth factor 1
iNOS	Inducible nitric oxide synthase
IPSC	Inhibitory post-synaptic potential
kDa	Kilodalton
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MAS5	Microarray Affymetrix suite 5.0
MCI	Mild cognitive impairment
mRNA	Messenger ribonucleic acid
MWM	Morris water maze
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptor
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo- <i>p</i> -dioxin
PCDF	Polychlorinated dibenzofuran
PKA	Protein kinase A
PKC	Protein kinase C
PS	Presenilin
RA	Retinoic acid

RBP	Retinol-binding protein
RMA	Robust Multi-array Averaging
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
T3	Triiodothyronine
T4	Thyroxin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TNF- α	Tumor necrosis factor- α
TTR	Transthyretin
VAD	Vitamin A deficiency

Chapter I

Introduction

Life expectancy in Canada, like in the other industrial countries, has markedly increased over the past decades. According to a recent health report of Statistics Canada (www.statcan.ca, n° 82-003-XIF), in 1951 the average life expectancy of men and women was 66.3 and 70.8 years respectively and has increased to 77.2 years for men and 82.1 years for women in 2002. Advances in medical research, better life conditions, aging of the baby-boomer generation as well as low birth-rate are all factors that have concomitantly contributed to aging of the population in the Western world. To decrease the social and economical impact associated with the aging of the population, major efforts are employed to find novel therapeutic avenues for age-related diseases.

Learning and memory disabilities are among the most common, debilitating and invalidating health problems associated with aging. Brain aging may lead to memory impairments related to disorders ranging from the benign senescent forgetfulness to the memory loss that characterizes Alzheimer's disease (AD) and other neurodegenerative disorders (Mattson & Magnus, 2006). Given that age is the most robust risk factor for AD (George-Hyslop, 2000) and early memory deficits occur long before other neuropathological hallmarks of AD (such as amyloid plaque formation and neurofibrillary tangles), discovery of novel genes related to memory loss that occurs during normal and pathological brain aging is the subject of active investigation. Since the completion of the Human Genome Project, there has been increasing interest to identify all the genes involved in memory processes. Considering the rapid aging of the population and given that one third of the population over 85 years old is suffering from AD (Lautenschlager et al., 1996), tremendous efforts are deployed to find new genes and mechanisms involved in age-related cognitive impairments.

The aim of this thesis is to investigate global alterations in gene expression in two animal models associated with learning and memory deficits. As seen in human, aged (24-months old) rats can naturally develop memory impairments and thus represent a useful model to investigate genes involved in long-term memory formation that are differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) animals following stimulation in a spatial

memory task (Quirion et al., 1995; Rowe et al., 1998). Rats treated with scopolamine, a muscarinic acetylcholine receptor antagonist, is a common psychopharmacological model which mimics memory deficits seen in young subjects similar to those occurring during aging (Stemmelin et al., 1999; Janas et al., 2005). Spatial memory in these two animal models was evaluated using the Morris water maze (MWM) task and genes altered following mnemonic stimulation were determined using a microarray approach. Selected genes were studied more extensively for their role in memory processing using pharmacological methods as well as transgenic and knock-out animals.

First, background information relevant to my project is introduced. An overview of the molecular and cellular mechanisms of memory is presented along with experiments designed to evaluate gene expression profiles associated with the cognitive capacities of the animals. Second, a review of the literature regarding aged rats and scopolamine-induced amnesia model are summarized. Among many neurotransmitter pathways that have been postulated to be associated with memory losses during aging, special attention is focused on disruption of the retinoid signalling pathway involved in age-related memory deficits. Since we observed that the retinol transporter transthyretin (TTR) is involved in the maintenance of memory capacities during aging (Brouillette & Quirion, 2007), the functional properties of this protein is discussed as well. In that perspective, we also reviewed the mnemonic deficiencies induced by dioxin, an environmental and food contaminant known to deplete retinol stocks. The potential implication of retinoids, TTR and dioxin in late onset AD is reviewed as well.

Scopolamine-induced amnesia was among the first animal models of AD used to mimic deficits in cholinergic neurotransmission observed in AD. The amnesic effects of scopolamine as well as the critical role of various muscarinic acetylcholine receptors in age-related memory deficits and AD are introduced. Since we observed that scopolamine alters various neurotransmitter systems besides the cholinergic one (Brouillette et al., 2007), interactions of this pathway with glutamatergic and GABAergic innervations in the hippocampus are reviewed

in relation to their capacities to modulate learning and memory functions. A special attention is given to GABA_B receptors and the glutamatergic related gene Homer1 that were shown to be markedly affected in our scopolamine-induced amnesia model (Brouillette et al., 2007). Finally, a brief summary and the major aims of the thesis conclude our review of the literature.

Chapter II

Review of the literature

1. Molecular and cellular mechanisms of memory formation

The ability to learn and remember involves complex processes based on the interplay between many genomic, molecular, cellular and environmental factors. New information acquired from experiences induces brain modulations to be retained and stored for short or long periods of time. The theory of memory that is currently the most widely accepted and sustained by a large body of evidence was elaborated by Hebb in 1949 (Hebb, 1949). The Hebbian process suggests that stabilization of neuronal changes associated with short-term memory leads to the formation of long-term memory. According to this hypothesis, new informations are temporarily stored by inducing post-translational modifications of pre-existing molecules. Stable forms of long-term plasticity require the induction of a cascade of genes and the synthesis of novel proteins during the consolidation phase to produce and maintain new synaptic connections necessary for permanent retention (Kandel, 2001).

New neurons that are continuously generated throughout adulthood in the dentate gyrus, a hippocampal subregion important in memory of facts and events, are also thought to be integrated into the neural circuitry within 3-4 weeks to participate in mnemonic functions (van Praag et al., 2002; Kee et al., 2007). Among other systems, the retinoid signalling pathway, which is critical for neurogenesis and neural differentiation throughout life, may have a key role in the formation and incorporation of new hippocampal neurons as well as in the development of novel synapses (Jacobs et al., 2006). Accumulating evidence suggests that adult neurogenesis in the hippocampus participates in various types of memory functions (Kee et al., 2007; Kitabatake et al., 2007).

Studies in diverse behavioural paradigms (Bourtchouladze et al., 1998; Igaz et al., 2002) indicate that long-term memory formation depends on two distinct temporal periods requiring gene expression and protein synthesis: immediately and several hours after newly learned information. Many immediate-early genes associated with the first phase have already been identified (Lanahan & Worley,

1998; Abel & Lattal, 2001) whereas more downstream genes expressed during the second time period are less known.

Components underlying changes in the strength of synaptic connections during memory processing are largely attributed to the extensively studied cellular model called long-term potentiation (LTP), a long-lasting enhancement in synaptic transmission that can be induced by brief neuronal stimulation. Since its first formal description (Bliss & Lomo, 1973), LTP has been widely, though not unequivocally, implicated in various forms of learning and memory (Larkman & Jack, 1995; Martin & Shapiro, 2000; Lisman & McIntyre, 2001). Recent data suggests that LTP has two phases (Fig. II.1). The early phase (lasting 1-3 hours) is characterized as being independent of protein synthesis. The late phase (consolidation: lasting many hours) is protein synthesis-dependent, and may be blocked by drugs that inhibit protein or mRNA synthesis.

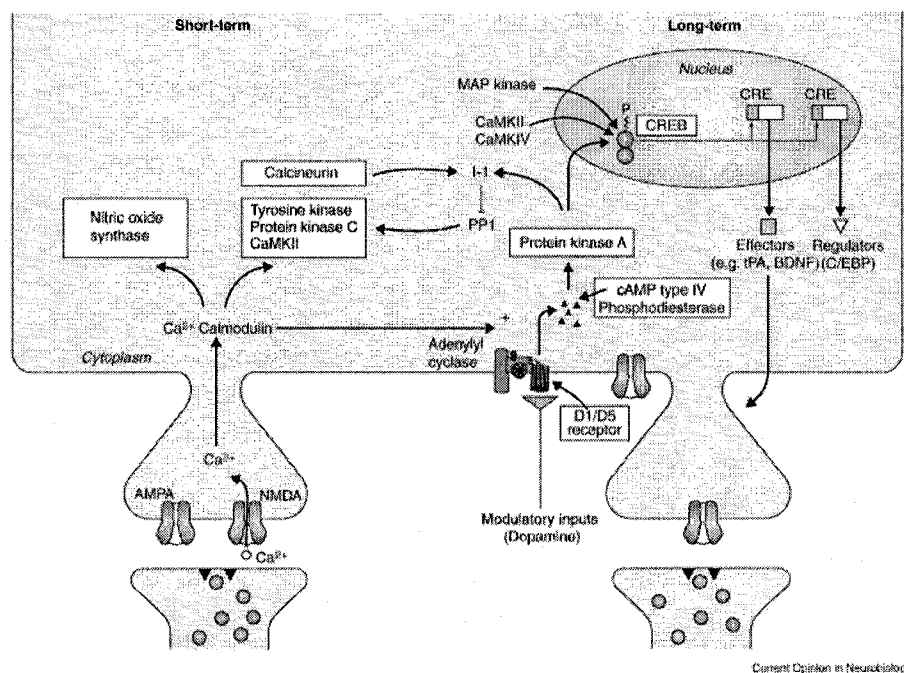


Fig. II.1. Molecular events that underlie the early and late phases of LTP. Among other events, stimulation of NMDA glutamate receptors, as a result of membrane depolarization through AMPA receptors and glutamate binding, allows calcium (Ca^{2+}) to enter the post-synaptic neurons leading to the induction of LTP.

Abbreviations: AMPA, *alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*; BDNF, *brain-derived neurotrophic factor*; CaMK, *CA²⁺-calmodulin-dependent protein kinase*; C/EBP, *CCAAT enhancer binding protein*; CRE, *cAMP response element*; CREB, *cAMP response element binding protein*; I-1, *protein phosphatase inhibitor-1*; MAP Kinase, *mitogen activated protein kinase*; NMDA, *N-methyl-D-aspartate*; PP1, *protein phosphatase-1*; tPA, *tissue plasminogen activator*. Adapted from Abel & Lattal (2001).

Studies of LTP in hippocampal area CA1 have focused on the signalling cascade initiated by the glutamate NMDA receptors. The activation of this receptor by glutamate is necessary, although not sufficient for the induction of LTP (Morris et al., 1990). NMDA receptors are activated only when the release of glutamate by pre-synaptic terminals is accompanied by substantial depolarization of post-synaptic cells through AMPA receptors. Among the immediate effects of Ca²⁺ influx through NMDA receptors, are the activation of nitric oxide synthase, calcineurin, protein kinase C (PKC) and CaMKII.

Long-lasting LTP occurs when adenylyl cyclase is activated to produce cAMP. This intermediate activates protein kinase A (PKA), which in turn recruits MAPK and both translocate into the nucleus to activate a transcriptional cascade initiated by CREB during or immediately following behavioural training (Dash et al., 1990). Once phosphorylated, CREB regulates the expression of immediate-early genes (IEG) such as C/EBP, a transcription factor known to regulate more downstream effector genes that lead to the growth of new synapses and/or prolonged strengthening and stabilization of synaptic facilitation (Taubenfeld et al., 2001). Decreased spatial memory abilities in aged rats are correlated to defective LTP in the hippocampus (Barnes & McNaughton, 1985; Bach et al., 1999; Tombaugh et al., 2002). Interestingly, a recent study has also shown that increased protein levels of CREB in the hippocampus prevented long-term memory deficits during normal aging in rats (Mouravlev et al., 2006).

2. The hippocampus: a brain area critical for memory

Studies on the anatomical localization of memory were conducted in the 1950s with epileptic patients by the neurosurgeon Wilder Penfield at the Montreal Neurological Institute. During mapping of the cortical surface in unanaesthetized subjects, his team discovered that electrical stimulations in certain subregions of the temporal cortex gave rise to memory-like perceptions (Penfield & Perot, 1963). The implication of these structures in memory processing was further investigated in 1957 by the neurosurgeon William Scoville and the psychologist Brenda Milner (Scoville & Milner, 1957), with the now famous H.M. patient. Following large bilateral removal of the medial temporal lobe (including the hippocampus) that relieved his epilepsy, H.M. was unable to form any new memories of people, facts or events from the time of his surgery whereas remote memories, such as those from his childhood, were unchanged. Despite severe memory deficits, his language and reasoning capacities were unchanged and his performance in an IQ test actually increased slightly.

More recent studies of amnesic patients and of animal models suggest that memory deficiencies in H.M. occurred primarily from damage to the hippocampus. Indeed, in humans as well as in animals, the hippocampus has been shown to be essential for the formation and recall of declarative memory (Hollup et al., 2001) and is necessary for consolidation of memory traces (Riedel et al., 1999). Declarative memory, also called explicit memory, can be further subdivided into episodic (events) and semantic (facts) memories. Spatial memories which have been extensively studied in animal models represent a form of declarative memory. The nature of hippocampal-dependent memory is not fully established but seems to require active exploration and integration of relationships among the items to be remembered.

Hippocampal pyramidal cells function as place neurons that are activated in response to animal location (O'Keefe, 1991). In any given environment, about half of the pyramidal cells fire when an animal occupies a specific area in its environment to form an "allocentric cognitive map" by using spatial cues available in the environment (Kesner et al., 1993). Functional brain imaging

studies in mice have demonstrated that recall of a spatial memory five days following learning recruits the hippocampus (Bontempi et al., 1999). Imaging studies in humans and rodents also confirmed that the hippocampus is implicated in spatial navigation (Maguire, 1997). Some evidence suggest that the hippocampus is especially vulnerable in the course of aging, and that age-related memory deficits involve a reduction in the functioning of the hippocampus (Uttl & Graf, 1993; Driscoll & Sutherland, 2005). Aged humans with long-lasting cortisol elevations show deficits in hippocampus-dependent memory tasks that correlate with size reduction of this structure (Lupien et al., 1998).

2.1. Anatomy and neurotransmitter systems of the hippocampus

The hippocampus is part of the limbic system, a region associated phylogenetically and architectonically to the primitive cortex (Amaral & Witter, 1989) (Fig. II.2). This brain area consists of a series of interconnected cells containing various neurotransmitters including glutamate, γ -aminobutyric acid (GABA), acetylcholine (ACh), noradrenaline (NA) and serotonin (5-HT) as well as many neuropeptides and gaseous transmitters (Izquierdo et al., 1993; Vizi & Kiss, 1998). Like other cortical regions, the neurochemical basis of the hippocampal architecture is related to excitatory glutamatergic neurons. These principal cells (pyramidal and granule neurons) represent 90% of hippocampal neurons, whereas the remaining 10% of hippocampal cells are inhibitory GABAergic interneurons (Freund & Buzsaki, 1996). Neurons containing other neurotransmitters have their cell bodies in other brain regions and send projections to the hippocampus to form synaptic and nonsynaptic interactions within this structure to regulate its functions (Vizi & Kiss, 1998).

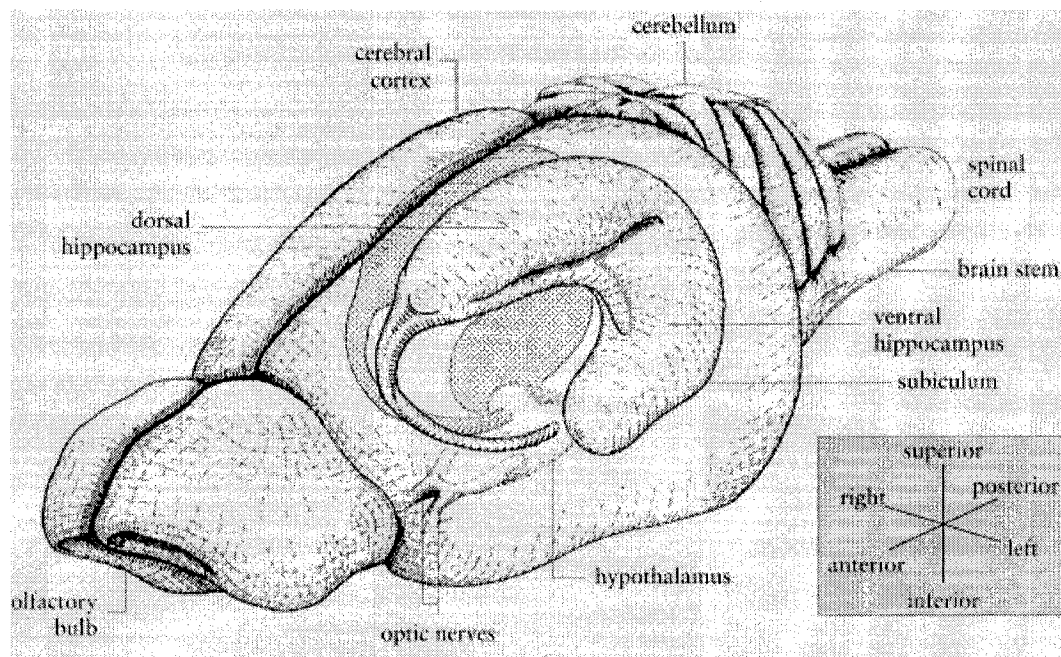


Fig. II.2. A three-dimensional organisation of the hippocampus in the rat brain. The hippocampus is a C-shaped structure that is often divided into its dorsal and ventral part. Adapted from Amaral & Witter (1989).

The glutamatergic pathway forms the trisynaptic loop of the hippocampal formation (Fig. II.3). Granule cells of the dentate gyrus receive glutamatergic afferent projections mainly from the entorhinal cortex and send their axons, the mossy fibers, to the dendrites of CA3 pyramidal cells. These neurons project to the dendritic region of CA1 pyramidal cells via the Schaffer collaterals. The main extrinsic projections arise from the CA1 area and terminate in the subiculum and entorhinal cortex, closing the cortico-hippocampocortical loop (Freund & Buzsaki, 1996).

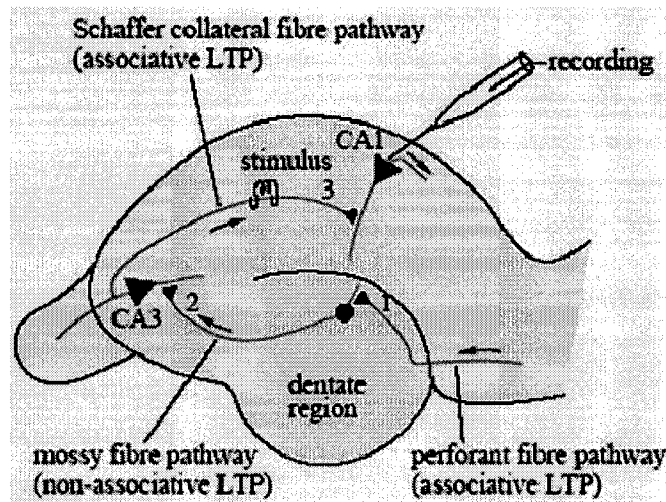


Fig. II.3. The trisynaptic loop of the hippocampus. The hippocampus has a characteristic laminar organisation: cross-section of the hippocampus revealed neurons that are packed into distinct layers. This brain area is composed of the hippocampus proper (CA1, CA2 and CA3) and the dentate gyrus. The intrahippocampal connections form a trisynaptic loop composed of the neurons of dentate gyrus, CA3 and CA1. The letters CA derive from the Latin words Cornu Ammonis; "Ammon's horn" in English. Adapted from Kandel & Pittenger (1999).

The principal cells of the hippocampus make synaptic contacts also on inhibitory GABAergic interneurons which form an organized neural network that regulates the synaptic activity of glutamatergic neurons. The cholinergic system, which is well known to be implicated in the acquisition of new memory (Deutsch, 1971; Jerusalinsky et al., 1997), sends abundant afferents that primarily originate from the medial septum and the diagonal band of Broca to every layer of the hippocampus (Frotscher & Leranth, 1985). The vast majority of these projections make nonsynaptic contacts (Umbriaco et al., 1995) with glutamatergic neurons (Freund & Buzsaki, 1996), while there is also evidence for synaptic contacts onto GABAergic interneurons (Leranth & Frotscher, 1987). In the mammalian brain, synaptic plasticity has been most intensively studied in the hippocampus and particularly at the Schaffer collateral synapses between CA3 and CA1 pyramidal cell fields although plasticity has been described at all synapses in this circuit (Kandel & Pittenger, 1999).

3. The Morris water maze task

The Morris water maze (MWM) is a well-characterized behavioural task used to evaluate spatial learning and memory capacities in rodents (Morris, 1984) (Fig. II.4). Cumulative evidence indicates the critical role of the hippocampal formation in the spatial processes of MWM learning (Rapp et al., 1987; van der Zee & Luiten, 1999). Lesion studies in animal models have shown that the dorsal hippocampus is more critical for spatial memory than the ventral area, and that hippocampus-lesioned rats are specifically impaired in hidden-platform MWM learning but not in visible-platform MWM learning (Moser et al., 1993; Moser et al., 1995).

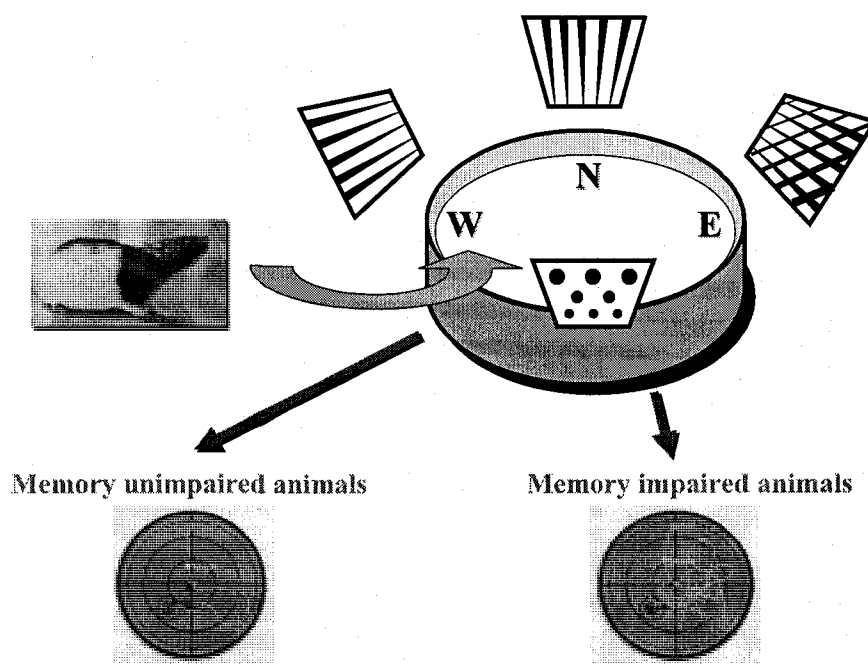


Fig. II.4. The Morris water maze task. During this simple aversively motivated behavioural task, rats or mice swim in a maze to find an escape platform by using distal visuo-spatial cues. Long-term mnemonic abilities are determined with the reference (long-term) memory version of the MWM task, where the hidden escape platform stays at the same location during all training days. A difficulty to find the hidden escape platform over the training days, which is translated in longer escape latencies, is considered to reflect long-lasting spatial memory deficits.

Similarly, human navigation in a computer-generated virtual maze revealed that subjects using spatial landmarks for navigation showed increased activation of the right hippocampus (Iaria et al., 2003). Following spatial MWM learning, formation of novel synapses was observed in the hippocampus of adult rats (Moser et al., 1994) and new neurons formed in the dentate gyrus seem to be preferentially recruited into circuits supporting spatial memory following MWM training (Kee et al., 2007).

Several authors have suggested that hippocampal LTP may be the neuronal basis for hippocampus-dependant memory (for review see Larkman & Jack, 1995). Indeed, the intracerebroventricular (icv) infusion of the NMDA receptor antagonist AP5 impairs hidden-platform MWM performance while blocking LTP in the dentate gyrus (Davis et al., 1992). Studies using genetically modified animals also show that alteration of the NMDA receptor (McHugh et al., 1996), CaMKII (Rotenberg et al., 1996), PKA (Rotenberg et al., 2000) and CREB (Bourtchuladze et al., 1994) induced abnormal LTP and impaired performance in the MWM.

4. Identification of novel memory genes using a microarray approach

Memory processing involves various signalling pathways composed of many molecular intermediates that can act on pre-existing proteins or regulates gene expression to synthesize new proteins. Moreover, interactions are also commonly observed between transduction pathways involved in learning and memory. These systems are implicated in specific aspects of memory functioning and influence each other in an organized and time-dependant manner to produce a fine tuning of synaptic plasticity underlying memory formation (Izquierdo et al., 1993). Because of this complexity, the identification of novel genes involved in learning and memory represents an enormous challenge that has been greatly facilitated by the latest advances in genomic research. In particular, genechip technology that has recently become available and the completion of the genome

sequencing of various species allow for the first time to investigate whole genome expression profiles of animal and human tissues (Marcotte et al., 2001). High-density microarrays may simultaneously and quantitatively compare thousands of genes differentially expressed between two cell populations. This approach is becoming increasingly popular and promises great biomedical progress as the technology is getting more accurate and sophisticated (Boguski & Jones, 2004).

The Affymetrix GeneChip[®] Rat Genome 230 2.0 array contained 28,757 different genes and is the most commonly used array to investigate gene expression profiles in this species. In the present thesis, these microarrays were used to identify novel downstream genes differentially expressed in the hippocampus following stimulation in the reference (long-term) spatial memory version of the MWM task in aged memory-impaired and scopolamine-induced amnesia rat models.

An important issue with this approach is the program used to analyse the microarray data. Depending on the software employed, the number of genes detected as differentially expressed will greatly vary as well as the amplitude of the fold change. The log₂ scale Robust Multi-array Averaging (RMA) method is a well-established microarray analysis commonly used to obtain a more restrictive list of genes with less pronounced fold change magnitudes compared to the Microarray Affymetrix Suite 5.0 (MAS5) default program (Irizarry et al., 2003b). In comparison to MAS5, RMA was shown to have a better precision in particular for lower expression values, to provide more consistent estimates of fold change and higher sensitivity to detect differential expression by lowering false positive rate (Irizarry et al., 2003a). Moreover, to obtain a more accurate gene expression estimates and confidence intervals, the use of individual microarray for each animal is preferable. Three independent replicates per group are generally acceptable to provide a reliable analysis of gene expression data using microarrays (Lee et al., 2000). Using this approach, together with an appropriate statistical analysis, we generated a consistent, restricted list of genes potentially implicated in memory processing. Candidate genes are then confirmed with independent quantitative real-time RT-PCR and various functional analyses.

5. Aged memory-impaired rats

Aged-associated memory deficits are not restricted to humans. Indeed, a subpopulation (~40%) of aged (24-months old) Long-Evans rats naturally show memory impairments when tested in the MWM task while others (~20%) perform as well as young adult (7-months old) control rats (Rowe et al., 1998; Brouillette & Quirion, 2007). Since a monogamous mating system is used with the Long-Evans strain to maximize the heterogeneity of their genetic background, marked individual differences in their mnemonic capacity is observed during normal aging. However, the identification of genes involved in the maintenance of normal cognitive processes during aging remains to be fully established. The performance of aged rats in the reference memory version of the MWM task has been used routinely to characterize aged memory-impaired (AI) and aged memory-unimpaired (AU) animals when compared to young adult rats (Gage et al., 1989; Issa et al., 1990; Frick et al., 1995; Rowe et al., 1998; Stemmelin et al., 2000). Decreased spatial memory abilities of aged rats are related to decline in the late phase of LTP in Schaffer collateral fibers of the hippocampus (Barnes & McNaughton, 1985; Bach et al., 1999; Tombaugh et al., 2002).

6. Signalling pathways involved in age-related memory deficits

Disruption of many neurotransmitter pathways has been suggested to be associated with memory loss during aging (Richter-Levin et al., 1995; Levin & Simon, 1998; van der Zee & Luiten, 1999; Law et al., 2000). Indeed, alterations in the modulation of the major neurotransmitters present in the hippocampus including ACh (Aubert et al., 1995; Quirion et al., 1995; Rowe et al., 2003), glutamate (Le Jeune et al., 1996; Magnusson, 1998), GABA (Froestl et al., 2004), noradrenaline (Collier et al., 1988; Sirvio et al., 1991), serotonin (Almaguer-Melian et al., 2005; Topic et al., 2007) and dopamine (Hersi et al., 1995; Bach et al., 1999) have been postulated to be involved to some extent in age-associated

memory impairments. In addition, various neurotrophic factors such as IGF-1 (Dore et al., 1997b), VGF (Sugaya et al., 1998), NGF (Auld et al., 2001; Niewiadomska et al., 2006) and BDNF (Silhol et al., 2007) may participate in altered mnemonic capacities during aging. Intracellular messengers including inositol phosphates and diacylglycerol (Parent et al., 1995), PKA (Ramos et al., 2003), PKC (Colombo & Gallagher, 2002), CREB (Mouravlev et al., 2006) as well as the retrograde messenger nitric oxide (Law et al., 2000) were also studied to possibly explain individual differences in cognitive capacities of aged rats.

These molecules represent only a few examples and further studies have yet to fully establish their respective roles in cognition to get a complete portrait of the mnemonic mechanisms altered during normal and pathological brain aging. Previous microarray studies have investigated global alteration of basal gene expression during the course of aging in humans (Lu et al., 2004) and animals (Blalock et al., 2003; Verbitsky et al., 2004). Very recently, our group (Brouillette & Quirion, 2007) and others (Rowe et al., 2007; Burger et al., 2007) have evaluated hippocampal genes differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) animals following a behavioural stimulation. These three studies provide complementary information regarding AI and AU rats since gene expression profiles were evaluated at different times (1, 6 and 24h) following memory stimulation in the MWM task.

7. Implication of the retinoid pathways in memory loss during aging

Our microarray study in aged rats (Brouillette & Quirion, 2007) strongly supports the small, growing body of evidence suggesting a key role for vitamin A (retinol) in age-related memory deficits. Our results, along with another report (Etchamendy et al., 2001), show that decline in the retinol pathway produces memory impairments during normal brain aging which was reversed by treatment with the small lipid retinoic acid (RA), the main active retinol metabolite. A Vitamin A poor diet has also been reported to induce spatial memory impairments

in rodents and to alter both LTP and LTD (Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002; Etchamendy et al., 2003). Similar results were obtained in mice lacking RA receptors β (RAR β) alone or concomitantly with retinoid X receptors γ (RXR γ) (Chiang et al., 1998).

7.1. Retinoid transport

Vitamin A is an essential nutritional element not produced within the body, and which is primarily ingested from products of animal origin (mostly as retinyl esters) and from plant products as beta-carotene. The amount of retinoids (vitamin A and its derivatives) in the brain depends on the diet, route of transport as well as synthesis and removal of active retinol metabolites. In the intestine, vitamin A is esterified to retinyl esters and then packed into chylomicrons before being secreted into the lymphatic system to reach storage tissues (Fig. II.5). Approximately 75% of the dietary vitamin A is taken up by hepatocytes in the liver. From these cells, retinol can be transferred to hepatic stellate cells where 80-90% of the vitamin A is stored as retinyl ester or secreted into the circulation bound to its specific transport protein, the retinol-binding protein (RBP).

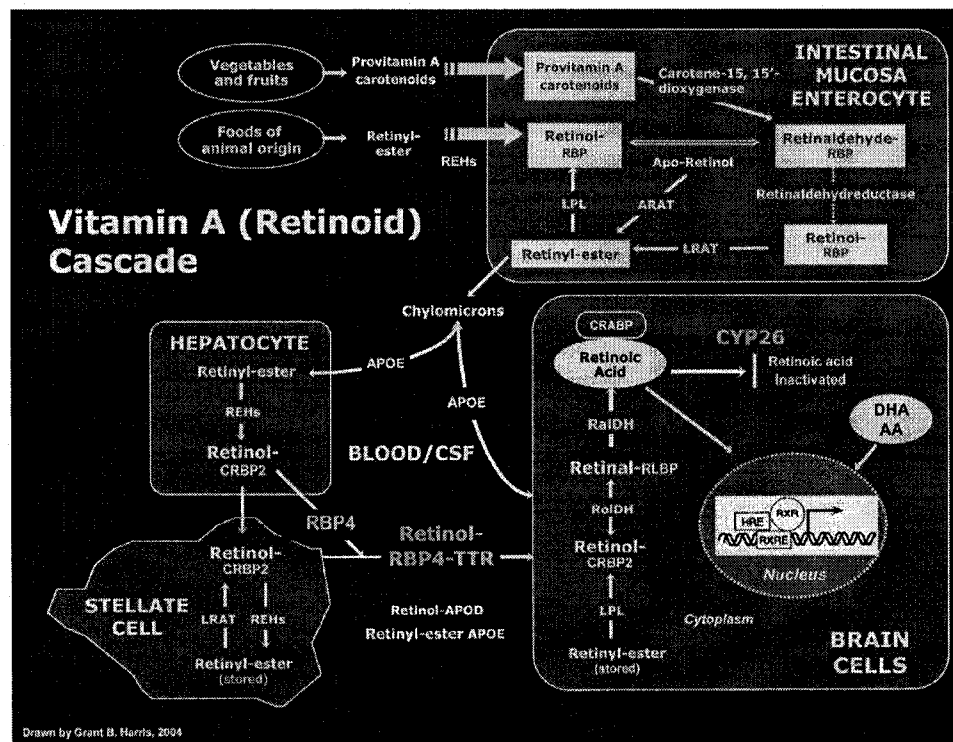


Fig. II.5. The retinoid cascade. Vitamin A originated from food is transported from intestine mainly to the liver to be stored, and then to target tissues as needed by the organism. **Abbreviations:** APOE, apolipoprotein E; ARAT, acyl coenzyme A:retinol acyltransferase; CRABP, cytoplasmic retinoic acid-binding protein; CRBP, cellular retinol-binding protein; DHA, docosahexaenoic acid; LPL, lipoprotein lipase; LRAT, lecithin:retinol acyltransferase; RalDH, retinaldehyde dehydrogenase; RBP, retinol-binding protein; REH, retinyl ester hydrolase; RLBP, retinal-binding protein; RolDH, retinol dehydrogenase; TTR, transthyretin. Adapted from Goodman (2006).

Retinol bound to RBP secreted by the liver represents 95-99% of the vitamin A present in the circulation. This binary complex binds to the plasma protein transthyretin (TTR) to form the major retinol transport route from liver storage to target tissues. Using liquid chromatography, 86% of the retinol-RBP complex was found to be linked to TTR in human plasma (Burri & Kutnink, 1989). TTR was shown to prevent renal glomerular filtration and influence the secretion of the retinol-RBP complex from liver (Monaco, 2000; van Bennekum et al., 2001). There is also a low concentration of retinyl esters associated with circulating lipoproteins including apolipoprotein E, lipocalin, apolipoprotein D as well as small amounts of circulating RA bound to albumin (Smith et al., 1973). Although retinol can also bind to serum albumin, mice lacking RBP were shown to be severely defective in retinol homeostasis (Quadro et al., 1999).

7.2. The retinol carrier transthyretin (TTR)

TTR is a 55 kDa homotetrameric protein strongly conserved between species with 93% homology between human and rodents, and can dissociate to form 30 kDa dimers or 15 kDa monomers (Sundelin et al., 1985; Murrell et al., 1992). The liver and choroid plexuses are the principal sites of synthesis and secretion of TTR (Herbert et al., 1986; Sousa et al., 2007), and the liver is the major site of degradation of TTR found both in the cerebrospinal fluid (CSF) and plasma (Makover et al., 1988). This protein has an essential role in the transport

of both thyroid hormone and retinol in serum and CSF. Indeed, TTR is the major thyroid hormone carrier in rodents with high-affinity binding for the thyroid hormones T3 and T4 (thyroxin) (Vranckx et al., 1990).

This transporter has also an independent binding site for RBP (Smith et al., 1970) and is the main carrier of retinol bound to RBP from liver stores through plasma to target tissues (Herbert et al., 1986). TTR detected in the CSF has been reported to be synthesized *de novo* within the choroid plexus rather than being selectively transported across the blood-CSF barrier (Weisner & Roethig, 1983; Herbert et al., 1986). The choroid plexus is an intraventricular epithelial tissue that forms the hemato-CSF barrier, providing vitamins, peptides and hormones to neurons. This structure is specialized in CSF production and secretes about 90% of CSF, whereas the remaining 10% comes from brain interstitial fluid drainage (Dohrmann, 1970). TTR represents 25% of total proteins synthesized by the choroid plexus, and 20% of total CSF proteins in humans (Weisner & Roethig, 1983).

RBP is able to carry retinol alone but since plasma TTR concentrations are normally in a 2 to 3 fold molar excess over those of RBP, most retinol-RBP in the circulation is bound to TTR in a 1:1 ratio to prevent filtration and elimination by kidney (Goodman, 1984). The importance of an adequate regulation of TTR is underscored by the fact that TTR is a single-copy gene that gives a single protein product, and no other analogous protein seems to be able to replace TTR in the transport of retinol (Dickson et al., 1985).

7.3. Metabolism of retinoids

After delivery to the target cells, retinol diffuses freely throughout the plasma membrane because of its lipophilic nature and is metabolized into RA in a two-step enzymatic pathway to exert biological activity (Molotkov et al., 2004) (Fig. II.6). Vitamin A is oxidized first to retinaldehyde by alcohol dehydrogenase (ADH), and short-chain dehydrogenase/reductase then can regenerate retinol. Retinol can also bind to cellular retinol-binding protein (CRBP) to limit its oxidation by ADH. Following oxidation of retinaldehyde into RA catalyzed by

aldehyde dehydrogenase (ALDH), RA can enter the nucleus and act as a ligand for RA receptors, or it can be metabolized by P450 (CYP) enzymes into 4-oxo-RA, which constitutes the first step in the degradation pathway leading to excretion. RA can bind to cellular RA-binding protein-I (CRABP-I) that catalyzes its degradation (Fiorella et al., 1993) or bind to CRABP-II which inversively sensitizes cells to the effect of RA (Dong et al., 1999).

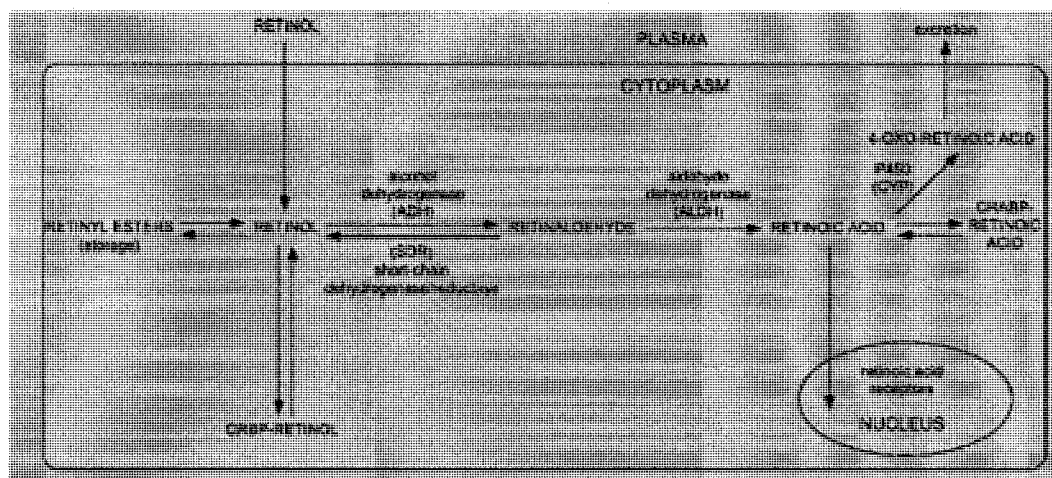


Fig. II.6. Retinol metabolism. Intracellular retinol can bind to CRBP with the equilibrium greatly in favour of the bound form. Retinol can be covalently linked to a fatty acid by LRAT to produce retinyl esters for storage, and a REH can regenerate retinol. Several forms of ADH and SDR catalyse oxidation of retinol and reduction of retinaldehyde respectively, and several forms of ALDH catalyse oxidation of retinaldehyde into RA. **Abbreviations:** ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; LRAT, lecithin:retinol acyltransferase; REH, retinyl ester hydrolase; SDR, short-chain dehydrogenase/reductase. Adapted from Molotkov et al. (2004).

The synthesis of RA has been reported in various brain areas including the hippocampus, olfactory bulbs, basal ganglia, blood vessels throughout the entire brain as well as meninges and choroid plexus which possess a high levels of RA-synthesizing enzymes (Zhao et al., 1996; Wagner et al., 2002). RA can also

function in a paracrine manner and diffuse from the liver and cross the blood-brain barrier because of its small size and lipophilic property. The subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus, two regions that maintain capacity for neurogenesis in the adult (van Praag et al., 2002), are particularly affected by RA diffusion from the closely apposed choroid plexus, meninges and blood vessels within the region of the hippocampal fissure (Zhao et al., 1996).

7.4. Retinoid receptors and their ligands

The action of retinoids is mainly mediated by nuclear receptors termed RAR ($\text{RAR}\alpha$, β and γ) and RXR ($\text{RXR}\alpha$, β and γ). These receptors are members of the steroid receptor family and they essentially function as transcription factors. Each subtype of receptor is encoded by a different gene which can undergo differential splicing, resulting in multiple receptor isoforms (for example, $\text{RAR}\beta 1$, $\text{RAR}\beta 2$, $\text{RAR}\beta 3$ and $\text{RAR}\beta 4$) (Lane & Bailey, 2005). This complexity gives rise to numerous receptor combinations as RARs heterodimerize with RXRs, whereas RXRs can homo- or heterodimerize with other nuclear receptors such as those for thyroid hormones, vitamin D and glucocorticoids (Kliwer et al., 1992).

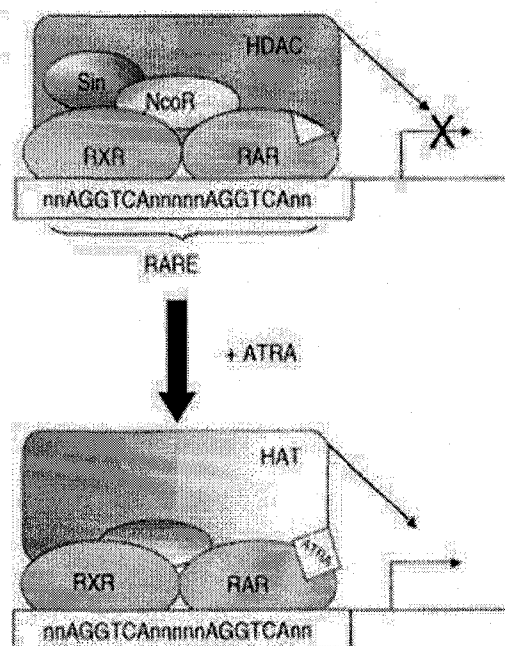


Fig. II.7. RA-mediated gene expression. ATRA regulates gene transcription via RARs and/or RXRs bound to RAREs. **Abbreviations:** ATRA, all-trans-RA; HAT, histone acetyl transferase; HDAC, histone deacetylase complex; NcoR, nuclear receptor corepressor; Sin, signal integrating protein; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor. Adapted from Lane & Bailey (2005)

Following the binding of specific retinoid ligands, these nuclear receptors interact with DNA on regulatory regions in the promoter sequence to regulate the expression of a broad array (> 300) of target genes (Clagett-Dame & Plum, 1997) (Fig. II.7). In the promoter region, RAR-RXR is known to bind to RA response elements (RAREs) while the RXR homodimer binds to retinoid X responsive elements (RXREs) (Soprano et al., 2004). Decreased expression (20-30%) of RAR β and RXR β/γ was observed in aged mice compared to control adults, and this reduction was reversed by the acute administration of RA (Enderlin et al., 1997b). The lower amount of these receptors in aged mice was suggested to result from inadequate bioavailability of RA to ensure the up-regulation of RA receptors, which is consistent with disruption of a major transport route of retinol involving TTR as observed in AI rats (Brouillette & Quirion, 2007).

RARs bind all-*trans*-RA (ATRA) with high affinity and 9-*cis*-RA with lower affinity (Soprano et al., 2004). RXRs bind 9-*cis*-RA (Mangelsdorf et al., 1992) as well as other non-retinoid ligands such as the brain-enriched lipid docosahexaenoic acid (DHA) (de Urquiza et al., 2000). The effects of vitamin A on gene transcription are mediated mostly by ATRA interactions with RARs since 9-*cis*-RA is undetectable in any tissue *in vivo* (Mic et al., 2003). Activation of the RAR-RXR heterodimers is controlled by RARs given that RXRs-selective ligands do not induce gene expression, a phenomenon termed RAR dominance (Kurokawa et al., 1994). However, once a RAR ligand is bound, RXR ligands can increase the transcriptional efficiency of the RAR-RXR heterodimers (Minucci et al., 1997).

Free RARs are complexed with histone deacetylase complexes (HDACs) and inhibit gene transcription. ATRA can also inhibit gene transcription through a RARE-independent mechanism by repressing the activity of the transcription complex AP-1 (Activating protein 1) (Schule et al., 1991; Li et al., 1996). On the other hand, AP-1 component proteins, Fos and Jun, can down-regulate the retinoid signal when present in high levels (Pfahl, 1993). Some other receptors such as COUP-TF, which is expressed in the hippocampus, can also compete with RAR-RXR to bind to RARE and prevent the induction of gene transcription by

RA (Tran et al., 1992). Unliganded RXRs are co-factors for non-steroid nuclear receptors including thyroid hormone receptors and the vitamin D receptors (Kliewer et al., 1992; Bugge et al., 1992). When exogenous RXR agonists are used, RXRs function as ligand-activated heterodimeric binding partners for various nuclear receptors including PPAR γ , LXR, FXR and the orphan receptor nur77/NGFB-1 (Perlmann & Jansson, 1995; Kliewer et al., 1995; Forman et al., 1995; Janowski et al., 1996).

7.5. Distribution of retinoid receptors in the hippocampus

The distinct temporal and spatial expression pattern of each retinoid receptor subtypes and isoforms suggest that they can regulate specific neuronal phenomena. In the adult brain, RAR α and RAR γ are widely distributed while high expression of RAR β is restricted to only few tissues (Table II.1). High levels of receptor transcripts and proteins for RAR α and RAR γ were detected in the hippocampus with an apparent absence of RAR β expression (Krezel et al., 1999). This study also found that RXR β proteins were expressed in the CA1-CA3 hippocampal region, and both RXR β and RXR α were present in the dentate gyrus. Another report indicated that the transcript for RXR γ is present diffusely in the hippocampus (Zetterstrom et al., 1999). It is interesting to note that RXR α transcripts were readily detected in some brain areas while the corresponding proteins were low or undetectable.

Table II.1. Distribution of RAR α , β and γ and RXR α , β , and γ receptor proteins in the mouse CNS

Brain area	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ
Hippocampus						
CA1 subfield	+++	—	+++	—*	++	—
CA2 subfield	+++	—	+++	—*	++	—
CA3 subfield	++	—	++	—*	+	—
Dentate gyrus	—	—	+++	+	+	—
Hilus of the dentate gyrus	—	—	+	+	—	—
Choroid plexus	+	++	++	++	++	—

The intensity of the nuclear immunoreaction signal was estimated as being either weak (+), medium (++) or strong (+++) in comparison to the strongest signal obtained with the cognate antibody. In all areas scored positive the percentage of immunostained cells was greater than 20%.

** Receptor transcripts were readily detected, but the corresponding proteins were either low or undetectable. Adapted from Krezel et al. (1999).*

7.6. Role of retinoic acid (RA) in learning and memory

The pleiotropic action of retinoids includes morphogenesis, vision, immunity, and fertility (Malik et al., 2000). RA is involved in the development of the central nervous system (CNS), cell differentiation (Maden, 2002), and in the determination of neurotransmitter phenotypes (Kobayashi et al., 1994; Berrard et al., 1995). In the adult brain, RA is implicated in the differentiation of glia and neurons in several cortical areas including the basal ganglia, olfactory bulbs, and particularly in the hippocampus where high levels of RA receptor signalling exist (Misner et al., 2001; Wagner et al., 2002; Luo et al., 2004). The action of RA on target genes containing RARE sequence in their promoter region occurs throughout the hippocampus during development but becomes progressively more restricted to the dentate gyrus with age (Luo et al., 2004). In this brain region, RA is suggested to regulate differentiation, cell survival and the production of new neurons from neural stem cells located in the subgranular zone in adult mice (Jacobs et al., 2006), thereby affecting learning and memory functions (Kitabatake et al., 2007). However, high concentrations of RA have also been demonstrated to suppress neurogenesis and hippocampal-dependent learning in adult mice (Crandall et al., 2004), suggesting that a fine regulation of RA homeostasis is critical to maintain normal cognitive functions.

Several lines of evidence indicate that RA could be involved in the production and the maintenance of new synapses necessary for long-term memory formation. Retinoids have been shown to increase neurite outgrowth from various neuronal systems including embryonic dorsal root ganglia, sympathetic neurons,

spinal cord, olfactory neurons as well as dissociated cerebra and retina explants (Clagett-Dame et al., 2006). RA was shown to induce neurites with dentrite-like properties in neuroblastoma cells (Wu et al., 1998) and to have a role in axonal elongation (Clagett-Dame et al., 2006). One mechanism by which RA can induce neurite outgrowth in the hippocampus may come from its ability to regulate the expression of neurotrophic factors such as NGF (Lee et al., 1990; Corcoran & Maden, 1999; Corcoran et al., 2000; Canon et al., 2004).

The action of RA in learning and memory could, at least partly, be mediated via a non-genomic mechanism, since RA can induce rapid and sustained phosphorylation of CREB in neuronal cells (Canon et al., 2004). CREB is known to contribute to neuronal differentiation (Ginty et al., 1994), brain neuroplasticity (Impey et al., 1998; Sgambato et al., 1998), neuronal survival (Bonni et al., 1999; Riccio et al., 1999) and memory as well (Bourtchuladze et al., 1994; Bozon et al., 2003; Mouravlev et al., 2006).

Many other genes regulated by RA are known to be involved in cognitive functions. Indeed, RA modulates gene expression of several structural or functional proteins involved in the synthesis, transport or degradation of neurotransmitters associated with learning and memory (Table II.2). Most of the neurotransmitters found in the hippocampus including glutamate, GABA, ACh and noradrenaline are affected by retinoid signalling pathways. Many receptors, channels and proteins that compose the intracellular cascades associated with synaptic plasticity and LTP are also regulated by RA.

For example, neurogranin (RC3) and growth-associated protein 43 (GAP43) are two proteins synthesized exclusively in neurons that were proposed to modulate synaptic plasticity by regulating calcium availability and calcium/calmodulin-dependent protein kinases (Gerendasy & Sutcliffe, 1997; Krucker et al., 2002). RC3 is a post-synaptic substrate of PKC involved in dendritic spine formation and is mainly expressed in the hippocampus, striatum and cerebral cortex (Gerendasy & Sutcliffe, 1997). Mice lacking the RC3 gene were shown to exhibit spatial memory impairments as well as deficits in hippocampal synaptic plasticity (Pak et al., 2000). Phosphorylation of RC3 has

also been reported during the maintenance phase of LTP (Chen et al., 1997). GAP43 is a pre-synaptic PKC substrate found in axonal growth-cone membrane. This protein has been implicated in different forms of synaptic plasticity including neurite outgrowth, regeneration and LTP (Gerendasy et al., 1995; Benowitz & Routtenberg, 1997). GAP43 immunostaining has been observed to be intense in fine fibrils found in the neuropil and neuritic processes of the senile plaques, whereas its level was shown to be reduced in the frontal cortex and hippocampus in AD (Bogdanovic et al., 2000).

Table II.2. Retinoid-regulated neuronal genes in the adult rodent brain

Gene	Effect of RA on expression	Cell system	Assay	References
Transporters				
Norepinephrine transporter	↑	PC12, SCG	mRNA, function	(Matsuoka et al., 1997)
Vesicular ACh transporter	↑	PC12, SN56, NG108	mRNA	(Berse & Blusztajn, 1995) (Berse & Blusztajn, 1997) (Dolezal et al., 2001)
Vesicular GABA transporter	↑	P19	mRNA	(Ebihara et al., 2003)
Metabolic enzymes				
Choline acetyltransferase ^P	↑	PC12, SN56, NG108-15, SCG	mRNA, protein, activity	(Kobayashi et al., 1994) (Pedersen et al., 1995) (Berse & Blusztajn, 1995) (Berse & Blusztajn, 1997) (Personett et al., 2000) (Dolezal et al., 2001)
Tyrosine hydroxylase	↓	SCG	mRNA, activity	(Berrard et al., 1993) (Kobayashi et al., 1994) (Cervini et al., 1994)
Dopamine β-hydroxylase	↓	SCG	activity	(Berrard et al., 1993) (Cervini et al., 1994)
Glutamic acid decarboxylase	↑	P19, NE-7C2	mRNA	(Bain et al., 1993) (Varju et al., 2002)
AChesterase	↑	P19	mRNA	(Coleman & Taylor, 1996)

G-protein coupled receptors				
Mu opioid receptor	↓/↑	SH-SY5Y	mRNA	(Jenab & Inturrisi, 2002)
Delta opioid receptor	↑	NG108-15	mRNA	(Beczowska et al., 1996)
Kappa opioid receptor	↓	P19	mRNA	(Bi et al., 2001) (Hu et al., 2002)
5-HT _{1A} receptor	↑	SN-48	mRNA	(Charest et al., 1993)
Dopamine D2 receptor ^v	↑	Striatum	mRNA, protein	(Samad et al., 1997) (Valdenaire et al., 1998)
Dopamine D2, D1, D5 receptors	↑	NT2	mRNA, function	(Sodja et al., 2002)
Ionotropic receptors				
Nicotinic AChR α3, α4, β2	↑	P19	mRNA, protein	(Cauley et al., 1996)
NMDA receptor (NR1)	↑	NG108-15	mRNA	(Beczowska et al., 1996)
Kainate receptor (GluR6)	↑	P19	mRNA	(Bain et al., 1996)
GABA _A receptor γ2	↑	P19	mRNA, function	(Reynolds et al., 1996)
Ion transport proteins				
K ⁺ channel (Kir 2.1)	↑	SH-SY5Y, SK-N-BE	mRNA, function	(Smith-Maxwell et al., 1991) (Arcangeli et al., 1998) (Tonini et al., 1999)
Na ⁺ /H ⁺ exchanger	↑	P19	mRNA, activity	(Dyck & Fliegel, 1995)
L-type Ca ²⁺ channel	↑	NG108-15, NT2	mRNA, function	(Kamp et al., 1995) (Gao et al., 1998)
N-type Ca ²⁺ channel	↑	NT2	mRNA, function	(Gao et al., 1998)
Cytoskeletal proteins				
Neurofilament (-L, -M, -H) proteins	↑	P19	mRNA, protein	(Chiu et al., 1995) (Paterno et al., 1997)
Dystrophin-associated proteins	↑	P19	mRNA	(Ceccarini et al., 2002)
Tenascin	↑	SH-SY5Y	protein	(Linnala et al., 1997)
Integrin α/β	↑	SH-SY5Y	protein	(Linnala et al., 1997)
Intracellular signalling molecules				
Phospholipase A2, C, D	↑	LA-N-1	activity	(Farooqui et al., 2004)
Arachidonic acid	↑	SK-N-BE	activity	(Petroni et al., 1996)
Neuronal nitric oxide synthase	↑	SN56	mRNA, protein, activity	(Personett et al., 2000)

Tissue transglutaminase	↑	SCG, SK-N-BE, SH-SY5Y	activity, mRNA	(Ando et al., 1996) (Melino et al., 1997) (Tucholski & Johnson, 2003)
Neuromodulin (GAP43)	↑	Striatum		(Husson et al., 2004)
Neurogranin (RC3) ^V	↑	SK-N-BE, striatum	mRNA	(Iniguez et al., 1994) (Husson et al., 2004)
Neuron-specific enolase ^P	↑	N-115, PC12	mRNA	(Matranga et al., 1993) (Sakimura et al., 1995)
Calbindin-D 28K	↑	PC12, D283	mRNA, protein	(Vyas et al., 1994) (Wang & Christakos, 1995)
Synapsins I, II, III	↑	NT2	mRNA	(Leypoldt et al., 2002)
Miscellaneous				
Gonadotropin-releasing hormone ^V	↓/↑	GT1-1, hypothalamus	mRNA	(Cho et al., 1998) (Cho et al., 2001)
Oxytocin ^V	↑	Neuro2A	mRNA	(Richard & Zingg, 1991)
Trk A / Trk B	↑	NB, P19	mRNA	(Lucarelli et al., 1995) (Salvatore et al., 1995)
Nerve growth factor	↑	PC12	mRNA	(Scheibe & Wagner, 1992)
Neural cell adhesion molecule	↑	P19, LA-N-5	Protein	(Husmann et al., 1989) (Cervello et al., 1997)
Presenilin 1	↑	SH-SY5Y	mRNA	(Flood et al., 2004)
Presenilin 2	↑	P19	mRNA	(Culvenor et al., 2000)
Apolipoprotein E	↓	NT2	mRNA	(Harris et al., 2004)
Amyloid protein precursor ^P	↑	P19, SH-SY5Y, PC12, hippocampus	mRNA, protein	(Konig et al., 1990) (Fukuchi et al., 1992) (Hung et al., 1992) (Lahiri & Nall, 1995) (Yang et al., 1998)
Tau ^P	↑	P19	mRNA	(Fukuchi et al., 1992) (Heicklen-Klein et al., 2000)
ADAM10	↑	SH-SY5Y	mRNA, activity	(Prinzen et al., 2005)
Insulin degrading enzyme	↑	SK-N-BE	mRNA, protein	(Melino et al., 1996)

The table summarizes data which show that RA can increase (↑) or decrease (↓) the expression of specific neuronal genes as assayed by mRNA, protein or functional activity in a variety of neuronal cell lines. ^P and ^V refer respectively to a putative or verified RARE in the promoter of the gene concerned.

Abbreviations: PC12, rat pheochromocytoma cells; SCG, rat superior cervical ganglia neurons; SN56, clonal cholinergic cell line derived from medial septum of mouse forebrain; SN-48, a murine septum × neuroblastoma fusion cell line; SH-SY5Y, human neuroblastoma cell line; GT1-1, mouse-derived hypothalamic GnRH neuronal cells; P19, embryonal carcinoma cells; NG108-15, mouse neuroblastoma X rat glioma hybrid cell line; LA-N-1/5, human neuroblastoma cell line; SK-N-BE, human neuroblastoma cell line; N-115, mouse neuroblastoma cell line; N2AB-1, mouse neuroblastoma cell line; NB, human neuroblastoma; D283, human medulloblastoma cell line; NT2, human teratocarcinoma cell line; NE-7C2, mouse p53-deficient neuroectodermal cell-line. Adapted from Lane & Bailey (2005).

Overall, RA may contribute to learning and memory in several ways. This small lipid acts mainly via genomic mechanisms but was also shown to possess a non-genomic action. Retinoid pathways are suggested to have a particular impact on the hippocampus by affecting synaptic plasticity and efficiency as well as by promoting the production of new synapses and the differentiation and survival of newborn cells. As it will be introduced below, RA was proposed to be implicated in the progression of late onset AD by regulating various components involved in deposition of amyloids, A β degradation, the formation of neurofibrillary tangles and memory deficits observed in this disease (for review see Lane & Bailey, 2005).

8. The environmental and food pollutant dioxin empties retinol stock

Another approach that can be used to further investigate the role of RA in learning and memory function is to induce vitamin A deficiency (VAD). Most studies have induced VAD by feeding animals with vitamin A-free diet which creates retinoid hypo-signalling after few weeks depending on the species and strain of animals used. VAD is more difficult to induce in mice than in rats since mouse pups are born with sufficient retinol stores to last their entire lives (McCaffery et al., 2003). Inadequate dietary vitamin A in mice decreases 20-30% expression of brain RARs after 27 weeks of VAD as observed in senescent mice (Enderlin et al., 1997a; Etchamendy et al., 2001).

A different method to empty retinol storage is to use compound known to interfere with retinoid turnover and metabolism. Dioxin, also known as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), is a widespread noxious environmental and food contaminant. The similar symptomatology of hypovitaminosis A and TCDD-exposed animals suggests that some toxic effects of this compound may be related to disruption of retinol transduction pathway (Thunberg et al., 1980). Mounting evidence indicate that dioxin interferes with retinoid turnover and metabolism by increasing retinoid mobilization from retinyl ester stores (Brouwer et al., 1989; Jurek et al., 1990; Van Birgelen et al., 1995; Kelley et al., 2000), altering retinol esterification (Nilsson et al., 2000; Hoegberg et al., 2003), changing tissue levels of RA (Kelley et al., 1998; Nilsson et al., 2000; Hoegberg et al., 2003), augmenting whole-body turnover of retinoids (Kelley et al., 1998) and increasing excretion of retinoid metabolites (Hakansson & Ahlborg, 1985; Brouwer et al., 1989).

A few reports have suggested that TCDD may alter memory capacities. Dioxin has been shown to repress excitatory post-synaptic potentials (EPSPs) in hippocampal CA1 field in rats (Hong et al., 1998). Moreover, air force veterans of the Vietnam war highly exposed to Agent Orange containing TCDD show deficits in various measures of memory performance (Michalek et al., 2001). Long-term

cognitive impairments have been observed following prenatal exposure to low levels of TCDD in rats (Seo et al., 1999; Powers et al., 2005) and monkey (Schantz & Bowman, 1989). Our study has revealed that neonatal administration of dioxin induces marked memory deficits in female mice (Brouillette & Quirion, Submitted). These cognitive impairments seem to be associated with the capacity of dioxin to alter retinoid metabolism since we observed that a RA treatment was able to reverse TCDD-induced memory deficits.

8.1. The pleiotropic action of dioxin is gender-specific

The gender-sensitive effects of TCDD on memory functioning revealed in our study has also been observed in other reports studying the effect of dioxin on cognitive abilities (Seo et al., 1999; Powers et al., 2005). Indeed, most of the pleiotropic effects of TCDD were shown to be gender-dependant (Mably et al., 1992; Wyde et al., 2001; Thornton et al., 2004; Myllymaki et al., 2005; Hojo et al., 2006) and several studies have suggested that it may occur through the inhibition of an estrogen pathway (Tritscher et al., 1996; Safe et al., 2000; Wormke et al., 2000; Wyde et al., 2001).

A large body of evidence indicates that estrogens are not only implicated in reproduction but also in cognitive functions such as learning and memory (Shughrue & Merchenthaler, 2000). Estrogen receptors are broadly expressed in the hippocampus and other brain regions involved in memory functioning including the neocortex and basal forebrain (Shughrue et al., 1997; Shughrue & Merchenthaler, 2000; Hart et al., 2001). Estrogens have been demonstrated to increase EPSPs modulated by NMDA and to decrease GABA-mediated IPSCs (Cordoba Montoya & Carrer, 1997; Rudick & Woolley, 2001; Gureviciene et al., 2003). High levels of estrogen induce various alterations in the hippocampus, including enhanced LTP, reduced LTD (Vouimba et al., 2000), increased dendritic spine density in CA1 field and dentate granular neurons (Woolley & McEwen, 1992; Miranda et al., 1999), increases in synaptic and spine-associated proteins (Murphy et al., 1998) as well as neurogenesis (Tanapat et al., 1999). Estrogens were also shown to activate gene expressions for CREB, NMDA

receptor 1 subunits, many subunit combinations of the GABA_A-benzodiazepine receptor system, glutamic acid decarboxylase (GAD) and choline acetyltransferase (McEwen & Alves, 1999). Although findings from behavioural studies investigating the effect of estrogen on cognitive functions have not reached consensus (Sherwin, 1994), some evidence suggests that estrogen treatment in aged female or young ovariectomized rodents can improve spatial and non-spatial memory capacities (Packard & Teather, 1997; Fugger et al., 1998; Gibbs, 1999; Rissanen et al., 1999; Chesler & Juraska, 2000; Sandstrom & Williams, 2001; Frick et al., 2002).

Estrogen pathways as also been hypothesis to be affected in AD (LeBlanc, 2002; Brinton, 2004; Baum, 2005). Indeed, estrogen deficiency has been shown to accelerate A β plaque formation (Yue et al., 2005), whereas E treatment prevents neural tau hyperphosphorylation in an animal model of AD (Alvarez-de-la-Rosa et al., 2005). Some epidemiological studies have suggested a protective role for estrogen therapy against AD in post-menopausal women (Brinton, 2004) while others have reported no benefit or potential deleterious effect (Shumaker et al., 2003).

Several reports have suggested cross-talk between dioxin and the estrogen signalling pathway. TCDD is a high affinity ligand for the aryl hydrocarbon receptor (AhR) that modulates various endocrine signalling pathways including inhibition of estrogen-induced responses in various cell types (Romkes et al., 1987; Umbreit & Gallo, 1988; Astroff et al., 1990; Astroff & Safe, 1990; DeVito et al., 1992). Moreover, DNA damage produced by dioxin was reported to be mediated via a mechanism involving estrogen (Wyde et al., 2001). Our study supports a close interaction between these two systems since, as seen with RA, a treatment with estrogen was able to suppress TCDD-induced memory impairments (Brouillette & Quirion, Submitted).

8.2. Source and noxious effects associated with exposure to dioxin

Although the exact mechanism of action of dioxin is not yet known, there is wealth of information on the toxicity of this compound. According to the World

Health Organization, TCDD is a highly noxious agent present in the air, water and soil mainly due to pollution generated in industrial countries from large-scale burning of municipal and medical wastes, fuel and wood burning, tobacco smoke, production of iron and steel, bleaching process of paper pulp, coal-fired electrical power generation and pesticides. Natural disasters such as forest fires and volcanic eruptions can also be a source of TCDD. This prototypical member of the halogenated aromatic hydrocarbons (HAHs) can also move into food chain and contaminate meat, milk products (breast milk) as well as fish and to lower levels fruits, vegetables and grains. Dioxin can remain stored in body fat for long periods of time and thus can accumulate in the organism. Its average half-life is 7 years and 9 months in adult humans and 20.8 days in Long-Evans rats (Pohjanvirta et al., 1990). The acute toxicity of TCDD in humans was suggested to be in the range of 70-300 µg/kg, and 10 pg/kg/day represents a safe lifetime exposure level (Rozman, 1989). The effect of dioxin varies greatly depending on age, sex, species and strain.

Several industrial accidents have exposed many individuals to high levels of TCDD and other HAHs including polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs). Extensive toxicology studies have established that TCDD and structurally similar congeners exert most of their effects by binding to nuclear AhRs, which in turn increase or decrease the expression of AhR-regulated genes (Schmidt & Bradfield, 1996). TCDD is the most toxic compound among the HAHs and produces various effects such as weight loss, thymic atrophy, dermal disorders, hepatotoxicity, carcinogenicity, teratogenicity, reproductive toxicity, immunotoxicity, and endocrine toxicity (Safe, 1990). Neurodevelopmental and cognitive deficits were observed in Japanese and Taiwanese children exposed to HAHs *in utero* or during breast-feeding (Kuratsune et al., 1971; Rogan et al., 1988). In Michigan, children born to mothers who were regular consumers of PCB-contaminated fish from the Great Lakes showed psychomotor and cognitive impairments that persisted through school age (Jacobson et al., 1990; Jacobson & Jacobson, 1996). Since TCDD is a prototypical member of the toxic contaminants HAHs, mechanisms underlying

dioxin-induced memory deficits could lead to potential novel targets to prevent noxious effects of pollutants on cognitive processing under normal and pathological conditions.

9. Role of retinoic acid signalling pathways in AD

9.1. Phenotypes and hallmarks of AD

AD is an age-related neurodegenerative disorder that currently affects nearly 2% of the population and one in three individuals over 85 years old in industrialized countries (Lautenschlager et al., 1996). AD represents the most common cause of dementia among people over 65 and is the eighth leading cause of death in the United States (Hebert et al., 2003). Prevalence studies have estimated that 25 million people worldwide had AD in 2000 (Wimo et al., 2006). The National Institute on Aging indicates that 4.5 million people in the U.S. alone are suffering from this devastating illness with current direct and indirect care costs estimated at least \$100 billion, annually. As the population continues to age, baby boomers reaching retirement and beyond, with the proportion of aged individuals in countries such as Japan expected to almost double over the next 50 years, the number of AD patients worldwide is expected to increase to 114 million by 2050 if new therapies do not emerge (Wimo et al., 2003).

AD is a dementia that primarily impairs episodic memory (Welsh et al., 1992; Artero et al., 2003) and as the disease progresses, other cognitive deficits manifest, particularly in attention and executive functions, semantic memory, language and spatial orientation (Perry & Hodges, 1999; Lambon Ralph et al., 2003). Neuronal and synaptic losses along with pathology of cholinergic basal forebrain neurons are considered the best correlates for memory decline in AD (Terry et al., 1991; Samuel et al., 1994). The severity of AD was shown to correlate with decreased choline acetyltransferase (ChAT) activity and reduced number of cholinergic neurons (Perry et al., 1978; Coyle et al., 1983; Etienne et al., 1986; Lehericy et al., 1993; but also see Perry et al., 1982).

An early event in AD is the neuronal loss that begins in the entorhinal cortex and extends to the hippocampus and eventually to the temporal cortex. The most vulnerable circuit is the medial perforant pathway that originates in the entorhinal cortex and terminates in the outer molecular layer of the dentate gyrus (Hyman et al., 1984; Hyman et al., 1986). Very mild cognitively impaired individuals have already lost 50% neurons in the entorhinal cortex, while in severe AD cases up to 90% neurons are lost in this brain region (Gomez-Isla et al., 1996). In contrast, elderly individuals with no cognitive impairments display no neuronal loss in the entorhinal cortex (Gomez-Isla et al., 1996). The CA1 hippocampal field, which is downstream to the input from the entorhinal cortex to the dentate gyrus, is also severely affected in brain aging and in AD (Braak & Braak, 1985).

Decreased synaptic density is the pathological feature that most closely correlates with the severity of memory impairment and ensuing dementia. Quantification using electron microscopy or immunohistochemical staining for synaptic markers has reported significant decrease in synaptic density in the association cortices and hippocampus of AD brain (Dekosky & Scheff, 1990; Terry et al., 1991). Moreover, the decrease in synapse number and density seem disproportionate to the loss of neuronal cell bodies (Davies et al., 1987; Dekosky & Scheff, 1990), suggesting that pruning of synaptic endings may precede the demise of the neuron in the disease process. Furthermore, some changes in synaptic markers in the brains of AD patients and transgenic mice model of AD suggest that synaptic function is compromised prior to the physical degeneration of the synapses (Palop et al., 2003; Westphalen et al., 2003).

The severe neurodegeneration observed in the entorhinal cortex in early AD is detected in the absence of significant amounts of extracellular plaque deposition and neurofibrillary tangles (NFTs), two other typical neuropathological hallmarks of the disease (Jellinger, 1998). Plaques have at their core amyloid- β ($A\beta$) peptides produced by the proteolytic cleavage of the transmembrane amyloid precursor protein (APP) by β - and γ -secretases (Haass, 2004). $A\beta$ is a normal soluble component of serum or CSF that was shown recently to be up-regulated

during neuronal and synaptic activity (Cirrito et al., 2005). In AD brains, $A\beta_{42}$ species, and to a lesser extent $A\beta_{40}$, tend to accumulate and form insoluble senile plaques. However, because amyloid deposits are also observed in some cognitively intact aged individuals, plaques are known to correlate poorly with cognitive status (Giannakopoulos et al., 2003).

NFTs consist mostly of a highly polymerized hyperphosphorylated form of microtubule-associated tau proteins (Masters et al., 1985). Some reports have shown that hyperphosphorylation induces intracellular aggregation and detachment of tau from microtubules that perturb axonal transport of synaptic vesicles and mitochondria, resulting in neuronal degeneration (Praprotnik et al., 1996; Terry, 1996). The presence of NFTs correlates better than $A\beta$ deposits with cognitive declines in AD but to a lesser degree than neuronal and synaptic losses (Gold et al., 2001). In most instances, the definitive diagnosis of AD can only be established by examining those neuropathological hallmarks at autopsy.

The most robust risk factors for AD are age and a positive family history (George-Hyslop, 2000). Genetic screens of families in which the disease occurs at an elevated frequency, familial AD (FAD), have revealed autosomal dominant mutations in three distinct genes: APP (Goate et al., 1991), presenilin 1 (PS1) (Campion et al., 1995; Sherrington et al., 1995) and presenilin 2 (PS2) (Sherrington et al., 1996). Mutations in APP and PS1 genes are the most frequent and penetrating (i.e. most carriers of the mutations develop AD before the age of 60), whereas PS2 mutations are rare and not fully penetrating. However, 99.5% of AD patients do not have mutations in any one of these three genes and FAD accounts for only about five percent of the total number of AD cases (Davies, 2000).

The vast majority of AD cases are idiopathic and are characterized by late onset (> 60 years of age). Even if the initiating events of the late onset AD are unknown, some genes have been identified that are associated with inherited susceptibility to sporadic AD. Among these factors are the ApoE4 allele (Rebeck et al., 1993; Poirier et al., 1993), alpha 2 macroglobulin (Scacchi et al., 2002) and interleukin-1 (Du et al., 2000). There is also evidence that statins could lower the

progression of AD by reducing the accumulation of cholesterol (Crisby et al., 2002). Microarray studies of AD hippocampi (Ginsberg et al., 2000; Colangelo et al., 2002; Blalock et al., 2004), entorhinal cortex and superior temporal gyrus (Pasinetti, 2001), frontal cortex (Yao et al., 2003), amygdala and cingulate cortex (Loring et al., 2001), and nucleus basalis (Mufson et al., 2002) have revealed possible novel target genes involved in various cellular phenomena such as apoptosis, metal ion homeostasis, inflammation, synaptic plasticity and neurotrophic support, among others. However, although a number of potentially important genes and processes have been identified, some methodological variations (including post-mortem conditions, subjects, statistics and platforms) across studies do not allow for consistent overview of the multiple cellular pathways likely to be affected in AD.

9.2. The amyloid hypothesis of AD

The amyloid cascade hypothesis suggests that accumulation of A β in the brain is central to the pathogenesis of AD (Hardy & Higgins, 1992; Selkoe, 2000). Initially, this theory proposed that senile plaques themselves were causing the disease. However, since some cognitively normal elderly can also have A β deposition and that senile plaques are observed long after marked neuronal loss in AD, the emphasis is now more on soluble oligomeric forms of A β (Hardy, 2006; Cole, 2006) (Fig. II.8).

Accordingly, toxic candidate A β species, which still needs to be identified, are suspected to arise at the very early stage of AD and initiate neuronal loss and memory deficits. Recently, extracellular accumulation of a 56 kDa soluble A β assembly (termed A β *56) was shown to correlate with the development of memory deficits but without producing any neuronal loss in an animal model of AD (Tg2576 mice) that expresses human APP containing the Indiana mutation (Lesne et al., 2006). Mechanisms by which toxic A β oligomers impair synaptic function apparently involve dysregulation of intracellular calcium signalling cascade (Mattson et al., 1992; LaFerla, 2002) and NMDA receptors (Snyder et al., 2005; Tanzi, 2005).

Interestingly, while the human correlate of A β *56 has yet be identified, intraneuronal A β has also been proposed to disrupt memory in a triple-transgenic model of AD (3xTg-AD) which expresses APP, PS1 and tau variants (Billings et al., 2005). However, if intraneuronal A β arises at the same time as cognitive deficits, its levels have been shown to decline with the progression of AD in both human and animal models (Cole, 2006). Moreover, contrary to expectation, the large amounts of amyloid deposits seen in various

transgenic mice models of AD have globally failed to produce significant neuronal losses, gross brain atrophy as well as NFTs unless when APP, tau and presenilin mutations are combined (Lee et al., 2007). Taken together, these results indicate that accumulation of A β is an important neuropathological hallmark of AD but may not represent the only causative event in most late onset sporadic cases.

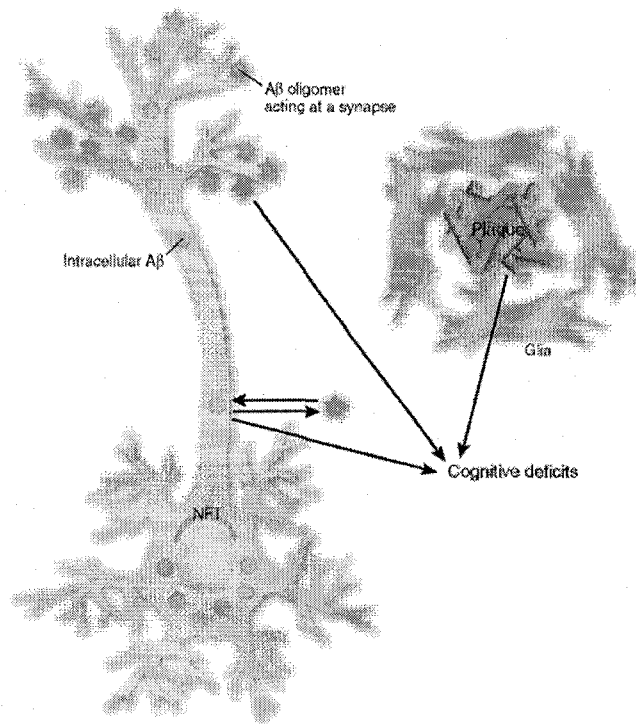


Fig. II.8. The amyloid cascade hypothesis of AD. A β accumulates as extracellular insoluble fibrils in plaques surrounded by glia. A β also aggregates within neurons or as soluble extracellular oligomers that bind to specific neuronal surface sites such as synapses. Unidentified plasma membrane receptors have been proposed to internalize extracellular A β species. The amyloid hypothesis suggests that intra- and/or extra-accumulation of A β may cause early cognitive deficits. Adapted from Cole (2006).

9.3. Disruption of retinoic acid homeostasis in AD

A small growing body of evidence suggest that the retinoid signalling pathway may be implicated in the development of late onset AD. The essential role of RA in memory functions as well as in synaptogenesis, differentiation and survival of newborn cells throughout life in brain regions affected severely and early in AD, such as the hippocampus, suggests a role for this pathway in the etiology of AD. Moreover, several genes involved in amyloid plaque processing (including PS1, PS2, ApoE, APP, ADAM10 and GAP43), A β degradation (insulin degrading enzyme), NFTs formation (tau) and memory loss (AChE, ChAT) observed in AD are regulated by retinoid signalling pathways (see Table II.2). Furthermore, retinol was shown to regulate CREB, a transcription factor known to be altered in AD (Tong et al., 2001; Palop et al., 2003), and dose-dependently inhibit the formation and extension of fibrillar A β (Ono et al., 2004).

Since age is the most robust risk factor for AD, it is likely that components affected in this disease are part of signalling pathways closely associated with brain aging. In the developing brain, two peaks of RA synthesis are observed perinatally in the choroid plexus to progressively decrease to reach stable levels in healthy individuals (Yamamoto et al., 1996; Yamamoto et al., 1999). As RA levels decrease in elderly compared to young individuals, perturbation in RA homeostasis may have a key impact on the aged brain especially during increased RA demand following cognitive challenges. Changes in the retinoid signalling pathway were shown to induce memory impairments in aged rats and mice, and RA treatments are able to restore cognitive capacities in both species (Etchamendy et al., 2001; Brouillette & Quirion, 2007).

Some studies have reported decreases in serum retinol levels in AD patients when compared to age-matched controls (Jimenez-Jimenez et al., 1999; Bourdel-Marchasson et al., 2001; Rinaldi et al., 2003). Hypo-functioning of the retinoid pathway induced by a dietary deficiency of vitamin A has also been shown to induce memory impairments and A β deposition as well as decreased expression of RAR α and ChAT in adult rat brain (Cocco et al., 2002; Corcoran et al., 2004).

Another report indicated that vitamin A deprived rats have altered levels of proteins involved in amyloidogenic processing and that subsequent administration of RA restores expression of RAR β , APP, β -secretase and APP carboxy-terminal fragment (Husson et al., 2006). Furthermore, the hypo-functioning of retinoid pathways produced by dysfunction of retinoid transport has been reported in late onset AD (Goodman & Pardee, 2003; Goodman, 2006).

9.4. Role of transthyretin in AD

TTR may be of special interest in the pathogenesis of AD since this retinol and thyroid hormone transporter has also been reported to rapidly form complexes with A β (Schwarzman et al., 1994) (Fig. II.9). While an earlier report has shown that the formation of amyloid fibrils is inhibited in the presence of CSF (Wisniewski et al., 1993), subsequent studies have identified TTR as the major A β binding protein in the CSF (Schwarzman et al., 1994; Carro et al., 2002). The sequestration of A β by TTR is suggested to regulate the biological activity

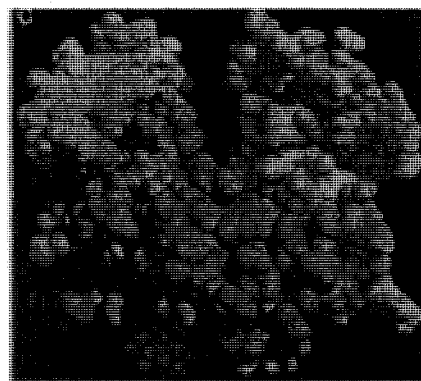


Fig. II.9. Computer graphic model of A β binding to TTR. Two A β molecules (orange) complex as a space filling model with monomers (blue and green) of TTR dimer. Adapted from Schwarzman et al. (1994).

of A β and to play a role in the transport and clearance of the peptide from the CNS (Schwarzman et al., 1994). Interestingly, TTR was shown to bind with higher affinity to the more amyloidogenic A β ₄₂ species (Tsuzuki et al., 2000), and thus decreased TTR gene expression may have a particular impact on the accumulation of A β ₄₂ as observed in AD.

Early studies have shown that AD patients have lower levels of CSF TTR (Riisøen, 1988; Serot et al., 1997; Merched et al., 1998), whereas higher levels of TTR in human CSF (Schwarzman et al., 1994) and AD mouse models (Carro et al., 2002) prevent A β aggregation and plaque formation. TTR levels have been also shown to negatively correlate with senile plaques formation in human

(Merched et al., 1998). Other reports have shown that in a mouse model of AD (Tg2576), the absence of amyloid plaques in young (6-months old) mice was associated with increased expression of TTR in the hippocampus (Stein & Johnson, 2002). When testing these mice at older age (18-months old), the neutralization of TTR was shown to increase the levels of A β and tau phosphorylation as well as neuronal loss and apoptosis in the CA1 neuronal field (Stein et al., 2004). Another study has reported that a mouse model of AD (ceAPP^{swe}/PS1 Δ E9 mice) housed under memory stimulating conditions shown marked up-regulation of TTR as well as reduction of A β levels and amyloid deposition (Lazarov et al., 2005).

The adequate regulation of TTR gene expression during aging may thus be essential for the clearance of A β and prevention of its accumulation, especially of the toxic fragment A β ₄₂. Moreover, TTR gene expression is also critical to the maintenance of RA homeostasis in order to limit any disturbance in neurogenesis, neural differentiation, synaptogenesis and synaptic plasticity required to sustain adequate memory functioning. Since increased A β production can only explain a small number of FAD cases and does not seem to contribute as much as to late onset AD, ineffective clearance of A β via altered TTR levels should be considered as a possible mechanism leading to A β peptide accumulation.

9.5. Possible role of the pollutant dioxin in AD

Both environmental and genetic factors contribute to the development of late onset AD (Richard & Amouyel, 2001). Twin studies estimate that heritability for AD varies between 60-80%, suggesting that environmental influences also play an important role in the progression and onset of dementia (Gatz et al., 2006). Environmental factors, including socioeconomic status, education, diet, and head injury are strong risk factors for AD especially in individuals having a genetic predisposition (Richard & Amouyel, 2001). Environmental and food toxins have also been suspected as potential risk factors although their impact remains to be fully established. Interestingly, TCDD-induced memory impairments are related to RA and estrogen signalling pathways that are suspected

to be associated with late onset AD (Brouillette & Quirion, Submitted). Indeed, estrogen deficiency has been shown to increase A β deposition (Yue et al., 2005), whereas treatment with this hormone prevents neural tau hyperphosphorylation in an animal model of AD (Alvarez-de-la-Rosa et al., 2005).

Our results showing that female rats are more sensitive to TCDD-induced amnesia is in agreement with the observation that the prevalence of AD is higher in women than men (Baum, 2005). Some epidemiological studies have suggested a protective role for estrogen replacement therapy against AD in post-menopausal women (Brinton, 2004) whereas others have reported no benefits or even deleterious effects (Shumaker et al., 2003). Therefore, it would be of interest to investigate if exposure to pollutants such as TCDD may, at least partly, explain these apparent discrepancies. The deleterious effect of TCDD on the major TTR-mediated transport route of retinol shown in our study (Brouillette & Quirion, Submitted) is also in agreement with defective retinoid transport reported in some AD patients (Goodman & Pardee, 2003) supporting a role for TTR deficits in AD.

9.6. Current treatments for AD

At the moment there is no cure for AD and treatments provide modest symptomatic benefits to some individuals with AD (Gauthier et al., 2003; Lleo et al., 2006). Only two types of drugs have been approved thus far for the treatment of AD. The findings of deficits in cholinergic neurotransmission in AD led to the development of cholinesterase inhibitors as the first approved treatment for AD's dementia symptoms (for review see Bartus et al., 1982; Cummings & Kaufer, 1996; Francis et al., 1999; Gauthier, 2006). The general mechanism of action for this class of agents (including donepezil, rivastigmine and galantamine) is to increase the availability of ACh by preventing its degradation through the inhibition of the catabolic enzyme acetylcholinesterase (for review see Giacobini, 2004).

Another approach to the treatment of AD is to inhibit glutamatergic neurotransmission. In 2004, the Food and Drug Administration (FDA) of the USA approved memantine, a non-competitive NMDA receptor antagonist, for treating

dementia symptoms in moderate to severe AD cases (Gauthier et al., 2006). Pharmacological studies suggested that memantine prevents excitotoxicity due to the high entry of extracellular calcium via NMDA receptors causing neuronal dysfunctions and death (Michaelis, 1998). Since memantine has low- to moderate-affinity for NMDA receptors, this drug leaves the calcium channel relatively open for neurotransmission at a low stimulation rate necessary for LTP and memory processes (Lipton, 2004).

Among other treatments that appear promising yet remain to prove their effectiveness are other cholinergic agents such as M1 muscarinic receptor agonists, antioxidants, nonsteroidal anti-inflammatory drugs, estrogen replacement therapy, anti-amyloid immunotherapy, statin treatments and neurotrophic agents (Gauthier, 2002; Koch et al., 2005; Lleo et al., 2006). Since extensive synaptic and neuronal loss is an early event in AD and is so far the best correlate of cognitive declines associated with the illness, one may predict that the identification of the primary events responsible for the neurodegeneration seen in AD will lead to the development of more effective therapies.

10. The scopolamine-induced amnesia model

Among the first animal models of AD were those attempting to mimic the deficits in cholinergic neurotransmission observed in the disease. Scopolamine treated rats are commonly used as a psychopharmacological model of memory dysfunctions (Lamberty & Gower, 1991; Nakajima et al., 1994) and have been extensively studied to establish the effectiveness of acetylcholinesterase inhibitors in the treatment of AD (Wu et al., 2003; van der Staay & Bouger, 2005). Scopolamine is a non-subtype selective muscarinic acetylcholine receptor antagonist that induces memory impairments in young subjects similar to those occurring during aging (Drachman & Leavitt, 1974; Weingartner, 1985), and its amnesic effect has been shown to increase with age (Ray et al., 1992b; Stemmelin et al., 1999).

The cholinergic system is involved in the acquisition of information during new learning (Deutsch, 1971; Jerusalinsky et al., 1997; Herrera-Morales et al., 2007) as well as in attention (Sarter & Bruno, 1997) (Fig. II.10). As discussed above, accumulating evidence suggests that an important factor in memory impairments associated with aging and AD relates to deficits in cholinergic neurotransmission (Davies & Maloney, 1976; Whitehouse et al., 1981; Bartus et al., 1982; Aubert et al., 1995; Levey, 1996; Araujo et al., 2005). Moreover, cholinergic muscarinic receptors are known to be essential in facilitating learning and memory functions (Quirion et al., 1995; van der Zee & Luiten, 1999; Rowe et al., 2003). Scopolamine-induced amnesia was shown to be linked to a decrease in central cholinergic neuronal activity following the blockade of various muscarinic receptors (Carli et al., 1997; Schon et al., 2005).

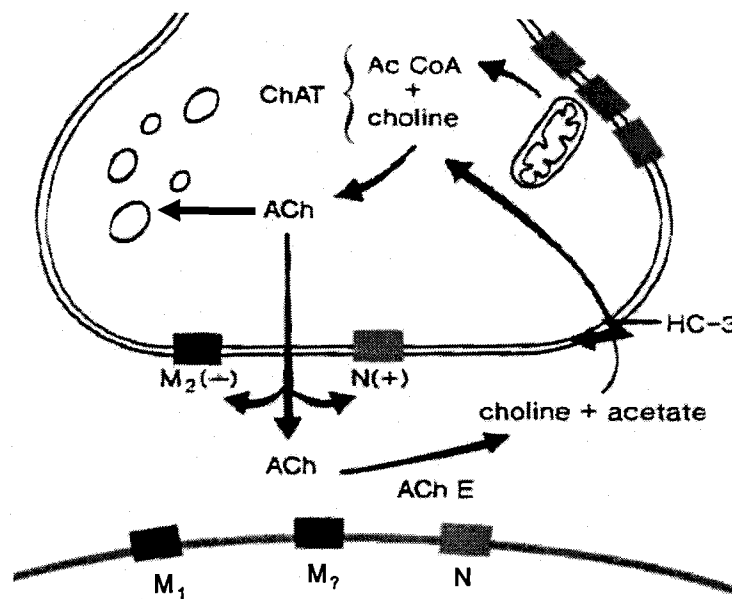


Fig. II.10. A model cholinergic synapse. *AcCoA and choline generate ACh in the cytoplasm via ChAT; ACh is then accumulated into synaptic vesicles. After exocytosis and binding to nicotinic (N) or muscarinic (M1, M2) receptors, ACh is rapidly degraded by AChE and the choline group retrieved by a Na⁺-coupled re-uptake pathway. Abbreviations: AcCoA, acetyl-CoA; ACh, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; HC-3, hemicholinium-3; M, muscarinic receptors; N, nicotinic receptors.*

Several studies have shown that systemic administration of scopolamine before acquisition, but not retrieval, impairs performance in several cognitive tasks in rodents (Decker et al., 1990; Devan et al., 2004; Janas et al., 2005), monkeys (Aigner et al., 1991; Tang et al., 1997) and humans (Atri et al., 2004; Schon et al., 2005). In the acquisition memory process of the MWM task, scopolamine is known to increase the escape latency to find the hidden platform (Nakajima et al., 1994; Chen et al., 2002; Janas et al., 2005).

10.1. Influence of nicotinic receptors on cholinergic, glutamatergic and GABAergic neurotransmission during memory processes

Several studies in humans and experimental animals have shown that nicotinic receptors are involved in diverse cognitive functions such as working memory, learning and attention (Levin et al., 2006). Neuronal nicotinic ACh receptors (nAChRs) are pentameric transmembrane ligand-gated ion channel receptors that produce excitatory synaptic transmission by allowing entry of cation (Na^+ and Ca^{2+}) into neurons (Seguela et al., 1993). Once ACh is released from the presynaptic terminals, one molecule of ACh binds to the α subunit to mediate fast depolarizing inward currents. The $\alpha 4\beta 2$ and $\alpha 7$ homo-oligomeric channels represent the major nAChR subtypes found in the brain (Changeux, 1990). Some studies have shown that nicotinic autoreceptors found on cholinergic terminals increase hippocampal ACh release (Araujo et al., 1988; Wilkie et al., 1996). Since ACh release was only slightly affected by selective nicotinic $\alpha 7$ subunit antagonist (Wilkie et al., 1996), the authors suggest that the nAChR mediating positive feedback of ACh release does not contain $\alpha 7$ subunit, but would be more likely of the $\alpha 3\beta 2$ or $\alpha 3\beta 4$ nicotinic receptor sub-types.

The cholinergic system, along with other neurotransmitters including glutamatergic and GABAergic systems, is integrated into the neural circuitry of the hippocampus to modulate learning and memory functions (White & McDonald, 2002). Considerable anatomical, electrophysiological and neurochemical evidence suggest close interactions between these three systems (Vizi & Kiss, 1998). The nAChRs containing $\alpha 7$ subunit expressed in mossy fiber

terminals have been shown to enhance glutamatergic transmission in the hippocampus (Gray et al., 1996). Moreover, an electrophysiological study reported that both nAChR $\alpha 4\beta 2$ and $\alpha 7$ present on GABAergic interneurons increase release of this inhibitory neurotransmitter, which in turn reduces post-synaptic current recorded from pyramidal cells (Alkondon et al., 1997).

In general, nicotinic agonists have been shown to improve certain forms of memory, whereas nicotinic antagonists and cholinergic lesions were reported to impair memory (Picciotto et al., 1995; Jones et al., 1999). As an example, local infusions of the $\alpha 7$ nicotinic receptor antagonist methyllycaconitine or the $\beta 2$ nicotinic receptor antagonist dihydro- β -erythroidine into the basolateral amygdala or hippocampus impair working memory of rats in the radial-arm maze (Levin et al., 1996; Levin et al., 2002; Nott & Levin, 2006). In animal studies, acute and chronic nicotine administration improve working memory, and nicotinic agonists were found to improve learning and memory in humans and non-human primates (Levin et al., 2006). Nicotinic agonists have been observed to facilitate memory in aged rats and monkeys (Buccafusco & Jackson, 1991; Arendash et al., 1995; Levin & Torry, 1996), although another study reported that chronic nicotine did not improve working memory in aged rats (Attaway et al., 1999). In addition, the stimulation of nicotinic receptors by nicotine has been shown to provide some improvement of attentional functions in both animals and humans (Mancuso et al., 1999; Stolerman et al., 2000; Rezvani & Levin, 2003; Kumari et al., 2003). Finally, although positive results were obtained early on following nicotine treatment in AD (for review see Newhouse et al., 1997), these results failed to be confirmed in larger studies (for review see Jones, 2003).

10.2. Influence of muscarinic receptors on cholinergic, glutamatergic and GABAergic neurotransmission during memory processes

Muscarinic receptors located in the hippocampus are of particular relevance for learning and memory processes (Bartus et al., 1982; Quirion et al., 1995; Sarter & Bruno, 1997; van der Zee & Luiten, 1999). Mounting evidence suggest that muscarinic receptors interact with other neurotransmitters such as

glutamatergic and GABAergic systems found in the dorsal hippocampus to modulate cognitive functions (Vizi & Kiss, 1998). M1 receptors are the most abundant muscarinic subtype receptors in the hippocampus (Table II.3), and is especially enriched in hippocampal CA1 area (Levey et al., 1995). These receptors were shown to play a key role in cognitive processes that require interactions between the hippocampus and cortex (Anagnostaras et al., 2003).

Table II.3. Localization of muscarinic ACh receptor subtypes in the brain

Molecular subtype	Regional abundance	Cellular localization	Synaptic localization
M1	Abundant in forebrain (neocortex, hippocampus, neostriatum)	Pyramidal neurons Striatal spiny neurons	Post- » Pre-synaptic
M2	Moderately abundant throughout brain	Cholinergic neurons, nonpyramidal neurons in cortex and hippocampus	Pre- » Post-synaptic
M3	Low levels throughout brain	Neuronal	Unknown
M4	Abundant in neostriatum, moderate levels in hippocampus and cortex	Striatal spiny neurons Associational and commissural hippocampal projections	Pre- and Post-synaptic
M5	Low levels in hippocampus, substantia nigra	Pyramidal neurons, substantia nigra pars compacta, microglia	Unknown

Adapted from Levey (1996).

Neurochemical data suggest that autoinhibition of ACh release in the hippocampus is mediated mainly via pre-synaptic M2 autoreceptors, and to a lesser extent M4 receptors (McKinney et al., 1993; Quirion et al., 1995; Vannucchi & Pepeu, 1995; Stillman et al., 1996; Kitaichi et al., 1999a; Kitaichi et al., 1999b). The inhibitory M2 and M4 receptors are also found on the commissural/associational glutamatergic and GABAergic pathways, whereas M1 and M3 receptors are expressed post-synaptically on glutamatergic neurons (Rouse & Levey, 1997). Muscarinic receptors seem to have a dual effect on inhibition induced by GABA. Indeed, enhancement of GABA-mediated IPSPs was suggested to be produced by M1 receptors located on GABAergic interneurons, whereas suppression of IPSPs is mediated by activation of pre-

synaptic M2 heteroreceptors (Ben Ari et al., 1981; Pitler & Alger, 1992; Behrends & ten Bruggencate, 1993).

Several studies have suggested that ACh can potentiate NMDA responses by activating post-synaptic M1 receptors (Harvey et al., 1993; Marino et al., 1998), but can also inhibit hippocampal glutamate release via the activation of M2 receptors (Marchi & Raiteri, 1989; Herrera et al., 1993). Another study has shown that decreased synaptic plasticity observed in M2 muscarinic receptor knock-out mice is attributed to increased strength of GABAergic inhibition of CA1 pyramidal neurons (Seeger et al., 2004). In agreement with these findings, our recent study has shown that scopolamine affected the expression of genes associated with various neurotransmitter systems besides the cholinergic one in the dorsal hippocampus (Brouillette et al., 2007). Indeed, we observed that GABA_B receptors and the glutamatergic component Homer1 are critical in the cascade of genes involved in scopolamine-induced amnesia (see Chapter V for details).

10.2.1. The GABA_B receptors

Neuroanatomical data have shown that virtually all the interneurons in the hippocampus are GABAergic and make synaptic contacts predominantly with glutamatergic principal cells, although a subgroup solely innervate other interneurons (Freund & Buzsaki, 1996; Acsady et al., 1996; Gulyas et al., 1996). In addition to those intrinsic connections, extrahippocampal GABAergic cells originating from the septum also innervate a broad array of hippocampal interneurons (Freund & Antal, 1988; Gulyas et al., 1990; Acsady et al., 1993). Moreover, some hippocampal GABAergic neurons send efferents to the medial septum and the vertical limb of the diagonal band of Broca (Alonso & Kohler, 1982; Toth et al., 1993), while others send commissural projections to the contralateral hippocampus (Ribak et al., 1986).

The release of GABA from these neurons inhibits neurotransmission by acting on two major GABA receptor subtypes: GABA_A and GABA_B. GABA_A receptors are transmembrane ligand-gated ion channel receptors that induce post-synaptic inhibition by causing an entry of chloride anions (Cl⁻) to compensate the

influx of sodium cations (Na^+) in stimulated neurons. GABA_B receptors are G-protein coupled receptors that mostly, but not exclusively, induce pre-synaptic inhibition by decreasing neurotransmitter release after blocking the entry of calcium (Ca^{2+}) and opening potassium (K^+) channels (Newberry & Nicoll, 1984). GABA_B autoreceptors expressed on GABAergic terminals in the hippocampus induce a negative feedback by reducing the release of endogenous GABA (Waldmeier et al., 1988; Davies et al., 1991; Khazipov et al., 1995). Hippocampal glutamate release was also shown to be inhibited by GABA_B heteroreceptors located pre-synaptically on glutamatergic terminals (Freund & Buzsaki, 1996).

Since GABAergic interneurons exert the main inhibitory action on principal glutamatergic cells, GABA is suggested to be essential in the modulation of synaptic efficacy and hippocampal-dependant memory capacities (Izquierdo et al., 1993). Several studies using various GABA_A or GABA_B receptor agonists or antagonists have shown alteration of cognitive performance in different behavioural paradigms (Myhrer, 2003). Accordingly, some GABA_B receptor antagonists were developed as cognitive enhancers (Bowery et al., 2002). In that regard, SGS742 is an orally active GABA_B receptor antagonist of particular interest since it is currently in clinical trials in patients suffering from mild cognitive impairments (MCI) and AD (Froestl et al., 2004). This compound was shown to enhance memory capacities of mice, rats and rhesus monkeys in various behavioural tasks (Mondadori et al., 1993; Mondadori et al., 1996; Helm et al., 2005). SGS742 was also observed to antagonize scopolamine-induced memory impairments in the MWM (Nakagawa & Takashima, 1997) and passive avoidance task (Bianchi & Panerai, 1993).

While the levels of GABA_A receptor subunits were shown to be relatively stable in the hippocampus of AD patients (Mizukami et al., 1998), the GABA_B receptor 1 subunit was reported to be increased at the onset of the illness (Iwakiri et al., 2005), as observed following scopolamine treatment (Brouillette et al., 2007). Interestingly, the modulatory effect of muscarinic receptors on GABAergic neurotransmission observed in scopolamine-treated rats was also seen in a mouse model of AD (Zhong et al., 2003).

10.2.2. The glutamatergic component Homer1

We obtained evidence that muscarinic receptors may be implicated in the regulation of the glutamatergic related gene Homer1 (Brouillette et al., 2007) (see also Chapter V for details). Homer1 is part of a family of evolutionarily conserved proteins naturally expressed in the brain (Xiao et al., 1998). Homer1a is generated by a non-classical splicing mechanism from the same transcript as Homer1b and 1c. Homer1a is a unique variant of Homer1 that is temporarily up-regulated following synaptic activity and it lacks the coiled-coil (CC) structure at the carboxy-terminal region which mediates specific self-association (Xiao et al., 1998; Kato et al., 1998). Conversely, Homer1b and 1c are constitutively expressed in the brain and dimerized to mediate the physical association of molecules involved in glutamatergic post-synaptic signalling, including group 1 metabotropic receptors (Brakeman et al., 1997), inositol trisphosphate (Tu et al., 1998) and NMDA receptors via the interaction with Shank proteins (Tu et al., 1999).

Following a synaptic stimulus, Homer1a is transiently up-regulated to compete with Homer1c and disassemble the signalling complex to allow synaptic plasticity associated with LTP and memory processes (Brakeman et al., 1997) (Fig. II.11). Thus, the short-spliced variant Homer1a is suggested to produce glutamatergic synapse rearrangement necessary for neuronal plasticity (Hennou et al., 2003), while the long-spliced variants Homer1b/1c were shown to be important for maintaining dendritic-spine structure and synaptic function (Sala et al., 2003). A fine regulation of the homeostatic balance between Homer1a and 1b/1c thus seems necessary for fully efficient synaptic plasticity required during memory processes.

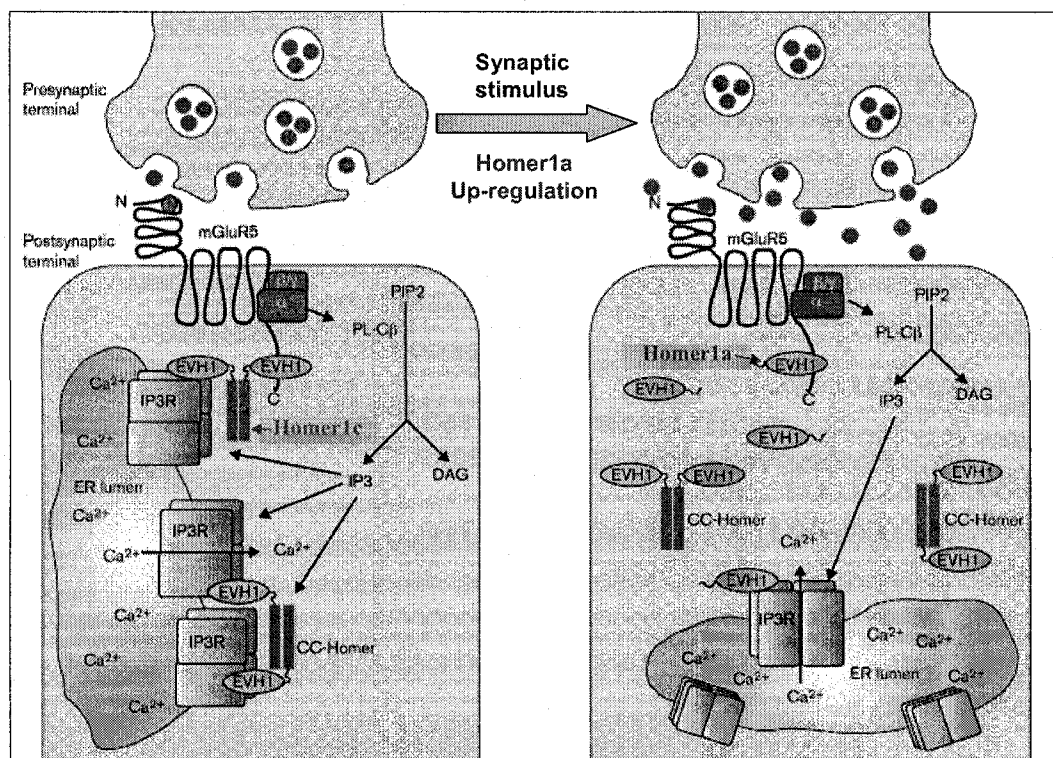


Fig. II.11. Homer1 regulates glutamatergic post-synaptic signalling. Constitutively expressed Homer1c proteins (CC-Homers) are localized at the post-synaptic terminals where they bind the carboxyl terminus of group I mGluRs and IP3Rs. Both receptors are physically coupled via Homer1c to form an efficient signalling complex that generates IP3 and releases Ca^{2+} from intracellular pools. Following synaptic stimulus, Homer1a is transiently up-regulated to disassemble the signalling complex needed for synaptic plasticity. **Abbreviations:** C, carboxyl terminus; DAG, diacylglycerol; EVH, ; Ena/VASP homology 1 domain; ER, endoplasmic reticulum; IP3R, inositol trisphosphate receptor; mGluR, metabolic glutamate receptor; N, amino terminus; PIP2, phosphatidylinositol bisphosphate; PL-C β , phospholipase C β . Adapted from Xiao et al. (2000).

Homer1a was shown to be induced following excitatory synaptic activity and involved in structural changes seen during the maintenance phase of LTP in hippocampal cell culture (Brakeman et al., 1997; Kato et al., 1997). The overexpression of Homer1a induced by transfection of vector in cultured hippocampal neurons has been reported to decrease the density and size of dendritic spines, the number of NMDA receptor clusters, the level of surface AMPA receptors as well as reducing AMPA and NMDA receptor post-synaptic currents (Sala et al., 2003). Conversely, another report has shown that the overexpression of Homer1a increased AMPA receptor function without affecting NMDA receptors and pre-synaptic glutamate release (Hennou et al., 2003). The up-regulation of Homer1a expression induced by BDNF and NMDA receptor stimulation has been reported to be mediated by the MAPK signalling pathway (Sato et al., 2001).

At the behavioural level, Homer1a gene expression has been observed to be increased in the hippocampus and neocortex following exploration of a novel environment (Vazdarjanova et al., 2002). Interestingly, a complete deletion of Homer1 was observed to produce memory deficits in the MWM task (Jaubert et al., 2006). In agreement with these results, our microarray data show increased gene expression of Homer1a in MWM trained versus naive rats, while levels of Homer1c were unaffected by spatial memory stimulation (Brouillette et al., 2007). We also found that a scopolamine treatment disrupts the temporal up-regulation of Homer1a seen in control rats following MWM task, suggesting that muscarinic receptors may be implicated in the regulation of Homer1a (see also Chapter V for details).

The recent approval of memantine for the treatment of AD (see page 43) has revealed the therapeutic potential of drugs targeting the glutamatergic system for the treatment of this disease (Kemp & McKernan, 2002). Interestingly, a microarray analysis recently demonstrated reduced Homer1a gene expression in a mouse model of AD (Dickey et al., 2003).

11. Summary and aims of this thesis

A large body of anatomical, neurochemical, electrophysiological and behavioural data have provided evidence for the existence of interactions between the various neurotransmitter systems expressed in the hippocampus for inducing synaptic plasticity required for learning and memory (Vizi & Kiss, 1998). Despite progress made in elucidating the molecular mechanisms underlying memory (Kandel, 2001; Silva, 2003), the identification of novel molecular and cellular components and their cross-talk to induce functional memory system remains to be fully established.

The major aim of this thesis was thus to investigate global alterations in gene expression in two animal models associated with memory deficits: 1) a cohort of aged Long-Evans rats which can naturally develop memory impairments during aging as seen in humans and 2) Long-Evans rats with memory impairments following systemic administration of scopolamine (a non-selective subtype muscarinic acetylcholine receptor antagonist). Global changes in hippocampal gene expression were investigated following spatial memory stimulation in the MWM task using a microarray approach. Differential expressions of genes of interest were further validated using quantitative real-time RT-PCR approach. Mechanisms underlying the implication of select genes in the two animal models were evaluated using pharmacological methods as well as transgenic and/or knock-out animals.

Among hippocampal genes differentially expressed between AI and AU rats following cognitive challenge, altered levels of TTR were associated with aged memory deficits (Brouillette & Quirion, 2007). Since TTR is a retinol transporter, we also used dioxin to deplete retinol stock and investigate its role in memory processing (Brouillette & Quirion, Submitted). Using the rat scopolamine-induced amnesia model, we observed that component associated with neurotransmitter systems besides the cholinergic one such as Homer1 and GABA_B receptors were differentially expressed (Brouillette et al., 2007). Altogether, the results summarized in this thesis are part of extensive active research investigating memory mechanisms altered during aging that could explain cognitive decline often observed in normal and pathological condition.

Chapter III

Transthyretin: a key gene involved in the maintenance of memory capacities during aging

Preface

We reported in this manuscript the first combined genetic, behavioural and molecular evidence that transthyretin (TTR), a retinol transporter shown to prevent the aggregation of neurotoxic beta-amyloid (A β) peptide, is a central component of the retinoid pathway involved in the maintenance of normal cognitive processes during aging.

In this study, we investigated genes differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) animals following stimulation in a spatial memory task. A major finding is that the decreased expression of the TTR gene following a mnemonic challenge was associated with memory deficits in aged rats. Moreover, the complete deletion of TTR in knock-out mice produced memory deficits during aging. We also reported on a possible mechanism associated with the role of TTR in the maintenance of normal cognitive abilities. The reversal of cognitive deficits by retinoic acid (RA) (which unlike retinol bypasses TTR transport) in TTR knock-out mice and aged rats suggests that the mechanism underlying the effect of TTR is related to its ability to regulate brain retinoid's availability and signalling pathways.

Since TTR is the main A β binding protein in the CSF and higher levels of TTR in human and mouse models prevent A β aggregation and plaque formation, decreased levels of TTR gene expression as reported in our study could result in both 1) defective retinoid transport that may account for memory deficits seen in Alzheimer's disease and 2) decreased sequestration of A β which will induce progressive amyloid plaque formation. Thus, losses of TTR may play a significant role in the progression of Alzheimer's disease.

**Transthyretin: a key gene involved in the maintenance of
memory capacities during aging**

Jonathan Brouillette ^a & Rémi Quirion ^{a,b,*}

Department of Psychiatry ^b and Neurology & Neurosurgery ^a, Douglas Mental
Health University Institute, McGill University, Montréal, Québec, Canada

*** Corresponding author:**

Prof. Rémi Quirion, PhD, OC, FRSC, CQ
Douglas Mental Health University Institute, McGill University
6875 LaSalle Boulevard
Montréal, Québec
H4H 1R3

Tel.: (514) 761-6131 (ext 2934)

Fax: (514) 762-3034

E-mail: remi.quirion@douglas.mcgill.ca

Keywords: aging; hippocampus; gene expression; transthyretin; retinoic acid;
Morris water maze

Abstract

Aging is often associated with decline of memory function. Aged animals, like humans, can naturally develop memory impairments and thus represent a useful model to investigate genes involved in long-term memory formation that are differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) animals following stimulation in a spatial memory task. We found that alterations in hippocampal gene expression of transthyretin (TTR), calcineurin, and NAD(P)H dehydrogenase quinone 2 (NQO2) were associated with memory deficits in aged animals. Decreased TTR gene expression could be attributed at least partially to diminish activity of C/EBP immediate-early gene cascade initiated by CREB since protein levels of C/EBP, a transcription factor regulating both TTR and NQO2 expression, was decreased in AI animals. Memory deficits were also found during aging in mice lacking TTR, a retinol transporter known to prevent amyloid- β aggregation and plaque formation as seen in Alzheimer's disease. Treatment with retinoic acid reversed cognitive deficits in these knock-out mice as well as in aged rats. Our study provides genetic, behavioural and molecular evidence that TTR is involved in the maintenance of normal cognitive processes during aging by acting on the retinoid signalling pathway.

Introduction

Brain aging is a complex process accompanied by molecular modifications that may lead to memory impairments related to disorders ranging from the benign senescent forgetfulness to the memory loss that characterizes Alzheimer's disease (AD) (Mattson & Magnus, 2006). Stable forms of long-term plasticity require the induction of a cascade of genes to produce and maintain structural changes associated with memory formation (Kandel, 2001). Despite progress that has been made in elucidating molecular mechanisms underlying memory formation (Silva, 2003), little is known about molecular events most relevant to memory deficits during brain aging. Global alteration of basal gene expression during the course of aging was recently evaluated in humans (Lu et al., 2004) and animals (Blalock et al., 2003; Verbitsky et al., 2004). However, a genome wide study investigating genes differentially expressed between aged memory-impaired (AI) and aged memory-unimpaired (AU) animals following a behavioural stimulation has yet to be performed.

High-density microarray represents a powerful tool to investigate hippocampal gene expression profiles in aged animals. Aged rats are a well-established natural model to study age-related memory deficits. The performances of aged rats in the reference memory version of the Morris water maze (MWM) task has been used routinely to characterize AI and AU rats when compared to young adult rats (Gage et al., 1989; Frick et al., 1995; Rowe et al., 1998; Stemmelin et al., 2000). Decreased spatial memory ability of aged rats was correlated to defective long-term potentiation (LTP) in the hippocampus (Barnes & McNaughton, 1985; Bach et al., 1999; Tombaugh et al., 2002). The hippocampus, a structure vulnerable in the course of aging (Driscoll & Sutherland, 2005), is essential for the formation and retrieval of spatial memory (Hollup et al., 2001) and is necessary for consolidation or long-term storage (Riedel et al., 1999).

Studies in various behavioural paradigms (Bourtchouladze et al., 1998; Igaz et al., 2002) have suggested that long-term memory formation depends on

two distinct temporal periods requiring gene expression and protein synthesis: shortly and several hours after training. Many immediate-early genes associated with the first phase have already been identified (Lanahan & Worley, 1998) but downstream genes expressed during the second time period have been studied to a lesser extent, especially in the context of aging.

This study combines behavioural, genetic and molecular approaches to investigate possible mechanisms underlying age-related memory deficits. First, we characterized individual memory capacities of young adult and aged animals at behavioural level with the MWM task. Using a microarray approach, we identified several known and novel downstream hippocampal genes related to age-associated memory deficits following stimulation in a spatial memory task. We then focused on genes differentially expressed between AI and AU rats and carried out a functional analysis using knock-out mice lacking transthyretin (TTR), one of the most markedly affected genes between the two groups. A role of TTR, in association with retinol-binding protein, is to transport retinol (vitamin A) from liver storage to target tissues where it can be metabolized into retinoic acid (RA) in response to physiological needs (Monaco, 2000). Some evidence thus far has suggested a role for perturbed RA pathway in age-related memory impairments (Chiang et al., 1998; Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002). However, the function of the retinol transporter TTR in mnemonic processing during aging has yet to be investigated. Our study identified genes altered between AI and AU animals, and provides evidence that TTR is a critical component of the retinoid pathway for the maintenance of memory capacities during aging.

Materials and Methods

Animals

Aged (A) and young adult (Y) male Long-Evans rats were purchased from Charles River Laboratories (St-Constant, QC, Canada). The animals were left undisturbed for a minimum of two months in our facility and behavioural testing of A and Y animals was started at the age of 24-months and 7-months respectively. Aging animals were monitored on a weekly basis by a veterinarian for weight loss, obesity, abscesses, cataracts, sebaceous cysts, tumours, malocclusions. Seventy-four healthiest rats were used in this study. Animals were housed in groups of two per cage and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Wild-type (WT; $n = 12$) and $TTR^{-/-}$ ($n = 18$) mice (F2 generation maintained on a mixed C57BL/6 x 129S background) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Drug administration

Retinoic acid (Sigma, St-Louis, MO) was dissolved in a vehicle solution containing polyethyleneglycol, NaCl (0.9%), and ethanol mixed in a proportion of 70:20:10 by volume. Daily administration of RA (150 $\mu\text{g/kg}$, sc) to 7-months old $TTR^{-/-}$ and WT mice as well as aged and young rats commenced four days prior to behavioural testing and continued until the end of testing in the MWM. An equal volume of vehicle solution was similarly administered to control animals. The treatment duration, time of injection (18:00 hrs), dose and route of administration used here have previously been shown to reverse age-related memory deficits in mice (Etchamendy et al., 2001). We used the same treatment in rats since the previous study was based on results showing differential gene expression of retinoic acid nuclear receptor in the brain of young, adult and aged rats (Enderlin et al., 1997a) suggesting similar drug pharmacokinetics in these two species.

Morris water maze task

Animals were required to find, in a 1.4 m diameter pool, a submerged platform (14 cm in diameter) located 1 cm below the surface of water (24°C), rendered opaque by the addition of skim milk powder. Animals were pseudo-randomly started from a different position at each trial and used distal visuo-spatial cues to find the hidden-platform that remained in the centre of the same quadrant throughout all training days (Morris, 1984). Animals were given three trials of 90 s per day with a 45 min inter-trial interval over five consecutive days. If the platform was not located within 90 s, animals were guided to it and remained there for 10 s before removal. One probe trial of 60 s was given 24 h after the acquisition phase on day 5 to a subset of six AI, AU, and Y rats or immediately after the end of the training to WT and $TTR^{-/-}$ mice to evaluate the number of times the animals crossed the previous location of the platform, time spent in the target quadrant, swimming speed, and swim pattern. The AI, AU, and Y rats chosen randomly for the probe trial were not the same as those used for microarray analysis since both the probe and hidden-platform trials are hippocampus-dependent and may interfere with gene expression. After each trial, animals were immediately placed under heat lamps to dry and prevent hypothermia. Five aged rats with a swimming speed lower than 16 cm/s on training day 3-5 were considered to have locomotor deficits and were eliminated from the study.

Aged animals ($n = 26$) falling between 0.5 and 2.0 standard deviations of the performance of Y rats were not used in any further tests. The experimenter was blind to mouse genotype when comparing $TTR^{-/-}$ and WT littermates (males, 3- and 7-months old). To control for possible effects due to circadian cycles, all trials were performed at approximately the same time of the day, between 11-15 h. Statistical significance of each test was assessed using the appropriate ANOVA design and simple main effects were analysed with F-test when the interaction was significant. Tukey's Honestly Significant Difference test was used as *post hoc* pair wise comparison test.

Microarray analysis

Coronal section (2 mm) used for microarray analysis were taken from the rostral end of the dorsal hippocampus (with choroid plexus) up to the tip of the ventral hippocampus. These coordinates are known to include all regions of the dorsal hippocampus proper (dentate gyrus, CA1, CA3). The dorsal hippocampus was dissected from the brain immediately after decapitation and stored at -80°C in RNAlater stabilization reagent until use. The frozen tissue was ground to be characterized for the different molecular analyses (microarray, real-time RT-PCR and western blot). Total RNA was isolated following the Qiagen's RNA isolation protocol (RNeasy Mini kit). The RNA quality and concentration were assessed with Agilent Technologies 2100 Bioanalyser. Quadruplicates of Affymetrix GeneChip® Rat Genome 230 2.0 array were used for each group. The microarray primary data have been deposited, using MIAMExpress, in the ArrayExpress database with the accession number E-MEXP-553. The raw intensity reading of each individual target on the Affymetrix microarray was processed and normalized by using the log₂ scale RMA method. Since RMA methods give less pronounced FC magnitude compared to Affymetrix Microarray Suite 5.0 (MAS5) analysis default program, a minimal FC of 1.5 is suggested as exclusion criteria to select genes and avoid false negatives (Irizarry et al., 2003a). Minus vs Average (MvA) plots were performed to control inter-animal variation within and between groups.

Quantitative real-time RT-PCR analysis

Two-step quantitative real-time RT-PCR was performed to confirm microarray results according to Applied Biosystems guidelines. Reverse transcription of total RNA was carried out with TaqMan™ Reagents and cDNA generated was quantified with the SYBR® Green PCR Core Reagents using the GeneAmp® 5700 Sequence Detection System (40 cycles). The endogenous control β -actin was used to normalize quantification of mRNA target, and non-specific amplifications were verified by dissociation curve. All the reactions were performed in triplicate. Gene-specific primer sequences used in this study are

shown in Table 3, which is published as supplemental material on the web site of Neurobiology of Aging.

Western blot analysis

Extracts from rat hippocampi were obtained by polytron homogenization in cold RIPA lysis buffer with protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Total protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of total protein corresponding to 30 μ g/lane were loaded on denaturing NuPAGE[®] Novex 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to Hybond-C membranes (Amersham Biosciences, UK) by electroblotting. Membranes were pretreated with 5% milk blotting buffer and then incubated with anti-C/EBP α (1/1000, ABR, Golden, CO), anti-C/EBP β (1/2000, ABR), anti-calcineurin α (1/20000, Sigma), or anti-actin (1/1000, Santa Cruz Biotechnology) antibody in 2% BSA solution overnight at 4°C. The membranes were then washed, treated with a secondary HRP-labeled anti-rabbit (1/5000) or anti-mouse (1/2000) antibody for 1 h, washed again and incubated with the Western Lightning Chemiluminescence Reagent *Plus* (PerkinElmer, Boston, MA). Membranes were exposed to Kodak BioMax MS film (Amersham Biosciences), and quantitative densitometric analysis was done using MCID image system. Statistical data were analyzed with one-way ANOVA followed by Tukey's Honestly Significant Difference test.

Results

Long-term spatial memory deficits are observed in a subpopulation of aged rats

Aged (A) animals (24-months old) were characterized as aged memory-impaired (AI) and aged memory-unimpaired (AU) rats when compared to young adult (Y) rats (7-months old) in the reference memory version of the Morris water maze, a hippocampus-dependant behavioural task (Morris, 1984). Over training days 3-5, aged Long-Evans rats ($n = 60$) with average escape latencies that were greater than 2 or less than 0.5 standard deviations from the mean of the young group were considered to be AI or AU rats respectively (Fig. 1a). A longer escape latency to find the hidden escape platform that was in a fixed location over all training days is considered to reflect long-term spatial memory deficits. The longer escape latency of AI rats is not attributed to slower swimming speed since the three groups show no significant difference in that regard (Y: 22.2 ± 0.9 s; AU: 21.2 ± 0.9 s; AI: 21.5 ± 0.9 s; mean $\pm$ SEM; $n = 6$; $p > 0.05$; one-way ANOVA). Amongst the animals tested, 38 % were designated as AI ($n = 23$) and 18 % as AU ($n = 11$).

Memory impairments of AI rats were validated the day after the end of training by performing a probe trial of 60 s in which the platform was removed. AI animals, unlike AU and Y rats, showed no preference for the quadrant that contained the platform (Fig. 1b) and fewer platform crossings (Fig. 1c). This trial was followed by a 60 s cued trial in which the platform was visible and AI rats show similar escape latency as AU and Y animals (6.5 ± 1.2 s; 6.6 ± 1.1 s; 6.3 ± 1.0 s respectively; mean $\pm$ SEM; $n = 6$; $p > 0.05$; one-way ANOVA), indicating the absence of sensorimotor or motivational deficits to escape from water. The extent to which animals swam close to the wall of the pool (thigmotaxis) is thought to be a measure of anxiety for rodents in the MWM task (Hodges, 1996). Divergence in escape latency is unlikely to be attributed to variation in the level of anxiety to escape since the percentage of the total time swam close to the pool wall (zone A) is not significantly different in rats that take much longer to escape

versus those that quickly find the platform (Fig. 1d). Similar percent time spent in the three equi-distant concentric zones indicates comparable swimming pattern strategies among the three groups (Fig. 1d). These results are consistent with previous report which indicates that AI and AU rats show comparable exploratory activity (i.e. number of rearings) in the open field and similar degree of anxiety in the elevated plus maze (Rowe et al., 1998; Schulz et al., 2002). Thus, aged animals demonstrate marked individual differences during training and probe trial in the MWM task which allows the characterization of aged rats with (AI) or without (AU) specific hippocampus-dependent memory impairments.

Microarray analysis of genes associated with age-related memory deficits

Gene expression profiles of the dorsal hippocampus, a key region involved in learning and memory in the MWM test (Moser et al., 1993), of each animal were investigated using individual Affymetrix high-density microarrays containing 28,757 different genes. Comparative gene expression was assessed 6 h after the last trial on day 5 using AI, AU, and Y rats (four animals per group) that best fit their respective average acquisition curves. Four aged and young adult naive control rats handled with identical procedure but not tested in the MWM were also used to determine basal gene expression levels under resting conditions. Another set of four AI rats were also sacrificed at 8 h post-training to provide an additional measure of genes differentially expressed during the second time period of *de novo* RNA synthesis required for long-term memory formation.

Fold change (FC) in gene expression between the various groups was determined with the $\log_2$ scale Robust Multi-array Averaging (RMA) method (Irizarry et al., 2003a). This well-established microarray analysis is commonly used to obtain a more restrictive and consistent list of genes compared to the MAS5 analysis default program (Irizarry et al., 2003b). A minimal FC of 1.5 derived from the average and a 95 % confidence interval (95 % CI) with a minimal bound of 1 FC for genes up-regulated or - 1 FC for genes down-regulated has to be reached to be considered as a candidate gene. To allow further

elimination of false positive and to generate an even more conservative list, genes selected also had a minimal FC of 1.5 derived from the median (data not shown).

To investigate possible molecular mechanisms underlying memory deficits among the aged population, we focused on genes differentially expressed between AI and AU animals (Table 1). Since complementary information regarding age-related memory deficits can also be drawn by comparing the pattern of gene expression across conditions, genes altered between the various groups can be found as supplemental Table 2 on the web site of Neurobiology of Aging. We found that TTR, protein phosphatase 3 (PPP3, also known as calcineurin), NAD(P)H dehydrogenase quinone 2 (NQO2), and one gene (1380552_at) identified only as transcribed locus were altered at both 6 and 8 h post-training in the two independent AI groups when compared to AU animals. Despite temporal variations in gene expression expected between 6 and 8 h post-training, four genes out of five at 6 h (80 %) were also shown to be differentially expressed at 8 h, indicating the effectiveness and reliability of the RMA microarray analysis. Furthermore, quantitative real-time RT-PCR was performed to confirm these microarray results and revealed parallel patterns of gene expression (Fig. 2). Since the role of calcineurin in the transition of short-term to long-term memory is already well documented (Mansuy et al., 1998; Lee & Ahnn, 2004) and selective ligands or transgenic models available to investigate more precisely the effect of NQO2 on memory are very limited, we focused on the role of TTR in hippocampus-dependent memory formation and maintenance in aging.

Differential expression of the late response gene TTR and NQO2 time-dependently overlap alteration in the protein level of C/EBP

To explain the differential expression of the late response (effector) gene TTR in AI rats 6 and 8 h post-training, we examined the protein expression of the immediate-early gene CCAAT enhancer binding protein (C/EBP) at those same time points. We focused on C/EBP since this transcription factor is known to regulate TTR as well as NQO2 and is shown to be critical for the formation of long-term memory (Alberini et al., 1994). C/EBP is part of the immediate-early

gene cascade initiated by cAMP response element binding protein (CREB) and once activated after training, C/EBP is suggested to regulate downstream effector genes such as TTR or NQO2 for the consolidation of new memory (Taubenfeld et al., 2001). Using an immunoblot approach, we observed that levels of either C/EBP α or β were decreased significantly in AI rats at 8 h post-training when compared to AU rats (Fig. 3). Since C/EBP α and β may synergise their regulatory actions on TTR gene expression by acting on the same promoter sequence, we observed significant differences in protein levels for both C/EBP α and β when analysed together in AI rats at 6 and 8 h post-training compared to AU rats. This result is taken as an indication that decreased expression of the late response gene TTR is time-dependently associated to decreased protein levels of C/EBP α and β , although we cannot rule out that the changes seen in the AI at 8 h may also be somehow related to decreased expression of C/EBP α and/or β in the AU (8 h) group.

Age-related memory deficits occurred in TTR^{-/-} mice

To confirm further the role of TTR in age-related memory deficits we next tested TTR^{-/-} mice in the MWM task. These mice were shown to display normal vision and no obvious phenotypic post-natal abnormalities as observed with vitamin A deficiency (Episkopou et al., 1993; Bui et al., 2001). This suggests the formation of compensatory mechanisms independent of the major retinol transport route involving TTR to maintain normal vitamin A status under resting physiological conditions (Wei et al., 1995).

We also found that 3-months old TTR^{-/-} mice tend to show a slight increase in escape latency in the MWM (Fig. 4a), and showed significant differences at 7-months (Fig. 4b). Spatial memory deficits in 7-months old TTR^{-/-} mice were confirmed during the probe trial performed after the end of training (Fig. 4c, d). WT and TTR^{-/-} mice show no significant difference in swimming speed and escape latency to a visible platform (Fig. 4e, f). The level of anxiety to escape from water is similar in the various groups and they all show comparable swimming pattern strategies according to the thigmotaxis measure and the time

swam in the three concentric zones (Fig. 4g). Furthermore, longer escape latency of $TTR^{-/-}$ mice is unlikely to be attributed to lower exploratory activity or depressive-like behaviours since deletion of TTR was previously associated with increased exploratory activity in the open field and reduced signs of depressive-like behaviours (Sousa et al., 2004). Results obtained with $TTR^{-/-}$ mice are consistent with our microarray data which suggest that decreased TTR expression leads to memory impairments with natural aging. The complete deletion of TTR could be responsible for the earlier memory deficits seen in $TTR^{-/-}$ mice when compared to 24-months old rats in which low endogenous expression levels of TTR are still observed.

Retinoic acid treatment reversed memory deficits in $TTR^{-/-}$ mice and aged rats

One of the key functions of TTR is to deliver retinol according to the needs of body from liver storage to target tissues where it can be metabolized into retinoic acid (RA) (Monaco, 2000). This active vitamin A metabolite acts on specific nuclear receptors to modulate a wide variety of biological processes including synaptic plasticity as well as LTP and long-term depression (LTD) (Chiang et al., 1998; Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002). We hypothesized that the mechanism sustaining the effect of TTR in spatial mnemonic processing is related to the bioavailability of RA to the brain tissue. We therefore administered RA (150 μ g/kg, sc, once per day) to 7-months old $TTR^{-/-}$ mice for four days prior to behavioural testing and every day until the end of testing. This treatment reverses memory deficits during the acquisition phase (Fig. 4b) and the probe trial (Fig. 4c, d) without affecting the swimming speed and escape latency to a visible platform (Fig. 4e, f).

To determine if the administration of RA could suppress memory impairments seen in aged rats we applied the same treatment to 24-months old rats and observed that RA treatment abolished aged-memory deficits during the training period (Fig. 5a) and the probe trial (Fig. 5b, c) without producing any significant difference in the swimming speed (Fig. 5d) and escape latency to a visible platform (Fig. 5e). These data are in agreement with a previous report

showing alleviation of age-related deficits in LTP and in a spatial discrimination task following the administration of RA in mice (Etchamendy et al., 2001). Furthermore, none of the RA treated rats were characterized as AI animals when taken individually (data not shown), which is in contrast to the 38 % of aged rats that naturally develop memory impairments without any treatment (see in the text Fig. 1). The effect of RA seems to be specific for the TTR deficiency rather than a non-selective cognitive enhancement since 3- and 7-months old WT mice (Fig. 4) as well as Y rats (Fig. 5) are not affected by the administration of RA. Hence, treatment with RA, which is transported mainly by albumin instead of TTR unlike retinol (Smith et al., 1973), can significantly reduce learning and memory deficits in both TTR^{-/-} mice and aged rats.

Figures

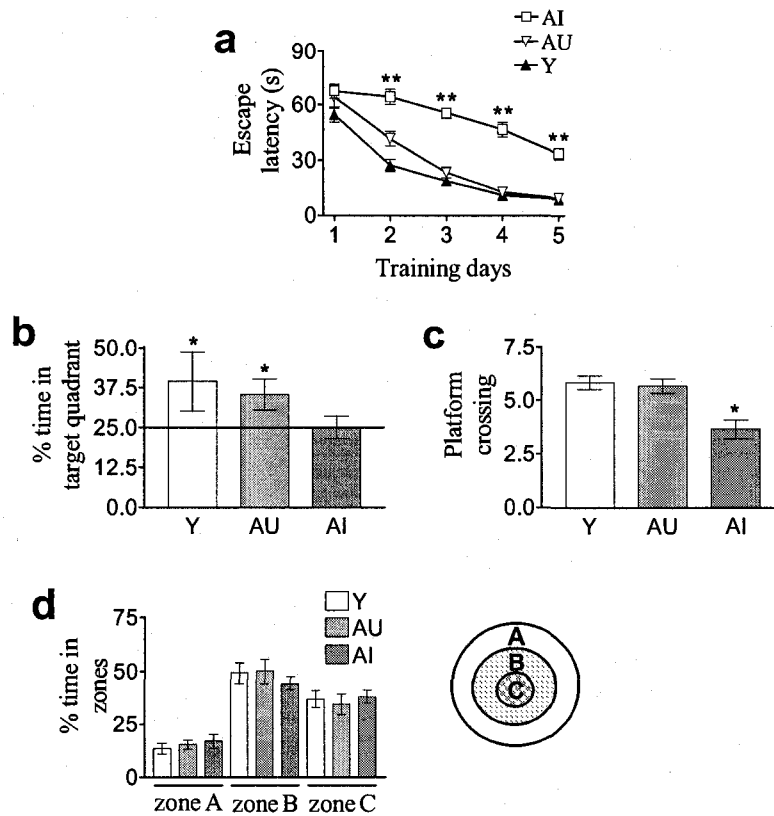


Fig.1. Spatial memory of young (7 mo) and aged (24 mo) rats in the MWM task. (a) Escape latencies before finding the hidden-platform (three trials of 90 s per day with a 45 min inter-trial interval) are longer for aged memory-impaired rats (AI; open squares, $n = 23$) than aged memory-unimpaired rats (AU; open triangles, $n = 11$) and young adult animals (Y; filled triangles, $n = 21$; ** $p < 0.01$; two-way ANOVA). (b) Percent time spent in the quadrant that previously contained the platform during the probe trial. Error bars ($\pm$ 95% confidence interval of the mean) of AI rats overlap the 25 % line which represents the chance level of being randomly in one of the four quadrants. Asterisk represents significant differences from the 25 % chance performance ($p < 0.05$). (c) AI rats crossed the previous location of the platform fewer times than AU and Y (* $p < 0.05$; one-way ANOVA) during the probe trial. (d) Percent time spent in each of the three concentric zones is not significantly different among the three groups (* $p < 0.05$; one-way ANOVA). All results, except for (b) are mean $\pm$ SEM.

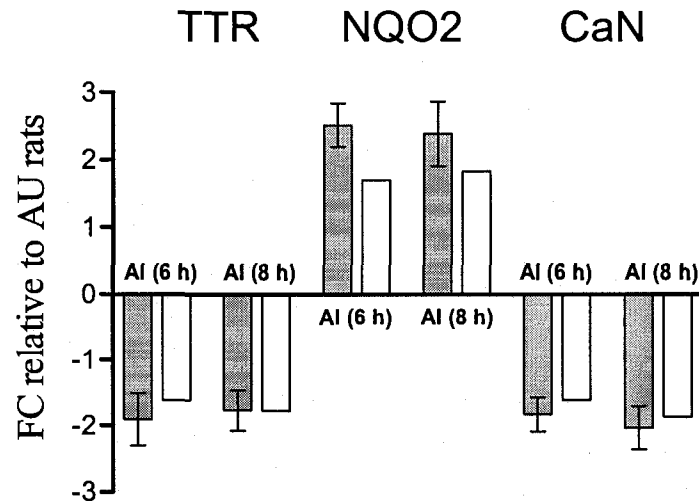


Fig. 2. Quantitative real-time RT-PCR and microarray analysis revealed parallel patterns of genes expression. Fold change (FC) in gene expressions of TTR, NQO2, and calcineurin (CaN) in aged memory-impaired rats (AI 6 and 8 h) relative to aged memory-unimpaired (AU) animals using real-time RT-PCR (filled bars) and microarrays (open bars). FC obtained by microarray with the RMA method is generally an underestimate of the FC found with real-time RT-PCR. FC with a positive sign indicates up-regulation of the gene in AI relative to AU rats whereas down-regulation is represented by a negative sign. Since the standard error for estimated FC is not valid in the $\log_2$ scale used for microarray data, statistical analysis (mean $\pm$ SEM) was only done with real-time RT-PCR data.

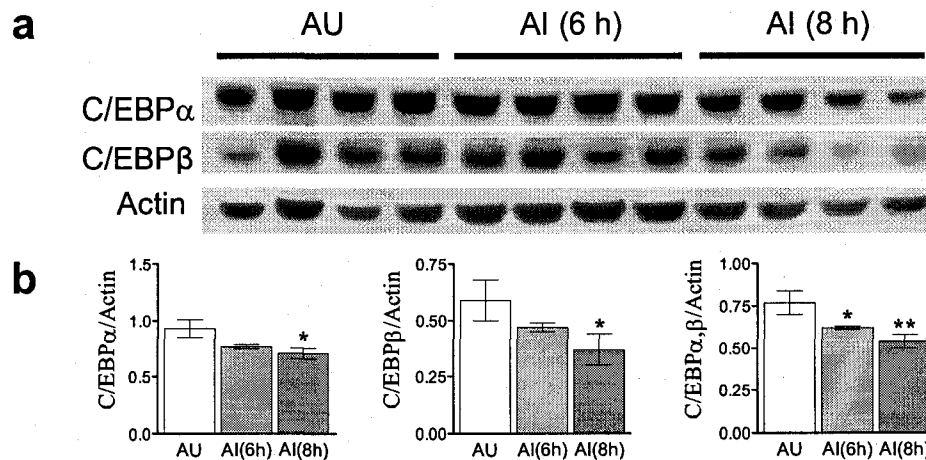


Fig. 3. Differential expression of the late response gene TTR and NQO2 time-dependently parallel decreased protein expressions of C/EBP α and/or C/EBP β . (a) Western blot of hippocampal extracts from the same aged memory-unimpaired (AU) rats and aged memory-impaired (AI 6 and 8 h) animals used for microarray and quantitative real-time RT-PCR analysis. The same blots were stained with anti-actin as well. (b) Densitometric analysis of the western blots shown in (a). Data are expressed as the ratio of C/EBP α /actin, C/EBP β /actin to normalize the values of the two isoforms with those of actin to account for slight variations in gel loading. Protein levels of either C/EBP α or C/EBP β isoforms were significantly decreased in AI rats at 8 h post-training compared to AU rats (* $p < 0.05$; one-way ANOVA). Combined protein levels of both C/EBP α and C/EBP β were significantly decreased in AI rats at 6 and 8 h post-training compared to AU rats (* $p < 0.05$; ** $p < 0.01$; one-way ANOVA). Each bar represents the mean $\pm$ SEM.

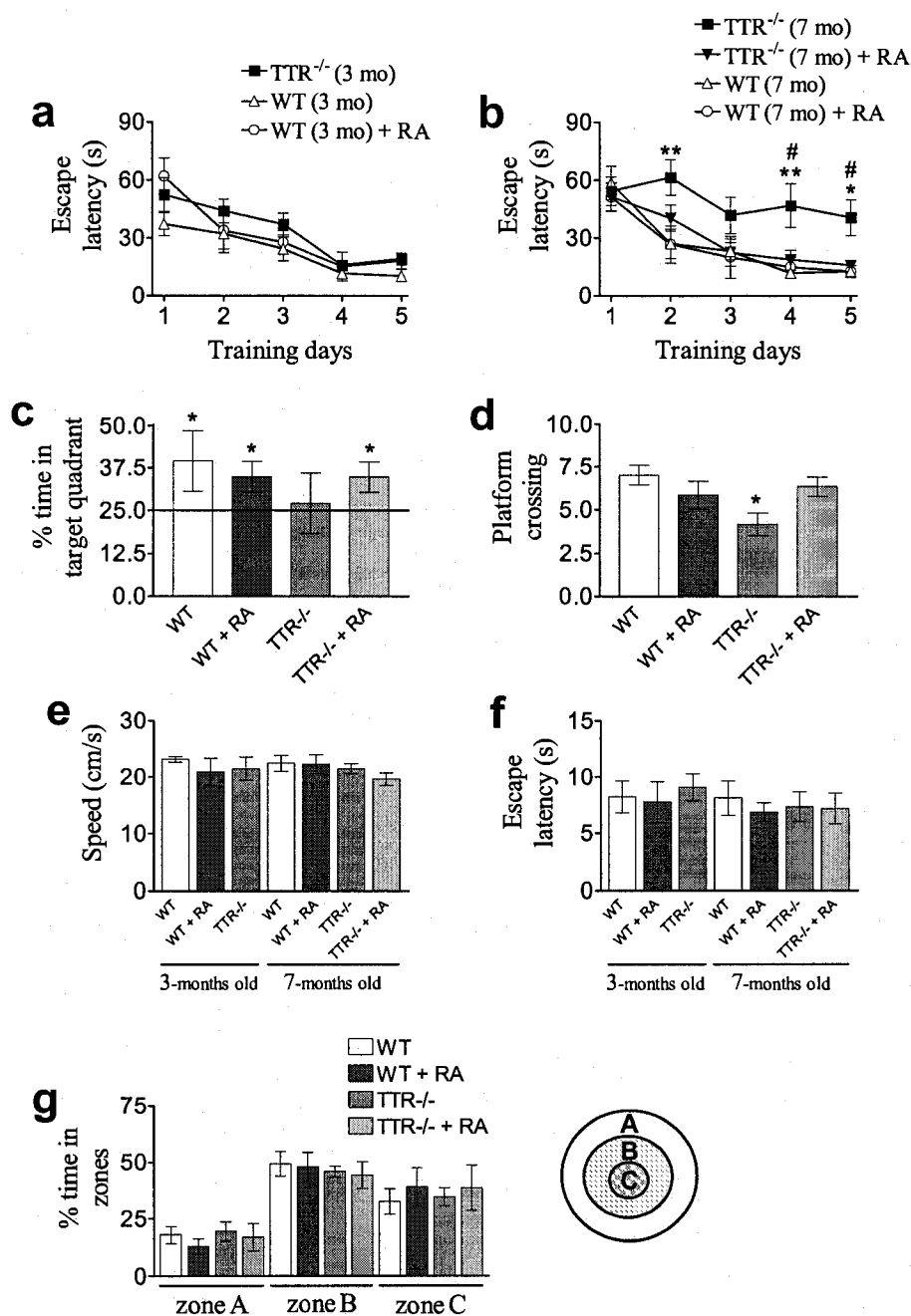


Fig. 4. Spatial memory deficits of 7-months old TTR^{-/-} mice are reversed by RA. (a) Escape latencies in hidden-platform test tend to be slightly longer for 3-months old TTR^{-/-} mice (filled squares, $n = 6$) and (b) show significant difference (* $p < 0.05$; ** $p < 0.01$; two-way ANOVA) in 7-months old TTR^{-/-} mice (filled

squares, $n = 6$) when compared to age-matched WT mice (open triangles, $n = 6$ for each age group). Administration of RA (filled triangles, 150 $\mu\text{g/kg}$, sc, once per day during nine days, $n = 6$) reverses memory deficits of 7-months old $\text{TTR}^{-/-}$ mice ($^{\#} p < 0.05$; two-way ANOVA) without affecting performance of 3- and 7-months old WT mice (open circles; $n = 6$; $p > 0.05$; two-way ANOVA). (c) $\text{TTR}^{-/-}$ mice overlap ($\pm 95\%$ confidence interval) the 25 % chance line of randomly being in one of the four quadrants during the probe trial ($* p < 0.05$). (d) $\text{TTR}^{-/-}$ mice crossed the previous platform location fewer times than WT mice ($* p < 0.05$; one-way ANOVA) during the probe trial, which is reversed by RA. (e) Treatment with retinoic acid produced no significant difference in swimming speed in 3- and 7-months old $\text{TTR}^{-/-}$ and WT mice during the probe trial and (f) similar escape latency to a visible platform. (g) Percent time spent in each of the three concentric zones is not significantly different in 7-months old mice ($* p < 0.05$; one-way ANOVA). All results, except for (c) are mean $\pm$ SEM.

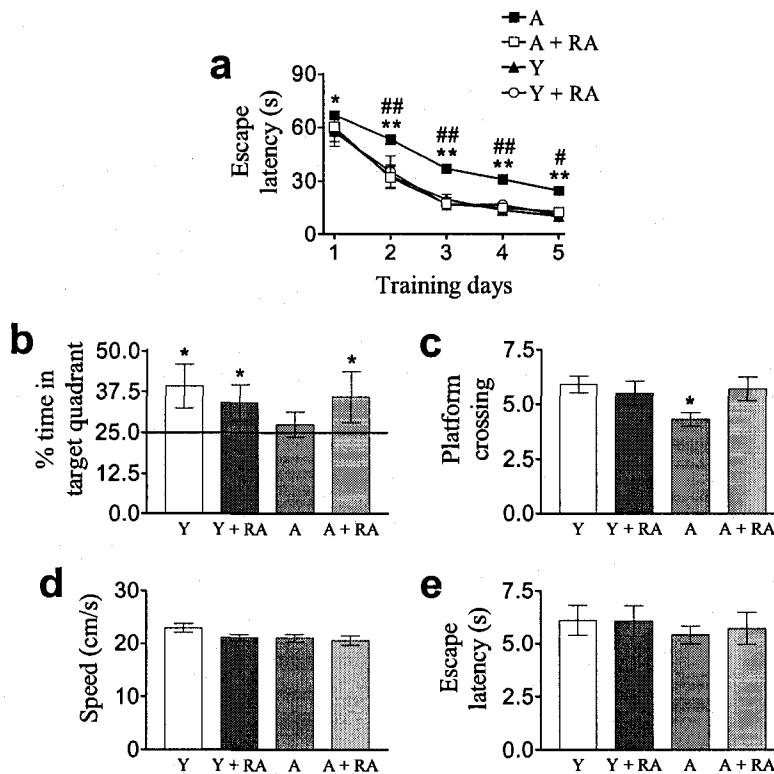


Fig. 5. Spatial memory impairments of aged rats are reversed by RA. (a) The total population of aged rats (A; filled squares, $n = 60$) tested in the hidden-platform task shows a longer escape latency than young adult animals (Y; filled triangles, $n = 10$; * $p < 0.005$; ** $p < 0.001$; two-way ANOVA). Injection of RA in A rats (open squares, 150 $\mu\text{g/kg}$, sc, $n = 10$) reversed spatial memory deficits (# $p < 0.05$; ## $p < 0.01$; two-way ANOVA) without affecting performance of Y rats (open circles; $n = 10$; $p > 0.05$; two-way ANOVA). (b) The time spent in the target quadrant during the probe trial ($\pm 95\%$ confidence interval) by the aged rats is around 25 % chance level, whereas A and Y animals treated with RA as well as Y rats ($n = 10$ for each group) swam longer in the target quadrant (* $p < 0.05$). (c) Aged rats crossed the previous site of the platform less frequently than A rats treated with RA (* $p < 0.05$; one-way ANOVA). (d) Treatment with retinoic acid produced no significant difference in swimming speed in aged and young adult animals during the probe trial and (e) similar escape latency to a visible platform. All results, except for (b) are mean $\pm$ SEM.

Tables

Table 1. Genes differentially expressed in AI (6 h) and AI (8 h) compared to AU animals

Genes altered in AI (6 h) relative to AU rats	Representative Public ID	Probe Set ID	FC	95 % CI
Transthyretin	NM_012681	1367598_at	- 1.61	- 1.14 : - 2.29
Protein phosphatase 3, catalytic A subunit, alpha isoform	BF415556	1395436_at	- 1.61	- 1.02 : - 2.59
NAD(P)H dehydrogenase quinone 2	AA945624	1374959_at	1.70	1.27 : 2.29
Transcribed locus	AI059603	1393615_at	1.67	1.04 : 2.66
Transcribed locus	AI555855	1380552_at	2.38	1.05 : 5.38
Genes altered in AI (8 h) relative to AU rats				
Transthyretin	NM_012681	1367598_at	- 1.78	- 1.10 : - 2.82
Protein phosphatase 3, catalytic A subunit, alpha isoform	BF415556	1395436_at	- 1.87	- 1.09 : - 3.17
NAD(P)H dehydrogenase quinone 2	AA945624	1374959_at	1.83	1.63 : 2.05
Casein kinase II, alpha 1 polypeptide	NM_053824	1387170_at	1.78	1.48 : 2.15
Pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	BI275435	1395020_at	2.20	1.07 : 4.53
Similar to RIKEN cDNA 1700040L02	AI171288	1373436_at	- 1.53	- 1.11 : - 2.10
Similar to Williams-Beuren syndrome deletion transcript 9 homolog	BF395914	1396420_at	- 1.62	- 1.00 : - 2.62
Transcribed locus	AI406369	1389996_at	1.71	1.02 : 2.86
Transcribed locus	AI555855	1380552_at	2.56	1.03 : 6.31

Genes up-regulated in AI (6 h) and AI (8 h) compared to AU rats are indicated by positive values whereas genes down-regulated are represented by a negative sign. Fold expression changes and bounds of the 95 % confidence interval shown in this Table are derived from the average measures. Gene probe set ID and representative public ID obtained from Affymetrix and NCBI respectively are provided.

Supplemental Table 2. Genes differentially expressed between the various groups

Genes altered in AU relative to A naive rats	Representative Public ID	Probe Set ID	FC	95 % CI
transthyretin	NM_012681	1367598_at	4.61	1.73 : 8.31
DNA-damage-inducible transcript 4-like	NM_080399	1368013_at	1.61	1.22 : 2.11
Beta-glo	BI287300	1371245_a_at	1.73	1.17 : 2.54
membrane frizzled-related protein (predicted)	BG374415	1377434_at	1.56	1.09 : 2.23
calcium/calmodulin-dependent protein kinase II alpha subunit	NM_012920	1368935_at	-1.64	-1.37 : -1.96
arrestin, beta 1	NM_012910	1368968_at	-1.51	-1.05 : -2.18
protein phosphatase 3, regulatory subunit B, alpha isoform, type 1	NM_017309	1369152_at	-1.51	-1.19 : -1.93
baculoviral IAP repeat-containing 4	AF304333	1369248_a_at	-1.67	-1.20 : -2.32
sodium channel, voltage-gated, type II, beta polypeptide	NM_012877	1369311_at	-1.63	-1.11 : -2.39
gamma-aminobutyric acid (GABA) B receptor 1	Y10369	1369371_a_at	-1.56	-1.01 : -2.41
opioid-binding protein/cell adhesion molecule-like	NM_053848	1369417_a_at	-1.63	-1.03 : -2.58
claudin 11	NM_053457	1369609_at	-1.53	-1.13 : -2.06
solute carrier family 1 (glial high affinity glutamate transporter), member 2	AY069978	1369694_at	-1.63	-1.21 : -2.18
myelin-associated oligodendrocytic basic protein and hypothetical gene supported by NM_012720	D28110	1370500_a_at	-1.71	-1.25 : -2.32
protein kinase, cAMP dependent, catalytic, beta (predicted)	D10770	1370746_at	-1.96	-1.19 : -3.21
FBJ murine osteosarcoma viral oncogene homolog	BF415939	1375043_at	-1.51	-1.06 : -2.15
low density lipoprotein receptor-related protein 11 (predicted)	BE095528	1375193_at	-1.51	-1.04 : -2.18
amyloid beta (A4) precursor-like protein 2	AI235240	1383096_at	-1.69	-1.10 : -2.60
putative aminopeptidase Fxna	BM387447	1384975_at	-1.74	-1.04 : -2.89
DEAH (Asp-Glu-Ala-His) box polypeptide 36 (predicted)	AA859982	1385871_at	-1.72	-1.08 : -2.73
potassium inwardly-rectifying channel, subfamily J, member 10	NM_031602	1387467_at	-1.59	-1.08 : -2.36
potassium voltage-gated channel, shaker-related subfamily, beta member 2	NM_017304	1387689_at	-1.69	-1.01 : -2.81
solute carrier family 6 (neurotransmitter transporter, glycine), member 9	M95413	1387693_a_at	-1.54	-1.34 : -1.77
potassium voltage-gated channel, subfamily Q, member 3	AF091247	1388052_a_at	-1.53	-1.24 : -1.89
Discs, large homolog 2 (Drosophila)	BE120660	1392196_at	-1.52	-1.19 : -1.95
potassium channel tetramerisation domain containing 12 (predicted)	AA800908	1392785_at	-1.64	-1.10 : -2.46
Similar to transmembrane 4 superfamily member 10	BG664080	1393171_at	-1.63	-1.17 : -2.27
similar to hypothetical protein DKFZp566A1524 (predicted)	AI502258	1394470_at	-1.61	-1.09 : -2.38
similar to Ac2-190 (predicted)	BF386788	1395036_at	-1.57	-1.05 : -2.37
GPI-anchored membrane protein 1 (predicted)	BF396243	1395173_at	-1.80	-1.21 : -2.66
similar to KIAA1189 protein (predicted)	BF567711	1395447_at	-1.69	-1.29 : -2.22
Similar to teratocyte-specific carboxylesterase	BF546710	1397569_at	-1.64	-1.27 : -2.12

Supplemental Table 2. Continuation

Genes altered in AU relative to Y rats	Representative Public ID	Probe Set ID	FC	95 % CI
transthyretin	NM_012681	1367598_at	3.28	1.16 : 6.85
heat shock 27kDa protein 1	NM_031970	1367577_at	1.72	1.24 : 2.38
connective tissue growth factor	NM_022266	1367631_at	1.59	1.09 : 2.31
glycoprotein (transmembrane) nmb	NM_133298	1368187_at	1.59	1.27 : 1.99
serine (or cysteine) peptidase inhibitor, clade G, member 1	AW915763	1372254_at	1.64	1.24 : 2.17
Hypothetical gene supported by NM_020088	BF418058	1375331_at	1.70	1.37 : 2.12
Calcium channel, voltage-dependent, gamma subunit 8	BE114019	1378151_at	1.60	1.19 : 2.15
Cytoplasmic FMR1 interacting protein 2 (predicted)	BF404462	1379150_at	1.51	1.22 : 1.87
Protease, serine, 35 (predicted)	AA866443	1379747_at	2.08	1.34 : 3.22
Slit homolog 3 (Drosophila)	BF386446	1381218_at	1.70	1.41 : 2.05
Hypothetical gene supported by NM_013150	BF386153	1381621_at	2.08	1.27 : 3.40
N-arginine dibasic convertase 1	BE115812	1382673_at	1.56	1.13 : 2.15
hypothetical protein LOC498544	AA800750	1389488_at	1.61	1.31 : 1.97
Ubiquitin specific protease 33 (predicted)	BE108244	1391770_at	1.61	1.19 : 2.18
Similar to KCNMA1 protein	BF413366	1396556_at	1.51	1.29 : 1.76
calcium/calmodulin-dependent protein kinase II alpha subunit	NM_012920	1368935_at	-1.60	-1.21 : -2.11
RAS guanyl releasing protein 1	NM_019211	1369129_at	-1.54	-1.07 : -2.21
protein phosphatase 3, regulatory subunit B, alpha isoform, type 1	NM_017309	1369152_at	-1.61	-1.20 : -2.16
baculoviral IAP repeat-containing 4	AF304333	1369248_a_at	-1.68	-1.25 : -2.25
sodium channel, voltage-gated, type II, beta polypeptide	NM_012877	1369311_at	-1.55	-1.16 : -2.05
gamma-aminobutyric acid (GABA) B receptor 1	Y10369	1369371_a_at	-1.74	-1.13 : -2.69
calcium/calmodulin-dependent protein kinase IV	M63334	1369753_at	-1.54	-1.27 : -1.87
homer homolog 1 (Drosophila)	AB003726	1370454_at	-1.70	-1.10 : -2.63
protein kinase, cAMP dependent, catalytic, beta (predicted)	D10770	1370746_at	-1.77	-1.03 : -3.05
NAD(P)H dehydrogenase quinone 2	AA945624	1374959_at	-1.75	-1.45 : -2.11
similar to mKIAA0738 protein	AA964744	1377580_at	-1.86	-1.20 : -2.90
amyloid beta (A4) precursor-like protein 2	AI235240	1383096_at	-1.68	-1.05 : -2.68
similar to B230212L03Rik protein	BG669823	1385626_at	-1.59	-1.30 : -1.93
proline-rich protein	BI278661	1385684_x_at	-1.63	-1.24 : -2.15
DEAH (Asp-Glu-Ala-His) box polypeptide 36 (predicted)	AA859982	1385871_at	-2.00	-1.17 : -3.41
Similar to hypothetical protein MGC52110	AA893212	1385889_at	-1.66	-1.38 : -1.99
potassium voltage-gated channel, subfamily Q, member 3	AF091247	1388052_a_at	-1.59	-1.29 : -1.95
asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase) (predicted)	AI070159	1392437_at	-1.68	-1.04 : -2.69
similar to cytosolic phosphoprotein DP58	AA924915	1393283_at	-1.58	-1.12 : -2.22
similar to hypothetical protein DKFZp566A1524 (predicted)	AI502258	1394470_at	-1.78	-1.16 : -2.72
leucine-rich PPR-motif containing (predicted)	BF289725	1395338_at	-1.59	-1.04 : -2.44
similar to cDNA sequence BC043098	BF562964	1395556_at	-1.72	-1.11 : -2.66
similar to ALEX3 protein	AI030168	1397960_at	-1.52	-1.18 : -1.98

Supplemental Table 2. Continuation

Genes altered in Y relative to Y naive rats	Representative Public ID	Probe Set ID	FC	95 % CI
apolipoprotein C-I	NM_012824	1368587_at	2.03	1.36 : 3.04
homer homolog 1 (Drosophila)	AB003726	1370454_at	1.66	1.27 : 2.17
Ectodermal-neural cortex 1	AA997271	1382710_at	1.66	1.15 : 2.39
potassium voltage-gated channel, shaker-related subfamily, beta member 2	NM_017304	1387689_at	1.52	1.22 : 1.91
Trinucleotide repeat containing 6 (predicted)	BI294732	1375664_at	-2.41	-2.07 : -2.80
Genes altered in A naive relative to Y naive rats				
vimentin	NM_031140	1367574_at	1.51	1.11 : 2.05
heat shock 27kDa protein 1	NM_031970	1367577_at	1.54	1.26 : 1.88
CD74 antigen (invariant polypeptide of major histocompatibility class II antigen-associated)	NM_013069	1367679_at	2.88	1.79 : 4.63
S100 calcium-binding protein A4	NM_012618	1367846_at	1.60	1.19 : 2.17
complement component 3	NM_016994	1368000_at	2.11	1.68 : 2.65
glycoprotein (transmembrane) nmb	NM_133298	1368187_at	1.76	1.42 : 2.17
acyl-Coenzyme A dehydrogenase, short/branched chain	NM_013084	1369526_at	1.68	1.18 : 2.39
RT1 class Ib, locus Aw2 and RT1 class Ia, locus A2 and RT1 class I, A3	AJ249701	1370428_x_at	1.90	1.45 : 2.50
protein kinase, cAMP dependent, catalytic, beta (predicted)	D10770	1370746_at	1.59	1.18 : 2.15
complement component 4a and complement component 4, gene 2	BI285347	1370892_at	1.62	1.41 : 1.85
serine (or cysteine) peptidase inhibitor, clade G, member 1	AW915763	1372254_at	1.67	1.35 : 2.06
leucine aminopeptidase 3 (predicted)	AA945172	1376579_at	1.56	1.24 : 1.96
Basic helix-loop-helix domain containing, class B2	AI548256	1379483_at	2.40	1.30 : 4.43
LOC499891	AI059089	1384058_at	1.83	1.47 : 2.27
Erythrocyte protein band 4.1-like 4b (predicted)	BE108011	1385604_at	1.57	1.27 : 1.93
aminoadipate aminotransferase	NM_017193	1387223_at	1.51	1.26 : 1.80
chemokine (C-X-C motif) ligand 12	AF189724	1387655_at	1.64	1.37 : 1.95
potassium voltage-gated channel, shaker-related subfamily, beta member 2	NM_017304	1387689_at	1.55	1.27 : 1.90
interferon induced transmembrane protein 3	BI285494	1387995_a_at	1.61	1.30 : 1.99
CD44 antigen	BI302830	1390659_at	1.54	1.31 : 1.81
BS69 protein	AA944574	1392846_at	1.54	1.23 : 1.93
Similar to transmembrane 4 superfamily member 10	BG664080	1393171_at	1.67	1.35 : 2.07
Fc receptor, IgG, low affinity III and Fc gamma receptor II beta	NM_053843	1398246_s_at	1.59	1.16 : 2.19
transthyretin	NM_012681	1367598_at	-2.29	-1.21 : -4.68
decorin	BM390253	1370956_at	-1.61	-1.18 : -2.18
Beta-glo	BI287300	1371245_a_at	-1.58	-1.11 : -2.25
peroxisomal membrane protein 4	AI232414	1383117_at	-1.66	-1.12 : -2.46

Genes up-regulated in the various groups relative to a control group are indicated by positive values whereas genes down-regulated are represented by a negative sign. Fold expression changes and bounds of the 95 % confidence interval shown in this Table are derived from the average measures obtained using microarray. Gene probe set ID and representative public ID obtained from Affymetrix and NCBI respectively is provided. Genes identified only as expressed sequence tags (ESTs) were not included in the table.

Supplemental Table 3. Gene-specific primers used for PCR amplification

Genes	5'- 3' Forward Primer	5'-3' Reverse Primer
Transthyretin	CGTACTGGAAGGCTCTTGGC	CCACCTCTGCGTATTCATGGA
NAD(P)H dehydrogenase, quinone 2	TGCTCTGGGTACAGCTGACAA	TGCCGTGCTTTAGGTCTCTTACT
Protein phosphatase 3, catalytic A subunit, α isoform	ACTGGCATGCTCCCCAGCGGA	GTGCCGTTAGTCTCTGAGGCG
beta-actin	TCCTGTGGCATCCATGAAACT	AACGTCACACTTCATGATGGAATT

Gene-specific primers were designed with the Primer Express software (Applied Biosystems, CA). The locations of the primers were chosen to cross an exon-exon junction to insure the specificity for RNA rather than nuclear DNA sequences.

Discussion

Our study provides genetic, behavioural and molecular evidence that TTR is involved in age-related memory deficits. The microarray analysis has revealed at two independent time points that TTR gene expression is lower in AI compared to AU animals following a mnemonic challenge and these results were confirmed by quantitative real-time RT-PCR. An immunoblot analysis done at those same time points indicates that the decreased expression of the late response gene TTR may be attributed, at least in part, to alteration in the protein expression of the immediate-early gene C/EBP, which is known to regulate TTR. The role of TTR in age-related memory deficits was further confirmed using knock-out mice lacking TTR which show spatial memory deficits during aging. Reversal of cognitive deficits with RA treatment in TTR^{-/-} mice and aged rats strongly suggests that the mechanism underlying the role of TTR in memory formation during aging is linked to its ability to regulate brain retinoid availability. Taken together, these results provide evidence that TTR is involved in the maintenance of memory capacities during aging.

While three independent replicates per group are generally acceptable to provide a reliable analysis of gene expression data using microarrays (Lee et al., 2000), here we employed four animals in each group. To obtain a more accurate gene expression estimates and confidence intervals, individual microarrays were used for each animal. Also a minimal FC of 1.5 derived from both the average and the median has to be reached, and a 95 % confidence interval (which gives less tight bounds than SEM measures) with a minimal bound of 1 or -1 FC for genes up- or down-regulated respectively has to be obtained. Furthermore, data of interest were confirmed with independent quantitative real-time RT-PCR. Functional analysis using TTR^{-/-} mice also validated the role of this gene in age-related memory deficits. Altogether, these results indicate that the microarray approach used in this study led to a consistent restricted list of candidate genes possibly implicated in age-associated memory impairments.

Decreased TTR expression as a key event in the development of memory impairments during aging

Dysfunctions of many neurotransmitter pathways have been postulated over the last decades to be associated with memory loss during aging. Our study strongly supports the small, growing body of evidence indicating a role of disturbed RA pathway in age-related memory deficits (Chiang et al., 1998; Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002). Most interestingly, we identified for the first time that TTR is a critical component of the complex RA signalling pathway that is associated with memory deficits during aging. Among all the genes present on high-density microarray, TTR gene expression was highlighted to be the most markedly affected RA component between the AI and AU. Given the essential role of TTR in the transport of retinol, decreased TTR gene expression may considerably alter the bioavailability of RA in response to the body's needs and thus may affect pleiotropic action of RA on brain. During an increased RA demand, as observed following stimulation in a spatial memory task, RA deficiency in the hippocampus may decrease memory performance by impairing synaptic plasticity (Misner et al., 2001), LTP and LTD (Chiang et al., 1998; Etchamendy et al., 2001), and neurogenesis (Snyder et al., 2005; Jacobs et al., 2006).

A previous report has shown that RA receptor β (RAR β) and retinoid X receptor γ (RXR γ) are critical for hippocampal LTP and LTD (Chiang et al., 1998). Decreased expression (20-30%) of RAR β and RXR β/γ was also observed in aged mice when compared to adults, and this reduction was reversed by acute administration of RA (Enderlin et al., 1997b). The lower amount of RAR β and RXR mRNA in aged mice was suggested to result from inadequate RA bioavailability to ensure the up-regulation of RA receptors, which is consistent with deficiency of the major transport route of retinol involving TTR observed in AI rats. Given the wide effects exerted by RA on the brain and the profound reversal of memory deficits produced by RA treatment in TTR^{-/-} mice and aged rats, the other genes differentially expressed between AI and AU rats identified in this study may be directly or indirectly influenced by RA.

The up-regulation of TTR expression may be especially important for aged rats to perform as well as young adult animals in a spatial memory task given that the basal gene expression level of TTR is markedly decreased with age (Supplemental Table 2). The importance of adequate TTR regulation is underscored by the fact that TTR is a single-copy gene that gives a single protein product, and no other protein seems to replace TTR in the transport of retinol (Dickson et al., 1985). Since deletion of TTR does not affect the uptake or storage of dietary retinol (Wei et al., 1995), it is unlikely that higher food intake of vitamin A would increase RA tissue pools in unrestrictive dietary condition.

Decreased expression of the late response gene TTR could be at least partially due to change in the protein expression of the immediate-early gene C/EBP

To explain differential expression of the effector gene TTR in AI rats 6 and 8 h post-training, we examined the protein expression of C/EBP at the same time points and found that lower amount of this transcription factor time-dependently overlap reduce gene expression of TTR. This result suggests that decreased TTR gene expression could be attributed at least partly to diminish activity of C/EBP immediate-early gene cascade initiated by CREB. Consistent with these data, a recent study has shown that elevation of hippocampal CREB protein levels prevented long-term memory deficits during normal aging in rats (Mouravlev et al., 2006). Our data support the hypothesis of a disruption of CREB signalling pathway in age-related memory impairments and identified TTR as a critical effector gene regulated by this signalling cascade which is involved in memory capacities during aging. Whereas the essential role of the C/EBP β isoform in the conversion of short- to long-term memory is already well documented (Yukawa et al., 1998; Chen et al., 2003), this study also provides evidence for a function of C/EBP α isoform in age-related memory abilities. Decreased protein levels of C/EBP α in AI rats suggest that C/EBP α is recruited downstream from CREB as a memory-enabling gene for hippocampal-based memory tasks during brain aging, and acts by altering the transcription of target

genes such as TTR and NQO2. Because of the well known temporal delay between gene expression and the synthesis of the corresponding protein product, it would be of interest in future studies to perform a detailed analysis of mRNA and protein levels at various time points post-training, for key mediators of the CREB signalling pathway.

Our study also reveals that calcineurin and NQO2 are associated to age-related memory deficits. Catalytic activity of calcineurin has been shown to be decreased during aging as well as in AD and was correlated to age-related memory deficits (Cook et al., 2005; Monti et al., 2005). NQO2 has been identified as a potential melatonin receptor 3 (*MT₃*) (Nosjean et al., 2000). This cytosolic receptor is, in fact, a flavoenzyme that provides cellular defence against the neurotoxicity induced by catecholamine-derived o-quinones and oxidative stress during redox cycling. NQO2 was proposed to be the molecular target of resveratrol, a neuroprotective molecule enriched in red wine (Buryanovskyy et al., 2004).

Role of TTR in Alzheimer's disease

TTR has been reported to be the major A β binding protein in the CSF (Schwarzman et al., 1994; Carro et al., 2002). As age is the most robust risk factor for AD (George-Hyslop, 2000), a disease where early hippocampal memory deficits occur long before the formation of amyloid plaques (Naslund et al., 2000; Kril et al., 2002), our observation that age-related decreases in hippocampal TTR expression affect learning and memory is especially intriguing, particularly in the understanding of the molecular alterations and progression of AD. Earlier studies have revealed that AD patients have lower levels of TTR (Serot et al., 1997; Merched et al., 1998), whereas higher levels of TTR in human CSF (Schwarzman et al., 1994) and AD mouse models (Carro et al., 2002) prevent A β aggregation and plaque formation. Other reports have shown that in a mouse model of AD, the absence of amyloid plaques and neuronal loss in young mice were associated with increased expression of TTR in the hippocampus (Stein & Johnson, 2002). Furthermore, in these aged mice, neutralization of TTR led to increased levels of

A β , tau phosphorylation, neuronal loss and apoptosis in the CA1 neuronal field (Stein et al., 2004).

A defective retinoid transport has been reported in some AD patients (Goodman & Pardee, 2003), and several genes involved in the formation of amyloid plaques (such as presenilin 1 and 2, apolipoprotein E, tau, and amyloid protein precursor) are regulated by retinoid signalling pathway (Lane & Bailey, 2005). Deficits in RA homeostasis due to decreased TTR levels may have a particular impact on the hippocampus, where high levels of RA signalling are required to regulate neurogenesis and neural differentiation (Jacobs et al., 2006). Altogether, these results suggest that TTR may play a role in the pathogenesis of AD and the decreased expression of TTR reported in our study could result in both 1) defective retinoid transport that may account for memory deficits and 2) decreased sequestration of A β which can induce a progressive increase in plaque formation. Current research is in progress in our laboratory to determine if complete deletion of TTR can reveal neuropathological hallmarks of AD in old TTR^{-/-} mice and if treatment with TTR could have an impact on the Alzheimer's phenotype.

Conclusion

The microarray approach used in this study reliably led to the identification of a restricted list of candidate genes possibly implicated in age-related memory deficits. Among those, TTR, NQO2, and calcineurin were differentially expressed at two time points in AI versus AU rats following stimulation in a spatial memory task. The retinol transporter TTR proves to be particularly interesting, as decreased expression levels were shown to be involved in the development of memory impairments during aging.

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to RQ and a student scholarship from CIHR to JB. We thank Doris Dea for technical assistance for real-time RT-PCR.

Disclosure statement: The authors declare that they have no actual or potential conflicts of interest. Animal care and handling procedures were approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care.

References

- Alberini CM, Ghirardi M, Metz R & Kandel ER (1994) C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. *Cell*, 76, 1099-1114.
- Bach ME, Barad M, Son H, Zhuo M, Lu YF, Shih R, Mansuy I, Hawkins RD & Kandel ER (1999) Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. *Proc Natl Acad Sci USA*, 96, 5280-5285.
- Barnes CA & McNaughton BL (1985) An age comparison of the rates of acquisition and forgetting of spatial information in relation to long-term enhancement of hippocampal synapses. *Behav Neurosci*, 99, 1040-1048.
- Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC & Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci*, 23, 3807-3819.
- Bourtchouladze R, Abel T, Berman N, Gordon R, Lapidus K & Kandel ER (1998) Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learn Mem*, 5, 365-374.
- Bui BV, Armitage JA, Fletcher EL, Richardson SJ, Schreiber G & Vingrys AJ (2001) Retinal anatomy and function of the transthyretin null mouse. *Exp Eye Res*, 73, 651-659.
- Buryanovskyy L, Fu Y, Boyd M, Ma Y, Hsieh TC, Wu JM & Zhang Z (2004) Crystal structure of quinone reductase 2 in complex with resveratrol. *Biochemistry*, 43, 11417-11426.
- Carro E, Trejo JL, Gomez-Isla T, LeRoith D & Torres-Aleman I (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. *Nat Med*, 8, 1390-1397.

- Chen A, Muzzio IA, Malleret G, Bartsch D, Verbitsky M, Pavlidis P, Yonan AL, Vronskaya S, Grody MB, Cepeda I, Gilliam TC & Kandel ER (2003) Inducible enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. *Neuron*, 39, 655-669.
- Chiang MY, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF & Evans RM (1998) An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron*, 21, 1353-1361.
- Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L & Fadda F (2002) Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience*, 115, 475-482.
- Cook CN, Hejna MJ, Magnuson DJ & Lee JM (2005) Expression of calcipressin1, an inhibitor of the phosphatase calcineurin, is altered with aging and Alzheimer's disease. *J Alzheimers Dis*, 8, 63-73.
- Dickson PW, Howlett GJ & Schreiber G (1985) Rat transthyretin (prealbumin). Molecular cloning, nucleotide sequence, and gene expression in liver and brain. *J Biol Chem*, 260, 8214-8219.
- Driscoll I & Sutherland RJ (2005) The aging hippocampus: navigating between rat and human experiments. *Rev Neurosci*, 16, 87-121.
- Enderlin V, Alfos S, Pallet V, Garcin H, Azais-Braesco V, Jaffard R & Huguieret P (1997a) Aging decreases the abundance of retinoic acid (RAR) and triiodothyronine (TR) nuclear receptor mRNA in rat brain: effect of the administration of retinoids. *FEBS Lett*, 412, 629-632.
- Enderlin V, Pallet V, Alfos S, Dargelos E, Jaffard R, Garcin H & Huguieret P (1997b) Age-related decreases in mRNA for brain nuclear receptors and target genes are reversed by retinoic acid treatment. *Neurosci Lett*, 229, 125-129.
- Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME & Robertson EJ (1993) Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proc Natl Acad Sci U S A*, 90, 2375-2379.

- Etchamendy N, Enderlin V, Marighetto A, Vouimba RM, Pallet V, Jaffard R & Higeret P (2001) Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J Neurosci*, 21, 6423-6429.
- Frick KM, Baxter MG, Markowska AL, Olton DS & Price DL (1995) Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiol Aging*, 16, 149-160.
- Gage FH, Dunnett SB & Bjorklund A (1989) Age-related impairments in spatial memory are independent of those in sensorimotor skills. *Neurobiol Aging*, 10, 347-352.
- George-Hyslop PH (2000) Genetic factors in the genesis of Alzheimer's disease. *Ann N Y Acad Sci*, 924, 1-7.
- Goodman AB & Pardee AB (2003) Evidence for defective retinoid transport and function in late onset Alzheimer's disease. *Proc Natl Acad Sci U S A*, 100, 2901-2905.
- Hodges H (1996) Maze procedures: the radial-arm and water maze compared. *Brain Res Cogn Brain Res*, 3, 167-181.
- Hollup SA, Kjelstrup KG, Hoff J, Moser MB & Moser EI (2001) Impaired recognition of the goal location during spatial navigation in rats with hippocampal lesions. *J Neurosci*, 21, 4505-4513.
- Igaz LM, Vianna MR, Medina JH & Izquierdo I (2002) Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci*, 22, 6781-6789.
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B & Speed TP (2003a) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*, 31, e15.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U & Speed TP (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, 4, 249-264.

- Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH & Evans RM (2006) Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A*, 103, 3902-3907.
- Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294, 1030-1038.
- Kril JJ, Patel S, Harding AJ & Halliday GM (2002) Neuron loss from the hippocampus of Alzheimer's disease exceeds extracellular neurofibrillary tangle formation. *Acta Neuropathol (Berl)*, 103, 370-376.
- Lanahan A & Worley P (1998) Immediate-early genes and synaptic function. *Neurobiol Learn Mem*, 70, 37-43.
- Lane MA & Bailey SJ (2005) Role of retinoid signalling in the adult brain. *Prog Neurobiol*, 75, 275-293.
- Lee JI & Ahnn J (2004) Calcineurin in animal behavior. *Mol Cells*, 17, 390-396.
- Lee ML, Kuo FC, Whitmore GA & Sklar J (2000) Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci U S A*, 97, 9834-9839.
- Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J & Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. *Nature*, 429, 883-891.
- Mansuy IM, Mayford M, Jacob B, Kandel ER & Bach ME (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell*, 92, 39-49.
- Mattson MP & Magnus T (2006) Ageing and neuronal vulnerability. *Nat Rev Neurosci*, 7, 278-294.
- Merched A, Serot JM, Visvikis S, Aguillon D, Faure G & Siest G (1998) Apolipoprotein E, transthyretin and actin in the CSF of Alzheimer's patients: relation with the senile plaques and cytoskeleton biochemistry. *FEBS Lett*, 425, 225-228.
- Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF & Evans RM (2001) Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci U S A*, 98, 11714-11719.

- Monaco HL (2000) The transthyretin-retinol-binding protein complex. *Biochim Biophys Acta*, 1482, 65-72.
- Monti B, Berteotti C & Contestabile A (2005) Dysregulation of memory-related proteins in the hippocampus of aged rats and their relation with cognitive impairment. *Hippocampus*, 15, 1041-1049.
- Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*, 11, 47-60.
- Moser E, Moser MB & Andersen P (1993) Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *J Neurosci*, 13, 3916-3925.
- Mouravlev A, Dunning J, Young D & During MJ (2006) Somatic gene transfer of cAMP response element-binding protein attenuates memory impairment in aging rats. *Proc Natl Acad Sci U S A*, 103, 4705-4710.
- Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P & Buxbaum JD (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *JAMA*, 283, 1571-1577.
- Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrang P, Canet E & Boutin JA (2000) Identification of the melatonin-binding site MT3 as the quinone reductase 2. *J Biol Chem*, 275, 31311-31317.
- Riedel G, Micheau J, Lam AG, Roloff EL, Martin SJ, Bridge H, de Hoz L, Poeschel B, McCulloch J & Morris RG (1999) Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat Neurosci*, 2, 898-905.
- Rowe WB, Spreekmeester E, Meaney MJ, Quirion R & Rochford J (1998) Reactivity to novelty in cognitively-impaired and cognitively-unimpaired aged rats and young rats. *Neuroscience*, 83, 669-680.
- Schulz D, Huston JP, Jezek K, Haas HL, Roth-Harer A, Selbach O & Luhmann HJ (2002) Water maze performance, exploratory activity, inhibitory avoidance and hippocampal plasticity in aged superior and inferior learners. *Eur J Neurosci*, 16, 2175-2185.

- Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK & . (1994) Transthyretin sequesters amyloid beta protein and prevents amyloid formation. *Proc Natl Acad Sci U S A*, 91, 8368-8372.
- Serot JM, Christmann D, Dubost T & Couturier M (1997) Cerebrospinal fluid transthyretin: aging and late onset Alzheimer's disease. *J Neurol Neurosurg Psychiatry*, 63, 506-508.
- Silva AJ (2003) Molecular and cellular cognitive studies of the role of synaptic plasticity in memory. *J Neurobiol*, 54, 224-237.
- Smith JE, Milch PO, Muto Y & Goodman DS (1973) The plasma transport and metabolism of retinoic acid in the rat. *Biochem J*, 132, 821-827.
- Snyder JS, Hong NS, McDonald RJ & Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. *Neuroscience*, 130, 843-852.
- Sousa JC, Grandela C, Fernandez-Ruiz J, de Miguel R, de Sousa L, Magalhaes AI, Saraiva MJ, Sousa N & Palha JA (2004) Transthyretin is involved in depression-like behaviour and exploratory activity. *J Neurochem*, 88, 1052-1058.
- Stein TD, Anders NJ, DeCarli C, Chan SL, Mattson MP & Johnson JA (2004) Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. *J Neurosci*, 24, 7707-7717.
- Stein TD & Johnson JA (2002) Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J Neurosci*, 22, 7380-7388.
- Stemmelin J, Lazarus C, Cassel S, Kelche C & Cassel JC (2000) Immunohistochemical and neurochemical correlates of learning deficits in aged rats. *Neuroscience*, 96, 275-289.

- Taubenfeld SM, Milekic MH, Monti B & Alberini CM (2001) The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. *Nat Neurosci*, 4, 813-818.
- Tombaugh GC, Rowe WB, Chow AR, Michael TH & Rose GM (2002) Theta-frequency synaptic potentiation in CA1 in vitro distinguishes cognitively impaired from unimpaired aged Fischer 344 rats. *J Neurosci*, 22, 9932-9940.
- Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC & Pavlidis P (2004) Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice. *Learn Mem*, 11, 253-260.
- Wei S, Episkopou V, Piantedosi R, Maeda S, Shimada K, Gottesman ME & Blaner WS (1995) Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. *J Biol Chem*, 270, 866-870.
- Yukawa K, Tanaka T, Tsuji S & Akira S (1998) Expressions of CCAAT/Enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca²⁺/calmodulin kinases activation in hippocampal neurons. *J Biol Chem*, 273, 31345-31351.

Chapter IV

The common environmental pollutant dioxin induced memory deficits by altering estrogen pathways and a major route of retinol transport involving transthyretin

Preface

Dysfunction of many neurotransmitter pathways has been postulated over the past few decades to be associated with memory losses occurring in aging. The role of the retinol transporter TTR in the maintenance of memory capacities during aging (Chapter III) supports the small, growing body of evidence indicating a role for altered RA pathways in age-related memory deficits (Chiang et al., 1998; Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002). To further investigate the effects of RA in learning and memory functions, we used the common environmental pollutant dioxin (also known as TCDD; 2,3,7,8-tetrachlorodibenzo-p-dioxin) to empty retinol storage (Kelley et al., 2000; Nilsson et al., 2000; Hoegberg et al., 2005). Since the role of TCDD in memory processes has not been studied in detail, the present report also aims at elucidating the potential action and mechanism implicating this toxic agent in various aspects of cognitive functions.

We observed that a treatment with TCDD induced robust memory impairments in female but not male mice. This gender-specific effect of TCDD on learning abilities was reversed by estrogen treatment. Furthermore, the administration of RA by itself or with estrogen abolished TCDD-induced memory deficits, suggesting significant cross-talk between these two signalling pathways in blocking the negative effects of TCDD on cognitive processes. The reduction of TCDD-induced cognitive impairments in TTR knock-out ($TTR^{-/-}$) mice also suggests that dioxin can alter memory function by disturbing the main route of retinol's delivery into the brain. Given that TCDD is a prototypical member of halogenated aromatic hydrocarbon contaminants, mechanisms underlying dioxin-induced memory impairments could lead to potential novel targets to block the deleterious effects of pollutants on normal cognitive processing.

The common environmental pollutant dioxin induced memory deficits by altering estrogen pathways and a major route of retinol transport involving transthyretin

Jonathan Brouillette ^a & Rémi Quirion ^{a,b,*}

Department of Psychiatry ^b and Neurology & Neurosurgery ^a, Douglas Mental Health University Institute, McGill University, Montréal, Québec, Canada

*** Corresponding author:**

Prof. Rémi Quirion, PhD, OC, FRSC, CQ
Douglas Mental Health University Institute, McGill University
6875 LaSalle Boulevard
Montréal, Québec
H4H 1R3

Tel.: (514) 761-6131 (ext 2934)

Fax: (514) 762-3034

E-mail: remi.quirion@douglas.mcgill.ca

Keywords: dioxin, spatial memory, estrogen, retinoic acid, transthyretin

Abstract

Many toxic environmental and food agents have been suspected to be potential risk factors in inducing memory disabilities under normal and pathological conditions. 2,3,7,8-tetrachlorodibenzo-p-dioxin (known as dioxin or TCDD) is a common and prototypical member of a class of noxious environmental and food contaminants called the halogenated aromatic hydrocarbons. Since the role of dioxin in memory processes has not been studied in detail, the present report aims at elucidating the role of this pollutant in the maintenance of cognitive function. We found that TCDD (50 $\mu\text{g/kg}$) induced spatial memory deficits in the Morris water maze (MWM) task in female but not male mice. This sex-dependant effect of dioxin seems to be related to the alteration of estrogen pathways, as treatment with 17 β -Estradiol-3-benzoate (E; 5 $\mu\text{g/day}$) reversed memory deficits induced by TCDD. We also observed that cognitive impairments produced by dioxin, which is known to interfere with retinoid turnover and metabolism, were abolished by retinoic acid (RA) treatment (150 $\mu\text{g/kg}$). The cognitive effects of E and RA treatments seem to derive from common rather than additive mechanisms since memory deficits produced by TCDD were fully reversed by these compounds when used separately or in combination. Attenuation of dioxin-induced memory deficits in mice lacking transthyretin (TTR) suggests that TCDD may be acting by affecting the major route of retinol transport involving TTR. Taken together, these results suggest that the environmental and food pollutant TCDD can induce memory deficits by altering the estrogen pathways and a main route of TTR-mediated retinol transport.

Introduction

According to the World Health Organization, dioxin is a widespread toxic environmental and food contaminant. This highly noxious agent is present in air, water and soil mainly because of pollution generated by industrialized societies such as large-scale burning of municipal and medical waste, fuel and wood burning, tobacco smoke, production of iron and steel, bleaching process of paper pulp, coal-fired electric power generation and pesticides. This prototypical member of the halogenated aromatic hydrocarbons (HAHs) can also move into food chain and contaminate meat, milk products (breast milk) as well as fish and to a lower level fruits, vegetables and grains. Since dioxin is stored in body fat, its half-life is very long (around 7-8 years in adult humans) and thus can accumulate in the organism over time (Pohjanvirta et al., 1990). The acute toxicity of TCDD in humans was shown to be in the range of 70-300 $\mu\text{g/kg}$, with 10 pg/kg/day representing a safe lifetime exposure level (Rozman, 1989). Several industrial accidents or natural disasters such as forest fires and volcanic eruptions have exposed many individuals to high levels of TCDD and other HAHs. It has been established that most of the effects exerted by TCDD and other HAHs are mediated through binding to the nuclear arylhydrocarbon receptor (AhR), which in turn increases or decreases the expression of AhR-regulated genes (Schmidt & Bradfield, 1996).

A few evidence suggest that this common noxious pollutant may alter memory capacities. Electrophysiological data have shown that TCDD represses excitatory post-synaptic potentials (EPSPs) in rat hippocampal slices (Hong et al., 1998). Air force veterans highly exposed to Agent Orange containing TCDD during the Vietnam War show deficits in several measures of memory functioning (Michalek et al., 2001). Long-term cognitive deficits have been reported following prenatal exposure to low levels of TCDD in monkeys (Schantz & Bowman, 1989) and rats (Seo et al., 1999; Powers et al., 2005). However, memory deficits in adult animals administered with TCDD remains to be investigated.

The mechanisms underlying the effect of this toxic agent on cognitive processes are also mostly unknown. A close association between disturbances in vitamin A (retinol) homeostasis and toxicity of dioxin was first proposed on the basis of similar symptoms seen in hypovitaminosis A and TCDD-exposed animals (Thunberg et al., 1980). Various studies have reported that TCDD interferes with retinoid turnover and metabolism by increasing retinoid mobilization from retinyl ester stores (Brouwer et al., 1989; Jurek et al., 1990; Van Birgelen et al., 1995; Kelley et al., 2000), altering retinol esterification (Nilsson et al., 2000; Hoegberg et al., 2003), changing tissue levels of retinoic acid (Kelley et al., 1998; Nilsson et al., 2000; Hoegberg et al., 2003), augmenting whole body turnover of retinoids (Kelley et al., 1998), and increasing excretion of retinoid metabolites (Hakansson & Ahlborg, 1985; Brouwer et al., 1989).

Retinol is stored in the liver as retinyl ester and secreted into the circulation bound to its specific transport protein, retinol-binding protein (RBP) in response to physiological needs. Retinol bound to RBP secreted by the liver represents 95-99% of the vitamin A present in the circulation. This binary complex binds to the plasma protein transthyretin (TTR) to form the major retinol transport route from its storage in the liver to target tissues. TTR was shown to prevent renal glomerular filtration and influence secretion of the retinol-RBP complex from the liver (Monaco, 2000). Once delivered to the target organs, retinol is metabolized into retinoic acid (RA) which acts on specific nuclear receptors to modulate a wide variety of biological processes including synaptic plasticity (Misner et al., 2001), neurogenesis (Snyder et al., 2005; Jacobs et al., 2006) as well as long-term potentiation (LTP) and long-term depression (LTD), two synaptic models of memory (Chiang et al., 1998; Etchamendy et al., 2001). The hypothesis that TCDD-induced memory deficits could be associated to its capacities to altered retinol metabolism by acting on its delivery route has yet to be investigated.

Several studies have reported that some effects of dioxin are sex-dependant and may occur through the inhibition of estrogen signalling pathways (Tritscher et al., 1996; Safe et al., 2000; Wormke et al., 2000; Wyde et al., 2001).

Considerable evidence suggest that estrogen is not only involved in reproduction but also in other neural mechanisms such as learning and memory (Sherwin, 1994). Estrogen receptors are widely distributed in the hippocampus and other brain areas implicated in memory processing (Shughrue et al., 1997; Shughrue & Merchenthaler, 2000; Hart et al., 2001). Electrophysiological data have shown that estrogen increases LTP and N-methyl-D-aspartate (NMDA)-mediated EPSPs, whereas it can attenuate the inhibition mediated by gamma-aminobutyric acid (GABA) (Cordoba Montoya & Carrer, 1997; Rudick & Woolley, 2001; Gureviciene et al., 2003). Although results from behavioural studies investigating the role of estrogen in the maintenance or improvement of memory capacities are somewhat controversial, evidence suggests that estrogen administered to young ovariectomized as well as aged female rodents can enhance spatial and non-spatial learning and memory (Packard & Teather, 1997; Gibbs, 1999; Rissanen et al., 1999; Sandstrom & Williams, 2001; Frick et al., 2002; Vaucher et al., 2002; but also Fugger et al., 1998; Chesler & Juraska, 2000). However, it remains to be established if dioxin can interact with estrogen pathways to alter cognitive abilities.

The present study investigates the potential role of TCDD on learning abilities and potential mechanisms relevant to this effect. We observed that TCDD induced robust memory impairments in the Morris water maze (MWM) task in female but not male mice. These deficits were abolished by treatment with 17 β -Estradiol-3-benzoate (E) and/or RA. Furthermore, a decrease of TCDD-induced memory deficits in TTR knock-out (TTR^{-/-}) mice suggest that this agent acts on a major route of TTR-mediated retinol transport to alter memory functions.

Materials and Methods

Animals

Wild-type (WT; $n = 77$) and $TTR^{-/-}$ mice ($n = 14$; F2 generation maintained on a mixed C57BL/6 x 129S background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in groups of maximum four per cage and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. The mice were bred in our animal facility and behavioural testing was started at 3-months of age. Animal care and handling procedures were approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Drug administration

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (ACP Chemicals, Inc., St-Leonard, Qc, Canada) was dissolved in corn oil (vehicle) and a single dose (50 $\mu\text{g/kg}$) was administered intraperitoneally (ip) to 3-months old mice 72 h before the first training day in the MWM, a time point at which difference in storage levels of retinol becomes evident (Thunberg et al., 1979). In addition, a separate group of 3-months old male mice ($n = 7$) received a single high dose (500 $\mu\text{g/kg}$) of TCDD 72 h before testing in the MWM. Control animals were injected with an equal volume of corn oil at the same time as the TCDD treated mice. The dose (50 $\mu\text{g/kg}$) and route of administration used here have previously been shown to change retinol bioavailability without lethality in mice with the same mixed C57BL/6 x 129S background (Hoegberg et al., 2005).

Retinoic acid (RA; Sigma, St-Louis, MO, USA) was dissolved in a vehicle solution containing polyethyleneglycol, NaCl (0.9%), and ethanol (70:20:10 by volume). RA was injected subcutaneously (sc) and administered daily in a single dose (150 $\mu\text{g/kg}$) to 3-months old mice. Treatments began four days prior to behavioural testing and continued until the end of testing in the MWM. An equal volume of vehicle solution was similarly administered to control animals. The treatment duration, time of injection (18:00 hrs), dose and route of administration

used here have previously been shown to reverse age-related memory deficits in mice (Etchamendy et al., 2001) and rats (Brouillette & Quirion, 2007).

17 β -Estradiol-3-benzoate (E; Sigma, St-Louis, MO, USA) was dissolved in sesame oil (vehicle). Daily administration of E (5 μ g/0.05 ml, sc) and vehicle (0.05 ml) started 5 days prior to behavioural testing and continued until the end of testing in the MWM. This form of estradiol was used since it is slowly metabolized leading to a relatively stable circulating estradiol levels following chronic administration at this dose (Frick et al., 2002). Estrogen pathway is known to crosstalk with RA signalling (Li & Ong, 2003; Li et al., 2004; Laganier et al., 2005), and both pathways are affected by TCDD (Lu et al., 1994; Toyoshiba et al., 2004; Widerak et al., 2006). Thus, we used gonadally intact female mice to avoid alteration of RA signalling and alteration of TCDD-induced memory deficits as seen in male mice. The treatment used in the present study was previously shown to improve spatial reference memory in aged female mice during the MWM task (Frick et al., 2002).

Morris water maze task

Spatial memory was determined using the reference (long-term) memory version of the MWM task, where the hidden escape platform remained in the centre of the same quadrant throughout all training days (Morris, 1984). In a 1.4 m diameter pool, mice were required to find a submerged platform (15 cm in diameter) located 1 cm below the surface of water (25°C), rendered opaque by the addition of skim milk powder. Animals were pseudo-randomly started from a different position at each trial and used distal visuo-spatial cues to find the hidden escape platform (Morris, 1984). Each animal were given three trials of 90 s per day with a 40 min inter-trial interval over five consecutive days. Animals were guided to the platform if not located within 90 s and all mice remained there for 10 s before removal.

After the acquisition phase on day five, mice were given one probe trial of 60 s in which the platform was removed from the pool to evaluate the number of times the animal crossed the previous location of the platform, time spent in the

target quadrant, and swimming speed. This trial was followed by one cued trial of 60 s in which the platform was visible to assess visual, sensorimotor or motivational deficits to escape from water. After each trial, animals were immediately placed under heat lamps to dry and prevent hypothermia. The experimenter was blind to the genotype and treatment performed on mice. Data derived from the MWM task were recorded on a computer using a video tracking system (HVS, Buckingham, UK).

All the animals ($n = 91$) were tested in one large experiment in the MWM to allow comparison and statistical analysis between each group. Because this study is composed of thirteen different groups, the data are presented in five graphs for clarity. Statistical significance was assessed using the two-way, one between, one within ANOVA design and simple main effects were analysed with F-test when the interaction was significant. Tukey's Honestly Significant Difference test was used as a *post hoc* pair wise comparison test when necessary. The alpha level used was $p < 0.05$.

Results

TCDD-induced memory deficits in MWM task

Female and male (3-months old) mice treated 72 h prior to MWM training with a single dose of TCDD (50 µg/kg, ip) and control animals treated with corn oil (vehicle) were tested in the reference memory version of the MWM task, where the hidden escape platform stayed at the same location during all training days (Fig. 1A and B). The two-way, one between, one within ANOVA conducted on the mean escape latencies of female mice yielded a significant group x days interaction ($F_{4,48} = 3.62$; $p < 0.0117$). Simple main effect tests revealed that female mice treated with TCDD showed longer escape latency on day two to five compared to control animals. Treatment with TCDD in male mice produced no significant effect on the mean escape latencies even after administration of a high dose of TCDD (500 µg/kg).

The effect of TCDD on learning abilities was validated immediately after the end of training by performing a probe trial of 60 s in which the platform was removed. Female mice treated with TCDD, unlike male and control female mice, showed no preference for the quadrant that previously contained the platform (Fig. 1C) and fewer platform crossings (Fig. 1D). The longer escape latency of TCDD treated mice cannot be attributed to slower swimming speed since no significant variations were observed in any groups (Fig. 1E). The various groups also show similar escape latencies during a 60 s cued trial in which the platform was visible, indicating the absence of visual, sensorimotor or motivational deficits to escape from water.

TCDD-induced memory deficits in female mice are reversed by estrogen treatment

Since TCDD has been shown to inhibit estrogen signalling pathways (Tritscher et al., 1996; Wormke et al., 2000; Wyde et al., 2001), we hypothesized that the cognitive deficits observed in female mice following TCDD treatment could be attributed to a disruption of this pathway. 17β-Estradiol-3-benzoate (E; 5

$\mu\text{g}/0.05\text{ ml}$, sc, once a day) was administered for five days prior to behavioural testing and every day until the end of testing to induce a stable, high level of circulating estradiol in female mice (Frick et al., 2002). This treatment suppresses memory deficits observed following exposure to dioxin during the acquisition phase (Fig. 2A). Learning deficits were confirmed during the probe trial and reversed by E treatment in TCDD treated mice (Fig. 2B and C). No significant changes were detected in swimming speed and escape latency to a visible platform in all groups (Fig. 2D and E).

Treatment with retinoic acid by itself or in combination with estrogen reversed TCDD-induced amnesia

To investigate if TCDD-induced memory deficits could be linked to its effects on retinol metabolism, we chronically administered retinoic acid (RA; $150\text{ }\mu\text{g}/\text{kg}$, sc, once a day) for four days prior to behavioural testing and every day until the end of testing. This treatment reversed memory impairments during the acquisition phase (Fig. 3A) and the probe trial (Fig. 3B and C) without altering swimming speed and escape latency to a visible platform (Fig. 3D and E). Co-treatment with RA and E (Fig. 4) improved learning abilities in a manner similar to either one by itself. Combination of these two treatments enhanced cognitive abilities of TCDD exposed mice during the training days (Fig. 4A) and probe trial (Fig. 4B and C) without altering swimming speeds and escape latencies to a visible platform (Fig. 4D and E). The acquisition curves obtained with RA and E injected alone or together was not significantly different, indicating common rather than additive effects.

TCDD-induced memory deficits are decreased in transthyretin knock-out mice

One of the key functions of TTR is to deliver retinol from its storage in the liver to target tissues where it can be metabolized into RA. To determine if the mechanism sustaining the effect of TCDD on cognition is dependent on the major retinol transport route involving TTR, we applied the same treatment with TCDD

(50 µg/kg, ip, 72 h prior to MWM training) to adult TTR knock-out (TTR^{-/-}) female mice. TCDD-induced memory impairments were not seen in TTR^{-/-} mice treated or not with TCDD during the training days (Fig. 5A). TTR^{-/-} mice injected with dioxin showed a slight but not significant increase in escape latency compared to control mice during the acquisition phase (Fig. 5A) and these results were confirmed during the probe trial (Fig. 5B and C). These data are in agreement with previous observation showing no significant memory deficits in 3-months old TTR^{-/-} male mice during the MWM task (Brouillette & Quirion, 2007). Performance of the various groups during the MWM task could not be attributed to the difference in the swimming speed (Fig. 5D) or to visual, sensorimotor or motivational deficits (Fig. 5E), as no significant differences were observed in the speed and escape latency to a visible platform.

Figures

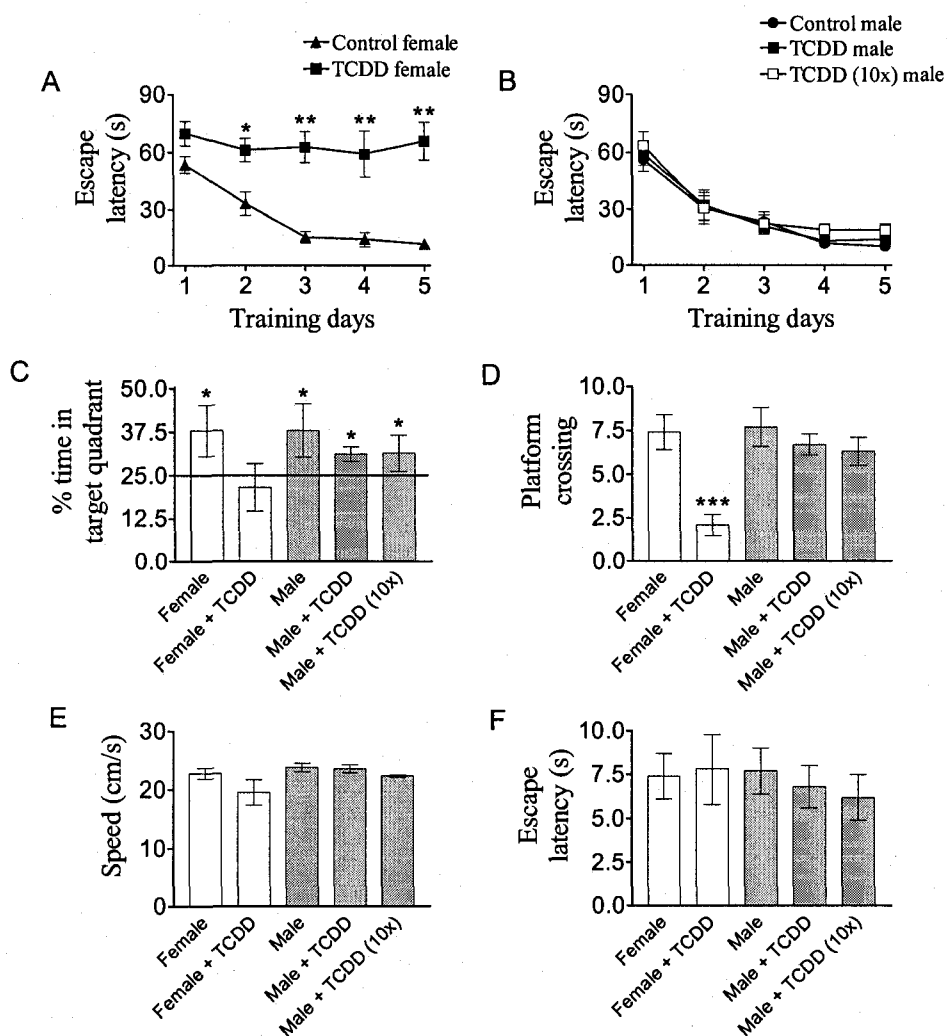


Fig. 1. TCDD exposed mice show spatial memory deficits in the MWM task.

(A) Escape latencies before finding the hidden platform (three trials of 90 s per day with a 40 min inter-trial interval) are longer in 3-months old female mice treated with TCDD (filled squares; 50 $\mu\text{g/kg}$; ip; 3 days before training; $n = 7$) than control animals injected with corn oil vehicle (filled triangles; $n = 7$; * $p < 0.05$, ** $p < 0.01$; two-way ANOVA). (B) The same dioxin treatment produces no change in 3-months old male mice (filled squares; $n = 7$) even after increasing the dose of TCDD by ten fold (500 $\mu\text{g/kg}$; open squares; $n = 7$) when compared to

control animals (filled circles; $n = 7$; $p > 0.05$; two-way ANOVA). **(C)** Percent time spent in the quadrant that previously contained the platform during the probe trial. Error bars ($\pm$ 95% confidence interval of the mean) of dioxin treated female mice overlap the 25% line which represents the chance level of being randomly in one of the four virtual quadrants whereas male mice treated or not with TCDD spent more time in the target quadrant. Asterisk represents significant differences from the 25% chance performance (* $p < 0.05$). **(D)** Female mice treated with dioxin crossed the previous location of the platform fewer times than control animals (***) $p < 0.001$; one-way ANOVA) during the probe trial. **(E)** Treatment with TCDD produced no significant difference in swimming speed during the probe trial and **(F)** similar escape latency to a visible platform among all the groups ($p > 0.05$; one-way ANOVA). All results, except for **(C)** are mean $\pm$ SEM.

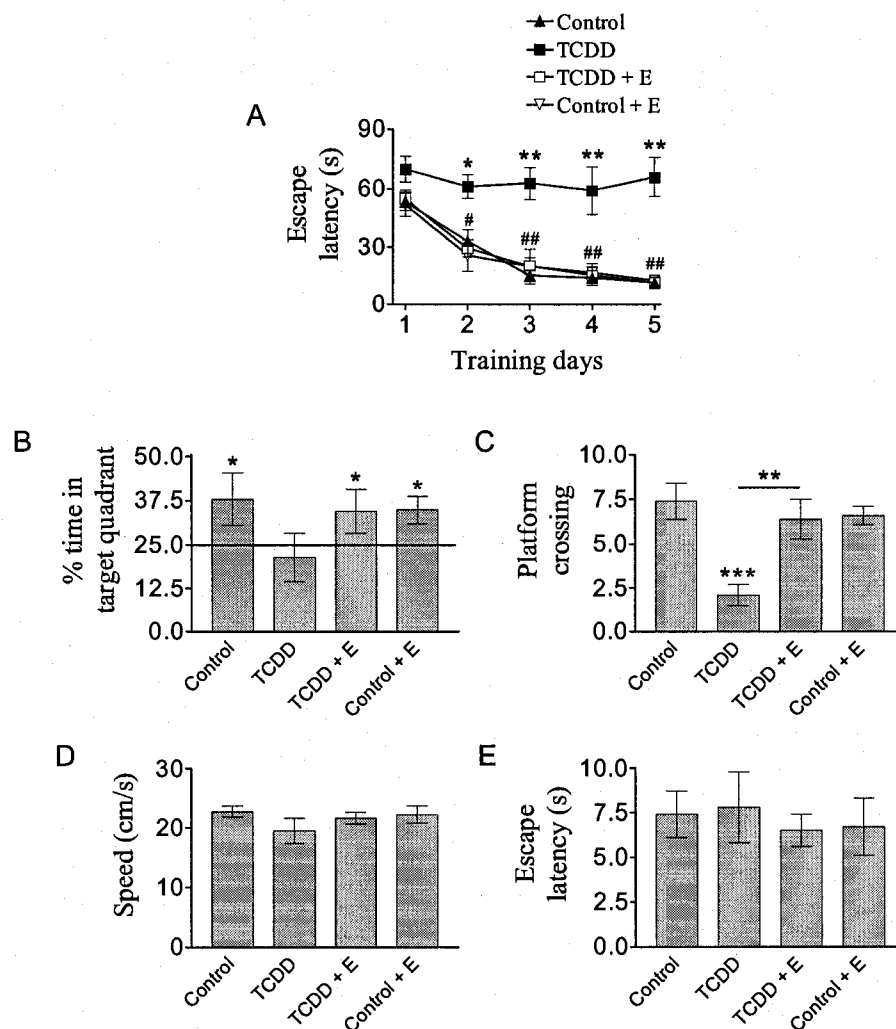


Fig. 2. Treatment with 17 β -Estradiol-3-benzoate (E) reversed memory deficits induced by TCDD in the MWM task. (A) Longer escape latencies before finding the hidden platform in dioxin treated female mice (filled squares; $n = 7$) compared to control animals (filled triangles; $n = 7$; * $p < 0.05$, ** $p < 0.01$; two-way ANOVA) were abolished with E treatment (open squares; 5 $\mu\text{g}/0.05 \text{ ml}$; sc; once a day for ten days; $n = 7$; # $p < 0.05$, ## $p < 0.01$; two-way ANOVA). The same E treatment in control mice (open triangles, $n = 7$) did not affect their performance ($p > 0.05$; two-way ANOVA). **(B)** The time spent in the target quadrant during the probe trial ($\pm$ 95% confidence interval) by TCDD treated mice is around 25% chance level, whereas mice co-administered with TCDD and E as well as control animals injected or not with E swam longer in the target

quadrant (* $p < 0.05$). (C) Mice exposed to dioxin crossed the previous site of the platform less frequently than control rodents (*** $p < 0.001$; one-way ANOVA) and this effect was suppressed by injection of E (** $p < 0.01$; one-way ANOVA). (D) Treatment with E produced no significant difference in swimming speed in control and TCDD exposed mice during the probe trial and (E) similar escape latency to a visible platform. All results, except for (B) are mean $\pm$ SEM.

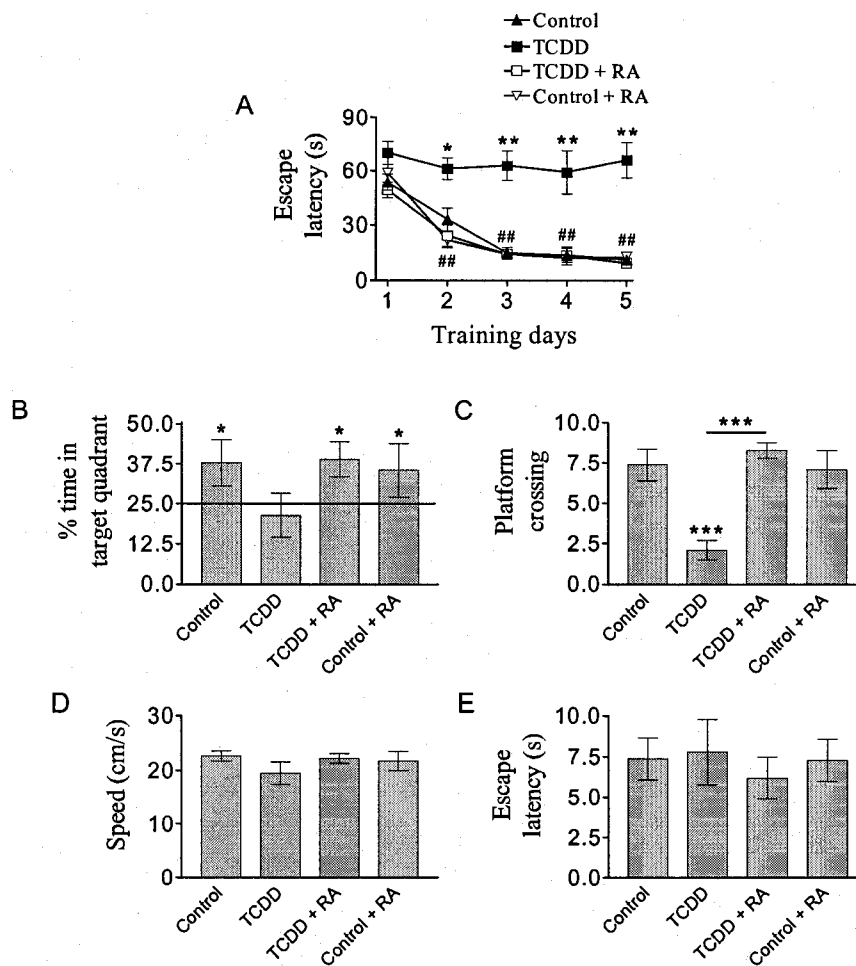


Fig. 3. Retinoic acid (RA) treatment suppressed memory impairments induced by TCDD in the MWM task. (A) Longer escape latencies before finding the hidden platform in TCDD treated female mice (filled squares; $n = 7$) compared to control animals (filled triangles; $n = 7$) were reversed by treatment with RA (open squares; 150 $\mu\text{g/kg}$; sc; once a day for nine days; $n = 7$; ## $p < 0.01$; two-way ANOVA). Performance of control mice was not altered by RA treatment (open triangles, $n = 7$; $p > 0.05$; two-way ANOVA). (B) Administration of RA in mice exposed to TCDD increased the time spent in the target quadrant (* $p < 0.05$; $\pm$ 95% confidence interval) and (C) the number of platform crossing during the probe trial (*** $p < 0.001$; one-way ANOVA). (D) RA treatment induced no significant difference in swimming speed in control and TCDD treated rodents during the probe trial and (E) similar escape latency to a visible platform. All results, except for (B) are mean $\pm$ SEM.

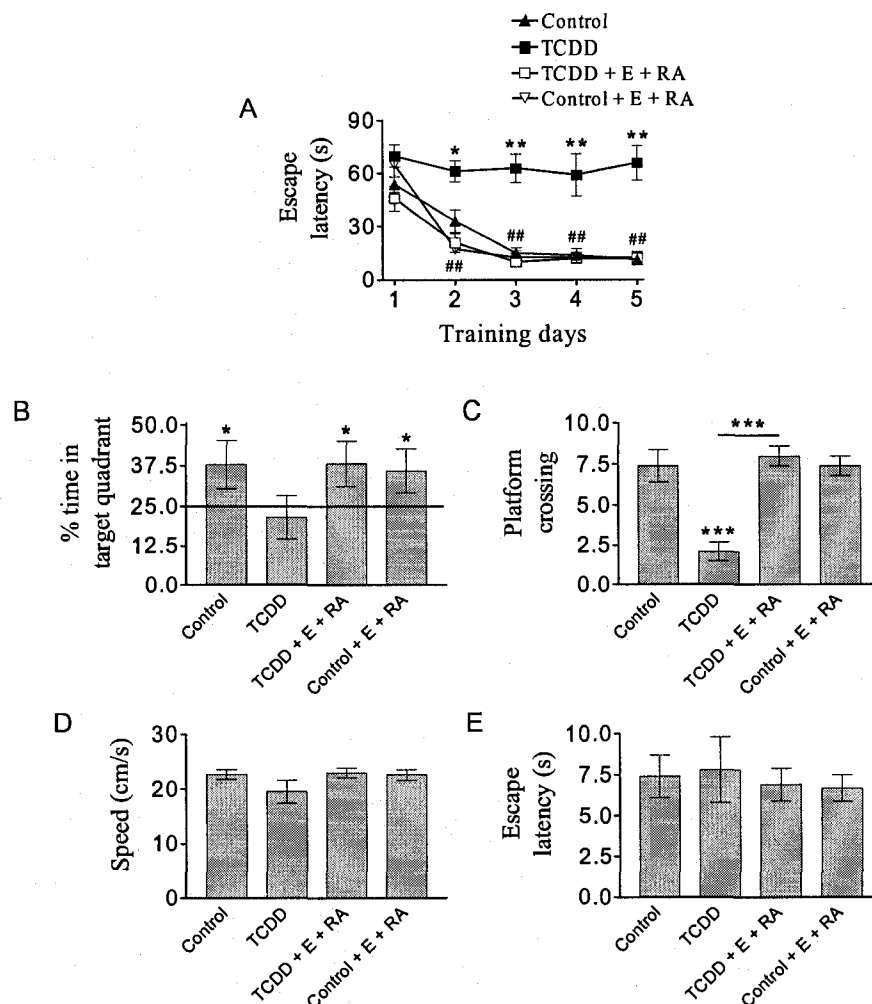


Fig. 4. Co-treatment with RA and E abolished memory deficits induced by TCDD in the MWM task. (A) Longer escape latencies in TCDD exposed female mice (filled squares; $n = 7$) were reversed by a co-treatment with RA and E (open squares; $n = 7$; $^{##}p < 0.01$; two-way ANOVA). Co-administration of RA and E in control mice (open triangles, $n = 7$) did not affect their acquisition curves ($p > 0.05$; two-way ANOVA). **(B)** Injection of RA and E in TCDD treated mice augmented the time spent in the target quadrant ($*p < 0.05$; $\pm$ 95% confidence interval) and **(C)** the number of platform crossing during the probe trial ($^{***}p < 0.001$; one-way ANOVA). **(D)** Co-treatment with RA and E produced comparable swimming speed in control and TCDD exposed mice during the probe trial and **(E)** similar escape latency to a visible platform. All results, except for **(B)** are mean $\pm$ SEM.

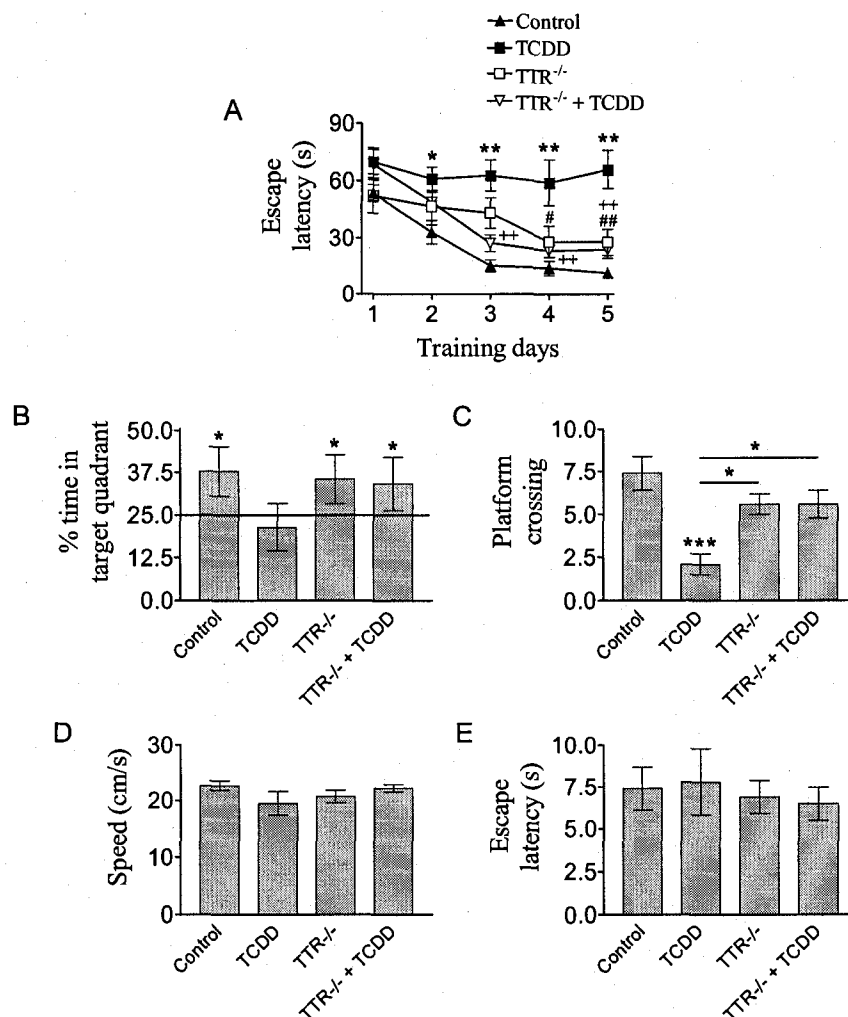


Fig. 5. TCDD-induced memory deficits are attenuated in TTR^{-/-} mice. (A) Longer escape latencies in TCDD exposed female mice (filled squares; $n = 7$) were decreased in TTR^{-/-} mice (open squares; $n = 7$; # $p < 0.05$; ## $p < 0.01$; two-way ANOVA) and when treated with dioxin (open triangles; $n = 7$; ++ $p < 0.01$; two-way ANOVA). (B) TTR^{-/-} mice injected with or not TCDD spent more time in the target quadrant (* $p < 0.05$; $\pm$ 95% confidence interval) and (C) cross the previous location of the platform more often than wild-type control animals exposed to dioxin during the probe trial (* $p < 0.05$; *** $p < 0.001$; one-way ANOVA). (D) TTR^{-/-} and control littermates with or without administration of TCDD had similar swimming speed during the probe trial and (E) comparable escape latency to a visible platform. All results, except for (B) are mean $\pm$ SEM.

Discussion

Normal cognitive functions can be altered by noxious pollutants from the environment and food. Studies on the deleterious mnemonic effects of TCDD, a prototypical member of the highly toxic HAHs, could reveal mechanisms through which this class of widespread environmental contaminants affect learning and memory processes. Our report provides behavioural and pharmacological evidence that TCDD induces severe memory deficits in female but not male mice. This gender-specific effect of TCDD on learning abilities was reversed by E treatment. Furthermore, RA injected alone or with E abolished TCDD-induced memory deficits, suggesting significant cross-talk between these two signalling pathways to the effects of TCDD on cognition. Reduction of TCDD-induced mnemonic impairments in $TTR^{-/-}$ mice indicated that dioxin can alter memory function by disturbing the main route of retinol delivery to the brain.

The severe cognitive impairments induced by a single dose of TCDD administered three days before behavioural testing indicates that this toxin can produce long-term cognitive disturbances. Our study provides the first evidence that administration of dioxin in young adult female mice induces marked memory deficits. This result is in accordance with previous reports showing that prenatal exposure to TCDD and other HAHs alter spatial memory capacities during adulthood in mice (Powers et al., 2005), rats (Seo et al., 1999), monkeys (Schantz & Bowman, 1989) and humans (Stewart et al., 2003). Decreased spatial memory performances of prenatally TCDD-exposed arylhydrocarbon receptor heterozygous ($AhR^{+/-}$) female mice suggest that dioxin-induced memory deficits are at least partially AhR dependent (Powers et al., 2005). Alternatively, TCDD can also penetrate into the brain (Cheng et al., 2002) and rapidly decrease EPSPs in the CA1 region of the hippocampus, indicating that this compound can also affect memory independently of AhR-linked gene induction (Hong et al., 1998) which requires several hours (Safe, 1995). Interestingly, NMDA receptors which are critical during synaptic plasticity underlying memory processes were shown to

be down-regulated in offspring following prenatal exposure to dioxin (Nayyar et al., 2003).

The present study is the first to demonstrate that the effect of TCDD on memory functioning is, at least partly, related to its action on estrogen pathways. Our findings is in agreement with reports showing that prenatal dioxin exposure decreased cognitive performance during adulthood in mice (Seo et al., 1999; Powers et al., 2005) and support a large body of evidence indicating that most of the pleiotropic effects of TCDD are gender-dependant in various species and strains (Mably et al., 1992; Wyde et al., 2001; Thornton et al., 2004; Myllymaki et al., 2005; Hojo et al., 2006). Several reports have suggested interactions between signalling pathways initiated by AhR and estrogen receptors (Tritscher et al., 1996; Wormke et al., 2000; Wyde et al., 2001). TCDD is a high affinity ligand for AhR that modulates numerous endocrine pathways including the inhibition of 17 β -Estradiol-induced responses in various cell types (Romkes et al., 1987; Umbreit & Gallo, 1988; Umbreit et al., 1988; Astroff et al., 1990; Astroff & Safe, 1990; DeVito et al., 1992). Moreover, DNA damage produced by TCDD was shown to occur *via* an estrogen-dependent mechanism (Wyde et al., 2001). In the present study, we observed that an increased level of E, which is derived from the aromatization of testosterone in male brain (Mooradian et al., 1987), was able to suppress TCDD-induced memory impairments. This finding strengthens the existence of functional interactions between these two pathways.

TCDD is well known to induce major changes in vitamin A turnover and metabolism. Dioxin can decrease retinoid levels by concomitantly enhancing mobilization of retinoids from hepatic storage sites into serum and by increasing their elimination from the kidneys (Brouwer et al., 1989). The positive effects on learning seen with the RA treatment in female mice injected with TCDD provide the first behavioural evidence that dioxin-induced cognitive deficits are linked, at least in part, to its capacity to empty retinoid stocks. Decreasing the bioavailability of RA following exposure to dioxin may lead to altered memory performance by impairing synaptic plasticity (Misner et al., 2001), LTP (Chiang

et al., 1998; Etchamendy et al., 2001), and neurogenesis (Snyder et al., 2005; Jacobs et al., 2006).

Interestingly, TCDD-induced memory deficits were not observed in $TTR^{-/-}$ mice. This result suggests that a complete blockade of the major retinol transport route involving TTR decreases the mobilization and elimination of retinoids. These data are consistent with a previous study showing that complete lack of TTR block secretion of the binary complex retinol-RBP from liver, which in turn decreases the plasma levels of both retinol and RBP to less than 6% of what was measured in wild-type mice (Wei et al., 1995). Moreover, reduced plasma levels of both retinol and RBP were also observed after exposure to 3,4,3',4'-tetrachlorobiphenyl, a HAH congener that directly sequesters TTR (Brouwer & Van den Berg, 1986; Brouwer et al., 1988). The viable alternate route of RA supply to maintain normal phenotype in $TTR^{-/-}$ mice (Episkopou et al., 1993) does not seem to be altered by TCDD since we observed no significant memory deficits in these mice following exposure to dioxin. The total absence of change on retinoid levels in the liver of $AhR^{-/-}$ mice following TCDD administration suggest that disruption of retinoid homeostasis by TCDD is mediated *via* AhR (Nishimura et al., 2005).

The reversal of TCDD-induced memory deficits by E or RA treatment alone or in combination suggests a close interaction between these systems in our model. Previous reports have shown that E can directly induce the expression of enzymes involved in the biosynthesis of RA in various cellular models (Li & Ong, 2003; Li et al., 2004). The estrogen receptor subtype α was also reported to activate RA receptor $\alpha 1$ ($RAR\alpha 1$) following E treatment (Laganiere et al., 2005), whereas RA and TCDD interact synergistically to alter estrogen's response (Lu et al., 1994). Gene network study also supports a direct link between RARs and AhR (Toyoshiba et al., 2004) while another report suggests that sequestration of $RAR\alpha$ corepressor by AhR is sufficient to activate $RAR\alpha$ in the absence of ligand (Widerak et al., 2006). Taken together, these data along with our results reveal the existence of multiple cross-talks between pathways regulated by TCDD, E and RA. Thus, alteration of both the estrogen system and the major route of TTR-

mediated retinol transport are most likely involved in memory deficits observed in our study following exposure to the environmental contaminant TCDD.

The behavioural approach used in this study provides evidence that the common noxious environmental and food agent TCDD can induce robust memory deficits in a gender-specific manner. RA and estrogen signalling pathways seem to interact through common mechanisms to reverse mnemonic disabilities induced by TCDD in female mice. HAHs such as TCDD are widespread pollutants that can accumulate in fat tissue and could exert long-term deleterious effects on various bodily functions including learning abilities. Given that TCDD is a prototypical member of the toxic contaminants HAHs, mechanisms underlying dioxin-induced memory impairments could lead to potential novel targets to prevent noxious effects of pollutants on normal cognitive processing.

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to R.Q. and a studentship award from CIHR to J.B.

References

- Astroff B, Rowlands C, Dickerson R & Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibition of 17 beta-estradiol-induced increases in rat uterine epidermal growth factor receptor binding activity and gene expression. *Mol Cell Endocrinol*, 72, 247-252.
- Astroff B & Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-dioxin as an antiestrogen: effect on rat uterine peroxidase activity. *Biochem Pharmacol*, 39, 485-488.
- Brouillette J & Quirion R (2007) Transthyretin: A key gene involved in the maintenance of memory capacities during aging. *Neurobiol Aging*, Epub ahead of print.
- Brouwer A, Hakansson H, Kukler A, Van den Berg KJ & Ahlborg UG (1989) Marked alterations in retinoid homeostasis of Sprague-Dawley rats induced by a single i.p. dose of 10 micrograms/kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology*, 58, 267-283.
- Brouwer A, Kukler A & Van den Berg KJ (1988) Alterations in retinoid concentrations in several extrahepatic organs of rats by 3,4,3',4'-tetrachlorobiphenyl. *Toxicology*, 50, 317-330.
- Brouwer A & Van den Berg KJ (1986) Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxine. *Toxicol Appl Pharmacol*, 85, 301-312.
- Cheng SB, Kuchiiwa S, Nagatomo I, Akasaki Y, Uchida M, Tominaga M, Hashiguchi W, Kuchiiwa T & Nakagawa S (2002) 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment induces c-Fos expression in the forebrain of the Long-Evans rat. *Brain Res*, 931, 176-180.
- Chesler EJ & Juraska JM (2000) Acute administration of estrogen and progesterone impairs the acquisition of the spatial morris water maze in ovariectomized rats. *Horm Behav*, 38, 234-242.

- Chiang MY, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF & Evans RM (1998) An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron*, 21, 1353-1361.
- Cordoba Montoya DA & Carrer HF (1997) Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. *Brain Res*, 778, 430-438.
- DeVito MJ, Thomas T, Martin E, Umbreit TH & Gallo MA (1992) Antiestrogenic action of 2,3,7,8-tetrachlorodibenzo-p-dioxin: tissue-specific regulation of estrogen receptor in CD1 mice. *Toxicol Appl Pharmacol*, 113, 284-292.
- Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME & Robertson EJ (1993) Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proc Natl Acad Sci U S A*, 90, 2375-2379.
- Etchamendy N, Enderlin V, Marighetto A, Vouimba RM, Pallet V, Jaffard R & Higeret P (2001) Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J Neurosci*, 21, 6423-6429.
- Frick KM, Fernandez SM & Bulinski SC (2002) Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. *Neuroscience*, 115, 547-558.
- Fugger HN, Cunningham SG, Rissman EF & Foster TC (1998) Sex differences in the activational effect of ERalpha on spatial learning. *Horm Behav*, 34, 163-170.
- Gibbs RB (1999) Estrogen replacement enhances acquisition of a spatial memory task and reduces deficits associated with hippocampal muscarinic receptor inhibition. *Horm Behav*, 36, 222-233.
- Gureviciene I, Puolivali J, Pussinen R, Wang J, Tanila H & Ylinen A (2003) Estrogen treatment alleviates NMDA-antagonist induced hippocampal LTP blockade and cognitive deficits in ovariectomized mice. *Neurobiol Learn Mem*, 79, 72-80.
- Hakansson H & Ahlborg UG (1985) The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the uptake, distribution and excretion of a single oral dose of

- [11,12-3H]retinyl acetate and on the vitamin A status in the rat. *J Nutr*, 115, 759-771.
- Hart SA, Patton JD & Woolley CS (2001) Quantitative analysis of ER alpha and GAD colocalization in the hippocampus of the adult female rat. *J Comp Neurol*, 440, 144-155.
- Hoegberg P, Schmidt CK, Fletcher N, Nilsson CB, Trossvik C, Gerlienke SA, Brouwer A, Nau H, Ghyselinck NB, Chambon P & Hakansson H (2005) Retinoid status and responsiveness to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking retinoid binding protein or retinoid receptor forms. *Chem Biol Interact*, 156, 25-39.
- Hoegberg P, Schmidt CK, Nau H, Ross AC, Zolfaghari R, Fletcher N, Trossvik C, Nilsson CB & Hakansson H (2003) 2,3,7,8-tetrachlorodibenzo-p-dioxin induces lecithin: retinol acyltransferase transcription in the rat kidney. *Chem Biol Interact*, 145, 1-16.
- Hojo R, Zareba G, Kai JW, Baggs RB & Weiss B (2006) Sex-specific alterations of cerebral cortical cell size in rats exposed prenatally to dioxin. *J Appl Toxicol*, 26, 25-34.
- Hong SJ, Grover CA, Safe SH, Tiffany-Castiglioni E & Frye GD (1998) Halogenated aromatic hydrocarbons suppress CA1 field excitatory postsynaptic potentials in rat hippocampal slices. *Toxicol Appl Pharmacol*, 148, 7-13.
- Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH & Evans RM (2006) Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A*, 103, 3902-3907.
- Jurek MA, Powers RH, Gilbert LG & Aust SD (1990) The effect of TCDD on acyl CoA:retinol acyltransferase activity and vitamin A accumulation in the kidney of male Sprague-Dawley rats. *J Biochem Toxicol*, 5, 155-160.
- Kelley SK, Nilsson CB, Green MH, Green JB & Hakansson H (1998) Use of model-based compartmental analysis to study effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A kinetics in rats. *Toxicol Sci*, 44, 1-13.

- Kelley SK, Nilsson CB, Green MH, Green JB & Hakansson H (2000) Mobilization of vitamin A stores in rats after administration of 2,3, 7,8-tetrachlorodibenzo-p-dioxin: a kinetic analysis. *Toxicol Sci*, 55, 478-484.
- Laganiere J, Deblois G & Giguere V (2005) Functional genomics identifies a mechanism for estrogen activation of the retinoic acid receptor alpha1 gene in breast cancer cells. *Mol Endocrinol*, 19, 1584-1592.
- Li XH, Kakkad B & Ong DE (2004) Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus. *Endocrinology*, 145, 4756-4762.
- Li XH & Ong DE (2003) Cellular retinoic acid-binding protein II gene expression is directly induced by estrogen, but not retinoic acid, in rat uterus. *J Biol Chem*, 278, 35819-35825.
- Lu Y, Wang X & Safe S (1994) Interaction of 2,3,7,8-tetrachlorodibenzo-p-dioxin and retinoic acid in MCF-7 human breast cancer cells. *Toxicol Appl Pharmacol*, 127, 1-8.
- Mably TA, Moore RW, Goy RW & Peterson RE (1992) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 2. Effects on sexual behavior and the regulation of luteinizing hormone secretion in adulthood. *Toxicol Appl Pharmacol*, 114, 108-117.
- Michalek JE, Akhtar FZ, Arezzo JC, Garabrant DH & Albers JW (2001) Serum dioxin and peripheral neuropathy in veterans of Operation Ranch Hand. *Neurotoxicology*, 22, 479-490.
- Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF & Evans RM (2001) Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci USA*, 98, 11714-11719.
- Monaco HL (2000) The transthyretin-retinol-binding protein complex. *Biochim Biophys Acta*, 1482, 65-72.
- Mooradian AD, Morley JE & Korenman SG (1987) Biological actions of androgens. *Endocr Rev*, 8, 1-28.

- Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*, 11, 47-60.
- Myllymaki SA, Haavisto TE, Brokken LJ, Viluksela M, Toppari J & Paranko J (2005) In utero and lactational exposure to TCDD; steroidogenic outcomes differ in male and female rat pups. *Toxicol Sci*, 88, 534-544.
- Nayyar T, Wu J & Hood DB (2003) Downregulation of hippocampal NMDA receptor expression by prenatal exposure to dioxin. *Cell Mol Biol (Noisy -le-grand)*, 49, 1357-1362.
- Nilsson CB, Hoegberg P, Trossvik C, Azais-Braesco V, Blaner WS, Fex G, Harrison EH, Nau H, Schmidt CK, van Bennekum AM & Hakansson H (2000) 2,3,7,8-tetrachlorodibenzo-p-dioxin increases serum and kidney retinoic acid levels and kidney retinol esterification in the rat. *Toxicol Appl Pharmacol*, 169, 121-131.
- Nishimura N, Yonemoto J, Miyabara Y, Fujii-Kuriyama Y & Tohyama C (2005) Altered thyroxine and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. *Arch Toxicol*, 79, 260-267.
- Packard MG & Teather LA (1997) Posttraining estradiol injections enhance memory in ovariectomized rats: cholinergic blockade and synergism. *Neurobiol Learn Mem*, 68, 172-188.
- Pohjanvirta R, Vartiainen T, Uusi-Rauva A, Monkkonen J & Tuomisto J (1990) Tissue distribution, metabolism, and excretion of ¹⁴C-TCDD in a TCDD-susceptible and a TCDD-resistant rat strain. *Pharmacol Toxicol*, 66, 93-100.
- Powers BE, Lin TM, Vanka A, Peterson RE, Juraska JM & Schantz SL (2005) Tetrachlorodibenzo-p-dioxin exposure alters radial arm maze performance and hippocampal morphology in female AhR mice. *Genes Brain Behav*, 4, 51-59.
- Rissanen A, Puolivali J, van Groen T & Riekkinen P, Jr. (1999) In mice tonic estrogen replacement therapy improves non-spatial and spatial memory in a water maze task. *Neuroreport*, 10, 1369-1372.
- Romkes M, Piskorska-Pliszczyńska J & Safe S (1987) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol*, 87, 306-314.

- Rozman K (1989) A critical view of the mechanism(s) of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Implications for human safety assessment. *Derm Beruf Umwelt*, 37, 81-92.
- Rudick CN & Woolley CS (2001) Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. *J Neurosci*, 21, 6532-6543.
- Safe S, Wormke M & Samudio I (2000) Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. *J Mammary Gland Biol Neoplasia*, 5, 295-306.
- Safe SH (1995) Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacol Ther*, 67, 247-281.
- Sandstrom NJ & Williams CL (2001) Memory retention is modulated by acute estradiol and progesterone replacement. *Behav Neurosci*, 115, 384-393.
- Schantz SL & Bowman RE (1989) Learning in monkeys exposed perinatally to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Neurotoxicol Teratol*, 11, 13-19.
- Schmidt JV & Bradfield CA (1996) Ah receptor signaling pathways. *Annu Rev Cell Dev Biol*, 12, 55-89.
- Seo BW, Sparks AJ, Medora K, Amin S & Schantz SL (1999) Learning and memory in rats gestationally and lactationally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Neurotoxicol Teratol*, 21, 231-239.
- Sherwin BB (1994) Estrogenic effects on memory in women. *Ann N Y Acad Sci*, 743, 213-230.
- Shughrue PJ, Lane MV & Merchenthaler I (1997) Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol*, 388, 507-525.
- Shughrue PJ & Merchenthaler I (2000) Estrogen is more than just a "sex hormone": novel sites for estrogen action in the hippocampus and cerebral cortex. *Front Neuroendocrinol*, 21, 95-101.
- Snyder JS, Hong NS, McDonald RJ & Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. *Neuroscience*, 130, 843-852.

- Stewart PW, Reihman J, Lonky EI, Darvill TJ & Pagano J (2003) Cognitive development in preschool children prenatally exposed to PCBs and MeHg. *Neurotoxicol Teratol*, 25, 11-22.
- Thornton AS, Oda Y, Stuart GR, Holcroft J & de Boer JG (2004) The dioxin TCDD protects against aflatoxin-induced mutation in female rats, but not in male rats. *Mutat Res*, 561, 147-152.
- Thunberg T, Ahlberg UG, Hakansson H, Krantz C & Monier M (1980) Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). *Arch Toxicol*, 45, 273-285.
- Thunberg T, Ahlberg UG & Johnsson H (1979) Vitamin A (retinol) status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch Toxicol*, 42, 265-274.
- Toyoshiba H, Yamanaka T, Sone H, Parham FM, Walker NJ, Martinez J & Portier CJ (2004) Gene interaction network suggests dioxin induces a significant linkage between aryl hydrocarbon receptor and retinoic acid receptor beta. *Environ Health Perspect*, 112, 1217-1224.
- Tritscher AM, Seacat AM, Yager JD, Groopman JD, Miller BD, Bell D, Sutter TR & Lucier GW (1996) Increased oxidative DNA damage in livers of 2,3,7,8-tetrachlorodibenzo-p-dioxin treated intact but not ovariectomized rats. *Cancer Lett*, 98, 219-225.
- Umbreit TH & Gallo MA (1988) Physiological implications of estrogen receptor modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Lett*, 42, 5-14.
- Umbreit TH, Hesse EJ, Macdonald GJ & Gallo MA (1988) Effects of TCDD-estradiol interactions in three strains of mice. *Toxicol Lett*, 40, 1-9.
- Van Birgelen AP, Van der KJ, Fase KM, Bol I, Poiger H, Brouwer A & Van den BM (1995) Subchronic dose-response study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in female Sprague-Dawley rats. *Toxicol Appl Pharmacol*, 132, 1-13.
- Vaucher E, Reymond I, Najaffe R, Kar S, Quirion R, Miller MM & Franklin KB (2002) Estrogen effects on object memory and cholinergic receptors in young and old female mice. *Neurobiol Aging*, 23, 87-95.

- Wei S, Episkopou V, Piantedosi R, Maeda S, Shimada K, Gottesman ME & Blaner WS (1995) Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. *J Biol Chem*, 270, 866-870.
- Widerak M, Ghoneim C, Dumontier MF, Quesne M, Corvol MT & Savouret JF (2006) The aryl hydrocarbon receptor activates the retinoic acid receptoralpha through SMRT antagonism. *Biochimie*, 88, 387-397.
- Wormke M, Stoner M, Saville B & Safe S (2000) Crosstalk between estrogen receptor alpha and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. *FEBS Lett*, 478, 109-112.
- Wyde ME, Wong VA, Kim AH, Lucier GW & Walker NJ (2001) Induction of hepatic 8-oxo-deoxyguanosine adducts by 2,3,7,8-tetrachlorodibenzo-p-dioxin in Sprague-Dawley rats is female-specific and estrogen-dependent. *Chem Res Toxicol*, 14, 849-855.

Chapter V

Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABA_B receptors in scopolamine-induced amnesia

Preface

In chapter III, we used aged rats to investigate genes involved in long-term memory formation that may be altered when cognitive deficits occur during aging. The role of RA signaling pathways in age-related memory impairments found in this first study was further investigated in chapter IV using dioxin to empty retinol stock and to determine mechanisms underlying the role of this compound on cognitive functions. In the present chapter, we used the scopolamine-induced amnesia model to identify candidate genes differentially expressed following stimulation in a spatial memory task. Scopolamine is a muscarinic acetylcholine receptor antagonist commonly used as a psychopharmacological model of amnesia which induces memory deficits that mimic those occurring during aging. In addition, by performing similar behavioural and microarray analyses as in chapter III, we were able to compare genes altered in aged rats and in scopolamine-treated rats (for details see chapter VI).

This genome wide study identified several novel hippocampal genes differentially expressed in scopolamine-treated rats and provided the first evidence that muscarinic receptors may be implicated in the regulation of the glutamatergic receptor related gene Homer1. The major finding of our study is that the stable overexpression of the glutamatergic component Homer1a or 1c in the hippocampus of adult rats induced by recombinant adeno-associated virus vector, abolished memory improvements produced by the GABA_B receptor antagonist SGS742 in scopolamine-treated rats.

Previous reports have suggested the essential role for Homer1 isoforms in synaptic plasticity. However, their role at the behavioural level has yet to be studied. Our findings begin to fill that gap and the candidate genes identified here represent potential novel targets to develop more effective therapeutic strategies for the treatment of cognitive impairments.

**Hippocampal gene expression profiling reveals the possible
involvement of *Homer1* and *GABA_B* receptors in
scopolamine-induced amnesia**

Jonathan Brouillette* Deborah Young† Matthew J. During† & Rémi Quirion*‡

Department of Neurology & Neurosurgery * and Department of Psychiatry ‡,
Douglas Mental Health University Institute, McGill University, Montréal, Canada

Department of Molecular Medicine and Pathology †, University of Auckland,
Auckland 1003, New Zealand

Corresponding author:

Prof. Rémi Quirion, PhD, OC, FRSC, CQ
Douglas Mental Health University Institute, McGill University
6875 LaSalle Boulevard
Montréal, Québec
H4H 1R3

Tel.: (514) 761-6131 (ext 2934)

Fax: (514) 762-3034

E-mail: remi.quirion@douglas.mcgill.ca

Keywords: scopolamine, memory, gene expression, *Homer1*, *GABA_B* receptor,
Morris water maze.

Abstract

Scopolamine treated rats are commonly used as a psychopharmacological model of memory dysfunction and have been extensively studied to establish the effectiveness of acetylcholinesterase inhibitors in the treatment of Alzheimer's disease. Scopolamine is a muscarinic acetylcholine receptor antagonist that induces memory deficits in young subjects similar to those occurring during aging. The amnesic effect of scopolamine is well established but the molecular and cellular mechanisms that sustain its neuropharmacological action are still unclear. The present genome wide study investigates hippocampal gene expression profiling in scopolamine treated adult rats following stimulation in a spatial memory task. Using microarray and quantitative real-time RT-PCR approaches, we identified several genes previously known to be associated with memory processes (*Homer1*, *GABA_B* receptor, *Egr1*, *prodynorphin*, *Vgf*) and multiple novel candidate genes possibly involved in cognition (including *Camkk2*, *Dusp5* and *6*, *glycophorin C*) that were altered following scopolamine treatment. Moreover, we found that stable overexpression of glutamatergic components *Homer1a* and *1c* in the hippocampus of adult rats induced by recombinant adeno-associated virus vector abolished memory improvement produced by the *GABA_B* receptor antagonist SGS742 in scopolamine treated rats. Taken together, these results reveal novel genes and mechanisms involved in scopolamine-induced amnesia, and demonstrate the involvement of both GABA and glutamate neurotransmission in this animal model of cognitive dysfunctions.

Introduction

The cholinergic system is involved in the acquisition (encoding) of new memory (Deutsch, 1971; Jerusalinsky et al., 1997) as well as in attention (Sarter & Bruno, 1997). Cumulative evidence suggests that a key factor in age-related memory deficits and Alzheimer's disease (AD) relates to deficits in cholinergic neurotransmission (Bartus et al., 1982; Aubert et al., 1995; Araujo et al., 2005). Cholinergic muscarinic receptors are known to be essential in facilitating learning and memory functions (Quirion et al., 1995; van der Zee & Luiten, 1999; Rowe et al., 2003). Memory impairment associated with scopolamine treatment was shown to be linked to a decrease in central cholinergic neuronal activity following the blockade of the muscarinic receptors (Carli et al., 1997; Schon et al., 2005). However, the molecular and cellular mechanisms implicated in the cascade of events underlying the amnesic effect of scopolamine remain to be fully established.

Several studies indicate that systemic administration of scopolamine before encoding, but not retrieval, impairs performance in a variety of memory tasks in rodents (Nakajima et al., 1994; Devan et al., 2004; Janas et al., 2005), monkeys (Aigner et al., 1991; Tang et al., 1997) and humans (Atri et al., 2004; Schon et al., 2005). In the acquisition memory process of the Morris water maze (MWM) task, scopolamine markedly increased the escape latency to find the hidden platform (Nakajima et al., 1994; Chen et al., 2002; Janas et al., 2005). Interestingly, scopolamine-induced memory deficits have been shown to increase with age (Ray et al., 1992; Stemmelin et al., 1999).

The hippocampus, especially the dorsal area (Moser et al., 1993), is essential for the formation and retrieval of spatial memory during the MWM task (Hollup et al., 2001) and is necessary for consolidation or long-term storage of memory traces (Riedel et al., 1999). The muscarinic receptors located in the hippocampus are of particular relevance for learning and memory processes (Bartus et al., 1982; Quirion et al., 1995; Sarter & Bruno, 1997; van der Zee & Luiten, 1999). This cholinergic component, along with other neurotransmitters

including the glutamatergic and GABAergic systems, is integrated into the neural circuitry of the dorsal hippocampus that modulates learning and memory functions (White & McDonald, 2002).

Stable forms of long-term plasticity require the induction of a cascade of genes immediately and several hours after training to produce and maintain structural changes associated with long-term memory formation (Bourtchouladze et al., 1998; Kandel, 2001; Igaz et al., 2002). High-density oligonucleotide microarray represents a powerful tool to investigate dorsal hippocampal gene expression profiles in scopolamine treated rats following stimulation in the reference (long-term) spatial memory version of the MWM task. This approach allows for the simultaneous examination of thousands of genes differentially expressed between scopolamine treated rats and control animals that performed the MWM task. Since genes expressed during the late response phase have been studied to a lesser extent than those induced directly after training, we focused on gene expression altered by scopolamine treatment 6 h after the last training trial in the MWM task. A similar approach was used recently by our group and proves to be efficient to investigate novel genes differentially expressed between aged memory-impaired and aged memory-unimpaired rats (Brouillette & Quirion, 2007).

In the present study, we identified several hippocampal genes already associated with memory formation as well as novel ones altered following scopolamine treatment. The transcriptional regulations of GABA_B receptor and glutamatergic component *Homer1* were shown to be markedly altered following our treatment. To investigate further key mechanisms altered by scopolamine in cholinergic, glutamatergic and GABAergic systems, we used non-pathogenic recombinant adeno-associated virus (rAAV) vector to induce long-lasting transgene expression of *Homer 1a* and *1c* in the hippocampus as well as the GABA_B receptor antagonist SGS742. Interestingly, memory improvements induced by SGS742 in scopolamine treated rats were reversed by the overexpression of Homer 1, revealing key interactions between these neurotransmitter systems in our model.

Material and Methods

Animals

Male Long-Evans rats ($n = 58$) were purchased from Charles River Laboratories (St-Constant, Canada) at 2 months of age and were housed in our animal facility until they were 7-months old. Animals were housed two per cage and maintained on a 12 h light/dark cycle schedule with *ad libitum* access to food (Purina Lab Chow) and water. Animal care, surgery and handling procedures were approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Drugs

(-)-Scopolamine hydrobromide (Sigma, St-Louis, MO, USA) was dissolved in physiological saline and administered (0.4 mg/kg) intraperitoneal (ip) 20 min before MWM training daily for four days. Control animals were administered an equal volume of physiological saline. The selective GABA_B receptor antagonist SGS742 (3-aminopropyl-*n*-butyl phosphinic acid) was generously provided by Dr. Thorsten Melcher (Saegis Pharmaceuticals, Inc., Half Moon Bay, CA, USA). SGS742, also known as CGP36742, was dissolved in physiological saline and administered (30 mg/kg) per oral (po) 90 min before daily training. The dose of SGS742 used here has been previously shown to reverse scopolamine-induced memory deficits in the MWM task in rats (Nakagawa & Takashima, 1997).

Morris water maze task

Spatial memory of Long-Evans rats was determined using the reference (long-term) memory version of the Morris water maze (MWM) task, a hippocampus-dependant behavioural task (Morris, 1984). Rats were required to find, in a 1.4 m diameter pool, a submerged platform (14 cm in diameter) located 2 cm below the surface of water (24°C), rendered opaque by the addition of skim milk powder. Animals were pseudo-randomly started from a different position at each trial and used distal visuo-spatial cues to find the hidden escape platform that

remained in the centre of the same quadrant throughout all training days (Morris, 1984). The rats were given three trials of 90 s per day with a 20 min inter-trial interval over four consecutive days. Animals were guided to the platform if not located within 90 s and all the rats remained there for 10 s before removal.

After the acquisition phase on day four, rats were given one probe trial of 60 s in which the platform was removed from the pool to evaluate the number of times the animal crossed the previous location of the platform, time spent in the target quadrant, and swimming speed. This trial was followed by one cued trial of 60 s in which the platform was visible to assess visual deficits and motivation to escape from water. Scopolamine and saline treated rats were sacrificed 6 hours after the last MWM trial on day 4. After each trial, animals were immediately placed under heat lamps to dry and prevent hypothermia. The experimenter was blind to treatment and surgery performed on rats. To control for possible effects due to circadian cycles, all trials were performed at approximately the same time of the day, between 11-13 h. Data derived from the MWM task were recorded on computer using a video tracking system (HVS, Buckingham, UK).

Statistical analysis

Statistical significance of each test was assessed using the appropriate ANOVA design and simple main effects were analysed with F-test when the interaction was significant. Tukey's Honestly Significant Difference test was used as a *post hoc* pair wise comparison test when necessary. The alpha level used was $p < 0.05$.

Microarray analysis

Coronal section (2 mm) used for microarray analysis were taken from the rostral end of the dorsal hippocampus up to the tip of the ventral hippocampus. These coordinates are known to include all regions of the dorsal hippocampus proper (dentate gyrus, CA1, CA3). The dorsal hippocampus was dissected from the brain immediately after decapitation and stored at -80°C in RNAlater stabilization reagent until use. The frozen tissue was ground and used for the

different molecular analyses. We used total dorsal hippocampal tissue instead of a more restricted brain region for microarray analysis. This approach provides information regarding widespread gene expression modification in the neurotransmitter systems involved in memory formation within this structure. Total RNA was isolated using RNeasy Lipid Tissue Kit with RNase-Free DNase Set (Qiagen, Valencia, CA, USA) to ensure maximal removal of DNA during RNA purification. The RNA quality and concentration were assessed with Agilent Technologies 2100 Bioanalyser. Quadruplicates of Affymetrix GeneChip® Rat Genome 230 2.0 array were used in each group of animals. The microarray primary data were deposited, using MIAMExpress, in the ArrayExpress database with the accession number E-MEXP-920. The raw intensity reading of each individual target on the Affymetrix microarray was processed and normalized by using the log₂ scale Robust Multi-array Averaging (RMA) method. Since the RMA method gives less pronounced FC magnitudes compared to the Affymetrix Microarray Suite 5.0 (MAS5) analysis default program, a minimal FC of 1.5 is suggested as exclusion criteria to select genes and avoid false negatives (Irizarry et al., 2003a). Minus versus Average (MvA) plots were performed to control inter-animal variation within and between groups.

Quantitative real-time RT-PCR analysis

Two-step quantitative real-time RT-PCR was performed to confirm microarray results according to Applied Biosystems guidelines. Reverse transcription of total RNA was carried out with TaqMan™ Reagents. The cDNA generated was directly quantified by measuring the increase in fluorescence with the SYBR® Green PCR Core Reagents during the PCR using the GeneAmp® 5700 Sequence Detection System (40 cycles). The endogenous control β -actin was used to normalize quantification of the mRNA target, and non-specific amplifications were verified by a dissociation curve. All the reactions were performed in triplicate. Gene-specific primer sequences used in this study are shown in supplemental Table 3, which is published as supplemental material on the website of Journal of Neurochemistry.

Generation of rAAV

The rat *Homer1a* and *Homer1c* coding sequences were amplified using whole-brain cDNA. The PCR products were expressed as an N-terminal fusion protein with a hemagglutinin (HA) tag into an rAAV backbone containing the 1.1 kb CMV enhancer/chicken β -actin (CBA) promoter, the woodchuck post-transcriptional regulatory element (WPRE), and the bovine growth hormone (bGH) polyA (rAAV-Homer1a, rAAV-Homer1c). The same rAAV-CBA-HA-WPRE-bGH backbone carrying no cDNA was used as control (rAAV-empty). The rAAV pseudotyped vectors (virions containing a 1 : 1 ratio of AAV1 and AAV2 capsid proteins with AAV2 intertrigeminal regions) were generated as described by (Hauck et al., 2003). Briefly, human embryonic kidney 293 (HEK) cells were transfected with the AAV *cis*-plasmid, the AAV1 (pH21) and AAV2 (pNLrep) helper plasmids (pF6), and the adenovirus helper plasmid by standard calcium phosphate transfection methods. Forty-eight hours after transfection, cells were harvested and the vector purified using heparin affinity columns (Sigma, St Louis, MO, USA) (During et al., 2003b). Genomic titers were determined using the ABI 7700 real-time PCR cycler (Applied Biosystems, Foster City, CA, USA) with primers designed to WPRE as described previously (Clark et al., 1999).

Surgical procedures and rAAV infusion

Animal surgery and rAAV vector administration were performed as described earlier (Klugmann et al., 2005). Briefly, rats were anaesthetized with sodium pentobarbital (60 mg/kg, ip). Two microliters of either rAAV-empty or rAAV-Homer1 (3×10^{12} viral particles) was mixed with 1 μ L of 20% mannitol, to enhance vector spread (Mastakov et al., 2001), and injected bilaterally into the dorsal hippocampus (-3.8 mm AP, ± 1.8 mm ML, -4.1 mm DV from bregma according to the Paxinos and Watson rat brain atlas) using a stereotaxic frame. Vectors were infused at a rate of 66 nL/min by a dual-piston pump (ESA 580; ESA, Chelmsford, ME, USA). Behavioral analysis was started 4 weeks after vector infusion when transgene protein expression had peaked to remain at stable levels

(During et al., 2003a). As previously shown (Klugmann et al., 2005), gene transfer and expression of the rAAV-Homer1 vectors into the dorsal hippocampus was confirmed by western blot after MWM analysis. Transgene expression was observed at least 2 mm on either side of the injection site and covered the entire dorsal hippocampus of the rAAV-Homer groups (Klugmann et al., 2005). Using HA immunoreactivity and cresyl violet counterstaining, this former study also demonstrated transgene expression in neuronal cell bodies and dendrites throughout the hippocampi of rAAV-Homer1 injected rats.

Western blot analysis

Extracts from rat hippocampi were obtained by polytron homogenization in cold RIPA lysis buffer with protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of total protein corresponding to 30 µg/lane were loaded on denaturing NuPAGE® Novex 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred to Hybond-C membranes (Amersham Biosciences, UK) by electroblotting. Membranes were pretreated with 5% milk blotting buffer and then incubated with anti-Homer1a, anti-Homer1c, or anti-actin (1/1000, Santa Cruz Biotechnology) antibody in 2% BSA solution overnight at 4°C. The membranes were then washed, treated with the appropriate secondary HRP-labeled antibodies for 1 h, washed again and incubated with the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA, USA). Membranes were exposed to Kodak BioMax MS film (Amersham Biosciences), and quantitative densitometric analysis was done using MCID image system. Statistical data were analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference test.

Results

Scopolamine-induced memory deficits in MWM task

Adult (7-months old) Long-Evans rats treated with scopolamine (0.4 mg/kg, ip, 20 min prior to daily training) and control saline treated animals were tested in the reference memory version of the MWM task, where the hidden escape platform stayed at the same location during all training days (Fig. 1a). A longer escape latency to find the hidden platform is considered to reflect long-term spatial memory deficits. The two-way, one between, one within ANOVA conducted on the mean escape latencies yielded a highly significant group x days interaction ($F_{(3,132)} = 9.31$; $p < 0.0001$). Simple main effect tests reveal that scopolamine treated rats showed longer escape latency on day two to four when compared to control animals. The longer escape latency of scopolamine treated rats is not attributed to slower swimming speed since those rats swam slightly faster than control animals ($p < 0.05$; unpaired t test) (Fig. 1b).

Memory impairments induced by scopolamine were validated immediately after the end of training by performing a probe trial of 60 s in which the platform was removed. Scopolamine treated animals, unlike saline treated rats, showed no preference for the quadrant that previously contained the platform (Fig. 1c) and fewer platform crossings (Fig. 1d). Saline and scopolamine treated rats show similar escape latencies during a 60 s cued trial in which the platform was visible (5.5 ± 0.5 s; 5.8 ± 0.9 s respectively; mean $\pm$ SEM; $p > 0.05$; unpaired t test), indicating the absence of visual, sensorimotor or motivational deficits to escape from water. Thus, scopolamine treatment produces marked impairment during training and the probe trial in the MWM task.

Genes differentially expressed in scopolamine treated rats following stimulation in MWM task

Gene expression profiles of the dorsal hippocampus of each animal were investigated using individual Affymetrix high-density oligonucleotide microarrays containing 28,757 different genes. Comparative gene expression was

assessed 6 h after the last trial on day 4 using saline and scopolamine treated rats (four animals per group). Because of minor individual differences in performance over the four days of training within the group treated with scopolamine or saline, we selected the rats with individual performance that best reflect the performance of the group for microarray analysis. This decreased individual difference in gene expression within groups. Four adult naive control rats handled by an identical procedure but not tested in MWM were also used to determine basal gene expression levels under resting conditions.

Fold change (FC) in gene expression between these groups of animals was determined with the log₂ scale Robust Multi-array Averaging (RMA) methods (Irizarry et al., 2003a). This well-established microarray analysis is commonly used to obtain a more restrictive and consistent list of genes compared to the MAS5 analysis default program (Irizarry et al., 2003b). To be considered as a candidate gene, a minimal FC of 1.5 derived from the average and a 95 % confidence interval with a minimal bound of 1 FC for genes up-regulated or -1 FC for genes down-regulated has to be reached. To allow further elimination of false positives and to generate an even more conservative list, genes selected also had a minimal FC of 1.5 derived from the median (data not shown).

To investigate late response (effector) genes implicated in long-term memory that are altered by scopolamine treatment, we focused on genes differentially expressed between scopolamine and saline treated rats 6 h after their last trial on day four in the MWM (Table 1). Since additional information regarding scopolamine-induced memory deficits can also be drawn by comparing the pattern of gene expression between trained and naive saline treated rats and also between scopolamine and naive saline treated rats, genes altered across these conditions can be found as supplemental Table 1 and 2 on the Journal of Neurochemistry website. We observed that scopolamine treatment altered several hippocampal genes already associated with memory formation such as *Homer1*, *GABA_B* receptor, *early growth response 1*, *prodynorphin*, and *VGF* (non-acronymic). Scopolamine treatment also affected the expression of novel memory susceptibility genes including *calcium/calmodulin-dependent protein kinase*

kinase 2, dual specificity phosphatase 5 and 6, glycophorin C, sulfotransferase family 1A, branched chain aminotransferase 1, CD3 antigen epsilon polypeptide, trinucleotide repeat containing 6, and gene similar to tumor protein D53. Quantitative real-time RT-PCR was performed on thirteen genes of interest to confirm microarray results and revealed parallel patterns of gene expression (Fig. 2).

Overexpression of Homer1a or 1c disrupts memory improvements induced by GABA_B receptor antagonist SGS742 in scopolamine treated rats

We observed that scopolamine also affected the expression of genes associated with various neurotransmitter systems beside the cholinergic one in the dorsal hippocampus. As our study provides evidence that muscarinic receptors may be implicated in the regulation of the glutamatergic component *Homer1* and given that convergent results in more than one probe set indicate that *Homer1a* gene expression is the most altered by scopolamine treatment, we focused next on the role of this gene in scopolamine-induced amnesia. *Homer1a* is an activity-inducible variant of *Homer1* that lacks dimerization capacity and thus acts as a natural dominant negative in dynamic competition with constitutively expressed *Homer1c* to modulate synaptic glutamatergic transmission (Xiao et al., 1998). Our microarray analysis revealed that basal gene expression level of *Homer1c* was not altered in the hippocampus following the spatial memory task (see supplemental Table 1), which is consistent with previous observation showing that *Homer1c* is not induced by neural activity (Xiao et al., 1998).

Since *Homer1a* is in dynamic competition with *Homer1c* to modulate synaptic plasticity following neuronal activity, we hypothesize that a stable and continuous overexpression of either *Homer1a* or *Homer1c* may disrupt the balance needed between these two isoforms to allow memory formation in scopolamine treated animals. Given that *Homer1a* is formed by a non-classical splicing mechanism from the same transcript as *Homer1c*, the respective roles of *Homer1a* and *1c* in scopolamine-induced amnesia were determine using local infusion of rAAV vectors carrying either isoforms instead of using *Homer1* knock-out mice. Despite the fact that *Homer1c* gene expression is not altered by

neuronal activity, the effect of *Homer1c* overexpression after scopolamine treatment was also determined to mimic the lack of *Homer1a* relative to *Homer1c* in scopolamine treated animals following neuronal stimulation as revealed by our microarray analysis.

Therefore, to test if disequilibrium in the homeostatic balance of *Homer1a* and *1c* may be implicated in scopolamine-induced amnesia, we determined if an overexpression of either *Homer1a* or *1c* in the hippocampus of adult rats was able to abolish memory improvements induced by SGS742, a GABA_B receptor antagonist known to reverse memory impairments following scopolamine treatment (Nakagawa & Takashima, 1997). This compound was selected to establish further the role of GABA_B receptor in scopolamine-induced amnesia as shown by our microarray analysis.

As reported previously (Klugmann et al., 2005), infusion of rAAV-*Homer1a* vector increased the overall protein expression of *Homer1a* in the dorsal hippocampus (Fig. 3), while endogenous *Homer1a* level ($96 \pm 7\%$) was not altered ($p > 0.05$; one-way ANOVA). Protein levels of *Homer 1b/c* were also markedly increased in the dorsal hippocampus following infusion of rAAV-*Homer1c* vector (Fig. 3).

When tested in the MWM task, animals infused with either rAAV-*Homer1a* or *1c* vector were able to reverse memory improvements induced by SGS742 in scopolamine treated rats (Fig. 4a and b). During the probe trial, preference for the target quadrant in animals co-treated with scopolamine and SGS742 was abolished by the overexpression of *Homer1a* and *1c* (Fig. 4c). Fewer platform crossings were also observed in rats co-injected with scopolamine and SGS742 when either *Homer1* isoforms were up-regulated in the hippocampus (Fig. 4d). Treatment with SGS742 produced no significant changes in swimming speed and escape latency to a visible platform (Figs 4e and f).

The negative effect of continuous (instead of transient) up-regulation of *Homer1a* or *Homer1c* on mnemonic capacities was also confirmed by infusing vector containing either isoforms in scopolamine treated rats (Fig. 5a). We observed that scopolamine treated rats infused with vector rAAV-*Homer1a* or *1c*

demonstrate robust memory deficits that were not significantly different from those seen in rats treated with scopolamine only. To determine if learning and memory dysfunction induced by stable up-regulation of *Homer1a* and *1c* is specific to scopolamine treated animals, we injected either *Homer1* isoforms in saline treated rats (Fig. 5b). The increased expression of *Homer1a* or *1c* produced no significant effect on memory abilities when compared to control rats injected with empty vector, which is in accordance with a recent report (Klugmann et al., 2005). Memory capacities of animals injected with rAAV-*Homer1a* or rAAV-*Homer1c* vector following treatment with scopolamine or saline were validated during the probe trial performed after the end of training (Figs 5c and d). Up-regulation of either *Homer1* isoforms produced no significant effect on swimming speed and escape latency to a visible platform in scopolamine and saline treated animals (Figs 5e and f), indicating the absence of sensorimotor, visual and motivational deficits.

Figures

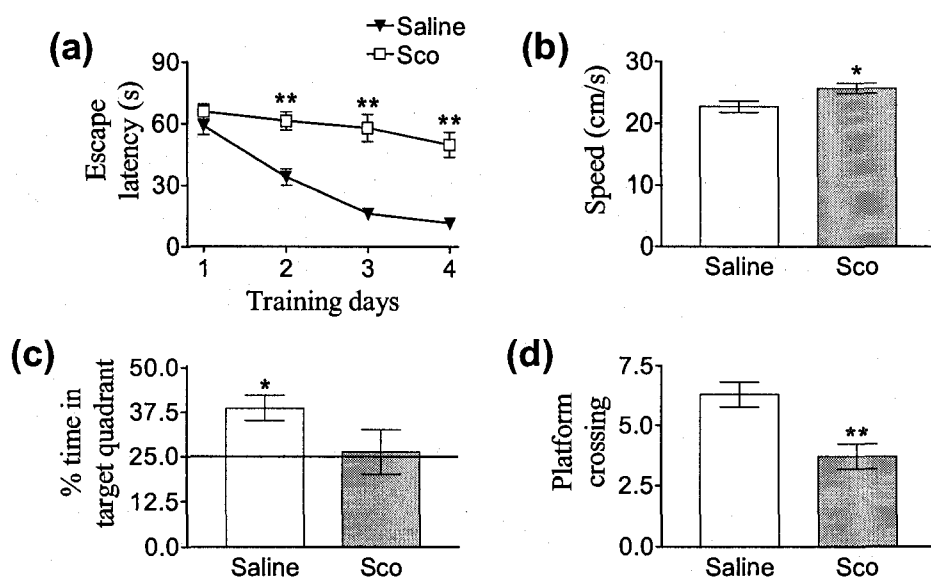


Fig. 1. Scopolamine treated rats show spatial memory deficits in the MWM task. (a) Escape latencies before finding the hidden platform (three trials of 90 s per day with a 20 min inter-trial interval) are longer for scopolamine (Sco) treated rats (open squares; 0.4 mg/kg; ip; 20 min before training; $n = 23$) than saline control animals (filled triangles; $n = 23$; ** $p < 0.01$; two-way ANOVA). (b) Swimming speeds recorded during the probe trial are slightly faster in scopolamine treated rats than control animals (* $p < 0.05$; unpaired t test). (c) Percent time spent in the quadrant that previously contained the platform during the probe trial. Error bars ($\pm$ 95% confidence interval of the mean) of scopolamine treated rats overlap the 25 % line which represents the chance level of being randomly in one of the four virtual quadrants. Asterisk represents significant differences from the 25 % chance performance (* $p < 0.05$). (d) Rats treated with scopolamine crossed the previous location of the platform fewer times than control rats (** $p < 0.005$; unpaired t test) during the probe trial. All results, except for (c) are mean $\pm$ SEM.

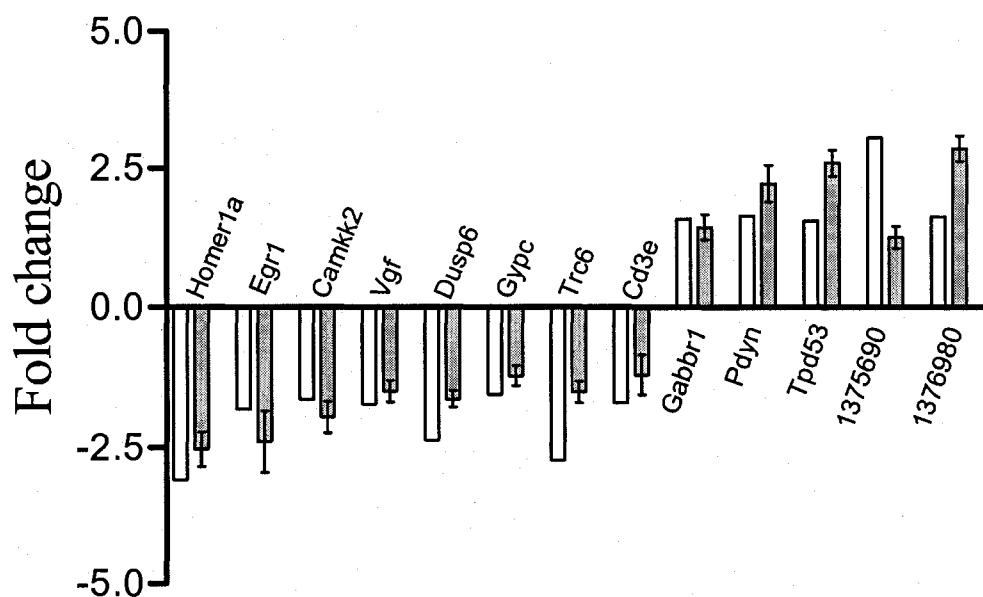


Fig. 2. Fold change obtained using quantitative real-time RT-PCR (filled bars) revealed parallel patterns of gene expression found with microarrays (open bars) between scopolamine and saline treated rats following stimulation in a spatial memory task. Fold expression change with a positive sign indicates up-regulation of the gene in scopolamine treated rats relative to saline control animals whereas down-regulation is represented by a negative sign. Since the standard error for estimated FC is not valid in the $\log_2$ scale used for microarray data, statistical analysis (mean $\pm$ SEM) was done with real-time RT-PCR data. *Egr1*, early growth response 1; *Camkk2*, calcium/calmodulin-dependent protein kinase kinase 2 beta subunit; *Vgf*, VGF nerve growth factor inducible; *Dusp6*, dual specificity phosphatase 6; *Gypc*, glycophorin C; *Trc6*, trinucleotide repeat containing 6; *Cd3e*, CD3 antigen epsilon polypeptide; *Gabbr1*, GABA_B receptor 1; *Pdyn*, prodynorphin; *Tpd53*, tumor protein D53.

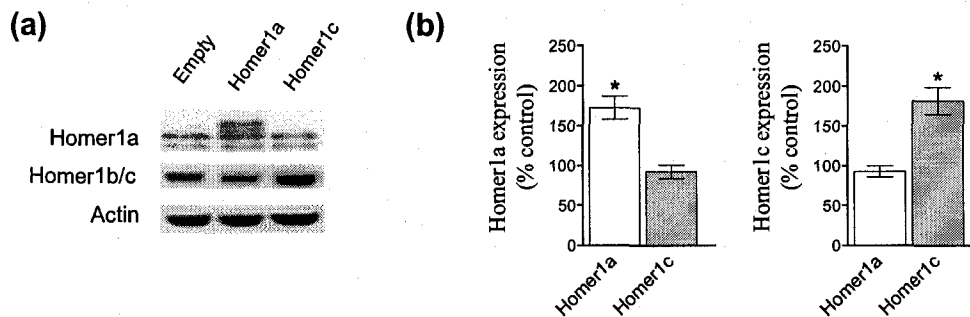


Fig. 3. Increased protein expressions of Homer1a and 1c following infusion of their respective rAAV-Homer1 vector into the dorsal hippocampus. (a) Representative western blot of dorsal hippocampal extracts from individual rats following MWM analysis. The same blots were stained with anti-actin to normalize the signals. Homer1a migrated as a doublet (28 and 29 kDa). The doublet at slightly increased size observed in rats injected with rAAV-Homer1a represents transgenic (HA-tagged) *Homer1a* expression whereas the lower doublet marks endogenous *Homer1a* expression. Since separation between the ectopic HA-tagged and native Homer 1b/c is negligible at 47 kDa, their respective protein levels could not be determined in rAAV-Homer1c group. **(b)** Densitometric analysis of the western blots shown in (a). Data are expressed as percentage of control $\pm$ SEM. Protein levels of Homer1a and 1c were significantly increased in rAAV-Homer1a and 1c group, respectively (* $p < 0.01$; one-way ANOVA).

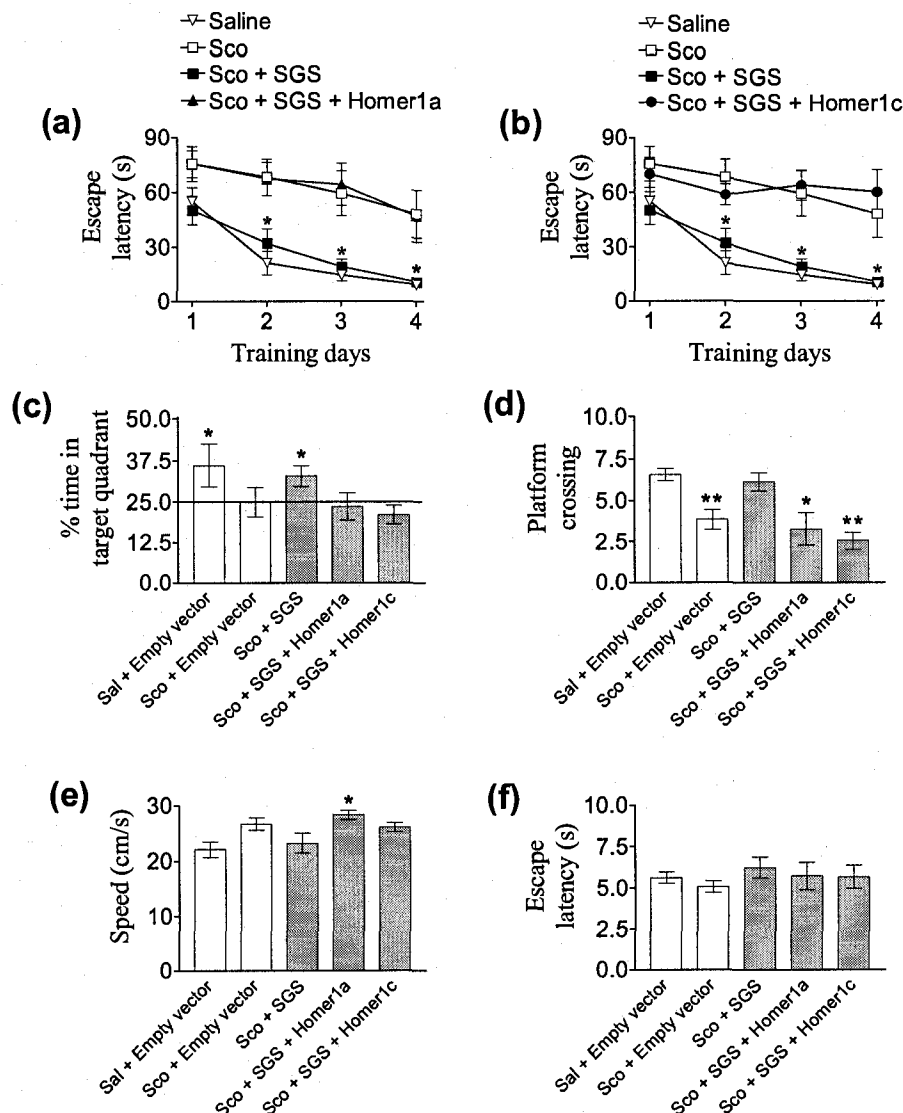


Fig. 4. Up-regulation of either *Homer1a* or *1c* reverses memory improvement produced by the GABA_B receptor antagonist SGS742 in scopolamine treated rats. (a) and (b) Scopolamine (Sco) treated rats (open squares; $n = 7$) show shorter escape latencies before finding the hidden platform after treatment with SGS742 (SGS) (filled squares; 30 mg/kg; po; 90 min before training; $n = 7$; $* p < 0.05$; two-way ANOVA). (a) Stable and continuous overexpression of *Homer1a* (filled triangles; $n = 7$) or (b) *Homer1c* (filled circles; $n = 7$) in scopolamine treated rats show no significant difference in escape latencies compared to scopolamine treated rats injected with empty vector ($p > 0.05$). (c) Sustained up-

regulation of either *Homer1a* or *1c* reverses preference for the target quadrant ($\pm$ 95% confidence interval) in rats co-administered with scopolamine and SGS742 (* $p < 0.05$). (d) Rats co-treated with scopolamine and SGS742 crossed the previous location of the platform fewer times when infused with rAVV-*Homer1a* or rAVV-*Homer1c* during the probe trial (* $p < 0.05$; ** $p < 0.01$; one-way ANOVA). (e) Swimming speeds are similar across all the groups with a slight difference in rats co-administered with scopolamine and SGS742 after the administration of rAVV-*Homer1a* vector (* $p < 0.05$; one-way ANOVA). (f) Escape latencies to a visible platform are comparable across all the groups ($p > 0.05$; one-way ANOVA). All results, except for (c) are mean $\pm$ SEM.

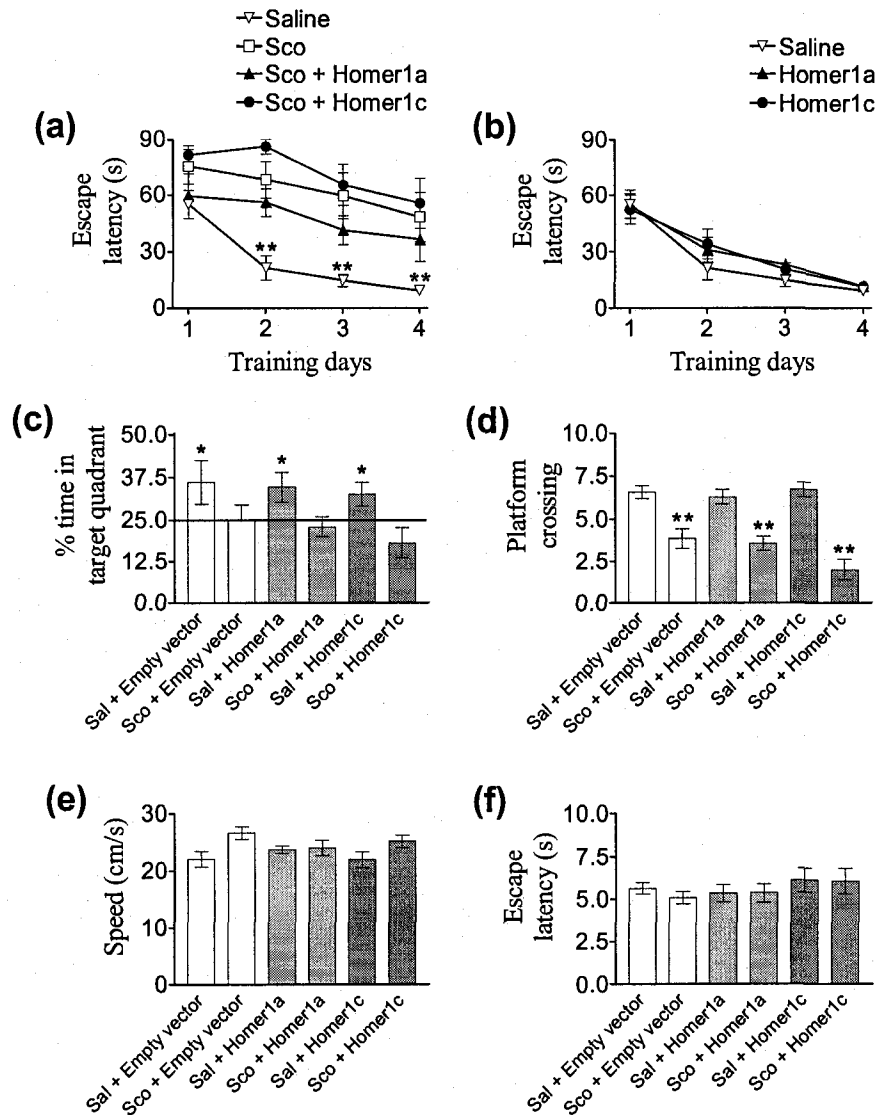


Fig. 5. Performance of scopolamine or saline treated rats in the MWM is not affected by the overexpression of either *Homer1a* or *1c* in the hippocampus of adult rats. (a) Longer escape latencies of scopolamine (Sco) treated rats relative to saline treated animals (** $p < 0.01$; two-way ANOVA) were not reversed by injection of rAVV-*Homer1a* (filled triangles; $n = 7$) or rAVV-*Homer1c* (filled circles; $n = 7$). (b) Infusion of rAVV-*Homer1a* (filled triangles; $n = 7$) or rAVV-*Homer1c* (filled circles; $n = 7$) in saline treated rats produced no change on the escape latencies of control animals ($p > 0.05$; two-way ANOVA). (c) Saline (Sal) treated rats infused with rAVV-*Homer1a*, rAVV-*Homer1c*, or rAVV-empty

vectors spent more time in the quadrant that previously contained the platform during the probe trial ($\pm$ 95% confidence interval; * $p < 0.05$) whereas scopolamine treated rats infused with vector containing *Homer1a* or *1c* or not show no preference for the target quadrant. (d) Injection of *Homer1a* and *1c* in saline treated rats did not alter the number of times animals crossed the previous location of the platform during the probe trial while scopolamine treated rats with or without stable overexpression of *Homer1a* or *1c* crossed the platform fewer times (** $p < 0.01$; one-way ANOVA). (e) Swimming speed and (f) escape latency to a visible platform are similar across all the groups ($p > 0.05$; one-way ANOVA). All results, except for (c) are mean $\pm$ SEM.

Tables

Table 1. Genes differentially expressed between scopolamine and saline treated rats following stimulation in a spatial memory task

Genes with decreased expression	Representative Public ID	Probe Set ID	FC	95 % CI
Homer1a (homolog) ^a	AF030088	1370997_at	-3.09	-1.73 : -5.54
Homer1a (homolog) ^a	AB003726	1370454_at	-2.85	-2.04 : -3.97
Dual specificity phosphatase 6	AI231350	1382778_at	-2.36	-1.59 : -3.51
Dual specificity phosphatase 5	NM_133578	1368124_at	-1.67	-1.20 : -2.33
Early growth response 1	NM_012551	1368321_at	-1.79	-1.38 : -2.34
VGF nerve growth factor inducible	NM_030997	1368359_a_at	-1.71	-1.55 : -1.89
Calcium/calmodulin-dependent protein kinase kinase 2, beta subunit	NM_031338	1368753_at	-1.62	-1.14 : -2.29
Glycophorin C	AA944212	1372273_at	-1.53	-1.31 : -1.78
Trinucleotide repeat containing 6 (predicted)	B1294732	1375664_at	-2.73	-1.25 : -5.98
CD3 antigen, epsilon polypeptide (predicted) ^a	AW915948	1379791_at	-1.85	-1.36 : -2.52
CD3 antigen, epsilon polypeptide (predicted) ^a	AA945909	1389997_at	-1.68	-1.21 : -2.35
Transcribed locus	BE109141	1381584_at	-2.07	-1.17 : -3.66
Transcribed locus	BI296482	1374035_at	-1.68	-1.23 : -2.30
Transcribed locus	AI030650	1380383_at	-1.65	-1.10 : -2.48
Transcribed locus	AA875536	1381242_at	-1.62	-1.02 : -2.59
Expressed sequence tag	BF390065	1380370_at	-1.73	-1.25 : -2.39
Expressed sequence tag	BI289762	1390174_at	-1.59	-1.39 : -1.82
Genes with increased expression				
Prodynorphin ^a	NM_019374	1369882_at	1.65	1.19 : 2.29
Prodynorphin ^a	BF412731	1383294_at	1.51	1.15 : 1.99
Sulfotransferase family 1A, phenol-preferring, member 1	AF394783	1370019_at	1.67	1.09 : 2.58
Gamma-aminobutyric acid (GABA) B receptor 1	AF312319	1388030_a_at	1.59	1.14 : 2.38
Branched chain aminotransferase 1, cytosolic	NM_017253	1369520_a_at	1.53	1.12 : 2.10
Cyclin-dependent kinase-like 4 (similar)	BG376193	1375495_at	2.68	1.34 : 5.37
Tumor protein D53 (similar)	AI231999	1372626_at	1.56	1.04 : 2.35
Transcribed locus, weakly similar to XP_576559.1 (predicted): similar to Ormdl1 protein	AI169620	1373053_at	1.92	1.35 : 2.73
Transcribed locus	AI138004	1375690_at	3.07	1.23 : 7.66
Transcribed locus	AA819409	1394643_at	1.96	1.19 : 3.22
Transcribed locus	BF285539	1376980_at	1.64	1.01 : 2.70
Transcribed locus	BM386385	1376911_at	1.62	1.01 : 2.62
Transcribed locus	BE097926	1375687_at	1.58	1.18 : 2.11
Expressed sequence tag	BF393907	1377056_at	1.63	1.12 : 2.38

Genes down-regulated in scopolamine treated rats compared to saline treated animals are indicated by a negative sign whereas genes up-regulated are represented by positive values. Fold changes (FC) and bounds of the 95 % confidence interval (95 % CI) are derived from the average measures obtained with the log₂ scale RMA methods to analyse microarray data. Gene probe set ID and representative public ID obtained from Affymetrix and NCBI respectively are provided.

^a More than one probe set obtained convergent results.

Supplemental Table 1. Genes differentially expressed between trained and naive saline treated rats

Genes with decreased expression	Representative Public ID	Probe Set ID	FC	95 % CI
Regulator of G-protein signaling 4*	NM_017214	1368505_at	-1.73	-1.11 : -2.70
Regulator of G-protein signaling 4*	U27767	1368506_at	-1.70	-1.10 : -2.61
Nuclear receptor subfamily 1, group D, member 1	M25804	1370816_at	-1.72	-1.42 : -2.07
Nuclear receptor subfamily 1, group D, member 2	U15660	1370540_at	-1.58	-1.28 : -1.94
Nuclear receptor subfamily 4, group A, member 3	NM_017352	1369217_at	-1.54	-1.27 : -1.87
Dopa decarboxylase	U31884	1368064_a_at	-1.91	-1.23 : -2.97
FERM domain containing 4B	AI713014	1384681_at	-1.76	-1.17 : -2.65
Kruppel-like factor 2	BM385790	1376569_at	-1.69	-1.23 : -2.34
Dual specificity phosphatase 1	U02553	1368146_at	-1.59	-1.11 : -2.29
TSC22 domain family 3	BF399517	1380777_at	-1.58	-1.03 : -2.42
Cocaine and amphetamine regulated transcript	NM_017110	1368585_at	-1.57	-1.13 : -2.20
Neurexophilin 3	BM382837	1374818_at	-1.56	-1.06 : -2.29
D site albumin promoter binding protein	AI230048	1387874_at	-1.53	-1.40 : -1.68
Ninjurin 2	NM_021595	1368135_at	-1.51	-1.25 : -1.83
CG12206-PA, isoform A (similar)	BF419840	1374367_at	-1.71	-1.07 : -2.75
Tumor protein D53 (similar)	AI231999	1372626_at	-1.54	-1.02 : -2.34
Transcribed locus	AI138004	1375690_at	-4.55	-2.98 : -6.96
Transcribed locus	AA819409	1394643_at	-2.92	-2.11 : -4.04
Transcribed locus	BI282169	1374787_at	-1.97	-1.16 : -3.34
Transcribed locus	BM386385	1376911_at	-1.89	-1.20 : -2.99
Transcribed locus	AI145951	1393585_at	-1.83	-1.31 : -2.54
Transcribed locus	BF285539	1376980_at	-1.68	-1.04 : -2.69
Transcribed locus	BG666908	1395000_at	-1.54	-1.23 : -1.92
Expressed sequence tag	AA944175	1389186_at	-1.88	-1.03 : -3.41
Genes with increased expression				
Homer1a (homolog)*	AB003726	1370454_at	3.81	2.71 : 5.35
Homer1a (homolog)*	AF030088	1370997_at	1.61	1.04 : 2.52
Prostaglandin-endoperoxide synthase 2	U03389	1368527_at	1.74	1.46 : 2.07
Guanine nucleotide binding protein, alpha 14	BI289045	1381557_at	1.67	1.46 : 1.91
Glycophorin C	AA944212	1372273_at	1.63	1.41 : 1.89
Protease, serine, 23	AI177099	1373152_at	1.58	1.20 : 2.08
SOX2 protein (similar)	BE120496	1396651_at	2.10	1.21 : 3.62
Cajalin 2 isoform a (similar)	BF403875	1396658_at	1.56	1.04 : 2.34
CD3 antigen, epsilon polypeptide (predicted)*	AW915948	1379791_at	1.74	1.49 : 2.04
CD3 antigen, epsilon polypeptide (predicted)*	AA945909	1389997_at	1.67	1.43 : 1.95
Ets variant gene 4 (E1A enhancer binding protein, E1AF) (predicted)	BE102969	1380168_at	1.54	1.15 : 2.05
LOC500720	BG670822	1398716_at	1.55	1.35 : 1.78
Transcribed locus	BE106273	1391334_at	1.90	1.08 : 3.34
Transcribed locus	AA926109	1383486_at	1.73	1.27 : 2.34
Transcribed locus	BF406606	1385087_at	1.69	1.33 : 2.16
Transcribed locus	BF549454	1391852_at	1.60	1.26 : 2.03
Expressed sequence tag	BI293026	1390369_at	2.15	1.33 : 3.47
Expressed sequence tag	BF390065	1380370_at	1.60	1.21 : 2.12

Genes down-regulated in trained saline treated rats compared to naive saline treated animals are indicated by a negative sign whereas genes up-regulated are represented by positive values. Fold expression changes and bounds of the 95 % confidence interval are derived from the average measures obtained with the log₂ scale RMA methods to analyse microarray data. Gene probe set ID and representative public ID obtained from Affymetrix and NCBI respectively are provided.

^a More than one probe set obtained convergent results.

Supplemental Table 2. Genes differentially expressed between scopolamine and naive saline treated rats

Genes with decreased expression	Representative Public ID	Probe Set ID	FC	95 % CI
Early growth response 1	NM_012551	1368321_at	- 1.64	- 1.35 : - 2.00
Early growth response 2	NM_053633	1387306_a_at	- 1.56	- 1.26 : - 1.93
Nuclear receptor subfamily 1, group D, member 1	M25804	1370816_at	- 1.77	- 1.47 : - 2.13
Nuclear receptor subfamily 4, group A, member 1	NM_024388	1386935_at	- 1.60	- 1.21 : - 2.11
Kruppel-like factor 2	BM385790	1376569_at	- 1.80	- 1.22 : - 2.66
Kruppel-like factor 4	NM_053713	1387260_at	- 1.64	- 1.22 : - 2.21
FBJ murine osteosarcoma viral oncogene homolog	BF415939	1375043_at	- 2.44	- 1.29 : - 4.62
Homer1a (homolog)	AF030088	1370997_at	- 1.92	- 1.09 : - 3.62
Activity regulated cytoskeletal-associated protein	NM_019361	1387068_at	- 1.85	- 1.39 : - 2.46
RM2 mRNA, partial sequence	BF390648	1392108_at	- 1.76	- 1.23 : - 2.51
Cocaine and amphetamine regulated transcript	NM_017110	1368585_at	- 1.63	- 1.09 : - 2.44
S100 calcium-binding protein A4	NM_012618	1367846_at	- 1.61	- 1.16 : - 2.22
Jun-B oncogene	NM_021836	1387788_at	- 1.58	- 1.10 : - 2.26
Dopa decarboxylase	U31884	1368064_a_at	- 1.53	- 1.24 : - 1.90
RGD1309360_predicted protein (similar)	AW527270	1391089_at	- 1.74	- 1.13 : - 2.69
Transcribed locus	AA899326	1392598_at	- 1.96	- 1.15 : - 3.33
Transcribed locus	BG670247	1390459_at	- 1.76	- 1.10 : - 2.82
Transcribed locus	AI030650	1380383_at	- 1.71	- 1.21 : - 2.42
Transcribed locus	BF281697	1374015_at	- 1.53	- 1.10 : - 2.12
Expressed sequence tag	BG380672	1394477_at	- 1.84	- 1.28 : - 2.63
Expressed sequence tag	BI289762	1390174_at	- 1.81	- 1.52 : - 2.14
Genes with increased expression				
Crystallin, alpha B	NM_012935	1370026_at	1.91	1.49 : 2.46
Sulfotransferase family 1A, phenol-preferring, member 1	AF394783	1370019_at	1.86	1.09 : 3.17
DnaJ (Hsp40) homolog, subfamily B, member 6	BM383877	1382024_at	1.65	1.15 : 2.38
Gap junction membrane channel protein beta 2	AI179953	1373386_at	1.56	1.15 : 2.12
Cyclin-dependent kinase-like 4 (similar)	BG376193	1375495_at	2.02	1.12 : 3.63
RGD1561072 (predicted)	BI288882	1383227_at	2.09	1.29 : 3.38
Deoxyguanosine kinase (predicted)	AI599463	1379435_at	1.75	1.18 : 2.60
Transcribed locus	BF402633	1375777_at	1.57	1.00 : 2.45
Expressed sequence tag	AW526768	1384082_at	2.19	1.27 : 3.79
Expressed sequence tag	BI293026	1390369_at	2.08	1.32 : 3.28
Expressed sequence tag	BF393907	1377056_at	1.58	1.10 : 2.28

Genes down-regulated in scopolamine treated rats compared to naive saline treated animals are indicated by a negative sign whereas genes up-regulated are represented by positive values. Fold expression changes and bounds of the 95 % confidence interval are derived from the average measures obtained with the $\log_2$ scale RMA methods to analyse microarray data. Gene probe set ID and representative public ID obtained from Affymetrix and NCBI respectively are provided.

Supplemental Table 3. Gene-specific primers used for PCR amplification

Genes	5'- 3' Forward Primer	5'- 3' Reverse Primer
Homer1a	GTGTCCACAGAAGCCAGAGAGGG	CTGTAGAGGACCCAGCTTCAGT
Egr1	GAGCCAAGTCCTTCTAGTCAGTAG	TGTGAGAGTTACAGTCGAGCAGTA
Camkk2	TGCGTTTCCATAGCAGCATG	GGCTCTACACACGTGCTTGGT
Vgf	ACTTCCTGGTCCCAAACGG	GGCTGGGAGACAGACACTTCA
Dusp6	CCACGAACATCATGGAGCAA	GATGCGACAGGTTGTGAGGAA
Gypc	TGGTCCCTGGGAGACTAATGA	GGTGGCGGCCATACTTGAC
Trc6	GGTTCACCGTGGGAACCTT	CCCTGCACTTTGAACAGGCT
Cd3e	TCGGAAGTCAAGGACAGTGGTT	CCTTGAGCTTTCTGTGTAGCAGAC
Gabbr1	GGCTCCTGGACGGATATGG	TGGAGCAGATTCGGACACAG
Pdyn	CCTTACCAGCGTCTCAGAGGA	CCAAACCTGCTGGAGAGACC
Tpd53	CCTGTCAGTGTGCAGAGCCA	TGGAGGCGACAGTCATCAAA
1375690	CTCAGTACGTGGCCCAACAGA	GAGTTGCCGGAGCGGTAAA
1376980	CCCAACACAGCAATTATTCTCAAC	TGCACGGAACCTTACGAATAGCA
Beta-actin	TCCTGTGGCATCCATGAAACT	AACGTCACACTTCATGATGGAATT

Gene-specific primers were designed with the Primer Express software (Applied Biosystems, CA). The locations of the primers were chosen to cross an exon-exon junction to insure the specificity for RNA rather than nuclear DNA sequences.

Discussion

Treatment with the muscarinic antagonist scopolamine is a well established model of learning and memory deficits that mimics those occurring during aging (Bartus et al., 1982; Ray et al., 1992; Stemmelin et al., 1999; Araujo et al., 2005). Determining the molecular and cellular mechanisms that sustain the amnesic effect of scopolamine could reveal critical defective components responsible for memory loss that occur during normal and pathological brain aging, such as in Alzheimer's disease. Using a microarray approach to perform whole-genome expression analysis, we identified several hippocampal genes that are differentially expressed in scopolamine treated rats following stimulation in a spatial memory task.

The present study is clearly distinct from a previous microarray analysis performed using scopolamine treated rats (Hsieh et al., 2003). In that report, hippocampal genes altered by scopolamine were analyzed in anesthetized Sprague-Dawley rats 30 min after injection of the drug without any learning and memory stimulation. They also used interspecies hybridization with human cDNA microarray containing a lower number of genes and injected a much higher dose of scopolamine (10 mg/kg) in lateral ventricle. In our study, we used a high-density microarray specific to rats to identify late response genes affected by lower dose of scopolamine (0.4 mg/kg, ip) following stimulation in a spatial memory task.

While three independent replicates per group are generally accepted to provide a reliable analysis of gene expression data using microarrays (Lee et al., 2000), here we employed four animals in each group. To obtain more accurate gene expression estimates and confidence intervals, individual Affymetrix microarrays were used for each animal. Moreover, a minimal FC of 1.5 derived from both the average and the median had to be reached, and a 95 % confidence interval (which gives less tight bounds than SEM measures) with a minimal bound of 1 or -1 FC for genes up- or down-regulated respectively had to be obtained. Gene results of interest were further confirmed with independent

quantitative real-time RT-PCR. Behavioral analysis of mice infused with rAAV vectors carrying either *Homer1a* or *1c* isoforms also validated the role of this gene in mnemonic deficits induced by scopolamine treatment. Altogether, these results indicate that the microarray approach used in this study led to a consistent restricted list of candidate genes possibly implicated in scopolamine-induced amnesia.

To our knowledge, our results provide the first evidence that muscarinic receptors may be implicated in the regulation of the glutamatergic related gene *Homer1*. Our microarray data shows that scopolamine treatment disrupts the rapid and temporal up-regulation of *Homer1a* seen in control rats following spatial memory stimulation. Because the *Homer1* gene encodes both *Homer1a* and *1c*, we performed local infusion of rAAV vectors carrying either *Homer1a* or *1c* isoforms into the hippocampus to determine their respective roles in scopolamine-induced amnesia. An up-regulation of *Homer1a* or *1c* suppressed memory improvements induced by the GABA_B receptor antagonist SGS742 in scopolamine treated rats strongly suggests that *Homer1* is an essential component in the cascade of genes involved in scopolamine-induced amnesia. This result also implies that neither a sustained up-regulation of the inducible *Homer1a* gene nor constitutively expressed *Homer1c* is favourable for fully efficient synaptic plasticity required during memory processes in scopolamine treated rats. A complete deletion of *Homer1* was also observed to produce memory deficits in the MWM task (Jaubert et al., 2006).

Transient induction of *Homer1a* has been shown to produce glutamatergic synapse rearrangement necessary for long-term potentiation (LTP) and memory formation (Brakeman et al., 1997), as supported by our microarray data showing increased gene expression of *Homer1a* in trained versus naive saline treated rats (see Supplemental Table 1). In contrast, we observed that stable, continuous expression of *Homer1a* reversed memory improvements induced by SGS742 in scopolamine treated animals. The same finding was observed when *Homer1c* was constantly overexpressed following vector infusion into the hippocampus. The inhibitory effects of *Homer1a* and *1c* on memory enhancement induced by

SGS742 seems to be specific to scopolamine treated rats rather than a non-selective cognitive deterioration since learning and memory abilities of saline treated rats are not altered by the overexpression of either *Homer1* isoforms.

In order to generate a highly efficient signalling complex, *Homer1c* must mediate the physical association of molecules involved in glutamatergic post-synaptic signalling, including group 1 metabotropic receptors (Brakeman et al., 1997), inositol trisphosphate (Tu et al., 1998) and NMDA receptor via the interaction with Shank proteins (Tu et al., 1999). Following a synaptic stimulus, *Homer1a* is transiently up-regulated to compete with *Homer1c* and disassemble the signalling complex to allow for the synaptic plasticity required for LTP and memory processes (Brakeman et al., 1997).

Taken together, these data suggests an essential role for *Homer1* isoforms in synaptic plasticity. However, their role at a behavioural level has not been studied in details. The results obtained here using the MWM task indicate that sustained, instead of transitory, overexpression of *Homer1a* may destabilize in a long-term manner the glutamatergic machinery localized in the post-synaptic compartment whereas sustained up-regulation of *Homer1c* strengthen the post-synaptic network and prevent synaptic plasticity. Accordingly, our results support a mechanism by which the transient up-regulation of *Homer1a* during neuronal activity leads to structural and functional reorganization by competing dynamically with constitutively expressed *Homer1c* (Brakeman et al., 1997; Tu et al., 1998; Tu et al., 1999).

Our study also revealed key interactions between hippocampal cholinergic, glutamatergic and GABAergic neurotransmissions during learning processes. A large body of anatomical, neurochemical and electrophysiological data have provided evidence for the existence of interactions between these systems (Vizi & Kiss, 1998), but the molecular and cellular components involved have yet to be fully established. Our data suggest that *GABA_B* receptors and the glutamatergic component *Homer1* are critical in the cascade of genes involved in scopolamine-induced amnesia. Interestingly, earlier data have shown that the activation of muscarinic receptors inhibits GABA release from hippocampal

interneurons (Ben Ari et al., 1981; Behrends & ten Bruggencate, 1993) and that the activation of pre-synaptic GABA_B receptors decreases hippocampal glutamate release (Freund & Buzsaki, 1996).

Many other genes previously associated or not with memory formation were differentially expressed following our scopolamine treatment. For example, *early growth response 1* (*Egr-1*, also known as *zif268*) is an inducible transcription factor implicated in the maintenance of the later phase of LTP as well as in memory consolidation or stabilization of long-term memory (Guzowski et al., 2001; Jones et al., 2001). Interestingly, the expression of *Egr-1* was shown to be activated by muscarinic and NMDA receptors (Cole et al., 1989; von der Kammer et al., 1998). Prodynorphin-derived peptides which modulate neurotransmitter release through the activation of presynaptic kappa opioid receptors have been shown to reverse scopolamine-induced memory impairments in mice (Ukai et al., 1995; Ukai et al., 1997). Previous reports have demonstrated that prodynorphin knock-out mice have diminished age-related memory deficits in the MWM task (Nguyen et al., 2005). An increased expression of this peptide family was also observed in the hippocampus of aged rats and is associated with memory deficits in the MWM task (Jiang et al., 1989; Kotz et al., 2004), which is consistent with our microarray data showing that increased prodynorphin expression is linked to memory impairments induced by a scopolamine treatment. The up-regulation of *VGF* in the hippocampus observed after learning as well as BDNF treatment suggests a role for this trophic factor in synaptic plasticity (Alder et al., 2003) and decreased expression of *VGF* was observed in the hippocampus of aged-memory impaired rats (Sugaya et al., 1998) and in scopolamine treated animals as shown in the present study.

Calcium/calmodulin-dependent protein kinase kinase 2 (*Camkk2*) as well as *dual specificity phosphatases* (*Dusp*) 5 and 6 are part of the mitogen-activated protein kinases (MAPKs) signalling pathways activated by both muscarinic and NMDA receptors. The MAPK cascade was also shown to up-regulate *Homer1a* expression following NMDA receptor stimulation (Sato et al., 2001). *Camkk2* phosphorylates and activates MAPKs whereas *Dusp5* and 6 induce the

dephosphorylation of MAPKs decreasing mitogenic signalling. Although the MAPKs cascade is well known to contribute to synaptic plasticity and long-term memory formation (Atkins et al., 1998; Blum et al., 1999), *Camkk2* as well as *Dusp5* and *6* represent novel candidate genes possibly involved in the regulation of learning and memory functions. Because of the well known temporal delay between gene expression and the synthesis of the corresponding protein product, it would be of interest in future studies to perform a detailed analysis of protein levels at various time points following mRNA transcription.

While the level of various GABA_A receptor subunits were shown to be relatively stable in the hippocampus of AD patients (Mizukami et al., 1998), the GABA_B receptor 1 subunit was shown to be increased at the onset of the illness (Iwakiri et al., 2005), as observed here following scopolamine treatment. Moreover, the modulation of GABAergic neurotransmission by muscarinic receptors was shown to be altered in a mouse model of AD (Zhong et al., 2003). The recent approval of memantine, a non-competitive NMDA receptor agonist, for the treatment of AD has revealed the therapeutic potential of drugs targeting the glutamatergic system in this disease (Kemp & McKernan, 2002). Interestingly, a microarray analysis revealed reduced mRNA expression of *Homer1a* in an AD mouse model (Dickey et al., 2003). The present study suggests that defects in glutamatergic transmission produced by alteration in the level of expression of *Homer1* isoforms could play a role in various forms of dementia including AD. Further studies are certainly warranted in that regard.

In summary, the microarray approach used in this study led to the identification of several candidate genes possibly implicated in scopolamine-induced amnesia. Of particular interest were *Homer1* and the GABA_B receptors. Indeed, we found that *Homer1a* and *1c* expressed in the dorsal hippocampus suppressed memory improvements induced by the GABA_B receptor antagonist SGS742 in scopolamine treated rats. These results and other gene families altered in our scopolamine-induced amnesia model suggest potential novel targets to develop more effective therapeutic strategies for the treatment of age-related cognitive impairments.

Acknowledgements

We thank Doris Dea for technical assistance in real-time RT-PCR. This work was supported by grants from the Canadian Institutes for Health Research (CIHR) to Rémi Quirion and a student scholarship from CIHR to Jonathan Brouillette.

References

- Aigner TG, Walker DL & Mishkin M (1991) Comparison of the effects of scopolamine administered before and after acquisition in a test of visual recognition memory in monkeys. *Behav Neural Biol*, 55, 61-67.
- Alder J, Thakker-Varia S, Bangasser DA, Kuroiwa M, Plummer MR, Shors TJ & Black IB (2003) Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J Neurosci*, 23, 10800-10808.
- Araujo JA, Studzinski CM & Milgram NW (2005) Further evidence for the cholinergic hypothesis of aging and dementia from the canine model of aging. *Prog Neuropsychopharmacol Biol Psychiatry*, 29, 411-422.
- Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM & Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. *Nat Neurosci*, 1, 602-609.
- Atri A, Sherman S, Norman KA, Kirchhoff BA, Nicolas MM, Greicius MD, Cramer SC, Breiter HC, Hasselmo ME & Stern CE (2004) Blockade of central cholinergic receptors impairs new learning and increases proactive interference in a word paired-associate memory task. *Behav Neurosci*, 118, 223-236.
- Aubert I, Rowe W, Meaney MJ, Gauthier S & Quirion R (1995) Cholinergic markers in aged cognitively impaired Long-Evans rats. *Neuroscience*, 67, 277-292.
- Bartus RT, Dean RL, III, Beer B & Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science*, 217, 408-414.
- Behrends JC & ten Bruggencate G (1993) Cholinergic modulation of synaptic inhibition in the guinea pig hippocampus in vitro: excitation of GABAergic interneurons and inhibition of GABA-release. *J Neurophysiol*, 69, 626-629.
- Ben Ari Y, Krnjevic K, Reinhardt W & Ropert N (1981) Intracellular observations on the disinhibitory action of acetylcholine in the hippocampus. *Neuroscience*, 6, 2475-2484.

- Blum S, Moore AN, Adams F & Dash PK (1999) A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J Neurosci*, 19, 3535-3544.
- Bourtchouladze R, Abel T, Berman N, Gordon R, Lapidus K & Kandel ER (1998) Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learn Mem*, 5, 365-374.
- Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL & Worley PF (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature*, 386, 284-288.
- Brouillette J & Quirion R (2007) Transthyretin: A key gene involved in the maintenance of memory capacities during aging. *Neurobiol Aging*, Epub ahead of print.
- Carli M, Luschi R & Samanin R (1997) Dose-related impairment of spatial learning by intrahippocampal scopolamine: antagonism by ondansetron, a 5-HT₃ receptor antagonist. *Behav Brain Res*, 82, 185-194.
- Chen Z, Xu AJ, Li R & Wei EQ (2002) Reversal of scopolamine-induced spatial memory deficits in rats by TAK-147. *Acta Pharmacol Sin*, 23, 355-360.
- Clark KR, Liu X, McGrath JP & Johnson PR (1999) Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum Gene Ther*, 10, 1031-1039.
- Cole AJ, Saffen DW, Baraban JM & Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature*, 340, 474-476.
- Deutsch JA (1971) The cholinergic synapse and the site of memory. *Science*, 174, 788-794.
- Devan BD, Sierra-Mercado D, Jr., Jimenez M, Bowker JL, Duffy KB, Spangler EL & Ingram DK (2004) Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacol Biochem Behav*, 79, 691-699.

- Dickey CA, Loring JF, Montgomery J, Gordon MN, Eastman PS & Morgan D (2003) Selectively reduced expression of synaptic plasticity-related genes in amyloid precursor protein + presenilin-1 transgenic mice. *J Neurosci*, 23, 5219-5226.
- During MJ, Cao L, Zuzga DS, Francis JS, Fitzsimons HL, Jiao X, Bland RJ, Klugmann M, Banks WA, Drucker DJ & Haile CN (2003a) Glucagon-like peptide-1 receptor is involved in learning and neuroprotection. *Nat Med*, 9, 1173-1179.
- During MJ, Young D, Baer K, Lawlor P & Klugmann M (2003b) Development and optimization of adeno-associated virus vector transfer into the central nervous system. *Methods Mol Med*, 76, 221-236.
- Freund TF & Buzsaki G (1996) Interneurons of the hippocampus. *Hippocampus*, 6, 347-470.
- Guzowski JF, Setlow B, Wagner EK & McGaugh JL (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. *J Neurosci*, 21, 5089-5098.
- Hauck B, Chen L & Xiao W (2003) Generation and characterization of chimeric recombinant AAV vectors. *Mol Ther*, 7, 419-425.
- Hollup SA, Kjelstrup KG, Hoff J, Moser MB & Moser EI (2001) Impaired recognition of the goal location during spatial navigation in rats with hippocampal lesions. *J Neurosci*, 21, 4505-4513.
- Hsieh MT, Hsieh CL, Lin LW, Wu CR & Huang GS (2003) Differential gene expression of scopolamine-treated rat hippocampus-application of cDNA microarray technology. *Life Sci*, 73, 1007-1016.
- Igaz LM, Vianna MR, Medina JH & Izquierdo I (2002) Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci*, 22, 6781-6789.
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B & Speed TP (2003a) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*, 31, e15.

- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U & Speed TP (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, 4, 249-264.
- Iwakiri M, Mizukami K, Ikonomovic MD, Ishikawa M, Hidaka S, Abrahamson EE, Dekosky ST & Asada T (2005) Changes in hippocampal GABABR1 subunit expression in Alzheimer's patients: association with Braak staging. *Acta Neuropathol (Berl)*, 109, 467-474.
- Janas AM, Cunningham SC, Duffy KB, Devan BD, Greig NH, Holloway HW, Yu QS, Markowska AL, Ingram DK & Spangler EL (2005) The cholinesterase inhibitor, phenserine, improves Morris water maze performance of scopolamine-treated rats. *Life Sci*, 76, 1073-1081.
- Jaubert PJ, Golub MS, Lo YY, Germann SL, Dehoff MH, Worley PF, Kang SH, Schwarz MK, Seeburg PH & Berman RF (2006) Complex, multimodal behavioral profile of the Homer1 knockout mouse. *Genes Brain Behav*, in press.
- Jerusalinsky D, Kornisiuk E & Izquierdo I (1997) Cholinergic neurotransmission and synaptic plasticity concerning memory processing. *Neurochem Res*, 22, 507-515.
- Jiang HK, Owyang VV, Hong JS & Gallagher M (1989) Elevated dynorphin in the hippocampal formation of aged rats: relation to cognitive impairment on a spatial learning task. *Proc Natl Acad Sci U S A*, 86, 2948-2951.
- Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S & Davis S (2001) A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci*, 4, 289-296.
- Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294, 1030-1038.
- Kemp JA & McKernan RM (2002) NMDA receptor pathways as drug targets. *Nat Neurosci*, 5 Suppl, 1039-1042.
- Klugmann M, Wymond SC, Leichtlein CB, Klaussner BK, Dunning J, Fong D, Young D & During MJ (2005) AAV-mediated hippocampal expression of short

- and long Homer 1 proteins differentially affect cognition and seizure activity in adult rats. *Mol Cell Neurosci*, 28, 347-360.
- Kotz CM, Weldon D, Billington CJ & Levine AS (2004) Age-related changes in brain proDynorphin gene expression in the rat. *Neurobiol Aging*, 25, 1343-1347.
- Lee ML, Kuo FC, Whitmore GA & Sklar J (2000) Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci U S A*, 97, 9834-9839.
- Mastakov MY, Baer K, Xu R, Fitzsimons H & During MJ (2001) Combined injection of rAAV with mannitol enhances gene expression in the rat brain. *Mol Ther*, 3, 225-232.
- Mizukami K, Ikonovic MD, Grayson DR, Sheffield R & Armstrong DM (1998) Immunohistochemical study of GABAA receptor alpha1 subunit in the hippocampal formation of aged brains with Alzheimer-related neuropathologic changes. *Brain Res*, 799, 148-155.
- Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*, 11, 47-60.
- Moser E, Moser MB & Andersen P (1993) Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *J Neurosci*, 13, 3916-3925.
- Nakagawa Y & Takashima T (1997) The GABA(B) receptor antagonist CGP36742 attenuates the baclofen- and scopolamine-induced deficit in Morris water maze task in rats. *Brain Res*, 766, 101-106.
- Nakajima M, Inui A, Miura M, Hirose Y, Okita M, Himori N, Baba S & Kasuga M (1994) Negative and positive effects of intracerebroventricular scopolamine on memory in mice undergoing passive avoidance and escape tests. *Brain Res Bull*, 34, 375-380.
- Nguyen XV, Masse J, Kumar A, Vijitruth R, Kulik C, Liu M, Choi DY, Foster TC, Usynin I, Bakalkin G & Bing G (2005) Prodynorphin knockout mice demonstrate diminished age-associated impairment in spatial water maze performance. *Behav Brain Res*, 161, 254-262.

- Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, Parent A, White N & Meaney MJ (1995) Facilitation of acetylcholine release and cognitive performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. *J Neurosci*, 15, 1455-1462.
- Ray PG, Meador KJ, Loring DW, Zamrini EW, Yang XH & Buccafusco JJ (1992) Central anticholinergic hypersensitivity in aging. *J Geriatr Psychiatry Neurol*, 5, 72-77.
- Riedel G, Micheau J, Lam AG, Roloff EL, Martin SJ, Bridge H, de Hoz L, Poeschel B, McCulloch J & Morris RG (1999) Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat Neurosci*, 2, 898-905.
- Rowe WB, O'Donnell JP, Pearson D, Rose GM, Meaney MJ & Quirion R (2003) Long-term effects of BIBN-99, a selective muscarinic M2 receptor antagonist, on improving spatial memory performance in aged cognitively impaired rats. *Behav Brain Res*, 145, 171-178.
- Sarter M & Bruno JP (1997) Trans-synaptic stimulation of cortical acetylcholine and enhancement of attentional functions: a rational approach for the development of cognition enhancers. *Behav Brain Res*, 83, 7-14.
- Sato M, Suzuki K & Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci*, 21, 3797-3805.
- Schon K, Atri A, Hasselmo ME, Tricarico MD, LoPresti ML & Stern CE (2005) Scopolamine reduces persistent activity related to long-term encoding in the parahippocampal gyrus during delayed matching in humans. *J Neurosci*, 25, 9112-9123.
- Stemmelin J, Cassel JC, Will B & Kelche C (1999) Sensitivity to cholinergic drug treatments of aged rats with variable degrees of spatial memory impairment. *Behav Brain Res*, 98, 53-66.
- Sugaya K, Greene R, Personett D, Robbins M, Kent C, Bryan D, Skiba E, Gallagher M & McKinney M (1998) Septo-hippocampal cholinergic and

- neurotrophin markers in age-induced cognitive decline. *Neurobiol Aging*, 19, 351-361.
- Tang Y, Mishkin M & Aigner TG (1997) Effects of muscarinic blockade in perirhinal cortex during visual recognition. *Proc Natl Acad Sci U S A*, 94, 12667-12669.
- Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M & Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron*, 23, 583-592.
- Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ & Worley PF (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron*, 21, 717-726.
- Ukai M, Itoh J, Kobayashi T, Shinkai N & Kameyama T (1997) Effects of the kappa-opioid dynorphin A(1-13) on learning and memory in mice. *Behav Brain Res*, 83, 169-172.
- Ukai M, Kobayashi T, Shinkai N, Shan-Wu X & Kameyama T (1995) Dynorphin A-(1-13) potently improves scopolamine-induced impairment of passive avoidance response in mice. *Eur J Pharmacol*, 274, 89-93.
- van der Zee EA & Luiten PG (1999) Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. *Prog Neurobiol*, 58, 409-471.
- Vizi ES & Kiss JP (1998) Neurochemistry and pharmacology of the major hippocampal transmitter systems: synaptic and nonsynaptic interactions. *Hippocampus*, 8, 566-607.
- von der Kammer H, Mayhaus M, Albrecht C, Enderich J, Wegner M & Nitsch RM (1998) Muscarinic acetylcholine receptors activate expression of the EGR gene family of transcription factors. *J Biol Chem*, 273, 14538-14544.
- White NM & McDonald RJ (2002) Multiple parallel memory systems in the brain of the rat. *Neurobiol Learn Mem*, 77, 125-184.
- Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ & Worley PF (1998) Homer regulates the association of

group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron*, 21, 707-716.

Zhong P, Gu Z, Wang X, Jiang H, Feng J & Yan Z (2003) Impaired modulation of GABAergic transmission by muscarinic receptors in a mouse transgenic model of Alzheimer's disease. *J Biol Chem*, 278, 26888-26896.

Chapter VI

General Discussion

1. The aim of this general discussion

The results presented in each manuscript have been discussed in detail in the previous chapters. The aim of this general discussion is to provide supplementary information on recent microarray studies using the AI rat model (Rowe et al., 2007; Burger et al., 2007; Brouillette & Quirion, 2007). Additionally, in light of altered TTR gene expression that we observed during aging (Brouillette & Quirion, 2007), we will discuss the potential role of TTR as a biomarker of aging as well as its implication in AD, the most common age-related neurodegenerative disorder. Since the role of TTR in the sequestration and clearance of A β has been discussed in the review of the literature (see section 9.4), we will focus here on studies showing association between altered TTR levels and factors influencing the risk of developing AD's related pathology. Finally, we will focus on mechanisms underlying scopolamine-induced amnesia and will compare gene expression profiles obtained in this animal model and in AI rats. A general conclusion with possible future research avenues then closes the thesis.

2. Hippocampal gene expression profile of aged memory-impaired rats

Similar to humans, individual differences in aged animals can naturally lead to memory impairments. Since stable forms of long-term plasticity require the induction of a cascade of genes to produce and maintain structural changes related to memory formation, genes differentially expressed between AI and AU rats following behavioural stimulation could, at least partly, reveal novel candidate genes involved in age-associated cognitive deficits. Our study (Brouillette & Quirion, 2007) along with two other recent reports (Rowe et al., 2007; Burger et al., 2007) have used microarray approach to investigate the simultaneous expression of thousands of hippocampal genes implicated in highly complex, multilevel memory networks that could explain individual differences in cognitive capacities of aged rats. However, because of important methodological differences only limited overlap in results is observed between these three studies.

The most critical difference between these protocols relates to the time point at which gene expression profiles were investigated. Several studies have indicated that long-term memory formation depends on two distinct temporal phases requiring gene expression and protein synthesis: shortly and several hours post-training of the animal in the behavioural task (Bourtchouladze et al., 1998; Quevedo et al., 1999; Igaz et al., 2002). Rowe et al (2007) have investigated gene profiles of aged rats 1 h after the last MWM trial to analyse alterations in the expression of immediate-early genes associated with the first phase. They also sacrificed aged rats 21 days post-training to detect differential gene expression between AI and AU animals under resting conditions. In our study, rats were sacrificed 6 and 8 h after the end of training to investigate changes in the expression of more downstream genes involved during the second time period. Burger et al (2007) studied hippocampal gene expression profiles of aged rats at 24 h, post-training.

Moreover, the results obtained in these reports can hardly be compared because of the type of analysis and controls used in each one of them. Rowe et al (2007) have selected genes altered in the AI group only if they differed in both the young and AU animals. This restriction has the disadvantage of eliminating many interesting genes that are differentially expressed between AI and AU rats but not in the young adult cohort. Burger et al (2007) used different controls and specifically excluded genes that differed with age. In our study, AI rats were compared directly to AU animals to avoid any *a priori* elimination of candidate genes.

The number of genes differentially expressed among the various groups also varies greatly among the three studies mainly because of the statistical programs used to analyse microarray data. For example, in our study, 27 genes were shown to be altered between non-trained aged and young rats using the RMA method of analysis. Burger et al (2007) have used BRB array tools 3.2.2 (*t* test with a significant threshold of $p < 0.005$) to identify 243 genes differentially expressed between these two same groups, whereas Rowe et al. (2007) detected 653 genes differentially expressed using MAS5 ($p < 0.05$) analysis default program. Despite

these methodological differences that prevent close comparison, all three studies demonstrated that alteration in the expression of many genes occur with age under non-trained conditions. Thus, individual variation in cognitive capacities of aged rats may depend mainly on differential gene expression seen at the basal level during aging. Alteration in gene transcription following memory challenge may thus compensate for age dependent variations to allow older animals to perform well in the MWM task. Hence, these three microarray studies may be seen as complementary to elucidate the multiple cellular pathways affected in age-associated cognitive impairments.

The families of genes highlighted in these three genome-wide studies vary greatly as well. By analysing only annotated genes (5331 out of 15923) present on the genechips, Rowe et al (2007) identified 746 genes that differed significantly across the Y, AU and AI groups 1 h following the last MWM trial on day 5. They also detected 847 genes that were differentially expressed across the three groups on day 21, post-training. To determine which cellular pathways and functional processes were potentially altered, they performed overrepresentation analyses on the identified genes using the Expression Analysis Systematic Explorer (EASE) program. With normal aging, Rowe et al (2007) found changes in genes associated with axonal growth, cytoskeletal assembly/transport, lipogenic/uptake pathways, immune/inflammatory response, lipid/protein degradation, cholesterol transport, transforming growth factors and cAMP signalling pathways. In AI animals, Rowe et al (2007) reported, among others, the down-regulation of immediate-early genes, wingless integration site, insulin, G-protein signalling, lipogenesis, and glucose utilization pathways on day 5 post-training. Notch2 (oligodendrocyte development) and myelination pathways were found to be up-regulated, particularly on day 21 post-training. In AU rats, various genes related to receptor/signal transduction pathways were observed to be up-regulated. However, because of the large number of genes differentially expressed between various groups, it is difficult to conclude which molecular and cellular pathways are primarily affected in age-related cognitive dysfunctions, especially since only

very few of these genes (only four) were validated by another independent method of analysis (immunohistochemistry).

After removing probe sets for which hybridization signal intensities were at/or below background levels, Burger et al (2007) detected 50 genes differentially expressed in the CA1 hippocampal field between AU and AI rats among the 4966 probe sets remaining on their genechips. These genes were classified into four groups based on their potential role in learning and memory: transcription, synaptic morphology, ion conductivity and protein modifications. Contrary to Rowe et al (2007), none of the genes identified are directly involved in apoptosis, inflammation, stress response or in the biosynthesis of lipids, protein or carbohydrates. Interestingly, a large number of these genes (21 of 50) are directly involved in neuronal or synaptic functions. However, one major issue with this study is that microarray analysis was performed 24 h post-training in the MWM task. Since the two distinct time points requiring gene expression for long-term memory formation were reported to take place shortly and around 8 h following training (Bourtchouladze et al., 1998; Igaz et al., 2002), it is difficult to interpret the genuine relevance of these genes in age-related memory deficits. Further studies at shorter time points are certainly warranted.

3. Hippocampal gene expression profiles of young and aged memory-unimpaired animals

In accordance with microarray experiments performed by Rowe et al (2007) and Burger et al (2007), our study revealed that young rats tested in the MWM did not show the same pattern of gene expression as AU animals despite similar acquisition curves. Indeed, the largest number ($n = 38$) of genes differentially expressed in our study was observed between these two groups. Several hypotheses can explain this observation. It is likely that young and aged rats used different navigational and/or molecular strategies to memorize the location of the hidden platform. Another explanation is that memory processing is more efficient in young animals and that alteration in gene transcription is achieved more

quickly after the last trial of the day. The consolidation of information learned during the first four training days could also be more stable in young rats compared to AU animals on day 5. Thus, even if both groups reached a plateau in their acquisition curves on day 4 and 5, better consolidation in young animals would decrease the number and/or amplitude of genes differentially expressed to induce synaptic plasticity associated with memory formation on day 5. This hypothesis is in accordance with the larger number of genes changed in young animals on day 4 ($n = 39$) (Brouillette et al., 2007) than on day 5 ($n = 5$) (Brouillette & Quirion, 2007) at the same time point (6 h), post-training. Interestingly, Homer1a was shown to be up-regulated on both days following memory stimulation. Thus, different consolidation profiles, in addition to variation in molecular and cellular pathways to elaborate navigational strategies, may explain differences in gene expression profiles between young and AU rats.

To determine further if the different patterns of gene expression observed between AU and young animals are attributed to more efficient memory processing in younger rats, a microarray analysis could be done in both groups at various time points after their last trial in the MWM task. A larger number of genes differentially expressed at earlier time points in young versus AU rats would suggest a faster processing to induce synaptic plasticity in young animals. According to the Hebbian process, well-learned information requires less extensive cascade of genes to produce new synapses since connections needed for permanent retention are already in place (Hebb, 1949; Skrebitsky & Chepkova, 1998). Thus, gene expression profiles of young and AU animals on day 3, 4 and 5 in the MWM task could be analyzed to determine if navigational information is consolidated at the same speed level in both groups. A smaller pool of gene expression changed in young rats when increasing the number of MWM trials could suggest a higher degree of consolidation compared to AU animals. Moreover, if the type of genes altered between young and AU rats vary greatly independent of the time points, one may argue that the molecular pathways involved are different between these groups. These experiments could also reveal differences in the efficacy of memory processing and/or consolidation if genes

altered are similar but differentially expressed at diverse time points between young and AU rats.

4. Role of transthyretin during aging and in AD

Our study has suggested that defective retinoid transport produced by decreased TTR expression is involved in the development of memory deficits during aging (Brouillette & Quirion, 2007). Indeed, we reported decreased TTR gene expression at the basal level in aged rats compared to adult animals as well as in age memory-impaired rats relative to aged memory-unimpaired rats. Age-related cognitive deficits were also detected in TTR^{-/-} mice. The effect of TTR in memory processing seems to be related to its ability to regulate brain retinoid availability since we observed that a treatment with RA was able to reverse the cognitive deficits observed in TTR^{-/-} mice and aged rats. Since TTR is known to sequester A β in addition to being involved in retinol transport, we will next discuss the impact of altered TTR gene expression during aging and its potential role in various neuropathological features of AD.

4.1. Transthyretin as a biomarker of aging

A general decline of biological functions is characteristic of the aging process, and although age-associated changes in protein levels are heterogeneous, most protein biosynthesis decreases during aging (Ward & Richardson, 1991; Vranckx et al., 1995). TTR levels are markedly up-regulated in developing choroid plexus (Makover et al., 1989) but then decrease in the CSF of adult humans (Larsen & DeLallo, 1989) as well as in choroid epithelial cells of adult rat brains (Kato et al., 1986). Another study reported decreased levels of TTR protein synthesis in choroid plexus and in newly secreted CSF in old sheep compared to young ones (Chen et al., 2005). Changes in plasma levels (Savu et al., 1991; Vranckx et al., 1995) or CSF/serum ratio (Garton et al., 1991) of TTR have been proposed as biomarkers of the aging process. All these findings are in agreement

with our study showing decreased TTR gene expression under resting conditions between young and aged rats (Brouillette & Quirion, 2007).

TTR has also been reported to be a potential marker for various neurological disorders. For example, concentrations of TTR in the CSF may serve as an indicator of CSF circulation disorders (Weisner & Roethig, 1983), and of choroid plexus tumors (Hasselblatt et al., 2006). Recently, TTR was identified as a potential CSF biomarker of frontotemporal dementia (Ruetschi et al., 2005), a neurodegenerative disorder showing symptoms bearing similarities with AD (Galasko & Marder, 2002). Interestingly, post-translational alterations of TTR detected in the CSF were also observed in AD patients (Biroccio et al., 2006). However, two studies have shown that CSF levels of TTR increased with age, with significantly lower levels in AD patients compared to age-matched controls (Serot et al., 1997; Merched et al., 1998). These results are similar to those we obtained in AU rats which showed a 3.28 fold increase of TTR gene expression relative to young animals, while the expression of this gene was reduced in AI compared to AU rats (Brouillette & Quirion, 2007). Aged individuals without neurological deficits who come to the hospital for lumbar punctures are exposed to a novel environment; a stress that may induce increases in TTR CSF levels as seen in AU rats following cognitive stimulation in the MWM task. This hypothesis is sustained by the observation that mice exposed to stimulating environments show up-regulation of TTR compared to animals maintained on standard housing conditions (Lazarov et al., 2005).

Decreased levels of TTR gene expression found in AI relative to AU rats suggest that a precise regulation of the expression of this gene during aging is essential to preserve intact memory capacities, which is further supported by age-related memory deficits observed in aged TTR^{-/-} mice (Brouillette & Quirion, 2007). Other microarray reports have found that TTR gene expression was also altered during the consolidation phase of long-term memory (Levenson et al., 2004) and in the generation of fear memory (Stork et al., 2001). Thus, TTR seems to be highly regulated and various substances have already been shown to modulate the expression/secretion of TTR in the brain. Indeed, TTR gene

expression was reported to be altered following treatment with Ginko biloba (Watanabe et al., 2001), omega-3 (Puskas et al., 2003; Kitajka et al., 2004) and the Asian medicinal herbal extract Yukmijihwang-tang (Rho et al., 2005). Interestingly, all these natural products have been proposed to have anti-aging properties and potential beneficial effects in the treatment of AD (Bastianetto et al., 2000a; Rho et al., 2005; Freund-Levi et al., 2006). Recently our group proposed that one mechanism by which red wine and tea-derived polyphenols such as resveratrol may exert their neuroprotective effect is by up-regulating TTR gene expression leading to the sequestration of A β peptides, which may in turn prevent the formation of toxic A β oligomers (Bastianetto et al., 2007).

4.2. Dual role of transthyretin in AD

Some evidence suggests that TTR may be involved in AD on the basis of its role as retinol transporter and A β sequestering protein (Fig. VI.1). Since no TTR gene mutations have been reported in AD (Palha et al., 1996), alterations in TTR gene expression during aging rather than specific mutations in this gene may be involved. For example, reduction in TTR gene expression during aging may have an impact on the onset of AD phenotype by affecting the bioavailability of retinol to brain tissues, and by decreasing the sequestration and elimination of A β from CNS parenchyma.

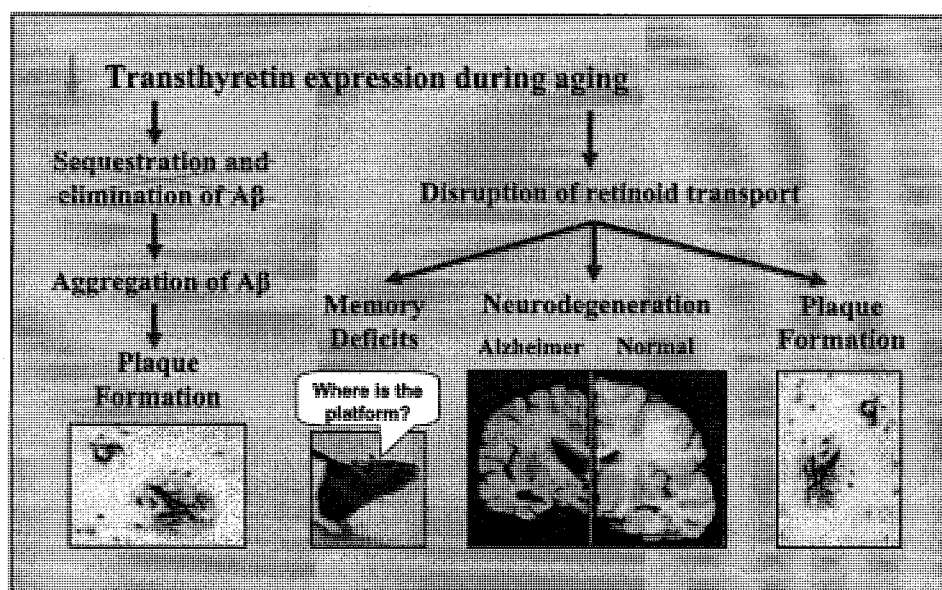


Fig. VI.1. The TTR cascade hypothesis of AD. Decreases in basal levels of TTR gene expression and/or following cognitive stimulation during brain aging reduce the sequestration of A β and the bioavailability of retinoic acid, which could increase the likelihood of developing AD-type pathologies.

TTR is the major A β binding protein in the CSF (Schwarzman et al., 1994; Carro et al., 2002), and it has been shown to bind A β_{42} with higher affinity than A β_{40} (Tsuzuki et al., 2000). Several studies have shown that A β_{42} has higher amyloidogenic and neurotoxic effects than A β_{40} (Selkoe, 2000; Zou et al., 2002; Zou et al., 2003), and that it is deposited early and selectively in senile plaques detected in AD brains (Iwatsubo et al., 1994; Selkoe, 2004). A β_{42} was suggested to be a major determinant in the onset of familial AD since high A β_{42} /A β_{40} ratio is observed in patients with presenilin mutations (Citron et al., 1997). Moreover, it has been reported that the level of A β_{42} increases exponentially in an age-dependent manner to induce neurotoxic damages in late-onset AD (Morishima-Kawashima et al., 2000). Thus, decreased TTR gene expression could lead to the preferential accumulation of A β_{42} as seen during aging and in AD, which in turn would promote plaque formation and amyloid pathology.

Since tight junctions are absent in most of ependyma linings of the ventricle (Abbott, 2004), exchanges between the CSF and interstitial fluid bathed neurons allow TTR to diffuse from the CSF to sequester extracellular A β . This notion is supported by increased levels of TTR colocalized with A β plaques observed in the extracellular space of the hippocampus in a mouse model of AD (APP_{sw} mice) and in post-mortem AD brains (Stein et al., 2004). Alternatively, TTR could be transported actively from the CSF to extracellular space by binding to membrane receptors present on ependymal cells (Kuchler-Bopp et al., 2000) as shown previously for other cell types (Smith et al., 1989; Divino & Schussler, 1990a; Divino & Schussler, 1990b). Following A β sequestration, TTR/A β complexes may be drained from the CNS and uptaken by the liver where both CSF and plasma TTR proteins are degraded (Makover et al., 1988). It is thus possible that decrease in basal levels of TTR during aging could favour the accumulation of

amyloid peptides in brain parenchyma; this event promoting the formation of senile plaques.

TTR is also an essential protein involved in the transport of retinol-RBP complex in serum and CSF (Herbert et al., 1986; Zheng et al., 2001). Thus, reduced levels of TTR during aging may decrease the bioavailability of RA to the brain (Brouillette & Quirion, 2007) and could, at least partly, be associated with the marked decline in hippocampal neurogenic capacities found in rodents and humans during aging (Taupin, 2006). Since newly generated hippocampal neurons are involved in learning and memory processing, even greater reductions in neurogenesis could contribute to the early cognitive deficits observed in AD (Ziabreva et al., 2006; Verret et al., 2007). Given that RA is involved in differentiation, neurogenesis, synaptogenesis and cell survival (Lane & Bailey, 2005; Jacobs et al., 2006), lower TTR gene expression following mnemonic challenges may lead to greater memory losses as seen in AD.

Interestingly, an overproduction of A β was recently shown to considerably diminish the survival of newborn hippocampal cells in adult mice (Haughey et al., 2002; Verret et al., 2007). The lack of A β sequestration due to inadequate TTR levels could worsen this phenomenon. Disturbed retinoid transport due to decreased TTR gene expression may also influence learning and memory processing by altering RA-dependent synaptogenesis (Lee et al., 1990; Wu et al., 1998; Corcoran & Maden, 1999; Clagett-Dame et al., 2006) which mediates synaptic plasticity associated with long-term memory formation. Interestingly, synaptic losses observed early in AD were shown to be the best predictor of memory decline in this disease (Terry et al., 1991).

Moreover, a lower functioning of the RA system induced by its inadequate transport and availability would likely have an important impact on the wide-array of genes regulated by RA, including those involved in amyloid processing, A β degradation, NFT formation, and memory functions (Lane & Bailey, 2005). Taken together, these data suggest that perturbations in TTR gene expression can induce a cascade of events that may influence the aging process as well as age-related neurodegenerative disorders such as AD.

4.3. Role of transthyretin in oxidative damage and calcium homeostasis

One theory of aging suggests that oxidative stress induced by reactive oxygen species (ROS) generated, for example, by metabolism, genomic alterations and cell death can cause cumulative damages (Harman, 1981). Other studies have shown that oxidative events are crucial for fibrillar A β toxicity (Mattson, 1995) since it can spontaneously generate free radicals and ROS such as hydrogen peroxide (H₂O₂) (Hensley et al., 1994; Huang et al., 1999). The oxidant effects of A β seem to be mediated, at least partly, by its interaction with redox-active metals including iron and copper (Rottkamp et al., 2001) that are associated with senile plaques in AD brains (Smith et al., 1997). In addition to decreasing the oxidative properties of A β by preventing its fibrillization and deposition, TTR could also have an impact on ROS formation by regulating bioavailability of retinol. Indeed, vitamin A deficiency has been shown to lead to the excessive accumulation of Ca²⁺ in rat brain (Rahman et al., 1996). Moreover, large Ca²⁺ influxes produced by glutamate receptor over activation were suggested to cause enhanced metabolic stress by producing excessive oxidative phosphorylation and ROS (Michaelis, 1998). This hypothesis is in agreement with the purported role of memantine in preventing excitotoxicity by blocking NMDA receptor over stimulation and herein reducing Ca²⁺ entry into the cell (Lleo et al., 2006).

4.4. Link between neuroinflammation and transthyretin in AD

Inflammatory processes are prominent neuropathologic features of AD (Heneka & O'Banion, 2007). However, the extent to which neuroinflammation contributes to the pathogenesis still needs to be fully established. Indeed, it is not clear whether inflammation in AD brains represents a protective reaction to neurodegeneration (Simard et al., 2006) or rather, a destructive process that contributes to further loss of brain functions (Bezzi et al., 2001; Brown & Bal-Price, 2003; Griffin, 2006). Nonetheless, some epidemiological studies have reported a decreased incidence of AD in subjects using anti-inflammatory drugs, especially the non-steroidal anti-inflammatory ones (NSAIDs) (in't Veld et al.,

2001; Szekely et al., 2004). However, clinical trials designed to inhibit inflammation have thus far failed in the treatment of AD patients (Aisen et al., 2003; ADAPT Research Group et al., 2007).

Interestingly, TTR gene expression in the liver has been reported to be decreased by 75% of its normal level 36 h following the induction of inflammation (Dickson et al., 1985). During inflammatory processes, reduced TTR levels could result in reduced availability of RA which has been shown to inhibit proinflammatory mediators such as tumor necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS) in microglia activated by A β and lipopolysaccharide (LPS) (Dheen et al., 2005). Thus, one mechanism by which decreased levels of TTR may lead to inflammation in AD is by resulting in the reduced availability of RA and consequently its anti-inflammatory effects on activated microglia (in addition to the effects of RA on neurogenesis and LTP as discussed above).

4.5. Susceptibility factors related to the development of AD and their relation to TTR

In our proposed TTR cascade hypothesis of AD (Fig VI.1), memory losses can be dissociated, at least partly, from A β accumulation. Indeed, if basal levels of TTR decrease excessively during aging, it may lead to the progressive accumulation and deposition of A β without causing significant memory deficits as seen in some aged individuals (Mann et al., 1992). Cognitive impairments would occur if TTR gene expression is not sufficiently up-regulated following memory stimulation to ensure the required levels of retinoid needed for the maintenance of normal learning and memory processes as seen in aged animals (Brouillette & Quirion, 2007). Such a temporal increase in the level of TTR would at the same time augment the clearance of A β . The A β sequestering action of TTR may be particularly important since A β was shown to be up-regulated during neuronal and synaptic activity (Cirrito et al., 2005). Thus, if A β production is increased during synaptic plasticity, its clearance by TTR thereafter may be essential to prevent its accumulation and toxic effects on neurons. This hypothesis is in accordance with

a report indicating that transgenic AD-like mice (ceAPPswe/PS1ΔE9 mice) housed under memory stimulating conditions shows marked up-regulation of TTR in association with reduced Aβ levels and amyloid deposition (Lazarov et al., 2005). The beneficial effect of increasing TTR expression following stimulating challenge is also in agreement with reports showing that frequent participation in cognitive activities (such as reading newspapers, playing cards, doing crossword puzzles) is associated with reduced risk of AD in elderly people (Wilson et al., 2002a; Wilson et al., 2002b). These reports support twin studies showing that demented twin had fewer years of education than his or her non-demented twin sibling (Gatz et al., 2001; Gatz et al., 2007). Many other protective factors of AD as well as environmental stimuli and physiopathological conditions which increase the risk of AD have been related to altered TTR gene expression and/or protein levels (see Table VI.1 for some examples of risk factors of AD, many of which have been shown to modulate TTR gene expression as discussed below in sections 4.6 to 4.10).

Table VI.1. Susceptibility factors related to the development of AD

Risk factors	Impact on TTR	References
Age	↓	(George-Hyslop, 2000; Blennow et al., 2006; Harman, 2006)
Family history of dementia	unknown	(George-Hyslop, 2000; Selkoe, 2000; Gatz et al., 2006)
Apolipoprotein E4 allele	unknown	(Rebeck et al., 1993; Poirier et al., 1993; Corder et al., 1993)
Hypo- and hyperthyroidism	unknown	(Yoshimasu et al., 1991; Ganguli et al., 1996; Kalmijn et al., 2000)
Diabetes mellitus (type II)	↓	(Leibson et al., 1997; Hassing et al., 2002; Arvanitakis et al., 2004)
Depression	↓	(Wetherell et al., 1999; Sweet et al., 2004; Rapp et al., 2006)
Down syndrome	↓	(Lai & Williams, 1989; Bush & Beail, 2004; Teipel & Hampel, 2006)
Parkinson's disease	unknown	(Selkoe, 2004; Aarsland et al., 2005; Galvin, 2006)
Traumatic brain injury	↑	(Lye & Shores, 2000; Jellinger, 2004; Szczygalski et al., 2005)

Table VI.1. Continuation

Hypertension	unknown	(Guo et al., 1999; Skoog & Gustafson, 2002; Skoog & Gustafson, 2006)
Stroke	unknown	(Richards et al., 2000; Skoog & Gustafson, 2003; Jellinger, 2004)
Obesity	unknown	(Richard & Amouyel, 2001; Gustafson et al., 2003)
Hypercholesterolemia	unknown	(Mielke et al., 2005; Sjogren et al., 2006; Panza et al., 2007)
Protective factors		
Education	↑	(Gatz et al., 2001; Wilson et al., 2002a; Wilson et al., 2002b; Gatz et al., 2007)
Moderate wine consumption	unknown	(Orgogozo et al., 1997; Obisesan et al., 1998; Bastianetto et al., 2000b)
Estrogen replacement therapy	↑	(Burns & Murphy, 1996; LeBlanc, 2002; Brinton, 2004; Henderson, 2006)
Anti-inflammatory drugs	unknown	(in't Veld et al., 2001; Szekely et al., 2004)

4.6. Relationship between apolipoprotein E and transthyretin in AD

The inheritance of the apolipoprotein E4 (apoE4) allele is now well established to be a major genetic risk factor for sporadic AD and to decrease significantly the age of onset of the disease (Rebeck et al., 1993; Poirier et al., 1993; Corder et al., 1993). *In vitro* and *in vivo* studies have shown that apoE derived from astrocytes (Carlsson et al., 1991) can bind to A β but to a lesser extent than TTR (Wisniewski & Frangione, 1992; Schwarzman et al., 1994; Holtzman, 2001). While TTR has been shown to prevent the formation of amyloid fibrils (Schwarzman et al., 1994; Link, 1995), apoE (and apoE4 in particular) seems to promote A β fibril formation and deposition (Wisniewski et al., 1994; Mazur-Kolecka et al., 1995). Thus, a key factor regulating A β deposition may originate from the competition between apoE and TTR to bind A β . This hypothesis is supported by a report showing that physiological concentrations of TTR inhibit A β accumulation induced by apoE3 and E4 (Mazur-Kolecka et al., 1995). The apoE4 allele would represent a higher risk factor for AD than other apoE isoforms since it has the greatest affinity for A β peptides (Strittmatter et al., 1993; Wisniewski et al., 1994). This hypothesis is in accordance with reports

showing that CSF apoE levels are increased during normal aging and to a greater extent in AD patients (Blain et al., 1997; Merched et al., 1997), while TTR is decreased in AD (Serot et al., 1997). The finding that TTR prevents apoE-induced accumulation of A β in cultured smooth muscle cells also supports the importance of competition between both carrier proteins of A β (Mazur-Kolecka et al., 1995).

ApoE proteins are possibly involved in retinol transport and may represent an alternate route to the major one involving TTR to deliver vitamin A to the CNS (Goodman, 2006). This alternate pathway may become more important during aging to compensate for decreased levels of TTR. On the other hand, the protective effect of apoE2 against AD could be attributed, at least in part, to its more efficient transport of retinol compared to apoE4 (Brown & Roberts, 1991; Dallongeville et al., 1992).

4.7. Hypothyroidism as a risk factor for AD

Decreased gene expression and binding capacity of the thyroid hormone triiodothyronine (T₃) have been reported in the brain of aged rats (De Nayer et al., 1991; Enderlin et al., 1997a). Population-based studies have shown that either hypo- or hyperthyroidism increase the risk of developing AD in the elderly (Yoshimasu et al., 1991; Ganguli et al., 1996; Kalmijn et al., 2000). Decreased levels of the thyroid hormone receptor c-ERB A α have also been observed in the hippocampus of AD patients (Sutherland et al., 1992). Moreover, reductions in the amounts of RAR α and γ receptors have been reported in hypothyroid patients (Feart et al., 2005b). The existence of a close relationship between RA and thyroid hormone receptor signalling is further supported by data demonstrating the up-regulation of RAR β and RXR β/γ by either RA or T₃ treatment (Feart et al., 2005a). Given that TTR is a key thyroid hormone carrier in serum and plasma (Vranckx et al., 1990), levels of this hormone would be greatly influenced by changes in TTR gene expression as observed during aging and in AD (Serot et al., 1997; Merched et al., 1998; Brouillette & Quirion, 2007). In agreement with this hypothesis, lower levels of T₃ and T₄ hormones were found in TTR^{-/-} mice (Episkopou et al., 1993).

4.8. Plasma transthyretin level is reduced in diabetes mellitus

Diabetes mellitus is characterized by either an impaired production of insulin due to primary islet cell death (type I) or by insulin resistance of normally insulin-responsive cells (type II) (Farag et al., 2007). Type II diabetes is often observed at later stages of life and, despite some controversial results, is a risk factor for the development of AD (Leibson et al., 1997; Hassing et al., 2002; Arvanitakis et al., 2004; Xu et al., 2004). Diabetes mellitus is also associated with cognitive impairments (Arvanitakis et al., 2004; Elias et al., 2005), whole brain and hippocampal atrophy (den Heijer et al., 2003; Schmidt et al., 2004), and increased risks for stroke and vascular dementia (Hassing et al., 2002; Xu et al., 2004). Diabetes mellitus is also linked to vitamin A deficiency since decreased plasma levels of RBP and retinol were observed in diabetic patients and streptozotocin-induced diabetes in animal models (Basu et al., 1989; Krempf et al., 1991). Similarly, reduced levels of serum retinol have been reported in AD patients compared to age-matched controls (Jimenez-Jimenez et al., 1999; Bourdel-Marchasson et al., 2001; Rinaldi et al., 2003).

Decreases in plasma vitamin A in both AD and diabetes could be due to reduced levels of TTR which is known to regulate the secretion of the retinol-RBP complex from the liver (Wolf, 1995; Monaco, 2000; van Bennekum et al., 2001). In support of this hypothesis, lower concentrations of plasma TTR were found in streptozotocin-induced diabetic rats compared to non-diabetic control animals (Tuitoek et al., 1996). Interestingly, drastic reductions in plasma RBP and retinol levels was also reported in mice lacking TTR (Wei et al., 1995). Decreased circulatory levels of RBP and retinol could not be attributed to decrease uptake or storage of dietary retinol since hepatic retinol concentrations were shown to be increased in diabetes mellitus (Basu & Basualdo, 1997) and to be similar in TTR^{-/-} mice compared to wild-type animals (Wei et al., 1995). Hence, TTR can be a factor linking both AD and diabetes-related pathologies.

4.9. Other susceptibility factors for AD and their relation to transthyretin

Some epidemiological studies have suggested a protective role for estrogen replacement therapy against AD in post-menopausal women (Burns & Murphy, 1996; LeBlanc, 2002; Brinton, 2004; Henderson, 2006) [although other studies have failed to report such beneficial effects, see Mulnard et al., 2000; Shumaker et al., 2003]. The mechanisms sustaining the protective effects of estrogen against AD are still unknown but may be related, at least partly, to synthesis and bioavailability of TTR. Indeed, estrogen treatment has been reported to increase TTR gene expression by acting on α and β estrogen receptors present in the choroid plexus (Tang et al., 2004).

Late-onset major depression is another risk factor for the development of AD and is likely related to neuropathological changes associated with AD (Sweet et al., 2004). It has been reported that lifetime history of major depression may increase amyloid and NFT pathology along with accelerating cognitive decline in AD patients (Rapp et al., 2006). Interestingly, CSF concentrations of TTR were shown to be lower in depressed patients compared to control subjects (Sullivan et al., 1999). Further studies are certainly warranted to determine if decreased levels of TTR could be involved in the likelihood of developing depression-like disorders.

Down syndrome (DS), or trisomy 21, is the most common identifiable genetic cause of mental retardation with increased risk of developing AD (Lai & Williams, 1989; Bush & Beal, 2004). Aging individuals with DS develop intraneuronal fibrillary tangles, extracellular senile plaques and similar pattern of age-related brain atrophy as observed in the early stages of AD (Teipel & Hampel, 2006). As DS patients age, they also develop other features often seen in AD such as thyroid dysfunctions (Prasher, 1999), cardiovascular disorders (Nagasaka et al., 1996) and depression (Merrick et al., 2006). Interestingly, both AD and Down syndrome patients were reported to have low serum levels of TTR (Elovaara et al., 1986) again suggesting a role for TTR in these pathologies.

Comorbidity has also been observed between Parkinson's disease and AD, although the nature of the link between these two pathologies remains to be fully established (Selkoe, 2004; Aarsland et al., 2005; Galvin, 2006). Interestingly, it was suggested that increased levels of CSF TTR may be responsible for clinical improvements seen in some Parkinson's patients who received adrenal medullary autotransplantation (Abram et al., 1990). Hence it would be of interest to study in details the link between Parkinson's disease and AD as related to TTR and RA. Animal models may be particularly useful in that regard.

Finally, levels of insulin-like growth factor 1 (IGF-1) have also been shown to decrease with age and to be altered in many neurodegenerative conditions including AD (Dore et al., 1997a; Arvat et al., 2000; Busiguina et al., 2000; Craft et al., 2000). IGF-1 is a circulating hormone structurally related to insulin that was recently shown to provide neuroprotection following physical exercise, to modulate neurogenesis and to induce neuronal excitability in the adult brain (Carro et al., 2000; Carro et al., 2001; Trejo et al., 2001). Interestingly, serum IGF-1 was reported to increase the clearance of A β by increasing brain levels of transthyretin (Carro et al., 2002). Thus, in addition to rescue neurons against A β neurotoxicity by acting on its specific receptors in the hippocampus (Dore et al., 1997a), IGF-1 may also prevent A β accumulation by enhancing the transport of TTR into the brain and/or the production of TTR by the choroid plexus (Carro et al., 2002).

4.10. Injuries to the choroid plexus as a major source of altered transthyretin levels during aging

The choroid plexus (CP) is an essential component involved in the maintenance of brain homeostasis. The CP rigorously restricts access to substances present in the bloodstream to protect brain microenvironment. It selects various vitamins, peptides, hormones and many other nutrients necessary for proper neuronal functioning. The CP also plays an essential role in the clearance of toxins and drugs. During development, the CP produces high amounts of RA, derived from retinol transported from the periphery, which plays

a critical role in regulation of neurogenesis and neuronal differentiation in several brain regions (Yamamoto et al., 1996; Redzic et al., 2005). In the adult, the CP is critically implicated in neuronal repair processes and in the restoration of brain homeostasis following stroke and traumatic brain injury, two pathophysiological conditions that increase the risk of developing AD (Lye & Shores, 2000; Jellinger, 2004; Szczygalski et al., 2005). The CP is most vulnerable to aging and shows decline in all aspects of its functions, including the synthesis and secretion of CSF and proteins with age (Redzic et al., 2005). Since TTR found in the CSF is almost exclusively synthesized by the CP and represents 25% of all proteins produced by this structure (Weisner & Roethig, 1983), conditions that alter CP functioning most likely have a direct impact on TTR synthesis. Because of its special synthesis and secretory patterns, TTR was proposed as a reliable biomarker of CSF circulation disorders (Weisner & Roethig, 1983)

4.10.1. Decreased activity of the choroid plexus during aging leads to the accumulation of A β and neurotoxic metals in the brain

Numerous factors can explain the decreasing functionality of the CP during aging. For example, the atrophy of the epithelium, thickening of basement membranes and stroma fibrosis in the CP occur during aging and are most prominent in AD (Serot et al., 2003). These changes are accompanied by decreased enzymatic activities of epithelial cells and a reduction of approximately 50% in the secretion of CSF in humans and animal models (May et al., 1990; Tanna et al., 1991; Preston, 2001). The rate of production of CSF was also reported to be decreased in AD (Serot et al., 2003). Although CSF's composition does not vary much during aging, slower CSF turnover in the elderly and in AD patients could have considerable impact on the clearance of A β . Interestingly, intraventricularly injected A β was shown to be cleared at 10.4 μ l/min in 3-months old rats but only at 0.71 μ l/min in 30-months old animals. This in turn increased brain amyloid content in old rats compared to younger ones (Preston, 2001).

Many metals have also been observed to accumulate in the brain during aging. To protect brain parenchyma, the CP was shown to sequester neurotoxic

metals (Zheng, 2001), and at least nine different heavy metals and metalloid ions have been reported to accumulate in the CP (Zheng, 2001). For example, increases in lead contents in the CP were found to be associated with age (Friedheim et al., 1983) leading to decrease production and secretion of TTR by the CP (Zheng et al., 1996). Decreased sequestering capacities of the CP during aging could also lead to accumulation of metals such as iron and copper found in senile plaques; two metals known to bind A β with high affinity and to induce oxidative stress (Smith et al., 1997; Cuajungco et al., 2000; Rottkamp et al., 2001).

4.10.2. Traumatic brain injury, hypertension and nicotine affect the functioning of the choroid plexus

The CP has also been shown to be critically important to reinstate brain microenvironment following traumatic and ischemic brain injuries (Emerich et al., 2005). Interestingly, increase in TTR gene expression was suggested to contribute to neuronal protection following a traumatic injury (Long et al., 2003). Increased in elevation of CSF TTR levels may be necessary to counteract the increased production of A β observed after head trauma (McKenzie et al., 1994). On the other hand, insufficient synthesis of TTR may contribute to cerebral A β deposition known to occur after brain trauma (Roberts et al., 1991; Clinton et al., 1991; Roberts et al., 1994). Moreover, changes in the functionality of the CP seen during aging and more extensively in AD may decrease TTR levels in the CSF, then leading to decreased ability to sequester and eliminate A β . Head trauma represents a significant environmental risk factor of AD as demonstrated by some epidemiological studies (Lye & Shores, 2000; Jellinger, 2004).

Interestingly, chronic hypertension in rats has been shown to induce major defects in the integrity of the blood-CSF barrier (Al Sarraf & Philip, 2003). Moreover, hypertension is associated with pathological manifestations of AD including plaque formation, NFTs and hippocampal atrophy (Sparks et al., 1995; Skoog et al., 1998; Petrovitch et al., 2000). Some studies have reported that high blood pressure precedes AD by decades, and high blood pressure levels reduce the

number of years before the onset of dementia. High blood pressure has also been related to the neuropathological manifestations of AD (Guo et al., 1999; Guo et al., 2001; Skoog & Gustafson, 2006), although some studies have failed to find protective effect of anti-hypertensive treatments in AD (Richards et al., 2000; in't Veld et al., 2001). Moreover, hypertension often clusters with stroke as well as vascular risk factors such as diabetes mellitus, obesity and hypercholesterolemia, which have all been associated with AD (Richard & Amouyel, 2001). Thus, one mechanism by which increased blood pressure may impact on the development of AD is by disturbing the transport from bloodstream to the CP, which may alter the synthesis and/or secretion of TTR.

It is known that the secretion of CSF and proteins by the CP is highly regulated by various neurotransmitters. Indeed, the CP is richly innervated, receiving adrenergic, cholinergic, peptidergic and serotonergic fibers (Lindvall & Owman, 1981). Cholinergic innervations have been shown to reach the secretory epithelium (Edvinsson et al., 1973) and the stimulation of nicotinic receptors was reported to increase the synthesis and secretion of TTR from the CP in rats (Li et al., 2000). These authors suggested that the protective effects of nicotine against AD noted in several epidemiological studies (Graves et al., 1991; van Duijn & Hofman, 1991; Brenner et al., 1993) may be associated, in part, with the increased production of TTR.

Taken together, these results suggest that various environmental factors and pathophysiological conditions may exacerbate and/or accelerate the "normal" reduction in CP activities seen in aged individuals, and thus reduce even more TTR gene expression and secretion. Genetic predisposition that influences the physiology of the CP during aging may also be related to AD. Hence, various phenomena can alter TTR levels and initiate the pathogenic cascade leading to AD. Since TTR appears to be a common link affected by all these genetic and non-genetic factors closely related to aging, it may explain the high prevalence of AD in the elderly and why age is the most robust risk factor associated with AD (George-Hyslop, 2000; Harman, 2006).

4.11. Possible future experiments to validate the key role of transthyretin in AD

As discussed above, TTR may be of particular relevance in the pathogenesis of AD since this protein is involved in the major route of retinol transport, in addition to act as a A β sequestering protein to prevent amyloid aggregation and plaque formation (Schwarzman et al., 1994; Carro et al., 2002). According to our proposed TTR cascade hypothesis of AD (Fig. VI.1), decreased basal level expression of TTR as well as following memory stimulation could result in both 1) defective retinoid transport that may account for memory impairments and 2) reduced sequestration and clearance of A β which can lead to increased A β toxicity and senile plaque formation.

To determine whether lower levels of TTR can exacerbate and accelerate the onset of an AD-like phenotype, many critical experiments still need to be performed. Research is currently in progress in our laboratory to determine if genetic ablation of TTR in a mouse model of AD (TgCRND8 mice) may alter the development of the AD-like phenotype. Most interestingly, in a brief communication, it was just reported that hemizygous reduction of TTR (TTR^{+/-}) lead to increase A β deposition in the cortex and hippocampus in a mouse model of AD (ceAPPswe/PS1 Δ E9 mice) (Choi et al., 2007). However, in a preliminary study we have failed thus far to detect any significant changes in the volume of amyloid burden in the cortex and hippocampus following complete (TTR^{-/-}) and partial (TTR^{+/-}) genetic ablations of TTR in 4- and 6-months old TgCRND8 mice (Brouillette, Chabot, Quirion, unpublished results). Since we used an antibody (6E10) which can detect soluble and insoluble forms of A β , we are currently performing another set of immunohistochemical experiments using an antibody (6F/3D) that selectively stains insoluble A β .

In addition, Choi et al (2007) reported elevated detergent-soluble and formic acid-soluble levels of A β peptides in the brains of ceAPPswe/PS1 Δ E9 mice with hemizygous deletion of TTR. Using an ELISA approach, we propose to extend these findings by quantifying soluble A β in the brain of TgCRND8 mice

with homo- and heterozygous ablation of TTR. Moreover, we plan to determine if the absence of TTR has an impact on the levels of NFTs, neuronal loss and memory capacities in TgCRND8 mice. Finally, it will be critical to establish if treatments with TTR or RA may counteract or at least attenuate AD-like pathogenesis in these mouse models.

5. Scopolamine-induced amnesia model

The administration of scopolamine in animals has been widely used as a psychopharmacological model of cognitive dysfunctions (Lamberty & Gower, 1991; Nakajima et al., 1994; Janas et al., 2005; van der Staay & Bouger, 2005; Brouillette et al., 2007). Since the deleterious effects of scopolamine on memory increase with age (Ray et al., 1992b; Stemmelin et al., 1999) and because cholinergic deficits are observed in AD (Whitehouse et al., 1981; Coyle et al., 1983; Etienne et al., 1986; Gauthier, 2006), treatment with scopolamine was among the first models used to mimic some of the features of AD, especially those related to learning and memory dysfunctions. Although scopolamine treatment is still commonly used as model, mechanisms underlying the full spectrum of actions on memory remains to be fully established (Flood & Cherkin, 1986; Sherman et al., 2003; Brouillette et al., 2007).

Systemic injections of scopolamine strongly impair encoding of new information but not retrieval of previously encoded ones. Scopolamine-induced amnesia has been associated with decreased cholinergic neuronal activity (Carli et al., 1997; Schon et al., 2005), although increased release of ACh is observed 30 to 40 min following a systemic injection of scopolamine (Mishima et al., 2000). This apparent contradiction can be explained by differential affinity of scopolamine for pre- and post-synaptic receptors. Indeed, this non- subtype selective muscarinic antagonist has been observed to have ten times more affinity for the post-synaptic M1-like than the pre-synaptic M2-like receptors (Szerb et al., 1977). Thus, even if scopolamine increases ACh release by blocking M2 and M4 autoinhibitory receptors, the action of ACh on post-synaptic neurons is suppressed by the

predominant action of scopolamine on excitatory post-synaptic M1 receptors (Szerb et al., 1977). As scopolamine induces much larger ACh release in the hippocampus of young rats compared to aged animals (Scali et al., 1995), it may explain the greater scopolamine-induced memory deficits observed in old animals (Ray et al., 1992a; Stemmelin et al., 1999). Moreover, decreases in basal ACh release were shown to be greater in AI than AU rats (Quirion et al., 1995). Increased levels of M2 receptors in the hippocampus of AI rats, and reversal of cognitive impairments by BIBN-99, a M2 receptor antagonist, in this animal model strongly suggest a critical role for muscarinic inhibitory autoreceptors in age-related memory deficits (Quirion et al., 1995).

Another mechanism underlying scopolamine-induced amnesia is likely associated to an effect on GABAergic neurotransmission. By blocking M2 and M4 inhibitory heteroreceptors located pre-synaptically on GABAergic interneurons (Rouse & Levey, 1997), a scopolamine treatment would enhance GABA-mediated IPSPs, which in turn would decrease glutamate release ultimately resulting in reduced LTP and cognitive abilities. A large body of anatomical, neurochemical, electrophysiological and behavioural results strongly suggests synergistic interactions between cholinergic, GABAergic and glutamatergic systems during cognitive processes involving the hippocampus (Freund & Buzsaki, 1996; Vizi & Kiss, 1998; Myhrer, 2003). Our study revealed that reducing cholinergic neurotransmission by scopolamine induces changes in GABAergic and glutamatergic systems associated with memory deficits (Brouillette et al., 2007). The induction of GABA_B gene expression that we observed 6 h post-training in scopolamine-treated rats possibly represents an adaptative mechanism to lower GABAergic inhibitory action on glutamatergic cells within the hippocampal formation. The reversal of scopolamine-induced amnesia by the GABA_B receptor antagonist SGS742 emphasizes the critical deleterious effects of excessive GABA-mediated IPSPs on cognition in this animal model (Brouillette et al., 2007). These observations are in accordance with a recent report suggesting that increased GABAergic inhibition may be

responsible for hippocampal synaptic plasticity impairments observed in muscarinic M2 receptors knock-out mice (Seeger et al., 2004).

Our microarray data also showed that the temporal up-regulation of Homer1a seen in saline control rats following spatial memory stimulation is disrupted following a scopolamine treatment. Since the transcription of Homer1a has been reported to be necessary for LTP and memory formation (Brakeman et al., 1997), the decreased expression of this gene in scopolamine-treated rats possibly results in reduced glutamatergic synaptic plasticity. Consistent with this hypothesis, we have shown that the lack of Homer1a relative to Homer1c suppressed memory improvements induced by SGS742 in scopolamine-treated animals (Brouillette et al., 2007). The over expression of Homer1a is apparently not favourable for efficient synaptic plasticity since a continuous, rather than transient, up-regulation of Homer1a using viral vectors abolished memory improvements induced by SGS742 in scopolamine-treated rats (Brouillette et al., 2007). Accordingly, a disequilibrium in the homeostatic balance between inducible Homer1a and constitutively expressed Homer1c may be of particular relevance in cognitive impairments induced by altered cholinergic neurotransmission.

Taken together, these results suggest that ACh is essential for normal learning and memory process and that the perturbation of this system by scopolamine is sufficient to impair the encoding of new information. Since the induction of LTP required binding of glutamate to NMDA receptors and substantial depolarization of post-synaptic cells through AMPA receptors, it suggests that cholinergic inputs to the hippocampus are critical to facilitate the depolarization of glutamatergic neurons in order to induce synaptic plasticity. Consistent with this hypothesis, the activation of M1 muscarinic receptors has been reported to potentiate NMDA receptor currents in hippocampal CA1 pyramidal cells (Marino et al., 1998). Moreover, the selective blockade of autoinhibitory M2 receptors also enhances glutamatergic transmission via the release of ACh, leading to the reversal of age-related cognitive deficits (Quirion et al., 1995). The release of ACh may also reduce GABAergic inhibition of

glutamatergic transmission by activating pre-synaptic M2 heteroreceptors located on GABAergic neurons (Ben Ari et al., 1981; Pitler & Alger, 1992; Behrends & ten Bruggencate, 1993). Moreover, it is also well-established that released ACh not only act on muscarinic but also on nicotinic receptors present in the hippocampus (Jones et al., 1999; Levin et al., 2006). The stimulation of nicotinic receptors can rapidly enhance glutamatergic transmission by directly increasing intracellular Ca^{2+} concentrations (McGehee et al., 1995). Hence, the “simple” scopolamine-induced amnesia model while primarily being associated to blockade of muscarinic receptors also leads to the alterations in various other systems and signalling pathways to produce its amnesia-related behaviours.

5.1. Age memory-impaired rats versus the scopolamine-induced amnesia models

Both AI animals and scopolamine-treated rats represent models of cognitive impairments. Although both animal models show poor performance in the MWM task, comparison between their respective gene expression profiles tend to mostly suggest differences in the cascade of events leading to spatial memory deficits. Rather surprisingly, none (may be except NQO2, see below) of the genes differentially expressed between AI and AU rats appear to be up- or down-regulated in scopolamine-treated rats. Decreased TTR mRNA expression and reversal of age-associated memory impairments by RA suggested that the retinoid signalling pathway is particularly affected in AI animals. However, no gene directly associated with the retinoid pathway was shown to be differentially expressed between scopolamine- and saline-treated rats. These somewhat unexpected results could relate to protocols used and the time points studied following stimulation in the MWM.

In any case, to uncover potentially significant data that could have been lost because of our stringent microarray analysis, scopolamine-treated rats were administered the same RA treatment used to reverse memory deficits in $\text{TTR}^{-/-}$ mice and aged rats (Brouillette & Quirion, 2007). In that context, a report has shown a decrease in hippocampal ACh release induced by scopolamine in vitamin

A-deficient rats (Cocco et al., 2002). However, the RA treatment failed to restore learning abilities in scopolamine-treated rats (Fig. VI.2). This result is in agreement with our microarray data showing no change in RA gene expression signalling pathways in the scopolamine-amnesia model (Brouillette et al., 2007).

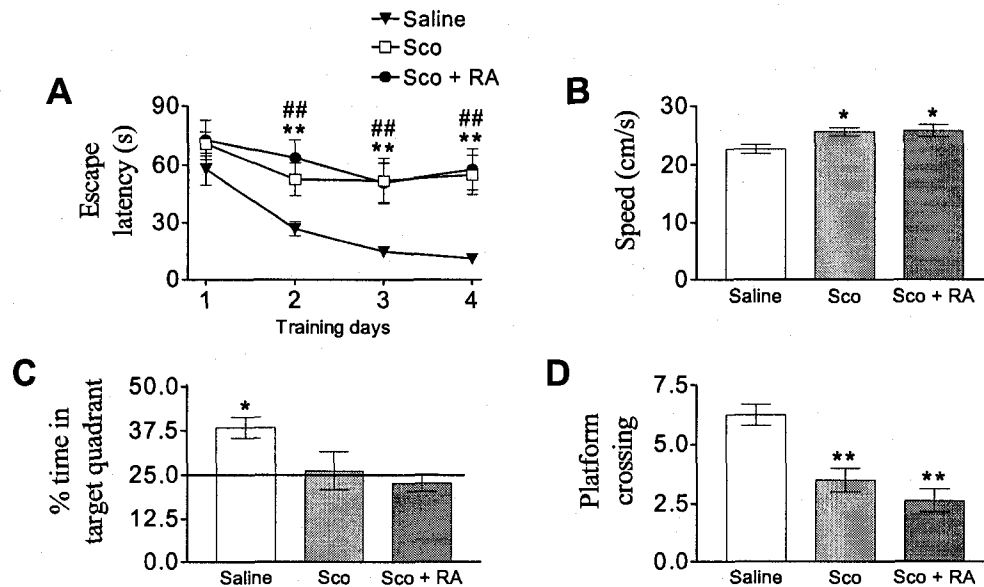


Fig. VI.2. Administration of RA failed to reverse scopolamine-induced amnesia in 7-months old rats. (A) Escape latencies before finding the hidden platform (three trials of 90 s per day with a 20 min inter-trial interval) are longer for scopolamine (Sco) treated rats (open squares; 0.4 mg/kg; ip; 20 min before training; $n = 8$) than saline control animals (filled triangles; $n = 8$; ** $p < 0.01$; two-way ANOVA). Scopolamine-treated rats injected with retinoic acid (RA; filled circles, 150 μ g/kg, sc, $n = 8$) also show longer escape latencies compared to control rats (## $p < 0.01$; two-way ANOVA). (B) Swimming speeds recorded during the probe trial are slightly faster in scopolamine treated rats injected with RA or not compared to control animals (* $p < 0.05$; one-way ANOVA). (C) Percent time spent in the quadrant that previously contained the platform during the probe trial. Error bars ($\pm$ 95% confidence interval of the mean) of scopolamine-treated rats administered with RA or not overlap the 25% line which represents the chance level of being randomly in one of the four virtual quadrants. Asterisk represents significant differences from the 25% chance

performance (* $p < 0.05$). **(D)** Rats treated with scopolamine crossed the previous location of the platform fewer times than control rats independently of RA treatment (** $p < 0.005$; one-way ANOVA) during the probe trial. All results, except for **(C)** are mean $\pm$ SEM.

Taken together, these results suggest that different cellular and molecular cascades are disrupted in the AI versus scopolamine-treated rat models.

6. Some future studies

To further characterize the role of TTR in age-associated memory deficits, it will be of interest to study aged (24-25-months old) TTR^{+/-} and TTR^{-/-} mice in the MWM as well as in other behavioural paradigms of learning and memory such as object recognition and attention to establish the role of TTR in various types of cognitive processes. These mice should also be tested in the open-field, accelerating rotarod as well as in the elevated plus maze tasks and forced swim test to investigate possible role of TTR in spontaneous motor activities, sensorimotor functions and anxiety, respectively. In addition, the two cellular models of synaptic plasticity and memory processing, LTP and LTD, should be investigated to determine if synaptic functioning is altered in TTR^{-/-} mice with age. These behavioural and electrophysiological experiments would nicely complement those summarized in this thesis (Brouillette & Quirion, 2007).

Microarray analysis of aged rats and animals treated with scopolamine uncovered various candidate genes possibly involved in learning and memory processes. Some of them should be studied in detail in the future. For example, in collaboration with Servier Research Institute in France, current research is in progress in our laboratory to investigate more extensively the role of NQO2 in cognition. Gene expression for this enzyme was shown to be increased in AI (6 and 8 h post-training) compared to AU rats (Brouillette & Quirion, 2007) as well as in scopolamine-treated animals when considering fold change derived from the median measures (Brouillette et al., 2007). Interestingly, NQO2 has been

identified as a potential melatonin receptor 3 (MT3) (Nosjean et al., 2000), and was proposed to be the molecular target of resveratrol, a neuroprotective molecule enriched in red wine (Buryanovskyy et al., 2004). Preliminary results suggest that the inhibition of the enzymatic activity of NQO2 is able to reverse scopolamine-induced amnesia and to prevent cell death in primary hippocampal cell cultures (Benoit, Bastianetto, Brouillette, Quirion, unpublished results). Moreover, to establish the distribution and comparative level of this enzyme in the brain of AI and AU rats as well as in scopolamine-treated animals, detailed immunohistochemical and western blot experiments are underway. NQO2 represents a novel candidate gene possibly involved in memory processing and our study may provide the first evidence that this enzyme is indeed relevant in age-related memory deficits. Its likely relationship with the melatonin receptor system provides further impetus in that regard.

Many other candidate genes uncovered in our studies could also be analyzed in detail in future experiments using molecular and pharmacological approaches. For example, gene expression for prodynorphin was shown to be increased in scopolamine-treated rats compared to saline-treated animals following stimulation in a spatial memory task. The processing and maturation of the prodynorphin gene precursor generate various peptides (dynorphin A, dynorphin B, dynorphin 32, α -neo-endorphin and β -neo-endorphin) which all predominantly act on κ -opioid receptors (Millan, 1990). The possible role of these peptides in learning and memory behaviours could be studied in prodynorphin knock-out mice recently developed by the group of Herbert Herzog in Australia (Sainsbury et al., 2007). The cognitive abilities of these mice could be evaluated in the MWM and other behavioural paradigms as well as in LTP and LTD cellular models of memory. Concomitantly, pharmacological studies could be performed by using selective κ -opioid receptor agonists and antagonists to scopolamine-treated rats and AI animals to determine their effects on learning behaviours. These functional studies, based on results previously obtained using our microarray approach could reveal a role for dynorphin-like peptides in memory functioning during normal and pathological brain aging.

The MWM protocol and microarray analysis used in this thesis could also be applied to other animal models of cognitive dysfunctions. In a previous study (see appendix III), we determined gene expression profiles of rats repeatedly treated with MK-801, a selective NMDA receptor antagonist known to induce learning impairments (O'Donnell et al., 2003). In that study, MK-801 (0.2mg/kg, ip) was injected twice a day for 14 days. Rats were initially tested in the MWM during days 1-3 of treatment and again (with a new platform location) on days 15-18 (starting 24 hours after the last drug administration). At that time we used Clontech AtlasTM Rat 1.2 array which contained only 1,176 genes compared to the Affymetrix GeneChip[®] Rat Genome 230 2.0 array containing 28,757 different genes used here. An average z-ratio method was used by O'Donnell et al (2003) to analyse differential gene expression levels compared to the RMA method used in this thesis. Moreover, microarray analysis was carried out 24 h after the last trial on day 18 instead of 6-8 h post-training as performed here. Because of all these experimental differences, results obtained by O'Donnell et al. (2003) cannot be directly compared to microarray data generated in this thesis. Thus, it would be of great interest to apply the protocol we elaborated in this thesis to investigate more extensively candidate genes altered in a MK-801-induced memory deficit model. Comparative analysis of data generated from three models (AI/AU, scopolamine and MK-801) could possibly reveal alterations in some genes for which the expression is similarly altered in all models, strengthening a possible role in cognitive behaviours. It would then be of great interest to also investigate the expression of these candidate genes in AD brain tissues and age-matched controls.

7. Conclusion

The major aim of this thesis was to investigate global alterations in gene expression in two animal models associated with memory dysfunctions. Using the scopolamine-induced amnesia rat model, we were able to reveal the existence of key interactions among hippocampal cholinergic, glutamatergic and GABAergic neurotransmissions during learning and memory processes (Brouillette et al., 2007). Dysfunctions of many neurotransmitter pathways have been postulated to occur during aging leading to cognitive declines. Our study with AI rats supports the small, growing body of evidence indicating a role for disturbed RA pathways in age-related memory deficits (Chiang et al., 1998; Etchamendy et al., 2001; Misner et al., 2001; Cocco et al., 2002). Interestingly, we identified that the retinol transporter TTR is a critical component of the complex RA signalling pathways associated with memory deficits during aging (Brouillette & Quirion, 2007). By acting on various neurotransmitter systems, the RA pathway may play a critical role in the maintenance of synaptic plasticity and memory capacities during aging.

Accumulating evidence now suggests that the well-established role of retinoids during neurodevelopment should be extended to mature and aging neurons (Maden, 2002). The critical effects of RA on the regulation of synaptogenesis, neurogenesis and neural differentiation throughout life in the dentate gyrus of the hippocampus could be of particular relevance in neurodegenerative diseases occurring during aging such as AD. In that context, the environmental and food pollutant dioxin, which is well known to alter turnover and metabolism of retinoids, could represent a potential additional risk factor in the development of age-related memory deficits. Taken together, the results summarized in this thesis uncover novel genes relevant to learning and memory processes. A better understanding of their functions could lead to the development of novel therapies to treat disease such as AD.

References

Review of the literature and General Discussion

- Aarsland D, Zaccai J & Brayne C (2005) A systematic review of prevalence studies of dementia in Parkinson's disease. *Mov Disord*, 20, 1255-1263.
- Abbott NJ (2004) Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. *Neurochem Int*, 45, 545-552.
- Abel T & Lattal KM (2001) Molecular mechanisms of memory acquisition, consolidation and retrieval. *Curr Opin Neurobiol*, 11, 180-187.
- Abram SR, Kruskal JB, Allen GS, Burns RS, Parker R & Tulipan N (1990) Alterations in prealbumin concentration after adrenal autotransplantation for Parkinson's disease. *Exp Neurol*, 108, 130-135.
- Acsady L, Arabadzisz D & Freund TF (1996) Correlated morphological and neurochemical features identify different subsets of vasoactive intestinal polypeptide-immunoreactive interneurons in rat hippocampus. *Neuroscience*, 73, 299-315.
- Acsady L, Halasy K & Freund TF (1993) Calretinin is present in non-pyramidal cells of the rat hippocampus--III. Their inputs from the median raphe and medial septal nuclei. *Neuroscience*, 52, 829-841.
- ADAPT Research Group, Lyketsos CG, Breitner JC, Green RC, Martin BK, Meinert C, Piantadosi S & Sabbagh M (2007) Naproxen and celecoxib do not prevent AD in early results from a randomized controlled trial. *Neurology*, 68, 1800-1808.
- Aigner TG, Walker DL & Mishkin M (1991) Comparison of the effects of scopolamine administered before and after acquisition in a test of visual recognition memory in monkeys. *Behav Neural Biol*, 55, 61-67.
- Aisen PS, Schafer KA, Grundman M, Pfeiffer E, Sano M, Davis KL, Farlow MR, Jin S, Thomas RG & Thal LJ (2003) Effects of rofecoxib or naproxen vs placebo on Alzheimer disease progression: a randomized controlled trial. *JAMA*, 289, 2819-2826.
- Al Sarraf H & Philip L (2003) Effect of hypertension on the integrity of blood brain and blood CSF barriers, cerebral blood flow and CSF secretion in the rat. *Brain Res*, 975, 179-188.

- Alkondon M, Pereira EF, Barbosa CT & Albuquerque EX (1997) Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. *J Pharmacol Exp Ther*, 283, 1396-1411.
- Almaguer-Melian W, Cruz-Aguado R, Riva CL, Kendrick KM, Frey JU & Bergado J (2005) Effect of LTP-reinforcing paradigms on neurotransmitter release in the dentate gyrus of young and aged rats. *Biochem Biophys Res Commun*, 327, 877-883.
- Alonso A & Kohler C (1982) Evidence for separate projections of hippocampal pyramidal and non-pyramidal neurons to different parts of the septum in the rat brain. *Neurosci Lett*, 31, 209-214.
- Alvarez-de-la-Rosa M, Silva I, Nilsen J, Perez MM, Garcia-Segura LM, Avila J & Naftolin F (2005) Estradiol prevents neural tau hyperphosphorylation characteristic of Alzheimer's disease. *Ann N Y Acad Sci*, 1052, 210-224.
- Amaral DG & Witter MP (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience*, 31, 571-591.
- Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnema NP, Nathanson NM & Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat Neurosci*, 6, 51-58.
- Ando M, Yamauchi M, Fujita K, Kakita M & Nagata Y (1996) Induction of tissue transglutaminase in rat superior cervical sympathetic ganglia following in vitro stimulation of retinoic acid. *Neurosci Res*, 24, 357-362.
- Araujo DM, Lapchak PA, Collier B & Quirion R (1988) Characterization of N-[3H]methylcarbamylcholine binding sites and effect of N-methylcarbamylcholine on acetylcholine release in rat brain. *J Neurochem*, 51, 292-299.
- Araujo JA, Studzinski CM & Milgram NW (2005) Further evidence for the cholinergic hypothesis of aging and dementia from the canine model of aging. *Prog Neuropsychopharmacol Biol Psychiatry*, 29, 411-422.

- Arcangeli A, Rosati B, Cherubini A, Crociani O, Fontana L, Passani B, Wanke E & Olivotto M (1998) Long-term exposure to retinoic acid induces the expression of IRK1 channels in HERG channel-endowed neuroblastoma cells. *Biochem Biophys Res Commun*, 244, 706-711.
- Arendash GW, Sanberg PR & Sengstock GJ (1995) Nicotine enhances the learning and memory of aged rats. *Pharmacol Biochem Behav*, 52, 517-523.
- Artero S, Tierney MC, Touchon J & Ritchie K (2003) Prediction of transition from cognitive impairment to senile dementia: a prospective, longitudinal study. *Acta Psychiatr Scand*, 107, 390-393.
- Arvanitakis Z, Wilson RS, Bienias JL, Evans DA & Bennett DA (2004) Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Arch Neurol*, 61, 661-666.
- Arvat E, Broglio F & Ghigo E (2000) Insulin-Like growth factor I: implications in aging. *Drugs Aging*, 16, 29-40.
- Astroff B, Rowlands C, Dickerson R & Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibition of 17 beta-estradiol-induced increases in rat uterine epidermal growth factor receptor binding activity and gene expression. *Mol Cell Endocrinol*, 72, 247-252.
- Astroff B & Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-dioxin as an antiestrogen: effect on rat uterine peroxidase activity. *Biochem Pharmacol*, 39, 485-488.
- Atri A, Sherman S, Norman KA, Kirchhoff BA, Nicolas MM, Greicius MD, Cramer SC, Breiter HC, Hasselmo ME & Stern CE (2004) Blockade of central cholinergic receptors impairs new learning and increases proactive interference in a word paired-associate memory task. *Behav Neurosci*, 118, 223-236.
- Attaway CM, Compton DM & Turner MD (1999) The effects of nicotine on learning and memory: a neuropsychological assessment in young and senescent Fischer 344 rats. *Physiol Behav*, 67, 421-431.
- Aubert I, Rowe W, Meaney MJ, Gauthier S & Quirion R (1995) Cholinergic markers in aged cognitively impaired Long-Evans rats. *Neuroscience*, 67, 277-292.

- Auld DS, Mennicken F & Quirion R (2001) Nerve growth factor rapidly induces prolonged acetylcholine release from cultured basal forebrain neurons: differentiation between neuromodulatory and neurotrophic influences. *J Neurosci*, 21, 3375-3382.
- Bach ME, Barad M, Son H, Zhuo M, Lu YF, Shih R, Mansuy I, Hawkins RD & Kandel ER (1999) Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. *Proc Natl Acad Sci U S A*, 96, 5280-5285.
- Bain G, Ramkumar TP, Cheng JM & Gottlieb DI (1993) Expression of the genes coding for glutamic acid decarboxylase in pluripotent cell lines. *Brain Res Mol Brain Res*, 17, 23-30.
- Bain G, Ray WJ, Yao M & Gottlieb DI (1996) Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem Biophys Res Commun*, 223, 691-694.
- Barnes CA & McNaughton BL (1985) An age comparison of the rates of acquisition and forgetting of spatial information in relation to long-term enhancement of hippocampal synapses. *Behav Neurosci*, 99, 1040-1048.
- Bartus RT, Dean RL, III, Beer B & Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science*, 217, 408-414.
- Bastianetto S, Brouillette J & Quirion R (2007) Neuroprotective Effects of Natural Products: Interaction with Intracellular Kinases, Amyloid Peptides and a Possible Role for Transthyretin. *Neurochem Res*, Epub ahead of print.
- Bastianetto S, Ramassamy C, Dore S, Christen Y, Poirier J & Quirion R (2000a) The Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. *Eur J Neurosci*, 12, 1882-1890.
- Bastianetto S, Zheng WH & Quirion R (2000b) Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br J Pharmacol*, 131, 711-720.
- Basu TK & Basualdo C (1997) Vitamin A homeostasis and diabetes mellitus. *Nutrition*, 13, 804-806.

- Basu TK, Tze WJ & Leichter J (1989) Serum vitamin A and retinol-binding protein in patients with insulin-dependent diabetes mellitus. *Am J Clin Nutr*, 50, 329-331.
- Baum LW (2005) Sex, hormones, and Alzheimer's disease. *J Gerontol A Biol Sci Med Sci*, 60, 736-743.
- Beczowska IW, Buck J & Inturrisi CE (1996) Retinoic acid-induced increase in delta-opioid receptor and N-methyl-D-aspartate receptor mRNA levels in neuroblastoma x glioma (NG108-15) cells. *Brain Res Bull*, 39, 193-199.
- Behrends JC & ten Bruggencate G (1993) Cholinergic modulation of synaptic inhibition in the guinea pig hippocampus in vitro: excitation of GABAergic interneurons and inhibition of GABA-release. *J Neurophysiol*, 69, 626-629.
- Ben Ari Y, Krnjevic K, Reinhardt W & Ropert N (1981) Intracellular observations on the disinhibitory action of acetylcholine in the hippocampus. *Neuroscience*, 6, 2475-2484.
- Benowitz LI & Routtenberg A (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci*, 20, 84-91.
- Berrard S, Faucon BN, Houhou L, Lamouroux A & Mallet J (1993) Retinoic acid induces cholinergic differentiation of cultured newborn rat sympathetic neurons. *J Neurosci Res*, 35, 382-389.
- Berrard S, Varoqui H, Cervini R, Israel M, Mallet J & Diebler MF (1995) Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter. *J Neurochem*, 65, 939-942.
- Berse B & Blusztajn JK (1995) Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor alpha, cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line. *J Biol Chem*, 270, 22101-22104.
- Berse B & Blusztajn JK (1997) Modulation of cholinergic locus expression by glucocorticoids and retinoic acid is cell-type specific. *FEBS Lett*, 410, 175-179.
- Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J & Volterra A (2001) CXCR4-activated

- astrocyte glutamate release via TNF α : amplification by microglia triggers neurotoxicity. *Nat Neurosci*, 4, 702-710.
- Bi J, Hu X, Loh HH & Wei LN (2001) Regulation of mouse kappa opioid receptor gene expression by retinoids. *J Neurosci*, 21, 1590-1599.
- Bianchi M & Panerai AE (1993) Reversal of scopolamine-induced amnesia by the GABAB receptor antagonist CGP 35348 in the mouse. *Brain Res Cogn Brain Res*, 1, 135-136.
- Billings LM, Oddo S, Green KN, McGaugh JL & LaFerla FM (2005) Intraneuronal A β causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*, 45, 675-688.
- Biroccio A, Del Boccio P, Panella M, Bernardini S, Di Ilio C, Gambi D, Stanzione P, Sacchetta P, Bernardi G, Martorana A, Federici G, Stefani A & Urbani A (2006) Differential post-translational modifications of transthyretin in Alzheimer's disease: a study of the cerebral spinal fluid. *Proteomics*, 6, 2305-2313.
- Blain H, Jeandel C, Merched A, Visvikis S & Siest G (1997) Apolipoprotein E level in cerebrospinal fluid increases with aging. *J Am Geriatr Soc*, 45, 1536.
- Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC & Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci*, 23, 3807-3819.
- Blalock EM, Geddes JW, Chen KC, Porter NM, Markesbery WR & Landfield PW (2004) Incipient Alzheimer's disease: microarray correlation analyses reveal major transcriptional and tumor suppressor responses. *Proc Natl Acad Sci U S A*, 101, 2173-2178.
- Blennow K, de Leon MJ & Zetterberg H (2006) Alzheimer's disease. *Lancet*, 368, 387-403.
- Bliss TV & Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol*, 232, 331-356.

- Bogdanovic N, Davidsson P, Volkman I, Winblad B & Blennow K (2000) Growth-associated protein GAP-43 in the frontal cortex and in the hippocampus in Alzheimer's disease: an immunohistochemical and quantitative study. *J Neural Transm*, 107, 463-478.
- Boguski MS & Jones AR (2004) Neurogenomics: at the intersection of neurobiology and genome sciences. *Nat Neurosci*, 7, 429-433.
- Bonni A, Brunet A, West AE, Datta SR, Takasu MA & Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science*, 286, 1358-1362.
- Bontempi B, Laurent-Demir C, Destrade C & Jaffard R (1999) Time-dependent reorganization of brain circuitry underlying long-term memory storage. *Nature*, 400, 671-675.
- Bourdel-Marchasson I, Delmas-Beauvieux MC, Peuchant E, Richard-Harston S, Decamps A, Reignier B, Emeriau JP & Rainfray M (2001) Antioxidant defences and oxidative stress markers in erythrocytes and plasma from normally nourished elderly Alzheimer patients. *Age Ageing*, 30, 235-241.
- Bourtchouladze R, Abel T, Berman N, Gordon R, Lapidus K & Kandel ER (1998) Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learn Mem*, 5, 365-374.
- Bourtchouladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G & Silva AJ (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell*, 79, 59-68.
- Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI & Enna SJ (2002) International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. *Pharmacol Rev*, 54, 247-264.
- Bozon B, Kelly A, Josselyn SA, Silva AJ, Davis S & Laroche S (2003) MAPK, CREB and zif268 are all required for the consolidation of recognition memory. *Philos Trans R Soc Lond B Biol Sci*, 358, 805-814.

- Braak H & Braak E (1985) On areas of transition between entorhinal allocortex and temporal isocortex in the human brain. Normal morphology and lamina-specific pathology in Alzheimer's disease. *Acta Neuropathol (Berl)*, 68, 325-332.
- Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL & Worley PF (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature*, 386, 284-288.
- Brenner DE, Kukull WA, van Belle G, Bowen JD, McCormick WC, Teri L & Larson EB (1993) Relationship between cigarette smoking and Alzheimer's disease in a population-based case-control study. *Neurology*, 43, 293-300.
- Brinton RD (2004) Impact of estrogen therapy on Alzheimer's disease: a fork in the road? *CNS Drugs*, 18, 405-422.
- Brouillette J & Quirion R (2007) Transthyretin: A key gene involved in the maintenance of memory capacities during aging. *Neurobiol Aging*, Epub ahead of print.
- Brouillette J, Young D, During MJ & Quirion R (2007) Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABA(B) receptors in scopolamine-induced amnesia. *J Neurochem*, 102, 1978-1989.
- Brouwer A, Hakansson H, Kukler A, Van den Berg KJ & Ahlborg UG (1989) Marked alterations in retinoid homeostasis of Sprague-Dawley rats induced by a single i.p. dose of 10 micrograms/kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology*, 58, 267-283.
- Brown AJ & Roberts DC (1991) The effect of fasting triacylglyceride concentration and apolipoprotein E polymorphism on postprandial lipemia. *Arterioscler Thromb*, 11, 1737-1744.
- Brown GC & Bal-Price A (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol Neurobiol*, 27, 325-355.
- Buccafusco JJ & Jackson WJ (1991) Beneficial effects of nicotine administered prior to a delayed matching-to-sample task in young and aged monkeys. *Neurobiol Aging*, 12, 233-238.

- Bugge TH, Pohl J, Lonnoy O & Stunnenberg HG (1992) RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J*, 11, 1409-1418.
- Burger C, Lopez MC, Feller JA, Baker HV, Muzyczka N & Mandel RJ (2007) Changes in transcription within the CA1 field of the hippocampus are associated with age-related spatial learning impairments. *Neurobiol Learn Mem*, 87, 21-41.
- Burns A & Murphy D (1996) Protection against Alzheimer's disease? *Lancet*, 348, 420-421.
- Burri BJ & Kutnink MA (1989) Liquid-chromatographic assay for free and transthyretin-bound retinol-binding protein in serum from normal humans. *Clin Chem*, 35, 582-586.
- Buryanovskyy L, Fu Y, Boyd M, Ma Y, Hsieh TC, Wu JM & Zhang Z (2004) Crystal structure of quinone reductase 2 in complex with resveratrol. *Biochemistry*, 43, 11417-11426.
- Bush A & Beail N (2004) Risk factors for dementia in people with down syndrome: issues in assessment and diagnosis. *Am J Ment Retard*, 109, 83-97.
- Busiguina S, Fernandez AM, Barrios V, Clark R, Tolbert DL, Berciano J & Torres-Aleman I (2000) Neurodegeneration is associated to changes in serum insulin-like growth factors. *Neurobiol Dis*, 7, 657-665.
- Campion D, Flaman JM, Brice A, Hannequin D, Dubois B, Martin C, Moreau V, Charbonnier F, Didierjean O, Tardieu S & . (1995) Mutations of the presenilin I gene in families with early-onset Alzheimer's disease. *Hum Mol Genet*, 4, 2373-2377.
- Canon E, Cosgaya JM, Scsucova S & Aranda A (2004) Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. *Mol Biol Cell*, 15, 5583-5592.
- Carli M, Luschi R & Samanin R (1997) Dose-related impairment of spatial learning by intrahippocampal scopolamine: antagonism by ondansetron, a 5-HT3 receptor antagonist. *Behav Brain Res*, 82, 185-194.

- Carlsson J, Armstrong VW, Reiber H, Felgenhauer K & Seidel D (1991) Clinical relevance of the quantification of apolipoprotein E in cerebrospinal fluid. *Clin Chim Acta*, 196, 167-176.
- Carro E, Nunez A, Busiguina S & Torres-Aleman I (2000) Circulating insulin-like growth factor I mediates effects of exercise on the brain. *J Neurosci*, 20, 2926-2933.
- Carro E, Trejo JL, Busiguina S & Torres-Aleman I (2001) Circulating insulin-like growth factor I mediates the protective effects of physical exercise against brain insults of different etiology and anatomy. *J Neurosci*, 21, 5678-5684.
- Carro E, Trejo JL, Gomez-Isla T, LeRoith D & Torres-Aleman I (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. *Nat Med*, 8, 1390-1397.
- Cauley K, Marks M, Gahring LC & Rogers SW (1996) Nicotinic receptor subunits alpha 3, alpha 4, and beta 2 and high affinity nicotine binding sites are expressed by P19 embryonal cells. *J Neurobiol*, 30, 303-314.
- Ceccarini M, Macioce P, Panetta B & Petrucci TC (2002) Expression of dystrophin-associated proteins during neuronal differentiation of P19 embryonal carcinoma cells. *Neuromuscul Disord*, 12, 36-48.
- Cervello M, D'Amelio L, Tesoro V, Rougon G & Matranga V (1997) Expression of PSA-N-CAM in human neuroblastoma cells induced to neuronal differentiation by retinoic acid. *Eur J Cell Biol*, 73, 270-275.
- Cervini R, Berrard S, Bejanin S & Mallet J (1994) Regulation by CDF/LIF and retinoic acid of multiple ChAT mRNAs produced from distinct promoters. *Neuroreport*, 5, 1346-1348.
- Changeux JP (1990) The TiPS lecture. The nicotinic acetylcholine receptor: an allosteric protein prototype of ligand-gated ion channels. *Trends Pharmacol Sci*, 11, 485-492.
- Charest A, Wainer BH & Albert PR (1993) Cloning and differentiation-induced expression of a murine serotonin_{1A} receptor in a septal cell line. *J Neurosci*, 13, 5164-5171.

- Chen RL, Athauda SB, Kassem NA, Zhang Y, Segal MB & Preston JE (2005) Decrease of transthyretin synthesis at the blood-cerebrospinal fluid barrier of old sheep. *J Gerontol A Biol Sci Med Sci*, 60, 852-858.
- Chen SJ, Sweatt JD & Klann E (1997) Enhanced phosphorylation of the postsynaptic protein kinase C substrate RC3/neurogranin during long-term potentiation. *Brain Res*, 749, 181-187.
- Chen Z, Xu AJ, Li R & Wei EQ (2002) Reversal of scopolamine-induced spatial memory deficits in rats by TAK-147. *Acta Pharmacol Sin*, 23, 355-360.
- Chesler EJ & Juraska JM (2000) Acute administration of estrogen and progesterone impairs the acquisition of the spatial morris water maze in ovariectomized rats. *Horm Behav*, 38, 234-242.
- Chiang MY, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF & Evans RM (1998) An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron*, 21, 1353-1361.
- Chiu FC, Feng L, Chan SO, Padin C & Federoff JH (1995) Expression of neurofilament proteins during retinoic acid-induced differentiation of P19 embryonal carcinoma cells. *Brain Res Mol Brain Res*, 30, 77-86.
- Cho S, Cho H, Geum D & Kim K (1998) Retinoic acid regulates gonadotropin-releasing hormone (GnRH) release and gene expression in the rat hypothalamic fragments and GT1-1 neuronal cells in vitro. *Brain Res Mol Brain Res*, 54, 74-84.
- Cho S, Chung J, Han J, Ju LB, Han KD, Rhee K & Kim K (2001) 9-cis-Retinoic acid represses transcription of the gonadotropin-releasing hormone (GnRH) gene via proximal promoter region that is distinct from all-trans-retinoic acid response element. *Brain Res Mol Brain Res*, 87, 214-222.
- Choi SH, Leight SN, Lee VM, Li T, Wong PC, Johnson JA, Saraiva MJ & Sisodia SS (2007) Accelerated Abeta deposition in APP^{swe}/PS1^{deltaE9} mice with hemizygous deletions of TTR (transthyretin). *J Neurosci*, 27, 7006-7010.

- Cirrito JR, Yamada KA, Finn MB, Sloviter RS, Bales KR, May PC, Schoepp DD, Paul SM, Mennerick S & Holtzman DM (2005) Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo. *Neuron*, 48, 913-922.
- Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St George HP & Selkoe DJ (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat Med*, 3, 67-72.
- Clagett-Dame M, McNeill EM & Muley PD (2006) Role of all-trans retinoic acid in neurite outgrowth and axonal elongation. *J Neurobiol*, 66, 739-756.
- Clagett-Dame M & Plum LA (1997) Retinoid-regulated gene expression in neural development. *Crit Rev Eukaryot Gene Expr*, 7, 299-342.
- Clinton J, Ambler MW & Roberts GW (1991) Post-traumatic Alzheimer's disease: preponderance of a single plaque type. *Neuropathol Appl Neurobiol*, 17, 69-74.
- Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L & Fadda F (2002) Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience*, 115, 475-482.
- Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG & Lukiw WJ (2002) Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J Neurosci Res*, 70, 462-473.
- Cole G (2006) A transgenic triple scores a home run. *Nat Med*, 12, 762-763.
- Coleman BA & Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. *J Biol Chem*, 271, 4410-4416.
- Collier TJ, Gash DM & Sladek JR, Jr. (1988) Transplantation of norepinephrine neurons into aged rats improves performance of a learned task. *Brain Res*, 448, 77-87.
- Colombo PJ & Gallagher M (2002) Individual differences in spatial memory among aged rats are related to hippocampal PKCgamma immunoreactivity. *Hippocampus*, 12, 285-289.

- Corcoran J & Maden M (1999) Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nat Neurosci*, 2, 307-308.
- Corcoran J, Shroot B, Pizzey J & Maden M (2000) The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *J Cell Sci*, 113 (Pt 14), 2567-2574.
- Corcoran JP, So PL & Maden M (2004) Disruption of the retinoid signalling pathway causes a deposition of amyloid beta in the adult rat brain. *Eur J Neurosci*, 20, 896-902.
- Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL & Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, 261, 921-923.
- Cordoba Montoya DA & Carrer HF (1997) Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. *Brain Res*, 778, 430-438.
- Coyle JT, Price DL & DeLong MR (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science*, 219, 1184-1190.
- Craft S, Asthana S, Schellenberg G, Baker L, Cherrier M, Boyt AA, Martins RN, Raskind M, Peskind E & Plymate S (2000) Insulin effects on glucose metabolism, memory, and plasma amyloid precursor protein in Alzheimer's disease differ according to apolipoprotein-E genotype. *Ann N Y Acad Sci*, 903, 222-228.
- Crandall J, Sakai Y, Zhang J, Koul O, Mineur Y, Crusio WE & McCaffery P (2004) 13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice. *Proc Natl Acad Sci U S A*, 101, 5111-5116.
- Crisby M, Carlson LA & Winblad B (2002) Statins in the prevention and treatment of Alzheimer disease. *Alzheimer Dis Assoc Disord*, 16, 131-136.
- Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, Huang X, Farrag YW, Perry G & Bush AI (2000) Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of abeta by zinc. *J Biol Chem*, 275, 19439-19442.

- Culvenor JG, Evin G, Cooney MA, Warden H, Sharples RA, Maher F, Reed G, Diehlmann A, Weidemann A, Beyreuther K & Masters CL (2000) Presenilin 2 expression in neuronal cells: induction during differentiation of embryonic carcinoma cells. *Exp Cell Res*, 255, 192-206.
- Cummings JL & Kaufer D (1996) Neuropsychiatric aspects of Alzheimer's disease: the cholinergic hypothesis revisited. *Neurology*, 47, 876-883.
- Dallongeville J, Lussier-Cacan S & Davignon J (1992) Modulation of plasma triglyceride levels by apoE phenotype: a meta-analysis. *J Lipid Res*, 33, 447-454.
- Dash PK, Hochner B & Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. *Nature*, 345, 718-721.
- Davies CA, Mann DM, Sumpter PQ & Yates PO (1987) A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. *J Neurol Sci*, 78, 151-164.
- Davies CH, Starkey SJ, Pozza MF & Collingridge GL (1991) GABA autoreceptors regulate the induction of LTP. *Nature*, 349, 609-611.
- Davies P (2000) A very incomplete comprehensive theory of Alzheimer's disease. *Ann N Y Acad Sci*, 924, 8-16.
- Davies P & Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet*, 2, 1403.
- Davis S, Butcher SP & Morris RG (1992) The NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5) impairs spatial learning and LTP in vivo at intracerebral concentrations comparable to those that block LTP in vitro. *J Neurosci*, 12, 21-34.
- De Nayer P, Rennotte B & Caucheteux D (1991) Thyroid hormone receptors in brain and liver during ageing. *Horm Metab Res*, 23, 12-14.
- de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjoball J & Perlmann T (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science*, 290, 2140-2144.

- Decker MW, Tran T & McGaugh JL (1990) A comparison of the effects of scopolamine and diazepam on acquisition and retention of inhibitory avoidance in mice. *Psychopharmacology (Berl)*, 100, 515-521.
- Dekosky ST & Scheff SW (1990) Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann Neurol*, 27, 457-464.
- den Heijer T, Vermeer SE, van Dijk EJ, Prins ND, Koudstaal PJ, Hofman A & Breteler MM (2003) Type 2 diabetes and atrophy of medial temporal lobe structures on brain MRI. *Diabetologia*, 46, 1604-1610.
- Deutsch JA (1971) The cholinergic synapse and the site of memory. *Science*, 174, 788-794.
- Devan BD, Sierra-Mercado D, Jr., Jimenez M, Bowker JL, Duffy KB, Spangler EL & Ingram DK (2004) Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacol Biochem Behav*, 79, 691-699.
- DeVito MJ, Thomas T, Martin E, Umbreit TH & Gallo MA (1992) Antiestrogenic action of 2,3,7,8-tetrachlorodibenzo-p-dioxin: tissue-specific regulation of estrogen receptor in CD1 mice. *Toxicol Appl Pharmacol*, 113, 284-292.
- Dheen ST, Jun Y, Yan Z, Tay SS & Ling EA (2005) Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. *Glia*, 50, 21-31.
- Dickey CA, Loring JF, Montgomery J, Gordon MN, Eastman PS & Morgan D (2003) Selectively reduced expression of synaptic plasticity-related genes in amyloid precursor protein + presenilin-1 transgenic mice. *J Neurosci*, 23, 5219-5226.
- Dickson PW, Howlett GJ & Schreiber G (1985) Rat transthyretin (prealbumin). Molecular cloning, nucleotide sequence, and gene expression in liver and brain. *J Biol Chem*, 260, 8214-8219.
- Divino CM & Schussler GC (1990a) Receptor-mediated uptake and internalization of transthyretin. *J Biol Chem*, 265, 1425-1429.
- Divino CM & Schussler GC (1990b) Transthyretin receptors on human astrocytoma cells. *J Clin Endocrinol Metab*, 71, 1265-1268.

- Dohrmann GJ (1970) The choroid plexus: a historical review. *Brain Res*, 18, 197-218.
- Dolezal V, Castell X, Tomasi M & Diebler MF (2001) Stimuli that induce a cholinergic neuronal phenotype of NG108-15 cells upregulate ChAT and VACHT mRNAs but fail to increase VACHT protein. *Brain Res Bull*, 54, 363-373.
- Dong D, Ruuska SE, Levinthal DJ & Noy N (1999) Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. *J Biol Chem*, 274, 23695-23698.
- Dore S, Kar S & Quirion R (1997a) Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. *Proc Natl Acad Sci U S A*, 94, 4772-4777.
- Dore S, Kar S, Rowe W & Quirion R (1997b) Distribution and levels of [125I]IGF-I, [125I]IGF-II and [125I]insulin receptor binding sites in the hippocampus of aged memory-unimpaired and -impaired rats. *Neuroscience*, 80, 1033-1040.
- Drachman DA & Leavitt J (1974) Human memory and the cholinergic system. A relationship to aging? *Arch Neurol*, 30, 113-121.
- Driscoll I & Sutherland RJ (2005) The aging hippocampus: navigating between rat and human experiments. *Rev Neurosci*, 16, 87-121.
- Du Y, Dodel RC, Eastwood BJ, Bales KR, Gao F, Lohmuller F, Muller U, Kurz A, Zimmer R, Evans RM, Hake A, Gasser T, Oertel WH, Griffin WS, Paul SM & Farlow MR (2000) Association of an interleukin 1 alpha polymorphism with Alzheimer's disease. *Neurology*, 55, 480-483.
- Dyck JR & Fliegel L (1995) Specific activation of the Na⁺/H⁺ exchanger gene during neuronal differentiation of embryonal carcinoma cells. *J Biol Chem*, 270, 10420-10427.
- Ebihara S, Obata K & Yanagawa Y (2003) Mouse vesicular GABA transporter gene: genomic organization, transcriptional regulation and chromosomal localization. *Brain Res Mol Brain Res*, 110, 126-139.

- Edvinsson L, Nielsen KC & Owman C (1973) Cholinergic innervation of choroid plexus in rabbits and cats. *Brain Res*, 63, 500-503.
- Elias MF, Elias PK, Sullivan LM, Wolf PA & D'Agostino RB (2005) Obesity, diabetes and cognitive deficit: The Framingham Heart Study. *Neurobiol Aging*, 26 Suppl 1, 11-16.
- Elovaara I, Maury CP & Palo J (1986) Serum amyloid A protein, albumin and prealbumin in Alzheimer's disease and in demented patients with Down's syndrome. *Acta Neurol Scand*, 74, 245-250.
- Emerich DF, Skinner SJ, Borlongan CV, Vasconcellos AV & Thanos CG (2005) The choroid plexus in the rise, fall and repair of the brain. *Bioessays*, 27, 262-274.
- Enderlin V, Alfos S, Pallet V, Garcin H, Azais-Braesco V, Jaffard R & Higuieret P (1997a) Aging decreases the abundance of retinoic acid (RAR) and triiodothyronine (TR) nuclear receptor mRNA in rat brain: effect of the administration of retinoids. *FEBS Lett*, 412, 629-632.
- Enderlin V, Pallet V, Alfos S, Dargelos E, Jaffard R, Garcin H & Higuieret P (1997b) Age-related decreases in mRNA for brain nuclear receptors and target genes are reversed by retinoic acid treatment. *Neurosci Lett*, 229, 125-129.
- Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME & Robertson EJ (1993) Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proc Natl Acad Sci U S A*, 90, 2375-2379.
- Etchamendy N, Enderlin V, Marighetto A, Pallet V, Higuieret P & Jaffard R (2003) Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling. *Behav Brain Res*, 145, 37-49.
- Etchamendy N, Enderlin V, Marighetto A, Vouimba RM, Pallet V, Jaffard R & Higuieret P (2001) Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J Neurosci*, 21, 6423-6429.

- Etienne P, Robitaille Y, Wood P, Gauthier S, Nair NP & Quirion R (1986) Nucleus basalis neuronal loss, neuritic plaques and choline acetyltransferase activity in advanced Alzheimer's disease. *Neuroscience*, 19, 1279-1291.
- Farag A, Karam J, Nicasio J & McFarlane SI (2007) Prevention of type 2 diabetes: an update. *Curr Diab Rep*, 7, 200-207.
- Farooqui AA, Antony P, Ong WY, Horrocks LA & Freysz L (2004) Retinoic acid-mediated phospholipase A2 signaling in the nucleus. *Brain Res Brain Res Rev*, 45, 179-195.
- Feart C, Mingaud F, Enderlin V, Husson M, Alfos S, Higuieret P & Pallet V (2005a) Differential effect of retinoic acid and triiodothyronine on the age-related hypo-expression of neurogranin in rat. *Neurobiol Aging*, 26, 729-738.
- Feart C, Vallortigara J, Higuieret D, Gatta B, Tabarin A, Enderlin V, Higuieret P & Pallet V (2005b) Decreased expression of retinoid nuclear receptor (RAR alpha and RAR gamma) mRNA determined by real-time quantitative RT-PCR in peripheral blood mononuclear cells of hypothyroid patients. *J Mol Endocrinol*, 34, 849-858.
- Fiorella PD, Giguere V & Napoli JL (1993) Expression of cellular retinoic acid-binding protein (type II) in Escherichia coli. Characterization and comparison to cellular retinoic acid-binding protein (type I). *J Biol Chem*, 268, 21545-21552.
- Flood F, Sundstrom E, Samuelsson EB, Wichager B, Seiger A, Johnston JA & Cowburn RF (2004) Presenilin expression during induced differentiation of the human neuroblastoma SH-SY5Y cell line. *Neurochem Int*, 44, 487-496.
- Flood JF & Cherkin A (1986) Scopolamine effects on memory retention in mice: a model of dementia? *Behav Neural Biol*, 45, 169-184.
- Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM & Weinberger C (1995) Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell*, 81, 687-693.

- Francis PT, Palmer AM, Snape M & Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry*, 66, 137-147.
- Freund TF & Antal M (1988) GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature*, 336, 170-173.
- Freund TF & Buzsaki G (1996) Interneurons of the hippocampus. *Hippocampus*, 6, 347-470.
- Freund-Levi Y, Eriksdotter-Jonhagen M, Cederholm T, Basun H, Faxen-Irving G, Garlind A, Vedin I, Vessby B, Wahlund LO & Palmblad J (2006) Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. *Arch Neurol*, 63, 1402-1408.
- Frick KM, Baxter MG, Markowska AL, Olton DS & Price DL (1995) Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiol Aging*, 16, 149-160.
- Frick KM, Fernandez SM & Bulinski SC (2002) Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. *Neuroscience*, 115, 547-558.
- Friedheim E, Corvi C, Graziano J, Donnelly T & Breslin D (1983) Choroid plexus as a protective sink for heavy metals? *Lancet*, 1, 981-982.
- Froestl W, Gallagher M, Jenkins H, Madrid A, Melcher T, Teichman S, Mondadori CG & Pearlman R (2004) SGS742: the first GABA(B) receptor antagonist in clinical trials. *Biochem Pharmacol*, 68, 1479-1487.
- Frotscher M & Leranth C (1985) Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. *J Comp Neurol*, 239, 237-246.
- Fugger HN, Cunningham SG, Rissman EF & Foster TC (1998) Sex differences in the activational effect of ERalpha on spatial learning. *Horm Behav*, 34, 163-170.
- Fukuchi K, Deeb SS, Kamino K, Ogburn CE, Snow AD, Sekiguchi RT, Wight TN, Piussan H & Martin GM (1992) Increased expression of beta-amyloid protein precursor and microtubule-associated protein tau during the

- differentiation of murine embryonal carcinoma cells. *J Neurochem*, 58, 1863-1873.
- Gage FH, Dunnett SB & Bjorklund A (1989) Age-related impairments in spatial memory are independent of those in sensorimotor skills. *Neurobiol Aging*, 10, 347-352.
- Galasko D & Marder K (2002) Picking away at frontotemporal dementia. *Neurology*, 58, 1585-1586.
- Galvin JE (2006) Cognitive change in Parkinson disease. *Alzheimer Dis Assoc Disord*, 20, 302-310.
- Ganguli M, Burmeister LA, Seaberg EC, Belle S & Dekosky ST (1996) Association between dementia and elevated TSH: a community-based study. *Biol Psychiatry*, 40, 714-725.
- Gao ZY, Xu G, Stwora-Wojczyk MM, Matschinsky FM, Lee VM & Wolf BA (1998) Retinoic acid induction of calcium channel expression in human NT2N neurons. *Biochem Biophys Res Commun*, 247, 407-413.
- Garton MJ, Keir G, Lakshmi MV & Thompson EJ (1991) Age-related changes in cerebrospinal fluid protein concentrations. *J Neurol Sci*, 104, 74-80.
- Gatz M, Mortimer JA, Fratiglioni L, Johansson B, Berg S, Andel R, Crowe M, Fiske A, Reynolds CA & Pedersen NL (2007) Accounting for the relationship between low education and dementia: A twin study. *Physiol Behav*.
- Gatz M, Reynolds CA, Fratiglioni L, Johansson B, Mortimer JA, Berg S, Fiske A & Pedersen NL (2006) Role of genes and environments for explaining Alzheimer disease. *Arch Gen Psychiatry*, 63, 168-174.
- Gatz M, Svedberg P, Pedersen NL, Mortimer JA, Berg S & Johansson B (2001) Education and the risk of Alzheimer's disease: findings from the study of dementia in Swedish twins. *J Gerontol B Psychol Sci Soc Sci*, 56, 292-300.
- Gauthier S (2002) Advances in the pharmacotherapy of Alzheimer's disease. *CMAJ*, 166, 616-623.
- Gauthier S (2006) Cholinesterase inhibitors in late-stage Alzheimer's disease. *Lancet Neurol*, 5, 468-469.

- Gauthier S, Emre M, Farlow MR, Bullock R, Grossberg GT & Potkin SG (2003) Strategies for continued successful treatment of Alzheimer's disease: switching cholinesterase inhibitors. *Curr Med Res Opin*, 19, 707-714.
- Gauthier S, Herrmann N, Ferreri F & Agbokou C (2006) Use of memantine to treat Alzheimer's disease. *CMAJ*, 175, 501-502.
- George-Hyslop PH (2000) Genetic factors in the genesis of Alzheimer's disease. *Ann N Y Acad Sci*, 924, 1-7.
- Gerendasy DD, Herron SR, Jennings PA & Sutcliffe JG (1995) Calmodulin stabilizes an amphiphilic alpha-helix within RC3/neurogranin and GAP-43/neuromodulin only when Ca^{2+} is absent. *J Biol Chem*, 270, 6741-6750.
- Gerendasy DD & Sutcliffe JG (1997) RC3/neurogranin, a postsynaptic calpacitin for setting the response threshold to calcium influxes. *Mol Neurobiol*, 15, 131-163.
- Giacobini E (2004) Cholinesterase inhibitors: new roles and therapeutic alternatives. *Pharmacol Res*, 50, 433-440.
- Giannakopoulos P, Herrmann FR, Bussiere T, Bouras C, Kovari E, Perl DP, Morrison JH, Gold G & Hof PR (2003) Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. *Neurology*, 60, 1495-1500.
- Gibbs RB (1999) Estrogen replacement enhances acquisition of a spatial memory task and reduces deficits associated with hippocampal muscarinic receptor inhibition. *Horm Behav*, 36, 222-233.
- Ginsberg SD, Hemby SE, Lee VM, Eberwine JH & Trojanowski JQ (2000) Expression profile of transcripts in Alzheimer's disease tangle-bearing CA1 neurons. *Ann Neurol*, 48, 77-87.
- Ginty DD, Bonni A & Greenberg ME (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell*, 77, 713-725.
- Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L & . (1991) Segregation of a missense mutation

- in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, 349, 704-706.
- Gold G, Kovari E, Corte G, Herrmann FR, Canuto A, Bussiere T, Hof PR, Bouras C & Giannakopoulos P (2001) Clinical validity of A beta-protein deposition staging in brain aging and Alzheimer disease. *J Neuropathol Exp Neurol*, 60, 946-952.
- Gomez-Isla T, Price JL, McKeel DW, Jr., Morris JC, Growdon JH & Hyman BT (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci*, 16, 4491-4500.
- Goodman AB (2006) Retinoid receptors, transporters, and metabolizers as therapeutic targets in late onset Alzheimer disease. *J Cell Physiol*, 209, 598-603.
- Goodman AB & Pardee AB (2003) Evidence for defective retinoid transport and function in late onset Alzheimer's disease. *Proc Natl Acad Sci U S A*, 100, 2901-2905.
- Goodman DS (1984) Overview of current knowledge of metabolism of vitamin A and carotenoids. *J Natl Cancer Inst*, 73, 1375-1379.
- Graves AB, van Duijn CM, Chandra V, Fratiglioni L, Heyman A, Jorm AF, Kokmen E, Kondo K, Mortimer JA, Rocca WA & . (1991) Alcohol and tobacco consumption as risk factors for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group. *Int J Epidemiol*, 20 Suppl 2, S48-S57.
- Gray R, Rajan AS, Radcliffe KA, Yakehiro M & Dani JA (1996) Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature*, 383, 713-716.
- Griffin WS (2006) Inflammation and neurodegenerative diseases. *Am J Clin Nutr*, 83, 470S-474S.
- Gulyas AI, Gorcs TJ & Freund TF (1990) Innervation of different peptide-containing neurons in the hippocampus by GABAergic septal afferents. *Neuroscience*, 37, 31-44.

- Gulyas AI, Hajos N & Freund TF (1996) Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J Neurosci*, 16, 3397-3411.
- Guo Z, Fratiglioni L, Viitanen M, Lannfelt L, Basun H, Fastbom J & Winblad B (2001) Apolipoprotein E genotypes and the incidence of Alzheimer's disease among persons aged 75 years and older: variation by use of antihypertensive medication? *Am J Epidemiol*, 153, 225-231.
- Guo Z, Fratiglioni L, Zhu L, Fastbom J, Winblad B & Viitanen M (1999) Occurrence and progression of dementia in a community population aged 75 years and older: relationship of antihypertensive medication use. *Arch Neurol*, 56, 991-996.
- Gureviciene I, Puolivali J, Pussinen R, Wang J, Tanila H & Ylinen A (2003) Estrogen treatment alleviates NMDA-antagonist induced hippocampal LTP blockade and cognitive deficits in ovariectomized mice. *Neurobiol Learn Mem*, 79, 72-80.
- Gustafson D, Rothenberg E, Blennow K, Steen B & Skoog I (2003) An 18-year follow-up of overweight and risk of Alzheimer disease. *Arch Intern Med*, 163, 1524-1528.
- Haass C (2004) Take five--BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J*, 23, 483-488.
- Hakansson H & Ahlborg UG (1985) The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the uptake, distribution and excretion of a single oral dose of [11,12-3H]retinyl acetate and on the vitamin A status in the rat. *J Nutr*, 115, 759-771.
- Hardy J (2006) Amyloid double trouble. *Nat Genet*, 38, 11-12.
- Hardy JA & Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science*, 256, 184-185.
- Harman D (1981) The aging process. *Proc Natl Acad Sci U S A*, 78, 7124-7128.
- Harman D (2006) Alzheimer's disease pathogenesis: role of aging. *Ann N Y Acad Sci*, 1067, 454-460.

- Harris FM, Tesseur I, Brecht WJ, Xu Q, Mullendorff K, Chang S, Wyss-Coray T, Mahley RW & Huang Y (2004) Astroglial regulation of apolipoprotein E expression in neuronal cells. Implications for Alzheimer's disease. *J Biol Chem*, 279, 3862-3868.
- Hart SA, Patton JD & Woolley CS (2001) Quantitative analysis of ER alpha and GAD colocalization in the hippocampus of the adult female rat. *J Comp Neurol*, 440, 144-155.
- Harvey J, Balasubramaniam R & Collingridge GL (1993) Carbachol can potentiate N-methyl-D-aspartate responses in the rat hippocampus by a staurosporine and thapsigargin-insensitive mechanism. *Neurosci Lett*, 162, 165-168.
- Hasselblatt M, Bohm C, Tatenhorst L, Dinh V, Newrzella D, Keyvani K, Jeibmann A, Buerger H, Rickert CH & Paulus W (2006) Identification of novel diagnostic markers for choroid plexus tumors: a microarray-based approach. *Am J Surg Pathol*, 30, 66-74.
- Hassing LB, Johansson B, Nilsson SE, Berg S, Pedersen NL, Gatz M & McClearn G (2002) Diabetes mellitus is a risk factor for vascular dementia, but not for Alzheimer's disease: a population-based study of the oldest old. *Int Psychogeriatr*, 14, 239-248.
- Haughey NJ, Nath A, Chan SL, Borchard AC, Rao MS & Mattson MP (2002) Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. *J Neurochem*, 83, 1509-1524.
- Hebb DO (1949) *The Organization of Behavior: A Neuropsychological Theory*. John Wiley & Sons, New York.
- Hebert LE, Scherr PA, Bienias JL, Bennett DA & Evans DA (2003) Alzheimer disease in the US population: prevalence estimates using the 2000 census. *Arch Neurol*, 60, 1119-1122.
- Heicklen-Klein A, Aronov S & Ginzburg I (2000) Tau promoter activity in neuronally differentiated P19 cells. *Brain Res*, 874, 1-9.

- Helm KA, Haberman RP, Dean SL, Hoyt EC, Melcher T, Lund PK & Gallagher M (2005) GABAB receptor antagonist SGS742 improves spatial memory and reduces protein binding to the cAMP response element (CRE) in the hippocampus. *Neuropharmacology*, 48, 956-964.
- Henderson VW (2006) Estrogen-containing hormone therapy and Alzheimer's disease risk: understanding discrepant inferences from observational and experimental research. *Neuroscience*, 138, 1031-1039.
- Heneka MT & O'Banion MK (2007) Inflammatory processes in Alzheimer's disease. *J Neuroimmunol*, 184, 69-91.
- Hennou S, Kato A, Schneider EM, Lundstrom K, Gahwiler BH, Inokuchi K, Gerber U & Ehrengruber MU (2003) Homer-1a/Ves1-1S enhances hippocampal synaptic transmission. *Eur J Neurosci*, 18, 811-819.
- Hensley K, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA & Butterfield DA (1994) A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci U S A*, 91, 3270-3274.
- Herbert J, Wilcox JN, Pham KT, Freneau RT, Jr., Zeviani M, Dwork A, Soprano DR, Makover A, Goodman DS, Zimmerman EA & . (1986) Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. *Neurology*, 36, 900-911.
- Herrera DG, Maysinger D & Goiny M (1993) Induction of c-FOS immunoreactivity in the hippocampus following potassium stimulation. *Neuroscience*, 52, 237-244.
- Herrera-Morales W, Mar I, Serrano B & Bermudez-Rattoni F (2007) Activation of hippocampal postsynaptic muscarinic receptors is involved in long-term spatial memory formation. *Eur J Neurosci*, 25, 1581-1588.
- Hersi AI, Rowe W, Gaudreau P & Quirion R (1995) Dopamine D1 receptor ligands modulate cognitive performance and hippocampal acetylcholine release in memory-impaired aged rats. *Neuroscience*, 69, 1067-1074.
- Hoegberg P, Schmidt CK, Nau H, Ross AC, Zolfaghari R, Fletcher N, Trossvik C, Nilsson CB & Hakansson H (2003) 2,3,7,8-tetrachlorodibenzo-p-dioxin induces

- lecithin: retinol acyltransferase transcription in the rat kidney. *Chem Biol Interact*, 145, 1-16.
- Hojo R, Zareba G, Kai JW, Baggs RB & Weiss B (2006) Sex-specific alterations of cerebral cortical cell size in rats exposed prenatally to dioxin. *J Appl Toxicol*, 26, 25-34.
- Hollup SA, Kjelstrup KG, Hoff J, Moser MB & Moser EI (2001) Impaired recognition of the goal location during spatial navigation in rats with hippocampal lesions. *J Neurosci*, 21, 4505-4513.
- Holtzman DM (2001) Role of apoe/Abeta interactions in the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy. *J Mol Neurosci*, 17, 147-155.
- Hong SJ, Grover CA, Safe SH, Tiffany-Castiglioni E & Frye GD (1998) Halogenated aromatic hydrocarbons suppress CA1 field excitatory postsynaptic potentials in rat hippocampal slices. *Toxicol Appl Pharmacol*, 148, 7-13.
- Hu X, Bi J, Loh HH & Wei LN (2002) Regulation of mouse kappa opioid receptor gene expression by different 3'-untranslated regions and the effect of retinoic acid. *Mol Pharmacol*, 62, 881-887.
- Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE & Bush AI (1999) The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry*, 38, 7609-7616.
- Hung AY, Koo EH, Haass C & Selkoe DJ (1992) Increased expression of beta-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage. *Proc Natl Acad Sci U S A*, 89, 9439-9443.
- Husmann M, Gorgen I, Weisgerber C & Bitter-Suermann D (1989) Up-regulation of embryonic NCAM in an EC cell line by retinoic acid. *Dev Biol*, 136, 194-200.
- Husson M, Enderlin V, Alfos S, Boucheron C, Pallet V & Huguieret P (2004) Expression of neurogranin and neuromodulin is affected in the striatum of vitamin A-deprived rats. *Brain Res Mol Brain Res*, 123, 7-17.

- Husson M, Enderlin V, Delacourte A, Ghenimi N, Alfos S, Pallet V & Higuieret P (2006) Retinoic acid normalizes nuclear receptor mediated hypo-expression of proteins involved in beta-amyloid deposits in the cerebral cortex of vitamin A deprived rats. *Neurobiol Dis*, 23, 1-10.
- Hyman BT, Van Hoesen GW, Damasio AR & Barnes CL (1984) Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science*, 225, 1168-1170.
- Hyman BT, Van Hoesen GW, Kromer LJ & Damasio AR (1986) Perforant pathway changes and the memory impairment of Alzheimer's disease. *Ann Neurol*, 20, 472-481.
- Iaria G, Petrides M, Dagher A, Pike B & Bohbot VD (2003) Cognitive strategies dependent on the hippocampus and caudate nucleus in human navigation: variability and change with practice. *J Neurosci*, 23, 5945-5952.
- Igaz LM, Vianna MR, Medina JH & Izquierdo I (2002) Two time periods of hippocampal mRNA synthesis are required for memory consolidation of fear-motivated learning. *J Neurosci*, 22, 6781-6789.
- Impey S, Smith DM, Obrietan K, Donahue R, Wade C & Storm DR (1998) Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat Neurosci*, 1, 595-601.
- in't Veld BA, Ruitenberg A, Hofman A, Stricker BH & Breteler MM (2001) Antihypertensive drugs and incidence of dementia: the Rotterdam Study. *Neurobiol Aging*, 22, 407-412.
- Iniguez MA, Morte B, Rodriguez-Pena A, Munoz A, Gerendasy D, Sutcliffe JG & Bernal J (1994) Characterization of the promoter region and flanking sequences of the neuron-specific gene RC3 (neurogranin). *Brain Res Mol Brain Res*, 27, 205-214.
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B & Speed TP (2003a) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*, 31, e15.

- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U & Speed TP (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, 4, 249-264.
- Issa AM, Rowe W, Gauthier S & Meaney MJ (1990) Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. *J Neurosci*, 10, 3247-3254.
- Iwakiri M, Mizukami K, Ikononovic MD, Ishikawa M, Hidaka S, Abrahamson EE, Dekosky ST & Asada T (2005) Changes in hippocampal GABABR1 subunit expression in Alzheimer's patients: association with Braak staging. *Acta Neuropathol (Berl)*, 109, 467-474.
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N & Ihara Y (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neuron*, 13, 45-53.
- Izquierdo I, Medina JH, Bianchin M, Walz R, Zanatta MS, Da Silva RC, Bueno e Silva, Ruschel AC & Paczko N (1993) Memory processing by the limbic system: role of specific neurotransmitter systems. *Behav Brain Res*, 58, 91-98.
- Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH & Evans RM (2006) Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A*, 103, 3902-3907.
- Jacobson JL & Jacobson SW (1996) Intellectual impairment in children exposed to polychlorinated biphenyls in utero. *N Engl J Med*, 335, 783-789.
- Jacobson JL, Jacobson SW & Humphrey HE (1990) Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. *J Pediatr*, 116, 38-45.
- Janas AM, Cunningham SC, Duffy KB, Devan BD, Greig NH, Holloway HW, Yu QS, Markowska AL, Ingram DK & Spangler EL (2005) The cholinesterase inhibitor, phenserine, improves Morris water maze performance of scopolamine-treated rats. *Life Sci*, 76, 1073-1081.

- Janowski BA, Willy PJ, Devi TR, Falck JR & Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*, 383, 728-731.
- Jaubert PJ, Golub MS, Lo YY, Germann SL, Dehoff MH, Worley PF, Kang SH, Schwarz MK, Seeburg PH & Berman RF (2006) Complex, multimodal behavioral profile of the Homer1 knockout mouse. *Genes Brain Behav*, in press.
- Jellinger KA (1998) The neuropathological diagnosis of Alzheimer disease. *J Neural Transm Suppl*, 53, 97-118.
- Jellinger KA (2004) Head injury and dementia. *Curr Opin Neurol*, 17, 719-723.
- Jenab S & Inturrisi CE (2002) Retinoic acid regulation of mu opioid receptor and c-fos mRNAs and AP-1 DNA binding in SH-SY5Y neuroblastoma cells. *Brain Res Mol Brain Res*, 99, 34-39.
- Jerusalinsky D, Kornisiuk E & Izquierdo I (1997) Cholinergic neurotransmission and synaptic plasticity concerning memory processing. *Neurochem Res*, 22, 507-515.
- Jimenez-Jimenez FJ, Molina JA, de Bustos F, Orti-Pareja M, Benito-Leon J, Tallon-Barranco A, Gasalla T, Porta J & Arenas J (1999) Serum levels of beta-carotene, alpha-carotene and vitamin A in patients with Alzheimer's disease. *Eur J Neurol*, 6, 495-497.
- Jones RW (2003) Have cholinergic therapies reached their clinical boundary in Alzheimer's disease? *Int J Geriatr Psychiatry*, 18, S7-S13.
- Jones S, Sudweeks S & Yakel JL (1999) Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci*, 22, 555-561.
- Jurek MA, Powers RH, Gilbert LG & Aust SD (1990) The effect of TCDD on acyl CoA:retinol acyltransferase activity and vitamin A accumulation in the kidney of male Sprague-Dawley rats. *J Biochem Toxicol*, 5, 155-160.
- Kalmijn S, Mehta KM, Pols HA, Hofman A, Drexhage HA & Breteler MM (2000) Subclinical hyperthyroidism and the risk of dementia. The Rotterdam study. *Clin Endocrinol (Oxf)*, 53, 733-737.

- Kamp TJ, Mitas M, Fields KL, Asoh S, Chin H, Marban E & Nirenberg M (1995) Transcriptional regulation of the neuronal L-type calcium channel alpha 1D subunit gene. *Cell Mol Neurobiol*, 15, 307-326.
- Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294, 1030-1038.
- Kandel ER & Pittenger C (1999) The past, the future and the biology of memory storage. *Philos Trans R Soc Lond B Biol Sci*, 354, 2027-2052.
- Kato A, Ozawa F, Saitoh Y, Fukazawa Y, Sugiyama H & Inokuchi K (1998) Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. *J Biol Chem*, 273, 23969-23975.
- Kato A, Ozawa F, Saitoh Y, Hirai K & Inokuchi K (1997) vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett*, 412, 183-189.
- Kato M, Soprano DR, Makover A, Kato K, Herbert J & Goodman DS (1986) Localization of immunoreactive transthyretin (prealbumin) and of transthyretin mRNA in fetal and adult rat brain. *Differentiation*, 31, 228-235.
- Kee N, Teixeira CM, Wang AH & Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nat Neurosci*, 10, 355-362.
- Kelley SK, Nilsson CB, Green MH, Green JB & Hakansson H (1998) Use of model-based compartmental analysis to study effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vitamin A kinetics in rats. *Toxicol Sci*, 44, 1-13.
- Kelley SK, Nilsson CB, Green MH, Green JB & Hakansson H (2000) Mobilization of vitamin A stores in rats after administration of 2,3, 7,8-tetrachlorodibenzo-p-dioxin: a kinetic analysis. *Toxicol Sci*, 55, 478-484.
- Kemp JA & McKernan RM (2002) NMDA receptor pathways as drug targets. *Nat Neurosci*, 5 Suppl, 1039-1042.
- Kesner RP, Bolland BL & Dakis M (1993) Memory for spatial locations, motor responses, and objects: triple dissociation among the hippocampus, caudate nucleus, and extrastriate visual cortex. *Exp Brain Res*, 93, 462-470.

- Khazipov R, Congar P & Ben Ari Y (1995) Hippocampal CA1 lacunosum-moleculare interneurons: modulation of monosynaptic GABAergic IPSCs by presynaptic GABAB receptors. *J Neurophysiol*, 74, 2126-2137.
- Kitabatake Y, Sailor KA, Ming GL & Song H (2007) Adult neurogenesis and hippocampal memory function: new cells, more plasticity, new memories? *Neurosurg Clin N Am*, 18, 105-13, x.
- Kitaichi K, Day JC & Quirion R (1999a) A novel muscarinic M(4) receptor antagonist provides further evidence of an autoreceptor role for the muscarinic M(2) receptor sub-type. *Eur J Pharmacol*, 383, 53-56.
- Kitaichi K, Hori T, Srivastava LK & Quirion R (1999b) Antisense oligodeoxynucleotides against the muscarinic m2, but not m4, receptor supports its role as autoreceptors in the rat hippocampus. *Brain Res Mol Brain Res*, 67, 98-106.
- Kitajka K, Sinclair AJ, Weisinger RS, Weisinger HS, Mathai M, Jayasooriya AP, Halver JE & Puskas LG (2004) Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. *Proc Natl Acad Sci U S A*, 101, 10931-10936.
- Kliwer SA, Lenhard JM, Willson TM, Patel I, Morris DC & Lehmann JM (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell*, 83, 813-819.
- Kliwer SA, Umesono K, Mangelsdorf DJ & Evans RM (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature*, 355, 446-449.
- Kobayashi M, Matsuoka I & Kurihara K (1994) Cholinergic differentiation of cultured sympathetic neurons induced by retinoic acid. Induction of choline acetyltransferase-mRNA and suppression of tyrosine hydroxylase-mRNA levels. *FEBS Lett*, 337, 259-264.
- Koch HJ, Haas S & Jurgens T (2005) On the physiological relevance of muscarinic acetylcholine receptors in Alzheimer's disease. *Curr Med Chem*, 12, 2915-2921.

- Konig G, Masters CL & Beyreuther K (1990) Retinoic acid induced differentiated neuroblastoma cells show increased expression of the beta A4 amyloid gene of Alzheimer's disease and an altered splicing pattern. *FEBS Lett*, 269, 305-310.
- Krempf M, Ranganathan S, Ritz P, Morin M & Charbonnel B (1991) Plasma vitamin A and E in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) adult diabetic patients. *Int J Vitam Nutr Res*, 61, 38-42.
- Krezel W, Kastner P & Chambon P (1999) Differential expression of retinoid receptors in the adult mouse central nervous system. *Neuroscience*, 89, 1291-1300.
- Krucker T, Siggins GR, McNamara RK, Lindsley KA, Dao A, Allison DW, De Lecea L, Lovenberg TW, Sutcliffe JG & Gerendasy DD (2002) Targeted disruption of RC3 reveals a calmodulin-based mechanism for regulating metaplasticity in the hippocampus. *J Neurosci*, 22, 5525-5535.
- Kuchler-Bopp S, Dietrich JB, Zaepfel M & Delaunoy JP (2000) Receptor-mediated endocytosis of transthyretin by ependymoma cells. *Brain Res*, 870, 185-194.
- Kumari V, Gray JA, ffytche DH, Mitterschiffthaler MT, Das M, Zachariah E, Vythelingum GN, Williams SC, Simmons A & Sharma T (2003) Cognitive effects of nicotine in humans: an fMRI study. *Neuroimage*, 19, 1002-1013.
- Kuratsune M, Yoshimura T, Matsuzaka J & Yamaguchi A (1971) Yusho, a poisoning caused by rice oil contaminated with polychlorinated biphenyls. *HSMHA Health Rep*, 86, 1083-1091.
- Kurokawa R, DiRenzo J, Boehm M, Sugarman J, Gloss B, Rosenfeld MG, Heyman RA & Glass CK (1994) Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature*, 371, 528-531.
- LaFerla FM (2002) Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. *Nat Rev Neurosci*, 3, 862-872.
- Lahiri DK & Nall C (1995) Promoter activity of the gene encoding the beta-amyloid precursor protein is up-regulated by growth factors, phorbol ester, retinoic acid and interleukin-1. *Brain Res Mol Brain Res*, 32, 233-240.

- Lai F & Williams RS (1989) A prospective study of Alzheimer disease in Down syndrome. *Arch Neurol*, 46, 849-853.
- Lamberty Y & Gower AJ (1991) Cholinergic modulation of spatial learning in mice in a Morris-type water maze. *Arch Int Pharmacodyn Ther*, 309, 5-19.
- Lambon Ralph MA, Patterson K, Graham N, Dawson K & Hodges JR (2003) Homogeneity and heterogeneity in mild cognitive impairment and Alzheimer's disease: a cross-sectional and longitudinal study of 55 cases. *Brain*, 126, 2350-2362.
- Lanahan A & Worley P (1998) Immediate-early genes and synaptic function. *Neurobiol Learn Mem*, 70, 37-43.
- Lane MA & Bailey SJ (2005) Role of retinoid signalling in the adult brain. *Prog Neurobiol*, 75, 275-293.
- Larkman AU & Jack JJ (1995) Synaptic plasticity: hippocampal LTP. *Curr Opin Neurobiol*, 5, 324-334.
- Larsen PD & DeLallo L (1989) Cerebrospinal fluid transthyretin in the neonate and blood-cerebrospinal fluid barrier permeability. *Ann Neurol*, 25, 628-630.
- Lautenschlager NT, Cupples LA, Rao VS, Auerbach SA, Becker R, Burke J, Chui H, Duara R, Foley EJ, Glatt SL, Green RC, Jones R, Karlinsky H, Kukull WA, Kurz A, Larson EB, Martelli K, Sadovnick AD, Volicer L, Waring SC, Growdon JH & Farrer LA (1996) Risk of dementia among relatives of Alzheimer's disease patients in the MIRAGE study: What is in store for the oldest old? *Neurology*, 46, 641-650.
- Law A, Dore S, Blackshaw S, Gauthier S & Quirion R (2000) Alteration of expression levels of neuronal nitric oxide synthase and haem oxygenase-2 messenger RNA in the hippocampi and cortices of young adult and aged cognitively unimpaired and impaired Long-Evans rats. *Neuroscience*, 100, 769-775.
- Lazarov O, Robinson J, Tang YP, Hairston IS, Korade-Mirnic Z, Lee VM, Hersh LB, Sapolsky RM, Mirnic K & Sisodia SS (2005) Environmental enrichment reduces Abeta levels and amyloid deposition in transgenic mice. *Cell*, 120, 701-713.

- Le Jeune H, Cecyre D, Rowe W, Meaney MJ & Quirion R (1996) Ionotropic glutamate receptor subtypes in the aged memory-impaired and unimpaired Long-Evans rat. *Neuroscience*, 74, 349-363.
- LeBlanc A (2002) Estrogen and Alzheimer's disease. *Curr Opin Investig Drugs*, 3, 768-773.
- Lee HG, Zhu X, Castellani RJ, Nunomura A, Perry G & Smith MA (2007) Amyloid-beta in Alzheimer disease: the null versus the alternate hypotheses. *J Pharmacol Exp Ther*, 321, 823-829.
- Lee HJ, Hammond DN, Large TH, Roback JD, Sim JA, Brown DA, Otten UH & Wainer BH (1990) Neuronal properties and trophic activities of immortalized hippocampal cells from embryonic and young adult mice. *J Neurosci*, 10, 1779-1787.
- Lee ML, Kuo FC, Whitmore GA & Sklar J (2000) Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci U S A*, 97, 9834-9839.
- Lehericy S, Hirsch EC, Cervera-Pierot P, Hersh LB, Bakchine S, Piette F, Duyckaerts C, Hauw JJ, Javoy-Agid F & Agid Y (1993) Heterogeneity and selectivity of the degeneration of cholinergic neurons in the basal forebrain of patients with Alzheimer's disease. *J Comp Neurol*, 330, 15-31.
- Leibson CL, Rocca WA, Hanson VA, Cha R, Kokmen E, O'Brien PC & Palumbo PJ (1997) Risk of dementia among persons with diabetes mellitus: a population-based cohort study. *Am J Epidemiol*, 145, 301-308.
- Leranth C & Frotscher M (1987) Cholinergic innervation of hippocampal GAD- and somatostatin-immunoreactive commissural neurons. *J Comp Neurol*, 261, 33-47.
- Lesne S, Koh MT, Kotilinek L, Kaye R, Glabe CG, Yang A, Gallagher M & Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*, 440, 352-357.
- Levenson JM, Choi S, Lee SY, Cao YA, Ahn HJ, Worley KC, Pizzi M, Liou HC & Sweatt JD (2004) A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel. *J Neurosci*, 24, 3933-3943.

- Levey AI (1996) Muscarinic acetylcholine receptor expression in memory circuits: implications for treatment of Alzheimer disease. *Proc Natl Acad Sci U S A*, 93, 13541-13546.
- Levey AI, Edmunds SM, Koliatsos V, Wiley RG & Heilman CJ (1995) Expression of m1-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. *J Neurosci*, 15, 4077-4092.
- Levin ED, Bradley A, Addy N & Sigurani N (2002) Hippocampal alpha 7 and alpha 4 beta 2 nicotinic receptors and working memory. *Neuroscience*, 109, 757-765.
- Levin ED, Kim P & Meray R (1996) Chronic nicotine working and reference memory effects in the 16-arm radial maze: interactions with D1 agonist and antagonist drugs. *Psychopharmacology (Berl)*, 127, 25-30.
- Levin ED, McClellon FJ & Rezvani AH (2006) Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)*, 184, 523-539.
- Levin ED & Simon BB (1998) Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology (Berl)*, 138, 217-230.
- Levin ED & Torry D (1996) Acute and chronic nicotine effects on working memory in aged rats. *Psychopharmacology (Berl)*, 123, 88-97.
- Leypoldt F, Flajolet M & Methner A (2002) Neuronal differentiation of cultured human NTERA-2cl.D1 cells leads to increased expression of synapsins. *Neurosci Lett*, 324, 37-40.
- Li JJ, Dong Z, Dawson MI & Colburn NH (1996) Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. *Cancer Res*, 56, 483-489.
- Li MD, Kane JK, Matta SG, Blaner WS & Sharp BM (2000) Nicotine enhances the biosynthesis and secretion of transthyretin from the choroid plexus in rats: implications for beta-amyloid formation. *J Neurosci*, 20, 1318-1323.

- Lindvall M & Owman C (1981) Autonomic nerves in the mammalian choroid plexus and their influence on the formation of cerebrospinal fluid. *J Cereb Blood Flow Metab*, 1, 245-266.
- Link CD (1995) Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A*, 92, 9368-9372.
- Linnala A, Lehto VP & Virtanen I (1997) Neuronal differentiation in SH-SY5Y human neuroblastoma cells induces synthesis and secretion of tenascin and upregulation of alpha(v) integrin receptors. *J Neurosci Res*, 49, 53-63.
- Lipton SA (2004) Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx*, 1, 101-110.
- Lisman JE & McIntyre CC (2001) Synaptic plasticity: a molecular memory switch. *Curr Biol*, 11, R788-R791.
- Lleo A, Greenberg SM & Growdon JH (2006) Current pharmacotherapy for Alzheimer's disease. *Annu Rev Med*, 57, 513-533.
- Long Y, Zou L, Liu H, Lu H, Yuan X, Robertson CS & Yang K (2003) Altered expression of randomly selected genes in mouse hippocampus after traumatic brain injury. *J Neurosci Res*, 71, 710-720.
- Loring JF, Wen X, Lee JM, Seilhamer J & Somogyi R (2001) A gene expression profile of Alzheimer's disease. *DNA Cell Biol*, 20, 683-695.
- Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J & Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. *Nature*, 429, 883-891.
- Lucarelli E, Kaplan DR & Thiele CJ (1995) Selective regulation of TrkA and TrkB receptors by retinoic acid and interferon-gamma in human neuroblastoma cell lines. *J Biol Chem*, 270, 24725-24731.
- Luo T, Wagner E, Grun F & Drager UC (2004) Retinoic acid signaling in the brain marks formation of optic projections, maturation of the dorsal telencephalon, and function of limbic sites. *J Comp Neurol*, 470, 297-316.
- Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NP, Thakur M, McEwen BS, Hauger RL & Meaney MJ (1998) Cortisol levels during human

- aging predict hippocampal atrophy and memory deficits. *Nat Neurosci*, 1, 69-73.
- Lye TC & Shores EA (2000) Traumatic brain injury as a risk factor for Alzheimer's disease: a review. *Neuropsychol Rev*, 10, 115-129.
- Mably TA, Moore RW, Goy RW & Peterson RE (1992) In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 2. Effects on sexual behavior and the regulation of luteinizing hormone secretion in adulthood. *Toxicol Appl Pharmacol*, 114, 108-117.
- Maden M (2002) Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci*, 3, 843-853.
- Magnusson KR (1998) Aging of glutamate receptors: correlations between binding and spatial memory performance in mice. *Mech Ageing Dev*, 104, 227-248.
- Maguire EA (1997) Hippocampal involvement in human topographical memory: evidence from functional imaging. *Philos Trans R Soc Lond B Biol Sci*, 352, 1475-1480.
- Makover A, Moriwaki H, Ramakrishnan R, Saraiva MJ, Blaner WS & Goodman DS (1988) Plasma transthyretin. Tissue sites of degradation and turnover in the rat. *J Biol Chem*, 263, 8598-8603.
- Makover A, Soprano DR, Wyatt ML & Goodman DS (1989) An in situ hybridization study of the localization of retinol-binding protein and transthyretin messenger RNAs during fetal development in the rat. *Differentiation*, 40, 17-25.
- Malik MA, Blusztajn JK & Greenwood CE (2000) Nutrients as trophic factors in neurons and the central nervous system: role of retinoic acid. *J Nutr Biochem*, 11, 2-13.
- Mancuso G, Warburton DM, Melen M, Sherwood N & Tirelli E (1999) Selective effects of nicotine on attentional processes. *Psychopharmacology (Berl)*, 146, 199-204.

- Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A & Evans RM (1992) Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev*, 6, 329-344.
- Mann DM, Jones D, South PW, Snowden JS & Neary D (1992) Deposition of amyloid beta protein in non-Alzheimer dementias: evidence for a neuronal origin of parenchymal deposits of beta protein in neurodegenerative disease. *Acta Neuropathol (Berl)*, 83, 415-419.
- Marchi M & Raiteri M (1989) Interaction acetylcholine-glutamate in rat hippocampus: involvement of two subtypes of M-2 muscarinic receptors. *J Pharmacol Exp Ther*, 248, 1255-1260.
- Marcotte ER, Srivastava LK & Quirion R (2001) DNA microarrays in neuropsychopharmacology. *Trends Pharmacol Sci*, 22, 426-436.
- Marino MJ, Rouse ST, Levey AI, Potter LT & Conn PJ (1998) Activation of the genetically defined m1 muscarinic receptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells. *Proc Natl Acad Sci USA*, 95, 11465-11470.
- Martin PD & Shapiro ML (2000) Disparate effects of long-term potentiation on evoked potentials and single CA1 neurons in the hippocampus of anesthetized rats. *Hippocampus*, 10, 207-212.
- Masters CL, Multhaup G, Simms G, Pottgiesser J, Martins RN & Beyreuther K (1985) Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO J*, 4, 2757-2763.
- Matranga V, Oliva D, Sciarrino S, D'Amelio L & Giallongo A (1993) Differential expression of neuron-specific enolase mRNA in mouse neuroblastoma cells in response to differentiation inducing agents. *Cell Mol Neurobiol*, 13, 137-145.
- Matsuoka I, Kumagai M & Kurihara K (1997) Differential and coordinated regulation of expression of norepinephrine transporter in catecholaminergic cells in culture. *Brain Res*, 776, 181-188.
- Mattson MP (1995) Degenerative and protective signaling mechanisms in the neurofibrillary pathology of AD. *Neurobiol Aging*, 16, 447-457.

- Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I & Rydel RE (1992) beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci*, 12, 376-389.
- Mattson MP & Magnus T (2006) Ageing and neuronal vulnerability. *Nat Rev Neurosci*, 7, 278-294.
- May C, Kaye JA, Atack JR, Schapiro MB, Friedland RP & Rapoport SI (1990) Cerebrospinal fluid production is reduced in healthy aging. *Neurology*, 40, 500-503.
- Mazur-Kolecka B, Frackowiak J & Wisniewski HM (1995) Apolipoproteins E3 and E4 induce, and transthyretin prevents accumulation of the Alzheimer's beta-amyloid peptide in cultured vascular smooth muscle cells. *Brain Res*, 698, 217-222.
- McCaffery PJ, Adams J, Maden M & Rosa-Molinar E (2003) Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. *Eur J Neurosci*, 18, 457-472.
- McEwen BS & Alves SE (1999) Estrogen actions in the central nervous system. *Endocr Rev*, 20, 279-307.
- McGehee DS, Heath MJ, Gelber S, Devay P & Role LW (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science*, 269, 1692-1696.
- McHugh TJ, Blum KI, Tsien JZ, Tonegawa S & Wilson MA (1996) Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. *Cell*, 87, 1339-1349.
- McKenzie JE, Gentleman SM, Roberts GW, Graham DI & Royston MC (1994) Increased numbers of beta APP-immunoreactive neurones in the entorhinal cortex after head injury. *Neuroreport*, 6, 161-164.
- McKinney M, Miller JH & Aagaard PJ (1993) Pharmacological characterization of the rat hippocampal muscarinic autoreceptor. *J Pharmacol Exp Ther*, 264, 74-78.
- Melino G, Draoui M, Bellincampi L, Bernassola F, Bernardini S, Piacentini M, Reichert U & Cohen P (1997) Retinoic acid receptors alpha and gamma

- mediate the induction of "tissue" transglutaminase activity and apoptosis in human neuroblastoma cells. *Exp Cell Res*, 235, 55-61.
- Melino G, Draoui M, Bernardini S, Bellincampi L, Reichert U & Cohen P (1996) Regulation by retinoic acid of insulin-degrading enzyme and of a related endoprotease in human neuroblastoma cell lines. *Cell Growth Differ*, 7, 787-796.
- Merched A, Blain H, Visvikis S, Herbeth B, Jeandel C & Siest G (1997) Cerebrospinal fluid apolipoprotein E level is increased in late-onset Alzheimer's disease. *J Neurol Sci*, 145, 33-39.
- Merched A, Serot JM, Visvikis S, Aguillon D, Faure G & Siest G (1998) Apolipoprotein E, transthyretin and actin in the CSF of Alzheimer's patients: relation with the senile plaques and cytoskeleton biochemistry. *FEBS Lett*, 425, 225-228.
- Merrick J, Merrick E, Lunskey Y & Kandel I (2006) A review of suicidality in persons with intellectual disability. *Isr J Psychiatry Relat Sci*, 43, 258-264.
- Mic FA, Molotkov A, Benbrook DM & Duester G (2003) Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc Natl Acad Sci U S A*, 100, 7135-7140.
- Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Prog Neurobiol*, 54, 369-415.
- Michalek JE, Akhtar FZ, Arezzo JC, Garabrant DH & Albers JW (2001) Serum dioxin and peripheral neuropathy in veterans of Operation Ranch Hand. *Neurotoxicology*, 22, 479-490.
- Mielke MM, Zandi PP, Sjogren M, Gustafson D, Ostling S, Steen B & Skoog I (2005) High total cholesterol levels in late life associated with a reduced risk of dementia. *Neurology*, 64, 1689-1695.
- Millan MJ (1990) Kappa-opioid receptors and analgesia. *Trends Pharmacol Sci*, 11, 70-76.
- Minucci S, Leid M, Toyama R, Saint-Jeannet JP, Peterson VJ, Horn V, Ishmael JE, Bhattacharyya N, Dey A, Dawid IB & Ozato K (1997) Retinoid X receptor

- (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. *Mol Cell Biol*, 17, 644-655.
- Miranda P, Williams CL & Einstein G (1999) Granule cells in aging rats are sexually dimorphic in their response to estradiol. *J Neurosci*, 19, 3316-3325.
- Mishima K, Iwasaki K, Tsukikawa H, Matsumoto Y, Egashira N, Abe K, Egawa T & Fujiwara M (2000) The scopolamine-induced impairment of spatial cognition parallels the acetylcholine release in the ventral hippocampus in rats. *Jpn J Pharmacol*, 84, 163-173.
- Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF & Evans RM (2001) Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci USA*, 98, 11714-11719.
- Mizukami K, Ikonomic MD, Grayson DR, Sheffield R & Armstrong DM (1998) Immunohistochemical study of GABAA receptor alpha1 subunit in the hippocampal formation of aged brains with Alzheimer-related neuropathologic changes. *Brain Res*, 799, 148-155.
- Molotkov A, Ghyselinck NB, Chambon P & Duester G (2004) Opposing actions of cellular retinol-binding protein and alcohol dehydrogenase control the balance between retinol storage and degradation. *Biochem J*, 383, 295-302.
- Monaco HL (2000) The transthyretin-retinol-binding protein complex. *Biochim Biophys Acta*, 1482, 65-72.
- Mondadori C, Jaekel J & Preiswerk G (1993) CGP 36742: the first orally active GABAB blocker improves the cognitive performance of mice, rats, and rhesus monkeys. *Behav Neural Biol*, 60, 62-68.
- Mondadori C, Mobius HJ & Borkowski J (1996) The GABAB receptor antagonist CGP 36,742 and the nootropic oxiracetam facilitate the formation of long-term memory. *Behav Brain Res*, 77, 223-225.
- Morishima-Kawashima M, Oshima N, Ogata H, Yamaguchi H, Yoshimura M, Sugihara S & Ihara Y (2000) Effect of apolipoprotein E allele epsilon4 on the initial phase of amyloid beta-protein accumulation in the human brain. *Am J Pathol*, 157, 2093-2099.

- Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods*, 11, 47-60.
- Morris RG, Davis S & Butcher SP (1990) Hippocampal synaptic plasticity and NMDA receptors: a role in information storage? *Philos Trans R Soc Lond B Biol Sci*, 329, 187-204.
- Moser E, Moser MB & Andersen P (1993) Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *J Neurosci*, 13, 3916-3925.
- Moser MB, Moser EI, Forrest E, Andersen P & Morris RG (1995) Spatial learning with a minislab in the dorsal hippocampus. *Proc Natl Acad Sci U S A*, 92, 9697-9701.
- Moser MB, Trommald M & Andersen P (1994) An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses. *Proc Natl Acad Sci U S A*, 91, 12673-12675.
- Mouravlev A, Dunning J, Young D & During MJ (2006) Somatic gene transfer of cAMP response element-binding protein attenuates memory impairment in aging rats. *Proc Natl Acad Sci U S A*, 103, 4705-4710.
- Mufson EJ, Counts SE & Ginsberg SD (2002) Gene expression profiles of cholinergic nucleus basalis neurons in Alzheimer's disease. *Neurochem Res*, 27, 1035-1048.
- Mulnard RA, Cotman CW, Kawas C, van Dyck CH, Sano M, Doody R, Koss E, Pfeiffer E, Jin S, Gamst A, Grundman M, Thomas R & Thal LJ (2000) Estrogen replacement therapy for treatment of mild to moderate Alzheimer disease: a randomized controlled trial. Alzheimer's Disease Cooperative Study. *JAMA*, 283, 1007-1015.
- Murphy DD, Cole NB, Greenberger V & Segal M (1998) Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. *J Neurosci*, 18, 2550-2559.

- Murrell JR, Schonert RG, Liepnieks JJ, Rosen HN, Moses AC & Benson MD (1992) Production and functional analysis of normal and variant recombinant human transthyretin proteins. *J Biol Chem*, 267, 16595-16600.
- Myhrer T (2003) Neurotransmitter systems involved in learning and memory in the rat: a meta-analysis based on studies of four behavioral tasks. *Brain Res Brain Res Rev*, 41, 268-287.
- Myllymaki SA, Haavisto TE, Brokken LJ, Viluksela M, Toppari J & Paranko J (2005) In utero and lactational exposure to TCDD; steroidogenic outcomes differ in male and female rat pups. *Toxicol Sci*, 88, 534-544.
- Nagasaka T, Shiozawa Z, Kobayashi M, Shindo K, Tsunoda S & Amino A (1996) Autopsy findings in Down's syndrome with cerebrovascular disorder. *Clin Neuropathol*, 15, 145-149.
- Nakagawa Y & Takashima T (1997) The GABA(B) receptor antagonist CGP36742 attenuates the baclofen- and scopolamine-induced deficit in Morris water maze task in rats. *Brain Res*, 766, 101-106.
- Nakajima M, Inui A, Miura M, Hirose Y, Okita M, Himori N, Baba S & Kasuga M (1994) Negative and positive effects of intracerebroventricular scopolamine on memory in mice undergoing passive avoidance and escape tests. *Brain Res Bull*, 34, 375-380.
- Newberry NR & Nicoll RA (1984) Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells. *Nature*, 308, 450-452.
- Newhouse PA, Potter A & Levin ED (1997) Nicotinic system involvement in Alzheimer's and Parkinson's diseases. Implications for therapeutics. *Drugs Aging*, 11, 206-228.
- Niewiadomska G, Baksalerska-Pazera M, Gasiorowska A & Mietelska A (2006) Nerve growth factor differentially affects spatial and recognition memory in aged rats. *Neurochem Res*, 31, 1481-1490.
- Nilsson CB, Hoegberg P, Trossvik C, Azais-Braesco V, Blaner WS, Fex G, Harrison EH, Nau H, Schmidt CK, van Bennekum AM & Hakansson H (2000) 2,3,7,8-tetrachlorodibenzo-p-dioxin increases serum and kidney retinoic acid

- levels and kidney retinol esterification in the rat. *Toxicol Appl Pharmacol*, 169, 121-131.
- Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrangé P, Canet E & Boutin JA (2000) Identification of the melatonin-binding site MT3 as the quinone reductase 2. *J Biol Chem*, 275, 31311-31317.
- Nott A & Levin ED (2006) Dorsal hippocampal $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptors and memory. *Brain Res*, 1081, 72-78.
- O'Donnell J, Stemmelin J, Nitta A, Brouillette J & Quirion R (2003) Gene expression profiling following chronic NMDA receptor blockade-induced learning deficits in rats. *Synapse*, 50, 171-180.
- O'Keefe J (1991) An allocentric spatial model for the hippocampal cognitive map. *Hippocampus*, 1, 230-235.
- Obisesan TO, Hirsch R, Kosoko O, Carlson L & Parrott M (1998) Moderate wine consumption is associated with decreased odds of developing age-related macular degeneration in NHANES-1. *J Am Geriatr Soc*, 46, 1-7.
- Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H & Yamada M (2004) Vitamin A exhibits potent anti-amyloidogenic and fibril-destabilizing effects in vitro. *Exp Neurol*, 189, 380-392.
- Orgogozo JM, Dartigues JF, Lafont S, Letenneur L, Commenges D, Salamon R, Renaud S & Breteler MB (1997) Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Rev Neurol (Paris)*, 153, 185-192.
- Packard MG & Teather LA (1997) Posttraining estradiol injections enhance memory in ovariectomized rats: cholinergic blockade and synergism. *Neurobiol Learn Mem*, 68, 172-188.
- Pak JH, Huang FL, Li J, Balschun D, Reymann KG, Chiang C, Westphal H & Huang KP (2000) Involvement of neurogranin in the modulation of calcium/calmodulin-dependent protein kinase II, synaptic plasticity, and spatial learning: a study with knockout mice. *Proc Natl Acad Sci U S A*, 97, 11232-11237.

- Palha JA, Moreira P, Wisniewski T, Frangione B & Saraiva MJ (1996) Transthyretin gene in Alzheimer's disease patients. *Neurosci Lett*, 204, 212-214.
- Palop JJ, Jones B, Kekonius L, Chin J, Yu GQ, Raber J, Masliah E & Mucke L (2003) Neuronal depletion of calcium-dependent proteins in the dentate gyrus is tightly linked to Alzheimer's disease-related cognitive deficits. *Proc Natl Acad Sci U S A*, 100, 9572-9577.
- Panza F, Capurso C, D'Introno A, Colacicco AM, De Candia D, Capurso A & Solfrizzi V (2007) Total cholesterol levels and the risk of mild cognitive impairment and Alzheimer's disease. *J Am Geriatr Soc*, 55, 133-135.
- Parent A, Rowe W, Meaney MJ & Quirion R (1995) Increased production of inositol phosphates and diacylglycerol in aged cognitively impaired rats after stimulation of muscarinic, metabotropic-glutamate and endothelin receptors. *J Pharmacol Exp Ther*, 272, 1110-1116.
- Pasinetti GM (2001) Use of cDNA microarray in the search for molecular markers involved in the onset of Alzheimer's disease dementia. *J Neurosci Res*, 65, 471-476.
- Paterno GD, Gillespie LL, Julien JP & Skup D (1997) Regulation of neurofilament L, M and H gene expression during retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells. *Brain Res Mol Brain Res*, 49, 247-254.
- Pedersen WA, Berse B, Schuler U, Wainer BH & Blusztajn JK (1995) All-trans- and 9-cis-retinoic acid enhance the cholinergic properties of a murine septal cell line: evidence that the effects are mediated by activation of retinoic acid receptor-alpha. *J Neurochem*, 65, 50-58.
- Penfield WW & Perot PP (1963) The brain's record of auditory and visual experience. *Brain*, 86, 595-696.
- Perlmann T & Jansson L (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev*, 9, 769-782.

- Perry EK, Tomlinson BE, Blessed G, Bergmann K, Gibson PH & Perry RH (1978) Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br Med J*, 2, 1457-1459.
- Perry RH, Candy JM, Perry EK, Irving D, Blessed G, Fairbairn AF & Tomlinson BE (1982) Extensive loss of choline acetyltransferase activity is not reflected by neuronal loss in the nucleus of Meynert in Alzheimer's disease. *Neurosci Lett*, 33, 311-315.
- Perry RJ & Hodges JR (1999) Attention and executive deficits in Alzheimer's disease. A critical review. *Brain*, 122 (Pt 3), 383-404.
- Personett D, Fass U, Panickar K & McKinney M (2000) Retinoic acid-mediated enhancement of the cholinergic/neuronal nitric oxide synthase phenotype of the medial septal SN56 clone: establishment of a nitric oxide-sensitive proapoptotic state. *J Neurochem*, 74, 2412-2424.
- Petroni A, Blasevich M, La Spada P, Papini N & Galli C (1996) Arachidonic acid synthesis and lipid metabolism in retinoic acid-differentiated neuroblastoma cells. *J Lipid Mediat Cell Signal*, 14, 39-44.
- Petrovitch H, White LR, Izmirilian G, Ross GW, Havlik RJ, Markesbery W, Nelson J, Davis DG, Hardman J, Foley DJ & Launer LJ (2000) Midlife blood pressure and neuritic plaques, neurofibrillary tangles, and brain weight at death: the HAAS. Honolulu-Asia aging Study. *Neurobiol Aging*, 21, 57-62.
- Pfahl M (1993) Nuclear receptor/AP-1 interaction. *Endocr Rev*, 14, 651-658.
- Picciotto MR, Zoli M, Lena C, Bessis A, Lallemand Y, Le Novere N, Vincent P, Pich EM, Brulet P & Changeux JP (1995) Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. *Nature*, 374, 65-67.
- Pitler TA & Alger BE (1992) Cholinergic excitation of GABAergic interneurons in the rat hippocampal slice. *J Physiol*, 450, 127-142.
- Pohjanvirta R, Vartiainen T, Uusi-Rauva A, Monkkinen J & Tuomisto J (1990) Tissue distribution, metabolism, and excretion of ¹⁴C-TCDD in a TCDD-susceptible and a TCDD-resistant rat strain. *Pharmacol Toxicol*, 66, 93-100.

- Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P & Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet*, 342, 697-699.
- Powers BE, Lin TM, Vanka A, Peterson RE, Juraska JM & Schantz SL (2005) Tetrachlorodibenzo-p-dioxin exposure alters radial arm maze performance and hippocampal morphology in female AhR mice. *Genes Brain Behav*, 4, 51-59.
- Praprotnik D, Smith MA, Richey PL, Vinters HV & Perry G (1996) Filament heterogeneity within the dystrophic neurites of senile plaques suggests blockage of fast axonal transport in Alzheimer's disease. *Acta Neuropathol (Berl)*, 91, 226-235.
- Prasher VP (1999) Down syndrome and thyroid disorders: a review. *Downs Syndr Res Pract*, 6, 25-42.
- Preston JE (2001) Ageing choroid plexus-cerebrospinal fluid system. *Microsc Res Tech*, 52, 31-37.
- Prinzen C, Muller U, Endres K, Fahrenholz F & Postina R (2005) Genomic structure and functional characterization of the human ADAM10 promoter. *FASEB J*, 19, 1522-1524.
- Puskas LG, Kitajka K, Nyakas C, Barcelo-Coblijn G & Farkas T (2003) Short-term administration of omega 3 fatty acids from fish oil results in increased transthyretin transcription in old rat hippocampus. *Proc Natl Acad Sci U S A*, 100, 1580-1585.
- Quadro L, Blaner WS, Salchow DJ, Vogel S, Piantedosi R, Gouras P, Freeman S, Cosma MP, Colantuoni V & Gottesman ME (1999) Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. *EMBO J*, 18, 4633-4644.
- Quevedo J, Vianna MR, Roesler R, de Paris F, Izquierdo I & Rose SP (1999) Two time windows of anisomycin-induced amnesia for inhibitory avoidance training in rats: protection from amnesia by pretraining but not pre-exposure to the task apparatus. *Learn Mem*, 6, 600-607.
- Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, Parent A, White N & Meaney MJ (1995) Facilitation of acetylcholine release and cognitive

- performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. *J Neurosci*, 15, 1455-1462.
- Rahman AS, Kimura M, Yokoi K, Naher TE & Itokawa Y (1996) Neurological disorder and excessive accumulation of calcium in brain of clinically vitamin A-deficient rats. *Biol Trace Elem Res*, 53, 57-64.
- Ramos BP, Birnbaum SG, Lindenmayer I, Newton SS, Duman RS & Arnsten AF (2003) Dysregulation of protein kinase a signaling in the aged prefrontal cortex: new strategy for treating age-related cognitive decline. *Neuron*, 40, 835-845.
- Rapp MA, Schnaider-Beeri M, Grossman HT, Sano M, Perl DP, Purohit DP, Gorman JM & Haroutunian V (2006) Increased hippocampal plaques and tangles in patients with Alzheimer disease with a lifetime history of major depression. *Arch Gen Psychiatry*, 63, 161-167.
- Rapp PR, Rosenberg RA & Gallagher M (1987) An evaluation of spatial information processing in aged rats. *Behav Neurosci*, 101, 3-12.
- Ray CA, Blin J, Chase TN & Piercey MF (1992a) Effects of cholinergic agonists on regional brain energy metabolism in the scopolamine-treated rat. *Neuropharmacology*, 31, 1193-1199.
- Ray PG, Meador KJ, Loring DW, Zamrini EW, Yang XH & Buccafusco JJ (1992b) Central anticholinergic hypersensitivity in aging. *J Geriatr Psychiatry Neurol*, 5, 72-77.
- Rebeck GW, Reiter JS, Strickland DK & Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron*, 11, 575-580.
- Redzic ZB, Preston JE, Duncan JA, Chodobski A & Szmydynger-Chodobska J (2005) The choroid plexus-cerebrospinal fluid system: from development to aging. *Curr Top Dev Biol*, 71, 1-52.
- Reynolds JN, Prasad A, Gillespie LL & Paterno GD (1996) Developmental expression of functional GABAA receptors containing the gamma 2 subunit in neurons derived from embryonal carcinoma (P19) cells. *Brain Res Mol Brain Res*, 35, 11-18.

- Rezvani AH & Levin ED (2003) Nicotinic-glutamatergic interactions and attentional performance on an operant visual signal detection task in female rats. *Eur J Pharmacol*, 465, 83-90.
- Rho S, Kang M, Choi B, Sim D, Lee J, Lee E, Cho C, Oh JW, Park S, Ko S, Shin M, Hong M & Bae H (2005) Effects of Yukmijihwang-tang derivatives (YMJd), a memory enhancing herbal extract, on the gene-expression profile in the rat hippocampus. *Biol Pharm Bull*, 28, 87-93.
- Ribak CE, Seress L, Peterson GM, Seroogy KB, Fallon JH & Schmued LC (1986) A GABAergic inhibitory component within the hippocampal commissural pathway. *J Neurosci*, 6, 3492-3498.
- Riccio A, Ahn S, Davenport CM, Blendy JA & Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science*, 286, 2358-2361.
- Richard F & Amouyel P (2001) Genetic susceptibility factors for Alzheimer's disease. *Eur J Pharmacol*, 412, 1-12.
- Richard S & Zingg HH (1991) Identification of a retinoic acid response element in the human oxytocin promoter. *J Biol Chem*, 266, 21428-21433.
- Richards SS, Emsley CL, Roberts J, Murray MD, Hall K, Gao S & Hendrie HC (2000) The association between vascular risk factor-mediating medications and cognition and dementia diagnosis in a community-based sample of African-Americans. *J Am Geriatr Soc*, 48, 1035-1041.
- Richter-Levin G, Canevari L & Bliss TV (1995) Long-term potentiation and glutamate release in the dentate gyrus: links to spatial learning. *Behav Brain Res*, 66, 37-40.
- Riedel G, Micheau J, Lam AG, Roloff EL, Martin SJ, Bridge H, de Hoz L, Poeschel B, McCulloch J & Morris RG (1999) Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat Neurosci*, 2, 898-905.
- Riisøen H (1988) Reduced prealbumin (transthyretin) in CSF of severely demented patients with Alzheimer's disease. *Acta Neurol Scand*, 78, 455-459.

- Rinaldi P, Polidori MC, Metastasio A, Mariani E, Mattioli P, Cherubini A, Catani M, Cecchetti R, Senin U & Mecocci P (2003) Plasma antioxidants are similarly depleted in mild cognitive impairment and in Alzheimer's disease. *Neurobiol Aging*, 24, 915-919.
- Rissanen A, Puolivali J, van Groen T & Riekkinen P, Jr. (1999) In mice tonic estrogen replacement therapy improves non-spatial and spatial memory in a water maze task. *Neuroreport*, 10, 1369-1372.
- Roberts GW, Gentleman SM, Lynch A & Graham DI (1991) beta A4 amyloid protein deposition in brain after head trauma. *Lancet*, 338, 1422-1423.
- Roberts GW, Gentleman SM, Lynch A, Murray L, Landon M & Graham DI (1994) Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J Neurol Neurosurg Psychiatry*, 57, 419-425.
- Rogan WJ, Gladen BC, Hung KL, Koong SL, Shih LY, Taylor JS, Wu YC, Yang D, Ragan NB & Hsu CC (1988) Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. *Science*, 241, 334-336.
- Romkes M, Piskorska-Pliszczynska J & Safe S (1987) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol*, 87, 306-314.
- Rotenberg A, Abel T, Hawkins RD, Kandel ER & Muller RU (2000) Parallel instabilities of long-term potentiation, place cells, and learning caused by decreased protein kinase A activity. *J Neurosci*, 20, 8096-8102.
- Rotenberg A, Mayford M, Hawkins RD, Kandel ER & Muller RU (1996) Mice expressing activated CaMKII lack low frequency LTP and do not form stable place cells in the CA1 region of the hippocampus. *Cell*, 87, 1351-1361.
- Rottkamp CA, Raina AK, Zhu X, Gaier E, Bush AI, Atwood CS, Chevion M, Perry G & Smith MA (2001) Redox-active iron mediates amyloid-beta toxicity. *Free Radic Biol Med*, 30, 447-450.
- Rouse ST & Levey AI (1997) Muscarinic acetylcholine receptor immunoreactivity after hippocampal commissural/associational pathway

- lesions: evidence for multiple presynaptic receptor subtypes. *J Comp Neurol*, 380, 382-394.
- Rowe WB, Blalock EM, Chen KC, Kadish I, Wang D, Barrett JE, Thibault O, Porter NM, Rose GM & Landfield PW (2007) Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelinogenic pathways with cognitive impairment in aged rats. *J Neurosci*, 27, 3098-3110.
- Rowe WB, O'Donnell JP, Pearson D, Rose GM, Meaney MJ & Quirion R (2003) Long-term effects of BIBN-99, a selective muscarinic M2 receptor antagonist, on improving spatial memory performance in aged cognitively impaired rats. *Behav Brain Res*, 145, 171-178.
- Rowe WB, Spreckmeester E, Meaney MJ, Quirion R & Rochford J (1998) Reactivity to novelty in cognitively-impaired and cognitively-unimpaired aged rats and young rats. *Neuroscience*, 83, 669-680.
- Rozman K (1989) A critical view of the mechanism(s) of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Implications for human safety assessment. *Derm Beruf Umwelt*, 37, 81-92.
- Rudick CN & Woolley CS (2001) Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. *J Neurosci*, 21, 6532-6543.
- Ruetschi U, Zetterberg H, Podust VN, Gottfries J, Li S, Hviid SA, McGuire J, Karlsson M, Rymo L, Davies H, Minthon L & Blennow K (2005) Identification of CSF biomarkers for frontotemporal dementia using SELDI-TOF. *Exp Neurol*, 196, 273-281.
- Safe S (1990) Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol*, 21, 51-88.
- Safe S, Wormke M & Samudio I (2000) Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. *J Mammary Gland Biol Neoplasia*, 5, 295-306.

- Sainsbury A, Lin S, McNamara K, Slack K, Enriquez R, Lee NJ, Boey D, Smythe GA, Schwarzer C, Baldock P, Karl T, Lin EJ, Couzens M & Herzog H (2007) Dynorphin knockout reduces fat mass and increases weight loss during fasting in mice. *Mol Endocrinol*, 21, 1722-1735.
- Sakimura K, Kushiya E, Ogura A, Kudo Y, Katagiri T & Takahashi Y (1995) Upstream and intron regulatory regions for expression of the rat neuron-specific enolase gene. *Brain Res Mol Brain Res*, 28, 19-28.
- Sala C, Futai K, Yamamoto K, Worley PF, Hayashi Y & Sheng M (2003) Inhibition of dendritic spine morphogenesis and synaptic transmission by activity-inducible protein Homer1a. *J Neurosci*, 23, 6327-6337.
- Salvatore AM, Cozzolino M, Gargano N, Galanti S, Levi A & Alema S (1995) Neuronal differentiation of P19 embryonal cells exhibits cell-specific regulation of neurotrophin receptors. *Neuroreport*, 6, 873-877.
- Samad TA, Krezel W, Chambon P & Borrelli E (1997) Regulation of dopaminergic pathways by retinoids: activation of the D2 receptor promoter by members of the retinoic acid receptor-retinoid X receptor family. *Proc Natl Acad Sci U S A*, 94, 14349-14354.
- Samuel W, Terry RD, DeTeresa R, Butters N & Masliah E (1994) Clinical correlates of cortical and nucleus basalis pathology in Alzheimer dementia. *Arch Neurol*, 51, 772-778.
- Sandstrom NJ & Williams CL (2001) Memory retention is modulated by acute estradiol and progesterone replacement. *Behav Neurosci*, 115, 384-393.
- Sarter M & Bruno JP (1997) Trans-synaptic stimulation of cortical acetylcholine and enhancement of attentional functions: a rational approach for the development of cognition enhancers. *Behav Brain Res*, 83, 7-14.
- Sato M, Suzuki K & Nakanishi S (2001) NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci*, 21, 3797-3805.

- Savu L, Vranckx R, Rouaze-Romet M, Maya M, Nunez EA, Treton J & Flink IL (1991) A senescence up-regulated protein: the rat thyroxine-binding globulin (TBG). *Biochim Biophys Acta*, 1097, 19-22.
- Scacchi R, Ruggeri M, Gambina G, Martini MC & Corbo RM (2002) Alpha2-macroglobulin deletion polymorphism and plasma levels in late onset Alzheimer's disease. *Clin Chem Lab Med*, 40, 333-336.
- Scali C, Vannucchi MG, Pepeu G & Casamenti F (1995) Peripherally injected scopolamine differentially modulates acetylcholine release in vivo in the young and aged rats. *Neurosci Lett*, 197, 171-174.
- Schantz SL & Bowman RE (1989) Learning in monkeys exposed perinatally to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Neurotoxicol Teratol*, 11, 13-19.
- Scheibe RJ & Wagner JA (1992) Retinoic acid regulates both expression of the nerve growth factor receptor and sensitivity to nerve growth factor. *J Biol Chem*, 267, 17611-17616.
- Schmidt JV & Bradfield CA (1996) Ah receptor signaling pathways. *Annu Rev Cell Dev Biol*, 12, 55-89.
- Schmidt R, Launer LJ, Nilsson LG, Pajak A, Sans S, Berger K, Breteler MM, de Ridder M, Dufouil C, Fuhrer R, Giampaoli S & Hofman A (2004) Magnetic resonance imaging of the brain in diabetes: the Cardiovascular Determinants of Dementia (CASCADE) Study. *Diabetes*, 53, 687-692.
- Schon K, Atri A, Hasselmo ME, Tricarico MD, LoPresti ML & Stern CE (2005) Scopolamine reduces persistent activity related to long-term encoding in the parahippocampal gyrus during delayed matching in humans. *J Neurosci*, 25, 9112-9123.
- Schule R, Rangarajan P, Yang N, Kliewer S, Ransone LJ, Bolado J, Verma IM & Evans RM (1991) Retinoic acid is a negative regulator of AP-1-responsive genes. *Proc Natl Acad Sci U S A*, 88, 6092-6096.
- Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK & . (1994) Transthyretin sequesters amyloid beta protein and prevents amyloid formation. *Proc Natl Acad Sci U S A*, 91, 8368-8372.

- Scoville WB & Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry*, 20, 11-21.
- Seeger T, Fedorova I, Zheng F, Miyakawa T, Koustova E, Gomeza J, Basile AS, Alzheimer C & Wess J (2004) M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. *J Neurosci*, 24, 10117-10127.
- Seguela P, Wadiche J, Dineley-Miller K, Dani JA & Patrick JW (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J Neurosci*, 13, 596-604.
- Selkoe DJ (2000) Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann N Y Acad Sci*, 924, 17-25.
- Selkoe DJ (2004) Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. *Nat Cell Biol*, 6, 1054-1061.
- Seo BW, Sparks AJ, Medora K, Amin S & Schantz SL (1999) Learning and memory in rats gestationally and lactationally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Neurotoxicol Teratol*, 21, 231-239.
- Serot JM, Bene MC & Faure GC (2003) Choroid plexus, aging of the brain, and Alzheimer's disease. *Front Biosci*, 8, s515-s521.
- Serot JM, Christmann D, Dubost T & Couturier M (1997) Cerebrospinal fluid transthyretin: aging and late onset Alzheimer's disease. *J Neurol Neurosurg Psychiatry*, 63, 506-508.
- Sgambato V, Pages C, Rogard M, Besson MJ & Caboche J (1998) Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. *J Neurosci*, 18, 8814-8825.
- Sherman SJ, Atri A, Hasselmo ME, Stern CE & Howard MW (2003) Scopolamine impairs human recognition memory: data and modeling. *Behav Neurosci*, 117, 526-539.
- Sherrington R, Froelich S, Sorbi S, Campion D, Chi H, Rogaeva EA, Levesque G, Rogaev EI, Lin C, Liang Y, Ikeda M, Mar L, Brice A, Agid Y, Percy ME, Clerget-Darpoux F, Piacentini S, Marcon G, Nacmias B, Amaducci L, Frebourg

- T, Lannfelt L, Rommens JM & George-Hyslop PH (1996) Alzheimer's disease associated with mutations in presenilin 2 is rare and variably penetrant. *Hum Mol Genet*, 5, 985-988.
- Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K & . (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, 375, 754-760.
- Sherwin BB (1994) Estrogenic effects on memory in women. *Ann N Y Acad Sci*, 743, 213-230.
- Shughrue PJ, Lane MV & Merchenthaler I (1997) Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol*, 388, 507-525.
- Shughrue PJ & Merchenthaler I (2000) Estrogen is more than just a "sex hormone": novel sites for estrogen action in the hippocampus and cerebral cortex. *Front Neuroendocrinol*, 21, 95-101.
- Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN, III, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S & Wactawski-Wende J (2003) Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA*, 289, 2651-2662.
- Silhol M, Arancibia S, Maurice T & Tapia-Arancibia L (2007) Spatial memory training modifies the expression of brain-derived neurotrophic factor tyrosine kinase receptors in young and aged rats. *Neuroscience*.
- Silva AJ (2003) Molecular and cellular cognitive studies of the role of synaptic plasticity in memory. *J Neurobiol*, 54, 224-237.
- Simard AR, Soulet D, Gowing G, Julien JP & Rivest S (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron*, 49, 489-502.
- Sirvio J, Riekkinen P, Jr., Valjakka A, Jolkkonen J & Riekkinen PJ (1991) The effects of noradrenergic neurotoxin, DSP-4, on the performance of young and aged rats in spatial navigation task. *Brain Res*, 563, 297-302.

- Sjogren M, Mielke M, Gustafson D, Zandi P & Skoog I (2006) Cholesterol and Alzheimer's disease--is there a relation? *Mech Ageing Dev*, 127, 138-147.
- Skoog I, Andreasson LA, Landahl S & Lernfelt B (1998) A population-based study on blood pressure and brain atrophy in 85-year-olds. *Hypertension*, 32, 404-409.
- Skoog I & Gustafson D (2002) Hypertension and related factors in the etiology of Alzheimer's disease. *Ann N Y Acad Sci*, 977, 29-36.
- Skoog I & Gustafson D (2003) Hypertension, hypertension-clustering factors and Alzheimer's disease. *Neurol Res*, 25, 675-680.
- Skoog I & Gustafson D (2006) Update on hypertension and Alzheimer's disease. *Neurol Res*, 28, 605-611.
- Skrebitsky VG & Chepkova AN (1998) Hebbian synapses in cortical and hippocampal pathways. *Rev Neurosci*, 9, 243-264.
- Smith FR, Raz A & Goodman DS (1970) Radioimmunoassay of human plasma retinol-binding protein. *J Clin Invest*, 49, 1754-1761.
- Smith JE, Milch PO, Muto Y & Goodman DS (1973) The plasma transport and metabolism of retinoic acid in the rat. *Biochem J*, 132, 821-827.
- Smith MA, Harris PL, Sayre LM & Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci USA*, 94, 9866-9868.
- Smith SS, Pino RM & Thouron CL (1989) Binding and transport of transthyretin-gold by the endothelium of the rat choriocapillaris. *J Histochem Cytochem*, 37, 1497-1502.
- Smith-Maxwell CJ, Eatock RA & Begenisich T (1991) Induction of K-channel expression in a neuroblastoma cell line. *J Neurobiol*, 22, 327-341.
- Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK & Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci*, 8, 1051-1058.
- Sodja C, Fang H, Dasgupta T, Ribocco M, Walker PR & Sikorska M (2002) Identification of functional dopamine receptors in human teratocarcinoma NT2 cells. *Brain Res Mol Brain Res*, 99, 83-91.

- Soprano DR, Qin P & Soprano KJ (2004) Retinoic acid receptors and cancers. *Annu Rev Nutr*, 24, 201-221.
- Sousa JC, Cardoso I, Marques F, Saraiva MJ & Palha JA (2007) Transthyretin and Alzheimer's disease: where in the brain? *Neurobiol Aging*, 28, 713-718.
- Sparks DL, Scheff SW, Liu H, Landers TM, Coyne CM & Hunsaker JC, III (1995) Increased incidence of neurofibrillary tangles (NFT) in non-demented individuals with hypertension. *J Neurol Sci*, 131, 162-169.
- Stein TD, Anders NJ, DeCarli C, Chan SL, Mattson MP & Johnson JA (2004) Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. *J Neurosci*, 24, 7707-7717.
- Stein TD & Johnson JA (2002) Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J Neurosci*, 22, 7380-7388.
- Stemmelin J, Cassel JC, Will B & Kelche C (1999) Sensitivity to cholinergic drug treatments of aged rats with variable degrees of spatial memory impairment. *Behav Brain Res*, 98, 53-66.
- Stemmelin J, Lazarus C, Cassel S, Kelche C & Cassel JC (2000) Immunohistochemical and neurochemical correlates of learning deficits in aged rats. *Neuroscience*, 96, 275-289.
- Stillman MJ, Shukitt-Hale B, Galli RL, Levy A & Lieberman HR (1996) Effects of M2 antagonists on in vivo hippocampal acetylcholine levels. *Brain Res Bull*, 41, 221-226.
- Stolerman IP, Mirza NR, Hahn B & Shoaib M (2000) Nicotine in an animal model of attention. *Eur J Pharmacol*, 393, 147-154.
- Stork O, Stork S, Pape HC & Obata K (2001) Identification of genes expressed in the amygdala during the formation of fear memory. *Learn Mem*, 8, 209-219.
- Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS & Roses AD (1993) Apolipoprotein E: high-avidity binding to

- beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A*, 90, 1977-1981.
- Sugaya K, Greene R, Personett D, Robbins M, Kent C, Bryan D, Skiba E, Gallagher M & McKinney M (1998) Septo-hippocampal cholinergic and neurotrophin markers in age-induced cognitive decline. *Neurobiol Aging*, 19, 351-361.
- Sullivan GM, Hatterer JA, Herbert J, Chen X, Roose SP, Attia E, Mann JJ, Marangell LB, Goetz RR & Gorman JM (1999) Low levels of transthyretin in the CSF of depressed patients. *Am J Psychiatry*, 156, 710-715.
- Sundelin J, Melhus H, Das S, Eriksson U, Lind P, Tragardh L, Peterson PA & Rask L (1985) The primary structure of rabbit and rat prealbumin and a comparison with the tertiary structure of human prealbumin. *J Biol Chem*, 260, 6481-6487.
- Sutherland MK, Wong L, Somerville MJ, Handley P, Yoong L, Bergeron C & McLachlan DR (1992) Reduction of thyroid hormone receptor c-ERB A alpha mRNA levels in the hippocampus of Alzheimer as compared to Huntington brain. *Neurobiol Aging*, 13, 301-312.
- Sweet RA, Hamilton RL, Butters MA, Mulsant BH, Pollock BG, Lewis DA, Lopez OL, Dekosky ST & Reynolds CF, III (2004) Neuropathologic correlates of late-onset major depression. *Neuropsychopharmacology*, 29, 2242-2250.
- Szczygielski J, Mautes A, Steudel WI, Falkai P, Bayer TA & Wirths O (2005) Traumatic brain injury: cause or risk of Alzheimer's disease? A review of experimental studies. *J Neural Transm*, 112, 1547-1564.
- Szekely CA, Thorne JE, Zandi PP, Ek M, Messias E, Breitner JC & Goodman SN (2004) Nonsteroidal anti-inflammatory drugs for the prevention of Alzheimer's disease: a systematic review. *Neuroepidemiology*, 23, 159-169.
- Szerb JC, Hadhazy P & Dudar JD (1977) Release of [3H]acetylcholine from rat hippocampal slices: effect of septal lesion and of graded concentrations of muscarinic agonists and antagonists. *Brain Res*, 128, 285-291.

- Tanapat P, Hastings NB, Reeves AJ & Gould E (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci*, 19, 5792-5801.
- Tang Y, Mishkin M & Aigner TG (1997) Effects of muscarinic blockade in perirhinal cortex during visual recognition. *Proc Natl Acad Sci U S A*, 94, 12667-12669.
- Tang YP, Haslam SZ, Conrad SE & Sisk CL (2004) Estrogen increases brain expression of the mRNA encoding transthyretin, an amyloid beta scavenger protein. *J Alzheimers Dis*, 6, 413-420.
- Tanna NK, Kohn MI, Horwich DN, Jolles PR, Zimmerman RA, Alves WM & Alavi A (1991) Analysis of brain and cerebrospinal fluid volumes with MR imaging: impact on PET data correction for atrophy. Part II. Aging and Alzheimer dementia. *Radiology*, 178, 123-130.
- Tanzi RE (2005) The synaptic Abeta hypothesis of Alzheimer disease. *Nat Neurosci*, 8, 977-979.
- Taubenfeld SM, Milekic MH, Monti B & Alberini CM (2001) The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. *Nat Neurosci*, 4, 813-818.
- Taupin P (2006) Neurogenesis in the adult central nervous system. *C R Biol*, 329, 465-475.
- Teipel SJ & Hampel H (2006) Neuroanatomy of Down syndrome in vivo: a model of preclinical Alzheimer's disease. *Behav Genet*, 36, 405-415.
- Terry RD (1996) The pathogenesis of Alzheimer disease: an alternative to the amyloid hypothesis. *J Neuropathol Exp Neurol*, 55, 1023-1025.
- Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA & Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*, 30, 572-580.
- Thornton AS, Oda Y, Stuart GR, Holcroft J & de Boer JG (2004) The dioxin TCDD protects against aflatoxin-induced mutation in female rats, but not in male rats. *Mutat Res*, 561, 147-152.

- Thunberg T, Ahlborg UG, Hakansson H, Krantz C & Monier M (1980) Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). *Arch Toxicol*, 45, 273-285.
- Tombaugh GC, Rowe WB, Chow AR, Michael TH & Rose GM (2002) Theta-frequency synaptic potentiation in CA1 in vitro distinguishes cognitively impaired from unimpaired aged Fischer 344 rats. *J Neurosci*, 22, 9932-9940.
- Tong L, Thornton PL, Balazs R & Cotman CW (2001) Beta -amyloid-(1-42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *J Biol Chem*, 276, 17301-17306.
- Tonini R, Mancinelli E, Balestrini M, Mazzanti M, Martegani E, Ferroni A, Sturani E & Zippel R (1999) Expression of Ras-GRF in the SK-N-BE neuroblastoma accelerates retinoic-acid-induced neuronal differentiation and increases the functional expression of the IRK1 potassium channel. *Eur J Neurosci*, 11, 959-966.
- Topic B, Willuhn I, Palomero-Gallagher N, Zilles K, Huston JP & Hasenohr RU (2007) Impaired maze performance in aged rats is accompanied by increased density of NMDA, 5-HT1A, and alpha-adrenoceptor binding in hippocampus. *Hippocampus*, 17, 68-77.
- Toth K, Borhegyi Z & Freund TF (1993) Postsynaptic targets of GABAergic hippocampal neurons in the medial septum-diagonal band of Broca complex. *J Neurosci*, 13, 3712-3724.
- Tran P, Zhang XK, Salbert G, Hermann T, Lehmann JM & Pfahl M (1992) COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol Cell Biol*, 12, 4666-4676.
- Trejo JL, Carro E & Torres-Aleman I (2001) Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. *J Neurosci*, 21, 1628-1634.
- Tritscher AM, Seacat AM, Yager JD, Groopman JD, Miller BD, Bell D, Sutter TR & Lucier GW (1996) Increased oxidative DNA damage in livers of 2,3,7,8-

- tetrachlorodibenzo-p-dioxin treated intact but not ovariectomized rats. *Cancer Lett*, 98, 219-225.
- Tsuzuki K, Fukatsu R, Yamaguchi H, Tateno M, Imai K, Fujii N & Yamauchi T (2000) Transthyretin binds amyloid beta peptides, Abeta1-42 and Abeta1-40 to form complex in the autopsied human kidney - possible role of transthyretin for abeta sequestration. *Neurosci Lett*, 281, 171-174.
- Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M & Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron*, 23, 583-592.
- Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ & Worley PF (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron*, 21, 717-726.
- Tucholski J & Johnson GV (2003) Tissue transglutaminase directly regulates adenylyl cyclase resulting in enhanced cAMP-response element-binding protein (CREB) activation. *J Biol Chem*, 278, 26838-26843.
- Tuitoek PJ, Ritter SJ, Smith JE & Basu TK (1996) Streptozotocin-induced diabetes lowers retinol-binding protein and transthyretin concentrations in rats. *Br J Nutr*, 76, 891-897.
- Umbreit TH & Gallo MA (1988) Physiological implications of estrogen receptor modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Lett*, 42, 5-14.
- Umbriaco D, Garcia S, Beaulieu C & Descarries L (1995) Relational features of acetylcholine, noradrenaline, serotonin and GABA axon terminals in the stratum radiatum of adult rat hippocampus (CA1). *Hippocampus*, 5, 605-620.
- Uttl B & Graf P (1993) Episodic spatial memory in adulthood. *Psychol Aging*, 8, 257-273.
- Valdenaire O, Maus-Moatti M, Vincent JD, Mallet J & Vernier P (1998) Retinoic acid regulates the developmental expression of dopamine D2 receptor in rat striatal primary cultures. *J Neurochem*, 71, 929-936.

- van Bennekum AM, Wei S, Gamble MV, Vogel S, Piantedosi R, Gottesman M, Episkopou V & Blaner WS (2001) Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *J Biol Chem*, 276, 1107-1113.
- Van Birgelen AP, Van der KJ, Fase KM, Bol I, Poiger H, Brouwer A & Van den BM (1995) Subchronic dose-response study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in female Sprague-Dawley rats. *Toxicol Appl Pharmacol*, 132, 1-13.
- van der Staay FJ & Bouger PC (2005) Effects of the cholinesterase inhibitors donepezil and metrifonate on scopolamine-induced impairments in the spatial cone field orientation task in rats. *Behav Brain Res*, 156, 1-10.
- van der Zee EA & Luiten PG (1999) Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. *Prog Neurobiol*, 58, 409-471.
- van Duijn CM & Hofman A (1991) Relation between nicotine intake and Alzheimer's disease. *BMJ*, 302, 1491-1494.
- van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD & Gage FH (2002) Functional neurogenesis in the adult hippocampus. *Nature*, 415, 1030-1034.
- Vannucchi MG & Pepeu G (1995) Muscarinic receptor modulation of acetylcholine release from rat cerebral cortex and hippocampus. *Neurosci Lett*, 190, 53-56.
- Varju P, Katarova Z, Madarasz E & Szabo G (2002) Sequential induction of embryonic and adult forms of glutamic acid decarboxylase during in vitro-induced neurogenesis in cloned neuroectodermal cell-line, NE-7C2. *J Neurochem*, 80, 605-615.
- Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF & Guzowski JF (2002) Experience-dependent coincident expression of the effector immediate-early genes *arc* and *Homer 1a* in hippocampal and neocortical neuronal networks. *J Neurosci*, 22, 10067-10071.
- Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC & Pavlidis P (2004) Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice. *Learn Mem*, 11, 253-260.

- Verret L, Jankowsky JL, Xu GM, Borchelt DR & Rampon C (2007) Alzheimer's-type amyloidosis in transgenic mice impairs survival of newborn neurons derived from adult hippocampal neurogenesis. *J Neurosci*, 27, 6771-6780.
- Vizi ES & Kiss JP (1998) Neurochemistry and pharmacology of the major hippocampal transmitter systems: synaptic and nonsynaptic interactions. *Hippocampus*, 8, 566-607.
- Vouimba RM, Foy MR, Foy JG & Thompson RF (2000) 17beta-estradiol suppresses expression of long-term depression in aged rats. *Brain Res Bull*, 53, 783-787.
- Vranckx R, Savu L, Lambert N, de Conchard GV, Grosse R, Mourey MS & Corman B (1995) Plasma proteins as biomarkers of the aging process. *Am J Physiol*, 268, R536-R548.
- Vranckx R, Savu L, Maya M & Nunez EA (1990) Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse. *Biochem J*, 271, 373-379.
- Vyas S, Michel PP, Copin MC, Biguet NF, Thomasset M & Agid Y (1994) Induction of calbindin-D 28K gene and protein expression by physiological stimuli but not in calcium-mediated degeneration in rat PC12 pheochromocytoma cells. *FEBS Lett*, 351, 53-57.
- Wagner E, Luo T & Drager UC (2002) Retinoic acid synthesis in the postnatal mouse brain marks distinct developmental stages and functional systems. *Cereb Cortex*, 12, 1244-1253.
- Waldmeier PC, Wicki P, Feldtrauer JJ & Baumann PA (1988) Potential involvement of a baclofen-sensitive autoreceptor in the modulation of the release of endogenous GABA from rat brain slices in vitro. *Naunyn Schmiedebergs Arch Pharmacol*, 337, 289-295.
- Wang YZ & Christakos S (1995) Retinoic acid regulates the expression of the calcium binding protein, calbindin-D28K. *Mol Endocrinol*, 9, 1510-1521.
- Ward W & Richardson A (1991) Effect of age on liver protein synthesis and degradation. *Hepatology*, 14, 935-948.

- Watanabe CM, Wolffram S, Ader P, Rimbach G, Packer L, Maguire JJ, Schultz PG & Gohil K (2001) The in vivo neuromodulatory effects of the herbal medicine ginkgo biloba. *Proc Natl Acad Sci U S A*, 98, 6577-6580.
- Wei S, Episkopou V, Piantedosi R, Maeda S, Shimada K, Gottesman ME & Blaner WS (1995) Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. *J Biol Chem*, 270, 866-870.
- Weingartner H (1985) Models of memory dysfunctions. *Ann N Y Acad Sci*, 444, 359-369.
- Weisner B & Roethig HJ (1983) The concentration of prealbumin in cerebrospinal fluid (CSF), indicator of CSF circulation disorders. *Eur Neurol*, 22, 96-105.
- Welsh KA, Butters N, Hughes JP, Mohs RC & Heyman A (1992) Detection and staging of dementia in Alzheimer's disease. Use of the neuropsychological measures developed for the Consortium to Establish a Registry for Alzheimer's Disease. *Arch Neurol*, 49, 448-452.
- Westphalen RI, Scott HL & Dodd PR (2003) Synaptic vesicle transport and synaptic membrane transporter sites in excitatory amino acid nerve terminals in Alzheimer disease. *J Neural Transm*, 110, 1013-1027.
- Wetherell JL, Gatz M, Johansson B & Pedersen NL (1999) History of depression and other psychiatric illness as risk factors for Alzheimer disease in a twin sample. *Alzheimer Dis Assoc Disord*, 13, 47-52.
- White NM & McDonald RJ (2002) Multiple parallel memory systems in the brain of the rat. *Neurobiol Learn Mem*, 77, 125-184.
- Whitehouse PJ, Price DL, Clark AW, Coyle JT & DeLong MR (1981) Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol*, 10, 122-126.
- Wilkie GI, Hutson P, Sullivan JP & Wonnacott S (1996) Pharmacological characterization of a nicotinic autoreceptor in rat hippocampal synaptosomes. *Neurochem Res*, 21, 1141-1148.

- Wilson RS, Bennett DA, Bienias JL, Aggarwal NT, Mendes De Leon CF, Morris MC, Schneider JA & Evans DA (2002a) Cognitive activity and incident AD in a population-based sample of older persons. *Neurology*, 59, 1910-1914.
- Wilson RS, Mendes De Leon CF, Barnes LL, Schneider JA, Bienias JL, Evans DA & Bennett DA (2002b) Participation in cognitively stimulating activities and risk of incident Alzheimer disease. *JAMA*, 287, 742-748.
- Wimo A, Jonsson L & Winblad B (2006) An estimate of the worldwide prevalence and direct costs of dementia in 2003. *Dement Geriatr Cogn Disord*, 21, 175-181.
- Wimo A, Winblad B, Aguero-Torres H & von Strauss E (2003) The magnitude of dementia occurrence in the world. *Alzheimer Dis Assoc Disord*, 17, 63-67.
- Wisniewski T, Castano E, Ghiso J & Frangione B (1993) Cerebrospinal fluid inhibits Alzheimer beta-amyloid fibril formation in vitro. *Ann Neurol*, 34, 631-633.
- Wisniewski T, Castano EM, Golabek A, Vogel T & Frangione B (1994) Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am J Pathol*, 145, 1030-1035.
- Wisniewski T & Frangione B (1992) Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neurosci Lett*, 135, 235-238.
- Wolf G (1995) Retinol transport and metabolism in transthyretin-"knockout" mice. *Nutr Rev*, 53, 98-99.
- Woolley CS & McEwen BS (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *J Neurosci*, 12, 2549-2554.
- Wormke M, Stoner M, Saville B & Safe S (2000) Crosstalk between estrogen receptor alpha and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. *FEBS Lett*, 478, 109-112.
- Wu G, Fang Y, Lu ZH & Ledeen RW (1998) Induction of axon-like and dendrite-like processes in neuroblastoma cells. *J Neurocytol*, 27, 1-14.

- Wu M, Newton SS, Atkins JB, Xu C, Duman RS & Alreja M (2003) Acetylcholinesterase inhibitors activate septohippocampal GABAergic neurons via muscarinic but not nicotinic receptors. *J Pharmacol Exp Ther*, 307, 535-543.
- Wyde ME, Wong VA, Kim AH, Lucier GW & Walker NJ (2001) Induction of hepatic 8-oxo-deoxyguanosine adducts by 2,3,7,8-tetrachlorodibenzo-p-dioxin in Sprague-Dawley rats is female-specific and estrogen-dependent. *Chem Res Toxicol*, 14, 849-855.
- Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ & Worley PF (1998) Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron*, 21, 707-716.
- Xu WL, Qiu CX, Wahlin A, Winblad B & Fratiglioni L (2004) Diabetes mellitus and risk of dementia in the Kungsholmen project: a 6-year follow-up study. *Neurology*, 63, 1181-1186.
- Yamamoto M, McCaffery P & Drager UC (1996) Influence of the choroid plexus on cerebellar development: analysis of retinoic acid synthesis. *Brain Res Dev Brain Res*, 93, 182-190.
- Yamamoto M, Ullman D, Drager UC & McCaffery P (1999) Postnatal effects of retinoic acid on cerebellar development. *Neurotoxicol Teratol*, 21, 141-146.
- Yang Y, Quitschke WW & Brewer GJ (1998) Upregulation of amyloid precursor protein gene promoter in rat primary hippocampal neurons by phorbol ester, IL-1 and retinoic acid, but not by reactive oxygen species. *Brain Res Mol Brain Res*, 60, 40-49.
- Yao PJ, Zhu M, Pyun EI, Brooks AI, Therianos S, Meyers VE & Coleman PD (2003) Defects in expression of genes related to synaptic vesicle trafficking in frontal cortex of Alzheimer's disease. *Neurobiol Dis*, 12, 97-109.
- Yoshimasu F, Kokmen E, Hay ID, Beard CM, Offord KP & Kurland LT (1991) The association between Alzheimer's disease and thyroid disease in Rochester, Minnesota. *Neurology*, 41, 1745-1747.

- Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y & Li R (2005) Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. *Proc Natl Acad Sci U S A*, 102, 19198-19203.
- Zetterstrom RH, Lindqvist E, Mata dU, Tomac A, Eriksson U, Perlmann T & Olson L (1999) Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur J Neurosci*, 11, 407-416.
- Zhao D, McCaffery P, Ivins KJ, Neve RL, Hogan P, Chin WW & Drager UC (1996) Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur J Biochem*, 240, 15-22.
- Zheng W (2001) Toxicology of choroid plexus: special reference to metal-induced neurotoxicities. *Microsc Res Tech*, 52, 89-103.
- Zheng W, Lu YM, Lu GY, Zhao Q, Cheung O & Blaner WS (2001) Transthyretin, thyroxine, and retinol-binding protein in human cerebrospinal fluid: effect of lead exposure. *Toxicol Sci*, 61, 107-114.
- Zheng W, Shen H, Blaner WS, Zhao Q, Ren X & Graziano JH (1996) Chronic lead exposure alters transthyretin concentration in rat cerebrospinal fluid: the role of the choroid plexus. *Toxicol Appl Pharmacol*, 139, 445-450.
- Zhong P, Gu Z, Wang X, Jiang H, Feng J & Yan Z (2003) Impaired modulation of GABAergic transmission by muscarinic receptors in a mouse transgenic model of Alzheimer's disease. *J Biol Chem*, 278, 26888-26896.
- Ziabreva I, Perry E, Perry R, Minger SL, Ekonomou A, Przyborski S & Ballard C (2006) Altered neurogenesis in Alzheimer's disease. *J Psychosom Res*, 61, 311-316.
- Zou K, Gong JS, Yanagisawa K & Michikawa M (2002) A novel function of monomeric amyloid beta-protein serving as an antioxidant molecule against metal-induced oxidative damage. *J Neurosci*, 22, 4833-4841.
- Zou K, Kim D, Kakio A, Byun K, Gong JS, Kim J, Kim M, Sawamura N, Nishimoto S, Matsuzaki K, Lee B, Yanagisawa K & Michikawa M (2003)

Amyloid beta-protein (Abeta)1-40 protects neurons from damage induced by Abeta1-42 in culture and in rat brain. *J Neurochem*, 87, 609-619.

Appendix I

Neuroprotective effects of natural products:
interaction with intracellular kinases, amyloid peptides
and a possible role for transthyretin

Neuroprotective Effects of Natural Products: Interaction with Intracellular Kinases, Amyloid Peptides and a Possible Role for Transthyretin

Stéphane Bastianetto · Jonathan Brouillette ·
Rémi Quirion

Accepted: 16 March 2007
© Springer Science+Business Media, LLC 2007

Abstract Various studies reported on the neuroprotective effects of natural products, particularly polyphenols, widely present in food and beverages. For example, we have shown that resveratrol, a polyphenol contained present in red wine and other foods, activates the phosphorylation of protein kinase C (PKC), this effect being involved in its neuroprotective action against A β -induced toxicity. Moreover, tea-derived catechin gallate esters inhibit the formation A β oligomers/fibrils, suggesting that this action likely contributes to their neuroprotective effects. Interestingly, the effects of polyphenols may be attributable, at least in part, to the presence of specific binding sites. Autoradiographic studies revealed that these binding sites are particularly enriched in choroids plexus in the rat brain. Interestingly, the choroid plexus secretes transthyretin, a protein that has been shown to prevent A β aggregation and that may be critical to the maintenance of normal learning capacities in aging. Taken together, these data suggest that polyphenols target multiple enzymes/proteins leading to their neuroprotective actions.

Keywords Alzheimer's disease · A β peptides · Resveratrol · Tea catechins · Transthyretin · Polyphenols · Neuroprotection · Beta-amyloid · PKC

Introduction

Various epidemiological studies have suggested that the consumption of polyphenols derived from fruits, vegetables and beverages may reduce the risk of age-related neurological disorders such as dementia of the Alzheimer's type (AD), stroke and Parkinson's disease [1–5]. In parallel, preclinical studies have focused on the pivotal role of polyphenols found in the ginkgo biloba extract (EGb 761, a natural extract that is prescribed in Europe for the treatment of cognitive deficits [6]) and red wine as they exert potent neuroprotective actions in various in vitro and animal models of toxicity [7–15]. For example, our group previously reported that the flavonoid fraction of EGb 761 and red wine-derived polyphenols known as quercetin, resveratrol and catechins protected hippocampal neurons against toxicity induced by the free radical donor sodium nitroprusside (SNP) [7] and beta-amyloid (A β) peptides found in AD [11, 15]. These studies also suggested that mechanisms underlying the neuroprotective action of these polyphenols were not solely attributable to their antioxidant properties but involved their abilities to modulate intracellular effectors such as protein kinase C (PKC) [10–12, 16] or to interact with A β peptides [15]. We briefly review here the interaction of polyphenols with intracellular signalling pathways and A β peptides, in relation with their neuroprotective abilities. We then discuss the role of transthyretin as a possible target of polyphenols and as relevant to its role in cognitive disorders related to amyloidogenesis.

Special issue dedicated to Dr. Moussa Youdim.

Stéphane Bastianetto, Jonathan Brouillette contributed equally to the manuscript.

S. Bastianetto · J. Brouillette · R. Quirion (✉)
Department of Psychiatry, Douglas Hospital Research Centre,
McGill University, 6875 LaSalle Boulevard, Verdun, Montreal,
QC, Canada H4H 1R3
e-mail: remi.quirion@douglas.mcgill.ca

Experimental procedure

Primary hippocampal cell cultures and A β -induced toxicity

Hippocampal cell cultures were prepared from E19 embryos obtained from Sprague–Dawley rats, as described in details elsewhere [11, 15]. Animal care was according to protocols and guidelines of the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Six-day old hippocampal cells were exposed to peptides A β _{25–35} (25 μ M), A β _{1–40} (5 μ M) or A β _{1–42} (25 μ M) for 24 h, in the presence or absence of different phenolic compounds, as described in details elsewhere [11, 15]. Cell viability was quantified 24 h later using the MTT colorimetric assay [11] whereas the extent of necrotic cells was evaluated using the fluorescence dye Sytox Green [15]. [³H]-Resveratrol binding assay and receptor autoradiography were performed as described in details elsewhere [11].

Water maze task and retinoic acid treatment to determined memory capacities of aged rats and transthyretin knock-out mice

Aged (24-months old) and young adult (7-months old) male Long–Evans rats were housed in groups of two per cage and maintained on a 12 h light/dark cycle with ad libitum access to food and water. Wild-type and transthyretin knock-out mice (F2 generation) were maintained on a mixed C57BL/6 $\times$ 129S background.

Retinoic acid was dissolved in a vehicle solution containing polyethyleneglycol, NaCl (0.9%), and ethanol mixed in a proportion of 70:20:10 by volume. Daily administration of RA (150 μ g/kg, sc) to 7-months old WT and TTR knock-out mice as well as aged and young rats commenced four days prior to behavioural testing and continued until the end of testing in the MWM.

Animals were required to find, in a 1.4 m diameter pool, a submerged platform located 1 cm below the surface of water (24°C), rendered opaque by the addition of skim milk powder. Animals were pseudo-randomly started from a different position at each trial and used distal visuo-spatial cues to find the hidden-platform that remained in the centre of the same quadrant throughout all training days. Animals were given three trials of 90 s per day with a 45 min inter-trial interval over five consecutive days. If the platform was not located within 90 s, animals were guided to it and remained there for 10 s before removal. Over training days 3–5, aged rats with average escape latencies that were greater than 2 or less than 0.5 standard deviations from the mean of the young group were considered to be aged memory-impaired and aged memory-unimpaired rats

respectively. One probe trial of 60 s was given after the acquisition phase on day 5 to evaluate the number of times the animals crossed the previous location of the platform, time spent in the target quadrant, swimming speed, and swim pattern. This trial was followed by a 60 s cued trial in which the platform was visible to measure sensorimotor ability and motivation to escape from water. The experimenter was blind to mouse genotype when comparing TTR knock-out and WT littermates. Statistical significance of each test was assessed using the appropriate ANOVA design and simple main effects were analysed with F-test when the interaction was significant. Tukey's Honestly Significant Difference test was used as post hoc pairwise comparison test.

Results

Neuroprotective effects of resveratrol against A β -induced toxicity

Han et al. reported in 2004 that resveratrol (20 μ M) protected hippocampal neurons before, during or post-exposure to either A β _{25–35}, A β _{1–40} or A β _{1–42}. These protective actions were shared by phorbol 12-myristate 13-acetate, PKC activator, whereas a pre-treatment with the PKC inhibitor, GF 109203X, blocked the effect of resveratrol [11]. In contrast, inhibitors of PI3K or MAP kinases such as LY294002 and PD98059, respectively, were ineffective [11]. Moreover, treatment of hippocampal cells with resveratrol (20 μ M) induced the phosphorylation of various isoforms of PKC (particularly the PKC- δ) and blocked the inhibitory effects of A β _{25–35} on the phosphorylation of PKC, indicating that this enzyme is involved in the protective effects of resveratrol in our model [11]. In contrast, resveratrol (up to 40 μ M) failed to modulate the phosphorylation of either Akt kinase, ERK1/ERK2 or MAP kinases, suggesting the specificity for its effects on PKC [11].

Neuroprotective effects of tea catechins gallate esters against A β -induced toxicity

Based on the purported preventive effects of tea against cognitive and neurodegenerative disorders [2, 17], we investigated the potential effectiveness of green and black tea extracts against A β toxicity in rat primary hippocampal cultured cells [15]. Both total extracts (5–25 μ g/ml) displayed neuroprotective action against toxicities induced by various A β peptides (A β _{25–35}, A β _{1–40} and A β _{1–42}). These effects were shared by the most abundant green tea catechin, epigallocatechin gallate (EGCG; 1–10 μ M), and to a lesser extent by epicatechin gallate (ECG; 1–20 μ M). Gallic acid, a phenolic acid present in black and green teas

[18], displays protective activity with a maximal protective effect at 10 μ M. In contrast, non-gallate forms of catechins (i.e. epicatechin and epigallocatechin) were ineffective in the same range of concentrations. Similarly, both extracts (5–25 μ g/ml) and EGCG (10 μ M) shared with EGb 761 (100 μ g/ml) the ability to block the toxic effects of the physiological fragment A β _{1–42}. Interestingly, western blot assay indicated that EGCG (10 μ M) inhibited soluble forms of A β (known as A β -derived diffusible neurotoxin ligands, ADDLs) [15] that have been reported to mediate A β _{1–42}-induced toxicity [19]. Moreover, the thioflavin (THT) fluorescent assay revealed that gallate forms of tea catechins and gallic acid, but not EC and EGC, inhibited the fibrilization of A β [15]. Taken together, these findings indicate that catechin gallates likely mediate the neuroprotective effects of tea extract, possibly through the presence of a gallic acid molecule in the hydroxyl group of catechins. These protective actions seem to be associated, at least in part, with their inhibitory action on the formation of A β fibrils/oligomers, in accordance with previous findings [10, 20]. Data are summarized in Table 1.

Identification of specific binding sites for polyphenols in the rat brain

Han et al. (2006) identified and characterized polyphenols binding sites in rat brain fractions using 20 nM [³H]-resveratrol. Significant [³H]-resveratrol binding sites were detected in plasma membrane fraction (PII fraction), and to a lesser extent in nuclear and cellular fractions. Moreover, treatment with trypsin significantly reduced [³H]-resveratrol binding to the PII fraction, suggesting the proteinous nature of specific [³H]-resveratrol binding sites [22]. Curve-fitting analysis revealed that [³H]-resveratrol specifically binds to a saturable and single class of sites with an apparent affinity (K_D) of 220 ± 80 nM. Using

quantitative autoradiography, specific [³H]-resveratrol binding sites were widely distributed in the rat brain, with highest levels seen in the choroid plexus and subfornical organ, and to a lesser extent in the hippocampus and cortex [22].

We then tested the effectiveness of stilbenes and catechins to protect hippocampal cells against A β _{25–35}-induced toxicity, in relationship with their affinity for specific [³H]-resveratrol binding sites. Interestingly, the affinity of polyphenols (i.e. EGCG and ECG), resveratrol and other stilbenes analogs (i.e. piceatannol) to compete against specific [³H]-resveratrol binding correlated very well ($r = 0.74$), with their neuroprotective activity against A β _{25–35}-induced toxicity. These results suggest that the neuroprotective action of polyphenols may be mediated by a common mechanism involving a specific plasma membrane protein [22].

Transthyretin as a possible target of promoting action of polyphenols

Transthyretin (TTR) is the major protein synthesized and secreted by the choroid plexus into the cerebrospinal fluid (CSF) and represents about 25% of total CSF proteins. TTR has been identified as the main A β binding protein in the CSF [23, 24]. Declining levels of CSF TTR were found to be associated with dementia of increasing severity in AD patients [25, 26]. Moreover, lack of amyloid plaques in young AD mouse model is associated with increased levels of TTR [27] whereas neutralization of TTR leads to increased A β , tau phosphorylation, neuronal loss and apoptosis in the hippocampus [28].

Recent unpublished data from our group also revealed that decreased levels of TTR may be critical to the development of memory impairments during aging (Brouillette and Quirion, submitted). A lower gene

Table 1 Summary of the involvement of protein kinase C and A β formation in the purported neuroprotective effects of polyphenols

Polyphenols	PKC	Inhibition of A β formation (soluble forms)	Inhibition of A β formation (insoluble forms)
Green tea extract	ND	Yes	Yes
Black tea extract	ND	ND	ND
Epigallocatechin gallate	Yes	Yes	Yes
Epicatechin gallate	ND	Yes	Yes
Epicatechin	ND	No	No
Epigallocatechin	ND	No	No
Gallic acid	ND	No	Yes
Resveratrol	Yes	No	Yes
Piceatannol	ND	Yes	Yes

ND: not determined

Taken from [10, 11, 15, 21]

expression of TTR was observed in aged memory-impaired (AI) rats when compared to aged memory-unimpaired (AU) animals following stimulation in a spatial memory task. Memory deficits were also found during aging in TTR knock-out mice. The mechanism underlying the role of TTR in the maintenance of normal cognitive abilities may be related to its capacity to transport retinol since cognitive deficits in TTR knock-out mice and aged rats were reversed by treatment with retinoic acid, an active vitamin A metabolite which acts on specific nuclear receptors to modulate a wide variety of biological processes including synaptic plasticity and long-term potentiation [29–31]. We also found that lower protein level of C/EBP, an immediate-early gene that regulate TTR, time-dependently overlap decreased TTR gene expression in AI rats, suggesting that alteration in transcription levels of TTR is attributed, at least in part, to altered C/EBP protein expression. C/EBP is part of the immediate-early gene cascade initiated by cAMP response element binding protein (CREB). Following its activation after training, C/EBP is suggested to regulate downstream effector genes such as TTR for the consolidation of new memory [32]. Interestingly, various studies have suggested that the activation of either PKC or PKA, via mitogen-activated protein kinase (MAPK), is an essential regulator of CREB phosphorylation in the hippocampus [33–36]. Taken together, these data suggest that red wine and tea-derived polyphenols could increase TTR gene expression by activating specific plasma membrane protein concentrated in the choroid plexus, leading to the activation of PKC, MAPK, CREB, and C/EBP signalling cascades. Increased TTR gene expression may in turn be involved in the maintenance of normal memory capacities by ensuring the availability of retinoic acid and the sequestration of A β . Experiments are currently in progress to validate this hypothesis.

Discussion

Red wine and tea-derived polyphenols such as resveratrol and catechin gallate esters displayed strong neuroprotective abilities in cultured hippocampal neuronal cells. The effect of resveratrol is mediated by the activation of PKC, supporting the hypothesis that the modulation of kinases plays an important role in the neuroprotective abilities of polyphenols, as previously reported for EGCG [10]. We cannot rule out other mechanisms since resveratrol and piceatannol have been reported to strongly inhibit A β polymerization [21], suggesting that stilbenes may directly interact with A β peptides. Finally, recent *in vitro* data demonstrate that resveratrol does not inhibit A β production, but instead promotes its intracellular degradation via a mechanism that involves the proteasome [37].

Green and black tea extracts shared with resveratrol the ability to protect hippocampal neurons against A β toxicity. EGCG and ECG are the most potent tea catechins in our model. These effects did not seem to be attributable to their antioxidant properties but rather to their abilities to inhibit the formation of A β fibrils. These findings are in accordance with previous studies showing that phenolic compounds display anti-amyloidogenic and fibril-destabilizing activities [38, 39]. Moreover, the most potent green tea catechin EGCG inhibited the formation of soluble, oligomers of A β known as ADDLs. Using the fluorescent dye Bis-ANS, preliminary data support the hypothesis that various polyphenols displaying neuroprotective ability (i.e. ECG, EGCG but not resveratrol) are also capable of inhibiting the formation of soluble A β . Given that oligomers of A β have been suggested to play a key role in A β toxicity, it is likely that the inhibitory action of phenolic compounds on the formation of A β fibrils/oligomers contributes, at least in part, to their neuroprotective actions in this model.

Binding studies revealed the presence of [^{3} H]-resveratrol sites at the level of plasma membrane fraction, and to a lesser extent in nuclear and cellular components. Interestingly, a strong correlation ($r = 0.74$) existed between the affinity and potency of various polyphenols tested in our binding studies and their neuroprotective action against A β -induced toxicity. Taken together, these data suggest that the neuroprotective effects of these polyphenols involve the activation of plasma membrane binding proteins.

Receptor autoradiography revealed that [^{3} H]-resveratrol binding sites are broadly distributed in the rat brain with highest levels found in the choroid plexus and subfornical organ, and to a lesser extent in the hippocampal formation. Interestingly, choroid plexus endothelial cells have been reported to be neuroprotective *in vitro* and *in vivo* models of neurotoxicity [40–42]. Moreover, choroid plexus is known to produce TTR, a protein that has been shown to sequester A β protein and to prevent A β aggregation [43]. Therefore, one mechanism whereby polyphenols may exert their neuroprotective effects is by the activation of TTR leading to the sequestration of A β peptides. In support of this hypothesis, an animal study has shown that dietary supplementation with the Ginkgo biloba extract EGb 761 modulated TTR mRNA levels in the rat hippocampus [44]. Moreover, resveratrol has recently been shown to inhibit monomeric TTR-induced cytotoxicity, suggesting that this stilbene is a good candidate for inhibiting TTR-related amyloidogenesis [45].

In summary, these findings support the role of polyphenols in the beneficial effects of red wine, tea, fruits and vegetables in reducing the risk for developing neurological disorders. Polyphenols likely modulate plasma membrane proteins and various intracellular effectors such

as PKC, and may directly interact with proteins that play a critical role in amyloidogenesis, particularly A β peptides.

Acknowledgments This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to R.Q. and a student scholarship from CIHR to J.B.

References

- Orgogozo JM, Dartigues JF, Lafont S, Letenneur L, Commenges D, Salomon R, Renaud S, Breteler MB (1997) Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Revue Neurologique* 153:185–192
- Pan T, Jankovic J, Le W (2003) Potential therapeutic properties of green tea polyphenols in Parkinson's disease. *Drugs Aging* 20:711–721
- Weinreb O, Mandel S, Amit T, Youdim MB (2004) Neurological mechanisms of green tea polyphenols in Alzheimer's and Parkinson's diseases. *J Nutr Biochem* 15:506–516
- Dauchet L, Amouyel P, Dallongeville J (2005) Fruit and vegetable consumption and risk of stroke: a meta-analysis of cohort studies. *Neurology* 65:1193–1197
- Dai Q, Borenstein AR, Wu Y, Jackson JC, Larson EB (2006) Fruit and vegetable juices and Alzheimer's disease: the Kame Project. *Am J Med* 119:751–759
- Le Bars PL (2003) Magnitude of effect and special approach to Ginkgo biloba extract EGB 761 in cognitive disorders. *Pharmacopsychiatry* 36(Suppl. 1):S44–S59
- Bastianetto S, Zheng WH, Quirion R (2000) Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br J Pharmacol* 131:711–720
- Bastianetto S, Quirion R (2001) Resveratrol and red wine constituents: evaluation of their neuroprotective properties. *Pharmacol News* 8:33–38
- Nagai K, Jiang MH, Hada J, Nagata T, Yajima Y, Yamamoto S, Nishizaki T (2002) (-)-Epigallocatechin gallate protects against NO stress-induced neuronal damage after ischemia by acting as an anti-oxidant. *Brain Res* 956:319–332
- Levites Y, Amit T, Mandel S, Youdim MB (2003) Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (-)-epigallocatechin-3-gallate. *FASEB J* 17:952–954
- Han YS, Zheng WH, Bastianetto S, Chabot JG, Quirion R (2004) Neuroprotective effects of resveratrol against beta-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. *Br J Pharmacol* 141:997–1005
- Mandel SA, Avramovich-Tirosh Y, Reznichenko L, Zheng H, Weinreb O, Amit T, Youdim MB (2005) Multifunctional activities of green tea catechins in neuroprotection. *Neurosignals* 14:46–60
- Mandel S, Weinreb O, Amit T, Youdim MB (2004) Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. *J Neurochem* 88:1555–1569
- Choi YB, Kim YI, Lee KS, Kim BS, Kim DJ (2004) Protective effect of epigallocatechin gallate on brain damage after transient middle cerebral artery occlusion in rats. *Brain Res* 1019:47–54
- Bastianetto S, Yao ZX, Papadopoulos V, Quirion R (2006) Neuroprotective effects of green and black teas and their catechin gallate esters against beta-amyloid-induced toxicity. *Eur J Neurosci* 23:55–64
- Levites Y, Amit T, Youdim MB, Mandel S (2002) Involvement of protein kinase C activation and cell survival/cell cycle genes in green tea polyphenol (-)-epigallocatechin 3-gallate neuroprotective action. *J Biol Chem* 277:30574–30580
- Kuriyama S, Hozawa A, Ohmori K, Shimazu T, Matsui T, Ebihara S, Awata S, Nagatomi R, Arai H, Tsuji I (2006) Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project I. *Am J Clin Nutr* 83:355–361
- Del Rio D, Stewart AJ, Mullen W, Burns J, Lean ME, Brighenti F, Crozier A (2004) HPLC-MSn analysis of phenolic compounds and purine alkaloids in green and black tea. *J Agric Food Chem* 52:2807–2815
- Klein WL (2002) Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem Int* 41:345–352
- Rezaei-Zadeh K, Shytte D, Sun N, Mori T, Hou H, Jeannot D, Ehrhart J, Townsend K, Zeng J, Morgan D, Hardy J, Town T, Tan J (2005) Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice. *J Neurosci* 25:8807–8814
- Riviere C, Richard T, Quentin L, Krisa S, Merillon JM, Monti JP (2006) Inhibitory activity of stilbenes on Alzheimer's beta-amyloid fibrils in vitro. *Bioorg Med Chem* [Epub ahead of print]
- Han YS, Bastianetto S, Dumont Y, Quirion R (2006) Specific plasma membrane binding sites for polyphenols, including resveratrol, in the rat brain. *J Pharmacol Exp Ther* 318:238–245
- Schwarzman AL, Gregori L, Vitek MP, Lyubski S, Strittmatter WJ, Enghilde JJ, Bhasin R, Silverman J, Weisgraber KH, Coyle PK (1994) Transthyretin sequesters amyloid beta protein and prevents amyloid formation. *Proc Natl Acad Sci USA* 91:8368–8372
- Carro E, Trejo JL, Gomez-Isla T, LeRoith D, Torres-Aleman I (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. *Nat Med* 8:1390–1397
- Merched A, Serot JM, Visvikis S, Aguilon D, Faure G, Siest G (1998) Apolipoprotein E, transthyretin and actin in the CSF of Alzheimer's patients: relation with the senile plaques and cytoskeleton biochemistry. *FEBS Lett* 425:225–228
- Serot JM, Christmann D, Dubost T, Couturier M (1997) Cerebrospinal fluid transthyretin: aging and late onset Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 63:506–508
- Stein TD, Johnson JA (2002) Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J Neurosci* 22:7380–7388
- Stein TD, Anders NJ, DeCarli C, Chan SL, Mattson MP, Johnson JA (2004) Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. *J Neurosci* 24:7707–7717
- Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L, Fadda F (2002) Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience* 115:475–482
- Etchamendy N, Enderlin V, Marighetto A, Vouimba RM, Pallet V, Jaffard R, Huguier P (2001) Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J Neurosci* 21:6423–6429
- Chiang MY, Misner D, Kempermann G, Schikorski T, Giguere V, Sucov HM, Gage FH, Stevens CF, Evans RM (1998) An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron* 21:1353–1361
- Taubenfeld SM, Milekic MH, Monti B, Alberini CM (2001) The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta. *Nat Neurosci* 4:813–818

33. Roberson ED, English JD, Adams JP, Selcher JC, Kondratieck C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J Neurosci* 19:4337–4348
34. Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, Storm DR (1998) Cross talk between ERK and PKA is required for Ca²⁺ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* 21:869–883
35. Huang YY, Martin KC, Kandel ER (2000) Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. *J Neurosci* 20:6317–6325
36. Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, Gallo V, Schinelli S (2001) cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. *J Biol Chem* 276:11487–11495
37. Marambaud P, Zhao H, Davies P (2005) Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. *J Biol Chem* 280:37377–37382
38. Ono K, Hasegawa K, Naiki H, Yamada M (2004) Anti-amyloidogenic activity of tannic acid and its activity to destabilize Alzheimer's beta-amyloid fibrils in vitro. *Biochim Biophys Acta* 1690:193–202
39. Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H, Yamada M (2003) Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease. *J Neurochem* 87:172–181
40. Borlongan CV, Skinner SJ, Geaney M, Vasconcellos AV, Elliott RB, Emerich DF (2004) Intracerebral transplantation of porcine choroid plexus provides structural and functional neuroprotection in a rodent model of stroke. *Stroke* 35:2206–2210
41. Borlongan CV, Skinner SJ, Geaney M, Vasconcellos AV, Elliott RB, Emerich DF (2004) Neuroprotection by encapsulated choroid plexus in a rodent model of Huntington's disease. *Neuroreport* 15:2521–2525
42. Watanabe Y, Matsumoto N, Dezawa M, Itokazu Y, Yoshihara T, Ide C (2005) Conditioned medium of the primary culture of rat choroid plexus epithelial (modified ependymal) cells enhances neurite outgrowth and survival of hippocampal neurons. *Neurosci Lett* 379:158–163
43. Tsuzuki K, Fukatsu R, Yamaguchi H, Tateno M, Imai K, Fujii N, Yamauchi T (2000) Transthyretin binds amyloid beta peptides, Abeta1–42 and Abeta1–40 to form complex in the autopsied human kidney—possible role of transthyretin for abeta sequestration. *Neurosci Lett* 281:171–174
44. Watanabe CM, Wolfram S, Ader P, Rimbach G, Packer L, Maguire JJ, Schultz PG, Gohil K (2001) The in vivo neuro-modulatory effects of the herbal medicine ginkgo biloba. *Proc Natl Acad Sci USA* 98:6577–6580
45. Reixach N, Adamski-Werner SL, Kelly JW, Koziol J, Buxbaum JN (2006) Cell based screening of inhibitors of transthyretin aggregation. *Biochem Biophys Res Commun* 348:889–897

Appendix II

Hippocampal gene expression profiles in
memory-impaired and memory-unimpaired aged rats

Hippocampal Gene Expression Profiles in Memory-Impaired and Memory-Unimpaired Aged Rats

Jonathan Brouillette¹, James O'Donnell¹, Oscar Díaz-Ruiz², Roger Godbout², Rémi Quirion^{1*}

¹ Douglas Hospital Research Centre, Department of Neurology & Neurosurgery, 6875 LaSalle boulevard, Montréal, Québec, Canada, H4H 1R3.

² Centre de biomédecine, Hôpital du Sacré-Cœur, Département de psychiatrie, 5400 Boul. Gouin ouest, Montréal, Québec, Canada, H4J 1C5.

Abstract

Individual difference is a well known feature of cognitive deficits that occur during brain aging, both in animal and man. However, the molecular and cellular mechanisms involved remain to be fully identified. Aged Long-Evans rats demonstrate marked individual differences in learning abilities and thus provide a unique model to investigate global alterations in gene expression using a cDNA microarray approach. In the present study, 24-25 months old rats were classified as aged memory-impaired (AI), aged moderately-impaired (AM) and aged memory-unimpaired (AU) animals relative to the performance of young rats in the Morris water maze test of spatial memory, a hippocampus-dependent learning task. The level of expression of genes in the hippocampus of the three groups of aged rats was assessed using cDNA microarray. Major differences were observed when comparing hippocampal gene expression profiles in the AI, AM and AU sub-groups. For example, the levels of expression of acetylcholinesterase, syntaxin A and carboxypeptidase E were markedly increased in the AI versus AU groups while the opposite was seen for NMDA receptor 2B, neurexin II beta-A precursor and tyrosine phosphatase-like protein. Differences between the AI and AM sub-groups were particularly evident for apolipoprotein D, gastric inhibitory polypeptide precursor and acetylcholine receptor protein beta 2 subunit (all three increased) while decreases in gene expression levels between AI versus AM rats were noted for brain hexokinase and neurexin II beta-A precursor. While it is still too early to claim that the observed differences in gene expression levels are directly related to altered learning abilities, our results may suggest new research avenues for a better understanding of features associated with cognitive disabilities in aging.

Keywords: Aging, Gene expression, cDNA microarray, Morris water maze, Hippocampus, Memory

Introduction

Learning and memory impairments are commonly observed in the elderly. Although cognitive decline does not invariably accompany aging, older people are at greater risk. Similarly, aged rodents exhibit marked heterogeneity in their learning and mnemonic capacities. Using the Morris water maze (MWM) to evaluate spatial learning, 24-25 months old Long-Evans rats can be

categorized as aged memory-impaired (AI), aged moderately-impaired (AM) and aged memory-unimpaired (AU) rats when compared to young (4-6 months) animals^{1,4}. The basis for such variability is unknown, but it is well documented that spatial memory abilities involved in hidden-platform MWM learning is hippocampus-dependent^{4,5} and can be impaired in aging. While several neurotransmitter systems are implicated in spatial learning⁷⁻¹⁰,

much remains to be established in regard to changes in intracellular mechanisms and gene transcription occurring with age, especially in relationship with cognitive abilities. In the present study, gene expressions in a rather unique cohort of aged rats with (AI, AM) or without (AU) learning deficits was characterized using the MWM were investigated using a cDNA microarray approach^{11,12}. Clusters of gene families were found to be differentially

#Correspondence: Dr. Rémi Quirion Douglas Hospital Research Centre 6875 LaSalle Boulevard Montréal, Québec H4H1R3
E-mail: quirem@douglas.mcgill.ca; Tel.: (514) 761-6131 (ext 2934); Fax: (514) 762-3034.

affected in the 3 sub-groups of aged rats used here suggesting their possible relevance to the maintenance (AU) or not (AI, AM) of adequate learning abilities with age.

Methods

Animals

Male Long-Evans rats (250 retired breeders) were purchased from Charles River (St. Constant, Québec) at 12 months of age and were housed in our vivarium until they were 24-25 months old. Thirty young rats (6 months old) were obtained at 3-4 months of age and were housed for 2-3 months prior to behavioral testing. Animals were housed in groups of two in cages (43x20x20cm) and maintained on a 12:12 light-dark schedule with ad libitum access to food (Purina Lab Chow) and water. Animal care and handling procedures were approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Morris water maze

Spatial memory was assessed using the MWM task^{3,4,13-15}. Briefly, the rats were required to find a submerged platform located 2.5 cm below the surface of water (rendered opaque by the addition of skim milk powder) in a 1.4 m diameter pool. Animals use only distal visuo-spatial cues available within the testing room to locate the submerged platform¹⁶. The rats were given 3 trials of 90 sec per day over 5 consecutive days. Animals were also assessed for motor, visual and motivational deficits on day 6 by raising the platform 2 cm above the surface of the water (visually cued condition). Four aged rats were unable to find the visible platform within 60 sec and were excluded from the study whereas twenty three aged rats died before the MWM trials. Average escape latency and distance were recorded along with swim paths using a video

tracking system (HVS, Buckingham, UK). Over training days 2-5, aged rats with average escape latencies that were greater than 2 or less than 0.5 standard deviations from the mean of the young group were considered to be memory-impaired (AI; n=55) or memory-unimpaired (AU; n=59), respectively. Animals whose mean escape latencies fell between these values were defined as moderately-impaired (AM; n=109) rats. Statistical significance was assessed using ANOVA and independent T-tests with Bonferroni correction.

RNA isolation, hybridization and imaging of atlas arrays

On day 6, rat brains were quickly removed by decapitation and hippocampi dissected, pooled and immediately stored at -80°C until use in RNeasy, an RNA stabilization solution (Ambion, Texas, USA). Total RNA was isolated from the entire samples of each rat group and DNase-I treated according to the Atlas Pure total RNA labeling system user manual from BD Biosciences Clontech (Palo Alto, California). The quality, integrity and quantity of the RNA from hippocampi were measured using UV absorption spectrophotometry and ribosomal RNA bands on electrophoresis.

Probes were generated by reverse transcription using the total RNA and ³²P-labeled dATP (Perkin-Elmer Life Sciences, Woodbridge, Ontario).

These probes were hybridized on Clontech Atlas Rat 1.2 arrays according to the Atlas cDNA expression arrays user manual from BD Biosciences Clontech. The array data were generated from three experiments using new membranes each time. After hybridization at 68°C for 24 h, membranes were exposed to Kodak Bio-Max MS film (VWR Canlab, Saint-Laurent-Montréal, Québec) using a Kodak high-energy intensifying screen (VWR Canlab) for at least three exposures per membrane (24 hours

to 4 days). Array images were acquired using an MCID System (Imaging Research, Saint-Catharines, Ontario).

Analysis of array data

Images of each array were first imported into AtlasImage software (Ver. 2.01; Clontech) for densitometric measurement of gene- and array-specific parameters taking into account film background. A simple ratio for each gene was then calculated using the normalized intensities and averaged with ratios over replicate array values. Only genes that show important change in hippocampal gene expression profiles were selected. Genes were removed if they show a change of 15% or smaller in expression.

Results

Animals were characterized as aged memory-impaired (AI), aged moderately-impaired (AM) and aged memory-unimpaired (AU) by comparing their performance with that of young (Y) animals during training days 2-5 in the MWM task. The AI group showed longer escape latency when compared to Y, AU and AM animals (fig. 1A). There was no significant change in the mean escape latency of aged and young rats when the platform was visible, suggesting the absence of significant motor, visual or motivational deficits (fig. 1B).

Hippocampal gene expression profile of the three groups of aged rats was then compared using a cDNA microarray approach (Table 1). Several genes are differently expressed between the AI and the two other groups shown here as average simple ratio compared to the AI group. The most upregulated gene is the acetylcholinesterase, which was increased by 36 fold in AI versus AU rats (Table 1). Syntaxin A, SNAP-25, GIP and carboxypeptidase E genes were also among the most over-expressed genes between these two groups of ani-

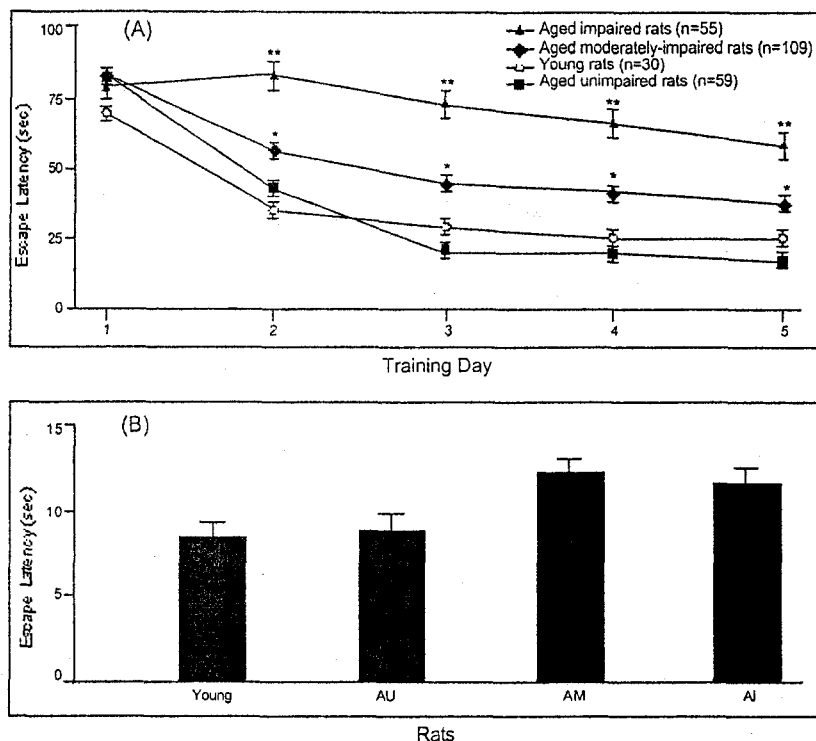


Figure 1. Performances of young (4 mo) and aged (24-25 mo) Long-Evans rats in the Morris water maze behavioral task. (A) Aged-impaired (AI), aged moderately-impaired (AM), and aged-unimpaired (AU) animals were identified as those whose mean escape latencies on training days 2-5 differed by > 2 (AI) or < 0.5 (AU) SD or between (AM) from the mean of young (Y) controls, respectively. * $p < 0.05$ and ** $p < 0.01$ compared to young animals, ANOVA and post-hoc t-test. (B) Escape latency when the platform was visible for 60 sec during the cued condition revealed no significant changes among the AI, AM, AU and Y rats, indicating no obvious motivational or visual deficits. All data are presented as mean $\pm$ S.E.M of the number of animals indicated in A.

mals. In contrast, gene expressions of NDKA, sodium/potassiumtransporting ATPase beta 1 subunit, brain hexokinase, mitochondrial ATP synthase beta subunit, neuronal acetylcholine nicotinic receptor protein beta 2 subunit, NMDA receptor 2B, neurexin II beta-A precursor, MIF, transducin beta-2 subunit and tyrosine phosphatase-like protein were apparently down-regulated in the AI versus AU animals.

Differences in gene expressions were also noted between the AI and AM subgroups (Table 1).

For example, apolipoprotein D (by 7 fold) and GIP (by 9 fold) expression levels are apparently markedly increased in the AI versus AM animals while the most significant decreases are seen for genes

such as brain hexokinase and neurexin II beta-A precursor.

Discussion

The present study confirms that clear individual differences are observed in learning abilities in aged Long-Evans rats with three broad sub-groups being characterized as aged memory impaired (AI), moderately impaired (AM) and unimpaired (AU). Moreover, multiple differences in hippocampal gene expression profiles (as revealed by cDNA microarray) were seen between these three groups of aged animals.

As reported earlier¹⁻⁴, marked individual differences were observed in the MWM task in aged Long-

Evans rats. Similar differences were noted in other learning tasks including versions of the MWM¹⁷⁻¹⁸ and dry mazes¹⁹⁻²². While various neurotransmitters⁷⁻¹⁰, neurotrophic factors^{13,23-25} and intracellular messengers²⁶⁻²⁸ have been studied to possibly explain individual differences in learning abilities in aged rats, much remain to be known as to key cascades of events leading to altered versus maintained cognitive abilities in this model.

A cDNA microarray analysis strategy was employed in the present study to investigate gene expression profile in the hippocampus of AI, AM and AU rats; this region is believed to be critical for acquisition and retrieval of spatial information as well as for memory consolidation and storage²⁹.

Interestingly, the most upregulated gene in the AI versus AU group was found to be the acetylcholinesterase, the main enzyme that hydrolyzes acetylcholine (ACh). This finding is in accordance with much evidence that have shown the critical role of ACh in the maintenance of normal cognitive functions^{10,15}. Moreover, acetylcholinesterase inhibitors are still the only drugs approved and widely used for the treatment of Alzheimer's disease.

Another gene that apparently increased substantially was syntaxin A. Its translated protein is known to mediate the docking of synaptic vesicles at the level of nerve terminals leading to neurotransmitter's exocytosis³⁰. SNAP-25, another gene implicated in regulated and constitutive exocytosis in neurons³¹, was also apparently increased in AI versus AU rats. Changes in gene expression levels of both SNAP-25 and syntaxin A could lead to significantly altered profiles of neurotransmitter releases. Other gene expressions that were apparently increased in the AI sub-group include GIP and carboxypeptidase E. The role of peptide GIP in the brain is mostly unknown while carboxypeptidases are known to be involved in the mat-

Table 1. Gene expression pattern observed in the hippocampus of aged memory-impaired (AI), moderately-impaired (AM) and memory-unimpaired (AU) rats.

Genbank number	Gene	AI AM	AI AU
D38492	neural adhesion molecule F3	2.40	0.29
M20035	prothymosin-alpha (PTMA)	3.38	2.54
X55572	apolipoprotein D	7.02	2.09
D13374	nucleoside diphosphate kinase A (NDKA)	0.88	0.14
X06942	A-raf proto-oncogene	1.85	0.46
J02701	sodium/potassium-transporting ATPase beta 1 subunit (ATP1B1)	1.24	0.07
M95734	syntaxin A	2.10	13.57
M95735	syntaxin B	1.48	2.02
AB003991	synaptosomal associated protein 25 (SNAP-25)	0.93	3.87
J04526	type 1 hexokinase (HK1); brain hexokinase	0.16	0.10
M19044	mitochondrial ATP synthase beta subunit (ATP5B)	0.96	0.14
S50879	acetylcholinesterase (ACHE)	0.42	36.46
M27905	60S ribosomal protein L21 (RPL21)	1.19	4.23
J02650	60S ribosomal protein L19 (RPL19)	1.05	4.19
L31622	neuronal acetylcholine receptor protein beta 2 subunit (NACHRB2)	3.87	0.40
M91562	NMDA Receptor 2B	0.96	0.08
AF005099	neuronal pentraxin receptor	2.35	0.42
M96377	neurexin II beta-A precursor	0.43	0.04
U62326	macrophage migration inhibitory factor (MIF)	0.86	0.16
L08831	gastric inhibitory polypeptide precursor (GIP); glucose-dependent insulinotropic polypeptide	9.71	4.40
M64300	extracellular signal-regulated kinase 2 (ERK2); mitogen-activated protein kinase 2 (MAPK2)	3.76	Nd
M16112	calcium/calmodulin-dependent protein kinase type II beta subunit (CAMK2B)	0.96	0.38
U34959	transducin beta-2 subunit; GTP-binding protein G(i)/G(s)/G(t) beta subunit 2 (GNB2)	0.76	0.08
D13125	neural visinin-like protein 2 (VILIP1; VSNL1)	1.98	2.56
M16736	growth-accentuating protein 43 (GAP43); neuromodulin	1.80	2.94
M31602	carboxypeptidase E (CPE)	1.30	5.32
L19181	receptor-linked protein tyrosine phosphatase (PTP-PS)	1.91	2.89
D38222	tyrosine phosphatase-like protein	1.10	0.05

Hippocampal cDNA array gene expression levels reaching criteria for changes sorted by average simple ratio following median normalization. Array hybridization was repeated three times. For gene grid assignment, refer to BD Atlas Rat 1.2 Array Gene List (PT3564-3) web site (http://www.clontech.com/atlas/genelists/7854-1_Ra12.pdf). For cDNA gene database, refer to BD Atlas gene lists version 5.0 (PT3593-CD) web site (<http://atlasinfo.clontech.com/search/initialization-action.do>). Nd, not determined.

uration of various peptide precursors and other metabolic activities^{32,33}. Further studies will be required to establish if they may play a direct role in cognitive functions.

Conversely, the level of the gene coding for neurexin II beta-A precursor decreased most significantly in AI compared to AU rats. Neurexins are neuronal cell surface receptors that likely perform multiple functions in the brain by participating in intercellular junctions and by act-

ing as signaling receptors³⁴. Interestingly, mRNA levels of neurexins II alpha and III alpha were found to be upregulated in CA1 and CA3 hippocampal neurons in response to a 15-min ischemic period³⁵. The apparent level of the gene encoding for the tyrosine phosphatase-like protein level also decreased in AI versus AU rats. This membrane protein is expressed only in the brain and may function as a negative regulator of PTPases³⁶.

Other genes of interest included those coding for the NMDA receptor 2B, the nicotinic receptor protein beta 2 subunit, sodium/potassium-transporting ATPase beta 1 subunit, brain hexokinase and mitochondrial ATP synthase beta subunit. Much evidence has shown the important roles of NMDA^{14,37,38} and nicotinic^{9,39} receptors in learning abilities. Similarly, altered cellular metabolic functions associated with the other genes mentioned above could

impact on the maintenance of appropriate learning abilities. Further studies are currently underway to verify this hypothesis.

Fewer genes apparently had modified levels of expression between the AI and AM sub-groups. This could be expected as learning abilities in the AM group is significantly but not dramatically distinct from that of the AI rats. Of particular interest is the up-regulation of GIP in the AI versus AM animals. As noted above, a similar increase was also seen between the AI and AU sub-groups suggesting that further investigations of this gene and its translated peptide are certainly warranted. Among the genes that are apparently most significantly down-regulated in the AI versus AM rats, neurexin II beta-A precursor and brain hexokinase are of particular interest as similarly affected in the AI versus AU sub-groups.

In summary, distinct profiles of expression of genes were observed in aged rats with varying degrees of learning abilities as monitored in the MWM task. While it is not possible to demonstrate that these changes are directly related to altered cognitive behaviors at this early stage in our studies, the present results suggest possible novel targets (GIP, hexokinase, neurexin II, etc) that could be considered as an attempt to better understand the molecular origins of differential learning profiles of AI, AM and AU rats.

Acknowledgment

Supported by a grant from the Canadian Institutes for Health Research (CIHR) to R. Quirion. J. Brouillette receives support from the CIHR and J. O'Donnell receives support from the Fonds de Formation des Chercheurs et l'Aide à la Recherche (FCAR) and the CIHR. R. Godbout is supported by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada.

References

- Gage F.H., Dunnett S.B., Björklund A., Spatial learning and motor deficits in aged rats. *Neurobiol Aging* 1984; 5: 43-8.
- Issa A.M., Rowe W., Gauthier S., Meaney M.J., Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. *J Neurosci* 1990; 10: 3247-54.
- Rowe W.B., Sreekmeester E., Meaney M.J., Quirion R., Rochford J., Reactivity to novelty in cognitively-impaired and cognitively-unimpaired aged rats and young rats. *Neuroscience* 1998; 83: 669-80.
- Stemmelin J., Lazarus C., Cassel S., Kelche C., Cassel J.C., Immunohistochemical and neurochemical correlates of learning deficits in aged rats. *Neuroscience* 2000; 96: 275-89.
- Hollup S.A., Kjelstrup K.G., Hoff J., Moser M.B., Moser E.I., Impaired recognition of the goal location during spatial navigation in rats with hippocampal lesions. *J Neurosci* 2001; 21: 4505-13.
- Morris R.G.M., Schenk F., Tweedie F., Jarrard L.E., Ibotenate lesions of hippocampus and/or subiculum: Dissociating components of allocentric spatial learning. *Eur J Neurosci* 1990; 2: 1016-28.
- Law A., Dore S., Blackshaw S., Gauthier S., Quirion R., Alteration of expression levels of neuronal nitric oxide synthase and haem oxygenase-2 messenger RNA in the hippocampi and cortices of young adult and aged cognitively unimpaired and impaired Long-Evans rats. *Neuroscience* 2000; 100: 769-75.
- Levin E.D., Simon B.B., Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology* 1998; 138: 217-30.
- Richter-Levin G., Canevari L., Bliss T.V., Long-term potentiation and glutamate release in the dentate gyrus: links to spatial learning. *Behav Brain Res* 1995; 66: 37-40.
- Van der Zee E.A., Luiten P.G., Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. *Prog Neurobiol* 1999; 58: 409-71.
- Geschwind D.H., Gregg J.P., Microarrays for the Neurosciences: an Essential Guide. MA: MIT Press Cambridge, 2002.
- Marcotte E.R., Srivastava L.K., Quirion R., DNA microarrays in neuropsychopharmacology. *Trends Pharmacol Sci* 2001; 22: 426-36.
- Dore S., Kar S., Rowe W., Quirion R., Distribution and levels of [125I]IGF-I, [125I]IGF-II and [125I]insulin receptor binding sites in the hippocampus of aged memory-unimpaired and -impaired rats. *Neuroscience* 1997; 80: 1033-40.
- Le Jeune H., Cecyre D., Rowe W., Meaney M.J., Quirion R., Ionotropic glutamate receptor subtypes in the aged memory-impaired and unimpaired Long-Evans rat. *Neuroscience* 1996; 74: 349-63.
- Quirion R., Wilson A., Rowe W., Aubert I., Richard J., Doods H., Parent A., White N., Meaney M.J., Facilitation of acetylcholine release and cognitive performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. *J Neurosci* 1995; 15: 1455-62.
- Brandeis R., Brandys Y., Yehuda S., The use of the Morris water maze in the study of memory and learning. *Int J Neurosci* 1989; 48: 29-69.
- Rapp P.R., Rosenberg R.A., Gallagher M., An evaluation of spatial information processing in aged rats. *Behav Neurosci* 1987; 101: 3-12.
- Stoelzel C.R., Stavnezer A.J., Denenberg V.H., Ward M., Markus E.J., The effects of aging and dorsal hippocampal lesions: performance on spatial and nonspatial comparable versions of the water maze. *Neurobiol Learn Mem* 2002; 78: 217-33.
- Noda Y., Yamada K., Nabeshima T., Role of nitric oxide in the effect of aging on spatial memory in rats. *Behav Brain Res* 1997; 83: 153-8.
- Oler J.A., Markus E.J., Age-related deficits on the radial maze and in fear conditioning: hippocampal processing and consolidation. *Hippocampus* 1998; 8: 402-15.
- Ward M.T., Stoelzel C.R., Markus E.J., Hippocampal dysfunction during aging II: deficits on the radial-arm maze. *Neurobiol Aging* 1999; 20: 373-80.
- Winocur G., Gagnon S., Glucose treatment attenuates spatial learning and memory deficits of aged rats on tests of hippocampal function. *Neurobiol Aging* 1998; 19: 233-41.
- Bimonte H.A., Nelson M.E., Granholm A.C., Age-related deficits as working memory load increases: relationships with growth factors. *Neurobiol Aging* 2003; 24: 37-48.
- Niewiadomska G., Komorowski S., Baksalerska-Pazera M., Amelioration of cholinergic neurons dysfunction in aged rats depends on the continuous supply of NGF. *Neurobiol Aging* 2002; 23: 601-13.
- Sugaya K., Greene R., Personett D., Robbins M., Kent C., Bryan D., Skiba E., Gallagher M., McKinney M., Septo-hippocampal cholinergic and neurotrophin markers in age-induced cognitive decline. *Neurobiol Aging* 1998; 19: 351-61.
- Colombo P.J., Gallagher M., Individual differences in spatial memory among aged rats are related to hippocampal PKCgamma immunoreactivity. *Hippocampus* 2002; 12: 285-9.
- Stenvers K.L., Lund P.K., Gallagher M., Increased expression of type I insulin-like growth factor receptor messenger RNA in rat hippocampal formation is associated with aging and behavioral impairment. *Neuroscience* 1996; 72: 505-18.
- Sugaya K., Chouinard M., Greene R., Robbins M., Personett D., Kent C., Gallagher M., McKinney M., Molecular indices of neuronal and glial plasticity in the hippocampal formation in a rodent model of age-induced spatial learning impairment. *J Neurosci* 1996; 16: 3427-43.
- Poucet B., Save E., Lenck-Santini P.P., Sensory and memory properties of hippocampal place cells. *Rev Neurosci* 2000; 11: 95-111.

30. Ohya A., Hosaka K., Komiya Y., Akagawa K., Yamauchi E., Taniguchi H., Sasagawa N., Kumakura K., Mochida S., Yamauchi T., Igarashi M., Regulation of exocytosis through Ca²⁺/ATP-dependent binding of autophosphorylated Ca²⁺/calmodulin-activated protein kinase II to syntaxin 1A. *J. Neurosci* 2002; 22: 3342-51.
31. Hepp R., Grant N.J., Chasserot-Golaz S., Aunis D., Langley K., The hypophysis controls expression of SNAP-25 and other SNAREs in the adrenal gland. *Neurocytol* 2001; 30: 789-800.
32. Jacob T.C., Kaplan J.M., The EGL-21 Carboxypeptidase E Facilitates Acetylcholine Release at *Caenorhabditis elegans* Neuromuscular Junctions. *J. Neurosci* 2003; 23: 2122-30.
33. Jin K., Graham S.H., Nagayama T., Goldsmith P.C., Greenberg D.A., Zhou A., Simon cecing enzyme carboxypeptidase E in the rat brain after global ischemia. *J Cereb Blood Flow Metab* 2001; 21: 1422-9.
34. Missler M., Fernandez-Chacon R., Sudhof T.C., The making of neurexins. *Neurochem* 1998; 71: 1339-47.
35. Sun H.B., Yokota H., Chi X.X., Xu Z.C., Differential expression of neurexin mRNA in CA1 and CA3 hippocampal neurons in response to ischemic insult. *Mol Brain Res* 2000; 84: 146-9.
36. Kambayashi Y., Takahashi K., Bardhan S., Inagami T., Cloning and expression of protein tyrosine phosphatase-like protein derived from a rat pheochromocytoma cell line. *Biochem J* 1995; 1: 331-5.
37. Adams M.M., Smith T.D., Moga D., Gallagher M., Wang Y., Wolfe B.B., Rapp P.R., Morrison J.H., Hippocampal dependent learning ability correlates with N-methyl-D-aspartate (NMDA) receptor levels in CA3 neurons of young and aged rats. *J Comp Neurol* 2001; 432: 230-43.
38. Clayton D.A., Grosshans D.R., Browning M.D., Aging and surface expression of hippocampal NMDA receptors. *J Biol Chem* 2002; 277: 14367-9.
39. Arendash G.W., Sengstock G.J., Sanberg P.R., Kem W.R., Improved learning and memory in aged rats with chronic administration of the nicotinic receptor agonist GTS-21. *Brain Res* 1995; 674: 252-9.

Appendix III

Gene expression profiling following chronic NMDA
receptor blockade-induced learning deficits in rats

Gene Expression Profiling Following Chronic NMDA Receptor Blockade-Induced Learning Deficits in Rats

JAMES O'DONNELL, JEANNE STEMMEIN, ATSUMI NITTA,
JONATHAN BROUILLETTE, AND RÉMI QUIRION*

Douglas Hospital Research Centre, Departments of Psychiatry, Pharmacology & Therapeutics and Neurology & Neurosurgery, Verdun-Montréal, Québec, Canada, H4H 1R3

KEY WORDS MK-801; hippocampus; striatum; cDNA microarray; learning deficits; Morris water maze

ABSTRACT Acute treatments with MK-801, a noncompetitive antagonist of the NMDA glutamate receptor, induce spatial memory deficits in rodents. In the present study, we developed a low-dose chronic MK-801 treatment regimen that induced persistent learning deficits (determined by the Morris water maze task) after administration of the drug (0.2 mg/kg) every 12 h for 14 days. To determine the impact of such a treatment, changes in mRNA expression were investigated in the hippocampi and striata of treated animals using a cDNA membrane array followed by Western blots. Genes whose expression levels were found to be most altered included preprolactin (downregulated) and mitogen-activated protein kinase (MAP kinase 1; upregulated) in the hippocampus, and acyl-CoA synthetase (downregulated) and apolipoprotein D (upregulated) in the striatum. Furthermore, MAP kinase 1 and proteasome subunit β precursor was found to meet selection criteria for upregulation in both the hippocampus and striatum. Among other genes found to be most changed in the hippocampus were protein kinase C β I and II, protein tyrosine phosphatase 1 β , neuropilin I and II, adenosine receptor A1, and metabotropic glutamate receptor 2/3. The impact of some gene expression alterations on their corresponding protein levels was studied next. In the hippocampus, protein kinase C β I and II, protein tyrosine phosphatase, neuropilin I and II, adenosine receptor A, metabotropic glutamate receptor 2/3, and in the striatum phosphatidyl inositol 4 kinase, mitogen-activated protein kinase 1, adenylyl cyclase II, dopamine receptors 1A and 2, and cytochrome C oxidase subunit Va gene and protein expression levels were found to be highly correlated. These results suggest the potential involvement of several genes and proteins in the neuropharmacological effects of MK-801 and possibly the persisting cognitive deficits induced by this repeated drug treatment. *Synapse* 50:171-180, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Learning and memory-related behaviors are complex processes based on the interplay between myriad genomic, molecular, and environmental events (Kandel, 2001; Matynia, 2001). Among the many neurotransmitter systems studied thus far, much evidence suggests key roles for glutamate (Bliss, 1990; Lee and Kesner, 2002; Morris, 1986, 1989; Richter-Levin, 1995), acetylcholine (Beninger, 1989; Van der Zee and Luiten, 1999; Levin and Simon, 1998), and nitric oxide (Haley et al., 1992; Law et al., 2000; Schuman and Madison, 1991) in cognition, and in the cascade of events leading to improved mnemonic capacities. One of the most studied cellular models of learning and memory is long-term potentiation (LTP), a phenomenon

known to occur during various forms of learning (Larkman and Jack, 1995; Lisman and McIntyre, 2001). During LTP, plastic changes occur in glutamatergic and NO-related neurotransmission (Grassi and Pettorossi, 2001; Larkman and Jack, 1995). However,

Contract grant sponsors: the Canadian Institutes for Health Research (CIHR) (to R.Q., J.O'D., J.B.), the Fonds de Formation des Chercheurs et l'Aide à la Recherche (FCAR) (to J.O'D.), the Bettencourt-Schueller Foundation (to J.S.).

J.O'D. and J.S. contributed equally to this work.

*Correspondence to: Rémi Quirion, Douglas Hospital Research Centre, 6875 LaSalle Boulevard, Verdun-Montréal, Québec, H4H 1R3, Canada. E-mail: quirem@douglas.mcgill.ca

Received 6 March 2003; Accepted 13 June 2003

DOI 10.1002/syn.10258

many of the detailed molecular events involved in LTP remain to be fully established. This is even more applicable for complex forms of cognitive behaviors.

In order to improve basic knowledge on the critical mechanisms involved in learning behaviors, various animal models have recently been developed, including transgenic mice with altered glutamatergic- (Myers, 1999; Seeburg, 2001) and NO- (Dere, 2001; Frisch, 2000; Huang and Fishman, 1996) related neurotransmission, various models of Alzheimer-type dementia based on the β -amyloid hypothesis (Dawson, 1999; Hsiao, 1998) as well as aged mammals since cognitive deficits are known to occur in a certain proportion of an aged cohort in several species (Albert, 1987; Shen, 1997). Learning deficits can also be induced pharmacologically by using inhibitors of, for example, glutamatergic (Kretschmer and Fink, 1999; Pitkanen, 1995; Whishaw and Auer, 1989) and cholinergic (Izquierdo, 1989) neurotransmission. However, in these latter models cognitive deficits are usually observed acutely (Kretschmer and Fink, 1999; Pitkanen, 1995), hence possibly involving different substrates compared to more chronic situations. In the present series of experiments, we first developed a chronic model of spatial, hippocampus-based learning deficits using a low daily dose regimen of MK-801, a known antagonist of the NMDA receptor blocking glutamatergic neurotransmission (Kretschmer and Fink, 1999; Pitkanen, 1995). A combined cDNA microarray-protein Western blot analysis strategy was then used to investigate genes correlated with the persisting behavioral deficits induced by the chronic inhibition of NMDA receptor neurotransmission. Clusters of gene families including various kinases were found to be particularly altered in our model, suggesting their potential involvement in chronic MK-801 treatment-induced learning deficits.

MATERIALS AND METHODS

Animals

Male rats (Long-Evans) used in these studies were obtained from Charles River (St. Constant, Québec) at 3–4 months of age (250–300 g) and were singly housed in cages 43 × 20 × 20 cm with environmental enrichment. They were maintained on a 12:12 light/dark schedule with access to food (Purina Lab Chow, St. Louis, MO) and water ad libitum. Animals were handled for several days prior to testing. All animal care and handling procedures were approved by McGill University Animal Care Committee and the Canadian Council for Animal Care.

Drugs

Rats in the experimental group ($n = 12$ /experiment) were given intraperitoneal (i.p.) injections of 0.2 mg/kg (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801 or dizocilpine) (Sigma-

Aldrich, Oakville, Ontario) using a solution of 1 mg/ml every 12 h for days 1–14. Rats in the control group ($n = 12$ /experiment) were given proportional volumes of isotonic saline vehicle (Abbott Laboratories, Montréal, Québec) using the same dosing regimen.

Water maze testing

The spatial memory of animals was assessed using the Morris water maze task (Stemmelin et al., 2000; Issa et al., 1990; Dore et al., 1997; Hersi et al., 1995; Issa et al., 1990; Le Jeune et al., 1996; Parent et al., 1995; Quirion et al., 1995; Rowe et al., 1998). Briefly, rats were given four trials per day to find a submerged platform located 2.5 cm below the surface of water (rendered opaque by the addition of skim milk powder) in a 1.6 m diameter pool. Animals use only distal visuospatial cues available within the testing room to locate the submerged platform. Rats were tested initially during days 1–3 of treatment and again (with a new platform location) on days 15–18 days (starting 24 h after the last drug administration). In order to eliminate the effects of learning on gene expression in this second series of tests (days 15–18), another set of animals was treated with either MK-801 or saline in an identical manner but were not subjected to the second set of behavioral tests. Animals were also assessed for motor, visual, and motivational deficits on day 4 by raising the platform 2 cm above the surface of the water (visually cued condition). Average escape latency and distance were recorded along with swim paths using a video tracking system (HVS, Buckingham, UK). Statistical significance was assessed using ANOVA and independent *t*-tests with Bonferroni correction.

RNA isolation and probe labeling

On day 19, animals were euthanized by decapitation. Brains were quickly removed and the hippocampi and striata were dissected, pooled, and immediately stored at -80°C until use in order to preserve RNA integrity. Tissues were subsequently homogenized in AtlasPure denaturing solution (BD Biosciences ClonTech, Palo Alto, CA) and total RNA was isolated. The quality, integrity, and quantity of the RNA from the hippocampi and striata of rats were determined (i.e., intact ribosomal RNA bands on electrophoresis and UV absorption spectrophotometry) and the RNA pooled (Marcotte et al., 2001). The total RNA was DNase-I (BD Biosciences ClonTech) treated, phenol-chloroform extracted, and ethanol precipitated. Complex probes were generated by reverse transcription using the total RNA, freshest possible ^{32}P -labeled dATP (Perkin-Elmer Life Sciences, Woodbridge, Ontario) and an Atlas gene-specific mix of oligonucleotide primers (ClonTech). Unincorporated radiolabeled nucleotides were removed with Nucleospin extraction spin columns

(ClonTech) and probe yields were quantified by liquid scintillation counting.

Hybridization and imaging of atlas arrays

Clontech Atlas Rat 1.2 arrays and reagents were used for analysis of gene expression according to the manufacturer's protocols. These membrane format arrays were chosen not only for initial investment considerations, but also because they are limited to known genes. Furthermore, array cDNA spots are short regions of cDNA selected for low homology to other genes, and gene-specific primers are used in probe syntheses. The array data in this report were generated from two identical experiments, each yielding two sets of array data for a total of four comparisons per structure (four treated group and four control group hybridizations). In addition, two sets of arrays per structure were performed on the control and experimental groups that were not assessed in the Morris maze on days 15–18. The results from these latter groups were used to eliminate gene expression changes induced by changes in learning on days 15–18. Each hybridization was performed using new membranes each time. The membranes for each drug condition were separately prehybridized in ExpressHyb buffer (ClonTech), and the ^{32}P -labeled probes from above were denatured, diluted with sheared salmon testes DNA (Sigma-Aldrich), and an equal amount added to each of the membranes. Hybridization at 68°C was allowed to proceed for 18 h, after which the membranes were washed and exposed to Kodak Bio-Max MS film (VWR Canlab, Saint-Laurent-Montréal, Québec) using a Kodak high-energy intensifying screen (VWR Canlab) for at least three exposures per membrane (14 h to 14 days). Array images were acquired using an MCID System (Imaging Research, Saint-Catharines, Ontario).

Analysis of array data

Images of each array were first imported into Atlasimage software (v. 1.5; ClonTech) for densitometric measurement of gene- and array-specific parameters such as intensity and global background levels and visual validation of spot quality. Raw intensity values were exported to Microsoft Excel. A z-score normalization method based on a z-ratio for the difference between means was first used to normalize each array (Becker et al., 2001; Guilford and Fruchter, 1973). This provides an indication of statistical significance of the expression changes. The raw intensity values were first converted to z-scores as described by Becker et al. (2001). Data were filtered to eliminate genes detected showing z-scores of less than 2 and greater than -2 for two or more experiments. From these z-scores, expression ratios (z-ratios) were calculated. Z-normalization is internal to an individual array and may allow comparability of array results across experiments. Genes

were selected based on significant z-ratios. In addition, for each set of arrays a traditional normalization factor was determined by dividing the average total intensity by the true total intensity. Each original intensity value was then multiplied by the normalization factor. A simple ratio for each gene was then calculated using the normalized intensities and averaged with ratios over replicate array values. Genes with a 15% or smaller difference were removed from the list. The remaining genes are the most consistently detected significantly changed gene expressions and therefore the best candidates for gene expression being modulated in this model. In order to eliminate differences in gene expression between MK-801 and vehicle-treated rats due to altered maze learning on days 15–18, gene expression changes observed for animals subjected to this series of tests was compared with changes observed for animals not subjected to them. Those changes observed in the former may be attributable to altered learning on days 15–18 rather than on persistent deficits due strictly to the effects of chronic MK-801 treatment. They were thus excluded from further analysis.

Western blotting

Proteins were extracted from dissected brain regions by homogenization and sonication in 10 volumes of ice-cold buffer; pH 7.4, Tris-HCl (50 mM), EDTA (1 mM) and protease inhibitors (Sigma-Aldrich) including leupeptin (1 μM), aprotinin (1 μM), and sodiumorthovanadate (0.2 mM). Samples were incubated at 4°C for 20 min. Protein content was assayed using a spectrophotometric assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as the standard. SDS 2× sample buffer (Novex, Carlsbad, CA) was added in equal volume. The sample was vortexed for 10 min and then centrifuged at 15,000g RCF for 15 min. The supernatant was then transferred to a new tube and boiled for 5 min prior to loading. Samples were next subjected to Tris-glycine gel electrophoresis and transferred to nitrocellulose by electroblotting. Blots were blocked using Blocking Solution (Novex) for 1 h at room temperature, before incubation for 1 h with the primary antibody (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA) or (Upstate, Waltham, MA) in blocking solution at room temperature. Blots were washed with antibody wash solution (Invitrogen, Carlsbad, CA) and water, incubated with alkaline-phosphatase-conjugated IgG secondary antibody (Invitrogen) in blocking buffer for 30 min at room temperature, washed, and immunoreactivity visualized on film by CDP star substrate (Invitrogen). Densitometry (Imaging Research MCID system, St. Catharines, Ontario) was used to quantify changes in protein levels. To control for inter-lane variability in protein loading, optical density values for protein bands were normalized to those of cor-

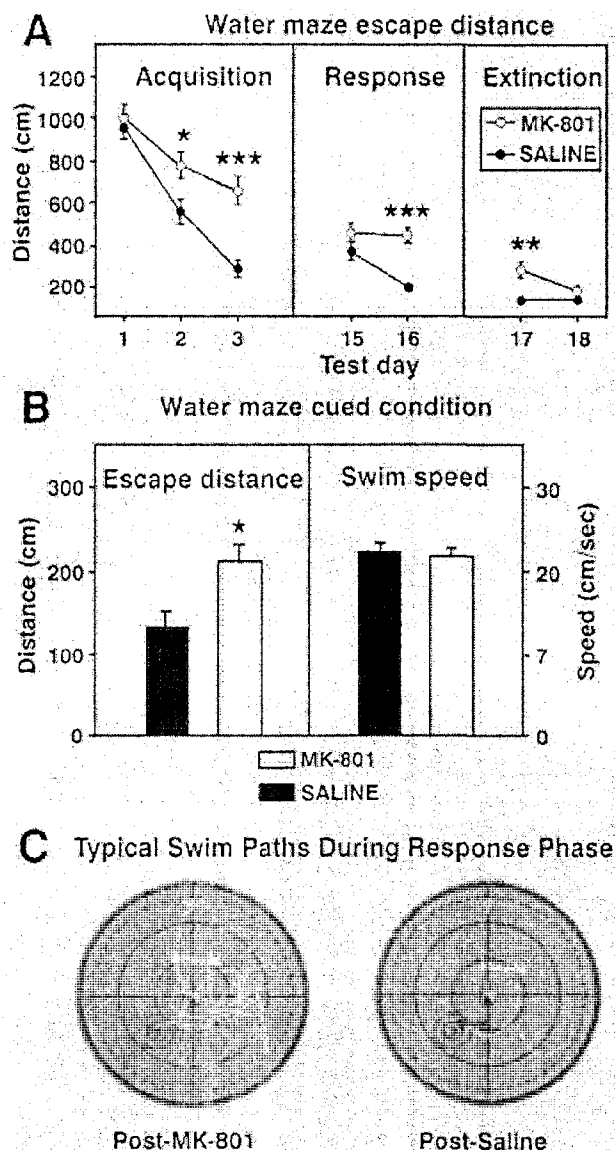


Fig. 1. Performances of chronic MK-801- and vehicle-treated Long-Evans rats as evaluated using the Morris water maze behavioral task. Escape distances in centimeters during acquisition, response and extinction phases of testing are shown (A). Escape distance and swim speed in the cued condition are also shown (B). Examples of typical swim patterns observed during the response phase are presented (C). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. controls, ANOVA and post-hoc t -tests.

responding tubulin bands, by co-incubating blots with an anti- β -tubulin antibody (1:800; Sigma).

RESULTS

Water maze performance

Rats treated with MK-801 (i.p. injection 20 min prior to testing) displayed impaired learning in the Morris water maze task compared to vehicle-treated animals as shown in Figure 1A. ANOVA on distance swam showed significant Drug ($F_{1,42} = 12.6$, $P < 0.001$) and

Day ($F_{2,84} = 47.2$, $P < 0.001$) effects, as well as a significant interaction between both factors ($F_{2,84} = 4.8$, $P < 0.05$). The interaction reflected decreases in the swim speeds which were less in MK-801-treated rats than vehicle-treated rats. All rats performed poorly on day 1. On days 2 and 3, MK-801 increased escape distance in the water maze ($P < 0.05$ and $P < 0.001$, respectively) compared to saline-treated animals. Twenty-four hours after the last injection, a long-term response of chronic MK-801 treatment was measured (days 15 and 16) (see Fig. 1C for representative swim paths). ANOVA on distance swam also showed significant Drug ($F_{1,42} = 23.2$, $P < 0.001$) and Day ($F_{1,42} = 5.7$, $P < 0.05$) effects, as well as a significant interaction between both factors ($F_{1,42} = 4.5$, $P < 0.05$). The interaction reflects evidence of a between-day decrease of the distance swam in vehicle-treated rats, while no improvement was observed in the MK-801-treated group. On day 15, the difference between vehicle and drug-treated groups was not significant. However, on day 16 MK-801 significantly impaired performances ($P < 0.001$). For the two following days (response extinction phase), distance swam of the treated group still significantly differed on day 17 ($P < 0.01$) but the difference was completely abolished on day 18. Two subjects were removed from the study because they were uncoordinated, ataxic, and unbalanced when MK-801 was administered. On day 4, immediately after acquisition learning, rats were tested for visually guided behavior with a raised platform in the water maze. Analysis showed that MK-801 induced an impairment in distance swam ($F_{1,42} = 6.5$, $P < 0.05$), but that swim speed did not differ significantly from vehicle-treated animals (Fig 1B).

Microarray results

In the hippocampus (Table I), 16 genes met the selection criteria (genes were selected if their z -ratios were greater than 2 or less than -2 and their median normalized change was greater than 15%) compared with 23 genes in the striatum (Table II). Among the genes altered were preprolactin (downregulated) and mitogen-activated protein kinase (MAP kinase or extracellular signal regulated kinase 1) (upregulated) in the hippocampus, and brain long-chain fatty acid-CoA ligase (acyl-CoA synthetase) (downregulated) and apolipoprotein D (upregulated) in the striatum. It is of note that MAP kinase I and proteasome β subunit precursor were found to meet selection criteria for upregulation in both the hippocampus and striatum (Tables I, II). Figure 2 shows radiographic images of representative membranes for each treatment group and brain structure.

Confirmation of differential protein expression

Hippocampus and striatum protein expression was analyzed using immunoblotting. Protein expression to

TABLE I. Gene expression pattern observed in the hippocampus of chronically treated MK-801 rats

Gene code	Gene name	Genbank accession no.	Average z-ratio	Average simple ratio after median normalization
E04d	preprolactin (Prl)	AF022935	-3.41	0.73
D11n	neuropilin	AF018957	-3.26	0.82
F12f	adenosine A1 receptor (ADORA1)	M64299	-2.79	0.79
E01a	insulin like growth factor II (IGF-II)	M13969	-2.26	0.83
D08n	NMDA receptor (NMDAR 1); glutamate receptor subunit ζ 1 precursor; NR1	X63255	-2.16	0.83
C06c	fatty acid amide hydrolase	U72497	2.15	1.77
D12j	glutamate metabotropic receptor 2 (mGluR2)	M92075	2.20	1.70
C11e	40S ribosomal protein S12	M18547	3.11	2.89
F10j	proteasome B subunit precursor; macropain B; multicatalytic endopeptidase complex B; proteasome chain 3; RN3; PSMB4	L17127	2.48	1.55
B08f	sodium/hydrogen exchange protein 1	M85299	3.19	2.52
C10l	acetylcholinesterase, T subunit, glycolipid-anchored	S50879	3.40	2.57
D13e	neuropilin 2	AF016297	3.81	1.96
E08k	protein kinase C B-I type (PKC-B I) + protein kinase C B-II type (PKC-B II)	M19007; X04440	4.32	1.80
E12f	protein tyrosine phosphatase 1 β	M33962	5.74	2.65
E08b	extracellular signal-regulated kinase 2 (ERK2); mitogen-activated protein kinase 2 (MAP kinase 2; MAPK2); p42-MAPK; ERT1	M64300	6.08	2.84
E08a	extracellular signal-regulated kinase 1 (MAP kinase 1); mitogen-activated protein kinase 1 (MAP kinase 1; MAPK1); insulin-stimulated microtubule-associated protein-2 kinase, MNK1; PRKM3; ERT2; p44-MAPK	M61177	6.26	3.02

Hippocampal cDNA array gene expression levels reaching criteria for changes sorted by average z-ratio and the median normalized expression ratios of the expression data are presented. Array hybridization was repeated four times over two complete experiments.

TABLE II. Gene expression pattern observed in the striatum of chronically treated MK-801 rats

Gene code	Gene	Genbank accession no.	Average z-ratio	Average median normalized expression ratio
C05d	brain long-chain fatty acid-CoA ligase (LACS); acyl-CoA synthetase	D10041	-3.20	0.43
F02g	PI4-K; phosphatidylinositol 4-kinase (230 kDa)	D83538	-2.79	0.46
D12b	non-processed neurexin II-B major, Neurexin II-B-A-precursor + Non-processed neurexin II- α , Neurexin II- α -B Precursor	M96377; M96376	-2.64	0.46
E14a	guanine nucleotide-binding regulatory, α subunit	J03773	-2.62	0.49
C04g	creatine kinase, ubiquitous, mitochondrial	X59737	-2.59	0.48
E10f	CamK II; calcium/calmodulin-dependent protein kinase brain type II B	M16112	-2.35	0.50
C03g	ATPase, subunit F, vacuolar (vati)	U43175	-2.32	0.51
C01j	synaptophysin, p38	X06655	2.36	2.05
F12g	adenosine A2A receptor (ADORA2A)	S47609	2.41	1.73
A02m	neural adhesion molecule F3, Rat neural adhesion molecule F3, Complete CDS	D38492	2.59	1.81
C12a	40S ribosomal protein S17 (RPS17)	K02933	3.58	2.37
F05f	neuromodulin; axonal membrane protein GAP43; PP46; B-50; protein F1; calmodulin-binding protein P-57	M61177	3.75	3.09
E08g	extracellular signal-regulated kinase 1; mitogen-activated protein kinase 1 (MAPK1); insulin-stimulated microtubule-associated protein-2 kinase; MNK1; PRKM3; ERT2; p44-MAPK	X62908	3.80	2.33
D04g	thyroid stimulating hormone receptor	M34842	4.24	2.86
F03f	adenylyl cyclase type II	J02942	4.42	3.78
D03c	D(2) dopamine receptor	M36831	4.65	3.91
F14j	neuronatin	U08290	4.73	3.25
D07a	neurotensin receptor type 2	X97121	4.85	2.93
A08d	microglobulin; B-2-microglobulin + prostaglandin receptor F2a	X16956; U26663	5.04	3.47
D08a	D(1A) dopamine receptor	M35077	5.15	4.95
C03j	cytochrome c oxidase, subunit Va, mitochondrial	X15030	5.41	3.50
F10j	proteasome B subunit precursor; macropain B; multicatalytic endopeptidase complex B; proteasome chain 3; RN3; PSMB4	L17127	5.46	3.77
A09n	apolipoprotein D	X55572	6.03	4.27

Striatal cDNA array gene expression levels reaching criteria for changes sorted by average z-ratio and the median normalized expression ratios of the expression data are presented. Array hybridization was repeated four times over two complete experiments.

be confirmed was chosen based on the degree of change in mRNA expression, the direction of the change (two suppressed and five induced), and, most critically, the availability of suitable, highly selective and specific antisera. In general, the magnitude of the changes in

protein expression was observed to be somewhat less than changes in mRNA expression (Fig. 3). However, they were always in the same direction. In the hippocampus, the repeated MK-801 treatment was found to decrease the expression of adenosine A1 receptor

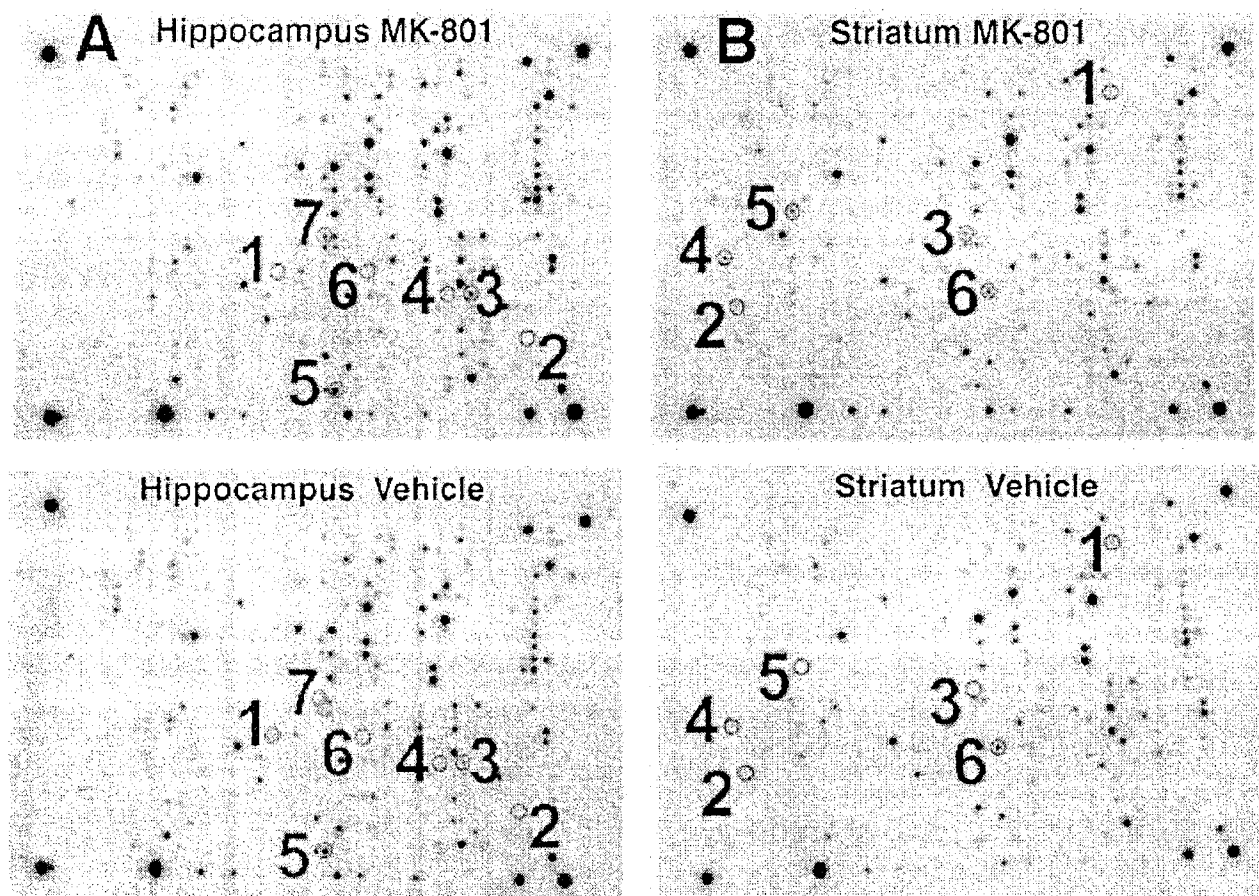


Fig. 2. cDNA array expression patterns in the hippocampus (A) and the striatum (B) of MK-801- and vehicle-treated rats. MCID images of Atlas Rat 1.2 arrays. Images from one experiment obtained from a 2-day radiographic screen exposure are shown. In A, the location on the array grid corresponding to hippocampal expression of preprolactin (1), proteosome β subunit precursor (2), neuromodulin (3), adenylyl cyclase II (4), protein kinase C β I and II (5), protein tyrosine

phosphatase 1β (6), and MAP kinase 1 (7) are shown. In B, the location corresponding to striatal expression of 1, brain long-chain fatty acid-CoA ligase (acyl-CoA synthetase) (1), neuromodulin (2), MAP kinase 1 (3), adenylyl cyclase II (4), proteosome β subunit precursor (5), apolipoprotein D (6) are shown. For gene grid assignment, refer to ClonTech's Atlas Rat 1.2 Array web site (http://www.clontech.com/atlas/genelists/7854-1_Ra12.pdf).

and neuropilin with ratios of 0.64 and 0.71, respectively. On the other hand, MK-801 was observed to increase the expression of protein tyrosine phosphatase (Protein tyrosine phosphatase 1β), protein kinase C (PKC) β I and II, neuropilin II, and the metabotropic glutamate receptor 2/3 with ratios of 1.20, 1.39, 1.40, 1.48, and 1.56, respectively. β -Tubulin was not altered by the repeated MK-801 treatment (Fig. 3). In the striatum, the repeated MK-801 treatment was found to decrease the expression of phosphatidyl inositol 4 kinase and to increase the expression of adenylyl cyclase II, mitogen-activated protein kinase I, cytochrome c, and dopamine receptors 1A and 2 with ratios of 0.5, 1.36, 1.62, 1.12, 1.68, and 1.40, respectively.

DISCUSSION

The key findings of the present study include 1) the demonstration that it is possible to induce lasting learning deficits using a repeated treatment regimen of

MK-801, an NMDA receptor antagonist; 2) the observation that such a treatment induces changes in a variety of genes in the hippocampus and striatum as examined by cDNA microarrays, with various genes associated with signal transduction being particularly altered; and 3) changes in the expression of several gene mRNAs are reflected by comparable alterations in the level of the translated proteins in the hippocampal formation. Taken together, these results suggest that the observed changes in the various genes and gene products are likely related in some way to the persistent behavioral effects induced by chronic treatment with the NMDA receptor antagonist.

We have shown here that a 14-day treatment with MK-801 induced lasting impairments in the Morris water maze task. Deficits were observed for at least up to 3 days after the last injection of the NMDA receptor antagonist. This far exceeds the reported plasma half-life of this drug, which is in the range of 1–4 h (Hucker,

Hippocampus			
	Veh. MK-801	Protein	mRNA
Adora A1		0.64	0.79
Neuropilin		0.71	0.82
PTPase 1 β		1.20	2.65
PKC β II		1.40	1.80
PKC β I		1.39	1.80
Neuropilin 2		1.48	1.96
mGlu 2/3		1.56	1.70
β -Tubulin		1.00	1.00
Striatum			
	Veh. MK-801	Protein	mRNA
PI4-K		0.50	0.46
MAPK-1		1.62	2.33
Cyt. C		1.12	3.50
A. Cyclase II		1.36	3.78
D2 DA-R		1.40	3.91
D1 DA-R		1.68	4.95
β -Tubulin		1.00	1.00

Fig. 3. Hippocampal and striatal mRNA and protein expression levels. In the upper panel (hippocampus), mRNA and protein expression ratios are shown for protein kinase C β I and II, protein tyrosine phosphatase 1 β , neuropilin I and II, adenosine receptor A1 and metabotropic glutamate receptor 2/3 and β -tubulin controls. In the lower panel (striatum), mRNA and protein expression ratios are

shown for phosphatidylinositol 4 kinase (PI4-K), mitogen-activated kinase 1 (MAPK-1), cytochrome C oxidase subunit Va (Cyt. C), adenylyl cyclase type II (A. Cyclase II) and dopamine receptors 2 and 1 (D2 DA-R and D1 DA-R). Results are presented as an expression ratio of MK-801-treated over vehicle-treated. For comparison, the mRNA expression ratios for these genes are also presented.

1983). Hence, our treatment induced a lasting deficit in spatial learning ability. While the acute blockade of the NMDA receptor is well known to hinder various forms of learning (Ahlander, 1999; Brosnan-Watters, 1996; Richter-Levin, 1995; McLamb, 1990), much less data are available on the impact of chronic inhibition of NMDA-associated neurotransmission using relatively low doses of an antagonist such as MK-801 (Gorter and de Bruin, 1992). Hence, the MK-801 treatment approach developed here, namely, 0.2 mg/kg i.p. every 12 h for 14 days, is novel and induced impaired cognitive abilities. We did not perform a detailed histological assessment of neurotoxicity in our model. However, we did not observe a pattern of gene expression suggesting an ongoing apoptotic or inflammatory process, although we recognize the limitation of our assumptions. Our hypothesis is that the noted impairments are due in part to the direct or downstream effects of changes in the expression of various gene products involved in the maintenance of normal cognitive abilities. Interestingly, the treated and control subjects were found to be significantly different in the visible platform trial. This finding has previously been shown to confound cognitive analysis of MK-801-treated subjects (Ahlander, 1999). This is likely to be due, in part, to the effect of the MK-801 treatment on striatum-based learning and we have noted certain changes in gene expression in this region (McDonald and White, 1994).

While several neurotransmitter systems have been suggested to be involved in spatial learning (Beninger, 1989; Law, 2000; Levin and Simon, 1998; Schuman and Madison, 1991; Van der Zee and Luiten, 1999), as well as in glutamate-induced memory abilities (Lee and Kesner, 2002; Morris, 1986, 1989; Richter-Levin, 1995), much remains to be established, particularly in terms of intracellular mechanisms. Accordingly, we used a cDNA microarray approach (Geschwind and Gregg, 2002; Marcotte, 2001) to globally explore a variety of gene and gene products as potential candidates. Transcriptomes were investigated in the hippocampus and the striatum, the former region being particularly associated with spatial learning in rodents (Kesner, 1987; Morris, 1990), while the latter is linked with the acquisition of visible platform learning (Ahlander, 1999). Interestingly, some gene products (MAP kinase, proteasome β subunit precursor) are similarly affected in both structures. It is suspected that presynaptic protein kinase activities support LTP in the hippocampus and that perturbation of presynaptic kinases can alter synaptic plasticity (Pavlidis, 2000). It is tempting to speculate that some of the observed gene changes may play an adaptative role to reverse some of the molecular events leading to impaired learning induced by a repeated MK-801 treatment.

Multiple gene mRNA products such as MAP kinase II, protein tyrosine phosphatase 1β , preprolactin, neuropilin I and II, NMDA receptor 1, metabotropic gluta-

mate receptor 2, adenosine receptor A1, acetylcholinesterase T-subunit, and protein kinase C- β (PKC- β) were uniquely affected in the hippocampus by the repeated MK-801 treatment. Evidence exists for a functional role of some of these in learning and memory. For example, mice lacking the NMDA receptor 1 gene were observed to have aberrant firing of CA1 pyramidal neurons correlating with improper representation of spatial place coding (McHugh, 1996). Moreover, the synaptic expression of NMDA receptor 1 subunit in the hippocampus was found to be specifically associated with memory formation of an inhibitory avoidance task (Cammarot, 2000). Furthermore, knockout mice deficient in the metabotropic glutamate receptor 2 were found to be deficient in hippocampal LTD, but unimpaired in the water maze (Yokoi, 1996).

Cholinergic systems have also been extensively shown to be primary actors in the modulation of learning and memory (Van der Zee, 1999). Furthermore, an adenosine receptor A1/A2 antagonist (theophylline) enhanced spatial learning in the radial arm maze in rats (Hauber and Bareiss, 2001). Suppression of adenosine action on its A1 receptors was also shown to be essential for the development of NMDA-dependent LTP in the hippocampus (de Mendonca and Ribeiro, 2000). In another study using the water maze, 6-month-old Long-Evans rats demonstrated a significant relationship between hippocampal PKC (α , β 2, and γ isoforms) content in subcellular fractions and performance in this spatial memory task (Colombo, 1997). Hence, the current study suggests that at least some of the gene expression changes are related to the longer than usual reference memory deficits displayed by rats treated for 14 days with MK-801. In addition, MAP kinase cascade activation in the hippocampus has been shown to be an important part of spatial learning (Selcher, 1999). However, to our knowledge, many other genes found to be differentially expressed such as protein tyrosine phosphatase 1β , neuropilin I and II, and preprolactin have not yet been reported to play a critical role in spatial learning behavior or LTP following an exposure to administration of MK-801. Future studies on the possible role of these genes and their products in these biological events could uncover new mechanisms associated with learning and memory-related behaviors.

Some genes, such as brain long-chain fatty acid-CoA ligase (LACS), phosphatidyl inositol 4-kinase (PI4-K), dopamine receptors 1A and 2, apolipoprotein D, and cytochrome c oxidase subunit Va were found to be differentially expressed only in the striatum. The role of the caudate and putamen in spatial learning and memory has not been fully elucidated, although it appears that it can be differentiated from that of the hippocampus (McDonald and White, 1993; Devan and White, 1999). In the water maze task, the striatum is suspected to be involved in acquiring a response to

directly swimming to a visible platform (McDonald and White, 1994). Further studies are required to determine the role of the observed gene expression changes in striatum-based learning tasks.

In the next series of experiments, we investigated whether the observed changes in gene expression led to altered protein levels. This is critical, as mRNA stability and turnover, as well as translational efficiency and posttranscriptional regulation, can lead to unaltered protein levels in spite of markedly up/downregulated mRNA transcripts (Lannes, 1995; Le Jeune, 1996). Most interestingly, several genes found to be differentially expressed in the hippocampus following a repeated MK-801 treatment resulted in altered protein levels. Among them, PKC- β 1 and 2, adenosine receptor A1, and metabotropic glutamate receptor 2 have previously been suggested to play important roles in LTP and/or spatial learning ability in rodents (McHugh, 1996). These results provide further support for the validity of our model and the chosen approach and suggest that other gene products such as neuropilin I and II, adenylyl cyclase II, and protein tyrosine phosphatase 1 β should be investigated. In the striatum, protein expression levels investigated also bore out predictions made based on the array data.

In summary, chronic treatment of rats with MK-801 results in the induction of persistent learning and memory impairments and is associated with structure-specific gene expression alterations in the hippocampus and the striatum. Genes involved in cell signal transduction such as kinases and phosphatases were profoundly affected by the treatment. Furthermore, altered protein levels for certain genes were found to correlate well with changes in mRNA expression levels in the hippocampus and the striatum. Future studies should now aim at establishing the role of the altered gene expression in learning deficits observed after the chronic MK-801 treatment. Recent progress in cognitive neuroscience suggested an important role for regulators of protein phosphorylation (kinases and phosphatases) in forming and maintaining memories in the mammalian brain. In light of this and the results of the present study, an examination of the role of hippocampal PTPase 1B and the MAP kinases is warranted.

REFERENCES

- Ahlander M, Misane I, Schott PA, Ogren SO. 1999. A behavioral analysis of the spatial learning deficit induced by the NMDA receptor antagonist MK-801 (dizocilpine) in the rat. *Neuropsychopharmacology* 21:414-426.
- Albert M, Duffy FH, Naeser M. 1987. Nonlinear changes in cognition with age and their neuropsychologic correlates. *Can J Psychol* 41:141-157.
- Becker KG, Teichberg D, Gray K, Vawter MP, Wood WHI, Cheadle C. 2001. cDNA microarrays in neuroscience: nylon membrane based arrays. In: Geschwind DH, Van Deerlin V, editors. *DNA microarrays: the new frontier in gene discovery and gene expression analysis*. Washington, DC: Society for Neuroscience. p 12-30.
- Beninger RJ, Wirsching EA, Jhamandas K, Boegman RJ. 1989. Animal studies of brain acetylcholine and memory. *Arch Gerontol Geriatr Suppl* 1:71-89.
- Bliss TV, Errington ML, Lynch MA. 1990. Long-term potentiation in the dentate gyrus in vivo is associated with a sustained increase in extracellular glutamate. *Adv Exp Med Biol* 268:269-278.
- Brosnan-Watters G, Wozniak DF, Nardi A, Olney JW. 1996. Acute behavioral effects of MK-801 in the mouse. *Pharmacol Biochem Behav* 53:701-711.
- Cammarota M, de Stein ML, Paratcha G, Bevilacqua LR, Izquierdo I, Medina JH. 2000. Rapid and transient learning-associated increase in NMDA NR1 subunit in the rat hippocampus. *Neurochem Res* 25:567-572.
- Colombo PJ, Wetsel WC, Gallagher M. 1997. Spatial memory is related to hippocampal subcellular concentrations of calcium-dependent protein kinase C isoforms in young and aged rats. *Proc Natl Acad Sci USA* 94:14195-14199.
- Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, O'Dowd G, Bowery BJ, Boyce S, Trumbauer ME, Chen HY, Van der Ploeg LH, Sirinathsinghji DJ. 1999. Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. *Neuroscience* 90:1-13.
- de Mendonca A, Ribeiro JA. 2000. Long-term potentiation observed upon blockade of adenosine A1 receptors in rat hippocampus is N-methyl-D-aspartate receptor-dependent. *Neurosci Lett* 291:81-84.
- Dere E, Frisch C, Souza Silva MA, Godecke A, Schrader J, Huston JP. 2001. Unaltered radial maze performance and brain acetylcholine of the endothelial nitric oxide synthase knockout mouse. *Neuroscience* 107:561-570.
- Devan BD, White NM. 1999. Parallel information processing in the dorsal striatum: relation to hippocampal function. *J Neurosci* 19:2789-2798.
- Dore S, Kar S, Rowe W, Quirion R. 1997. Distribution and levels of [125I]IGF-I, [125I]IGF-II and [125I]insulin receptor binding sites in the hippocampus of aged memory-unimpaired and -impaired rats. *Neuroscience* 80:1033-1040.
- Frisch C, Dere E, Silva MA, Godecke A, Schrader J, Huston JP. 2000. Superior water maze performance and increase in fear-related behavior in the endothelial nitric oxide synthase-deficient mouse together with monoamine changes in cerebellum and ventral striatum. *J Neurosci* 20:6694-6700.
- Geschwind DH, Gregg JP. 2002. *Microarrays for the neurosciences: an essential guide*. Cambridge, MA: MIT Press. p 63-108.
- Gorter JA, de Bruin JP. 1992. Chronic neonatal MK-801 treatment results in an impairment of spatial learning in the adult rat. *Brain Res* 580:12-17.
- Grassi S, Pettorossi VE. 2001. Synaptic plasticity in the medial vestibular nuclei: role of glutamate receptors and retrograde messengers in rat brainstem slices. *Prog Neurobiol* 64:527-553.
- Guilford JP, Fruchter B. 1973. *Fundamental statistics in psychology and education*. New York: McGraw-Hill.
- Haley JE, Wilcox GL, Chapman PF. 1992. The role of nitric oxide in hippocampal long-term potentiation. *Neuron* 8:211-216.
- Hauber W, Bareiss A. 2001. Facilitative effects of an adenosine A1/A2 receptor blockade on spatial memory performance of rats: selective enhancement of reference memory retention during the light period. *Behav Brain Res* 118:43-52.
- Hersi AI, Rowe W, Gaudreau P, Quirion R. 1995. Dopamine D1 receptor ligands modulate cognitive performance and hippocampal acetylcholine release in memory-impaired aged rats. *Neuroscience* 69:1067-1074.
- Hsiao K. 1998. Transgenic mice expressing Alzheimer amyloid precursor proteins. *Exp Gerontol* 33:883-889.
- Huang PL, Fishman MC. 1996. Genetic analysis of nitric oxide synthase isoforms: targeted mutation in mice. *J Mol Med* 74:415-421.
- Hucker HB, Hutt JE, White SD, Arison BH, Zacchei AG. 1983. Disposition and metabolism of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohept-5,10-imine in rats, dogs, and monkeys. *Drug Metab Dispos* 11:54-58.
- Issa AM, Rowe W, Gauthier S, Meaney MJ. 1990. Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. *J Neurosci* 10:3247-3254.
- Izquierdo I. 1989. Mechanism of action of scopolamine as an amnesic. *Trends Pharmacol Sci* 10:175-177.
- Kandel ER. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294:1030-1038.
- Kesner RP, Evans RB, Hunt MA. 1987. Further evidence in support of the neurobiological bases of an attribute model of memory: role of the hippocampus. *Int J Neurol* 21-22:184-196.
- Kretschmer BD, Fink S. 1999. Spatial learning deficit after NMDA receptor blockade and state-dependency. *Behav Pharmacol* 10:423-428.

- Lannes B, Bernard V, Bloch B, Micheletti G. 1995. Chronic treatment with dizocilpine maleate increases the number of striatal neurons expressing the D2 receptor gene. *Neuroscience* 65:431-438.
- Larkman AU, Jack JJ. 1995. Synaptic plasticity: hippocampal LTP. *Curr Opin Neurobiol* 5:324-334.
- Law A, Dore S, Blackshaw S, Gauthier S, Quirion R. 2000. Alteration of expression levels of neuronal nitric oxide synthase and haem oxygenase-2 messenger RNA in the hippocampi and cortices of young adult and aged cognitively unimpaired and impaired Long-Evans rats. *Neuroscience* 100:769-775.
- Le Jeune H, Cecyre D, Rowe W, Meaney MJ, Quirion R. 1996. Ionotropic glutamate receptor subtypes in the aged memory-impaired and unimpaired Long-Evans rat. *Neuroscience* 74:349-363.
- Lee I, Kesner RP. 2002. Differential contribution of NMDA receptors in hippocampal subregions to spatial working memory. *Nat Neurosci* 5:162-168.
- Levin ED, Simon BB. 1998. Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology (Berl)* 138:217-230.
- Lisman JE, McIntyre CC. 2001. Synaptic plasticity: a molecular memory switch. *Curr Biol* 11:R788-R791.
- Marcotte ER, Srivastava LK, Quirion R. 2001. DNA microarrays in neuropsychopharmacology. *Trends Pharmacol Sci* 22:426-436.
- Matynia A, Anagnostaras SG, Silva AJ. 2001. Weaving the molecular and cognitive strands of memory. *Neuron* 32:557-559.
- McDonald RJ, White NM. 1993. A triple dissociation of memory systems: hippocampus, amygdala, and dorsal striatum. *Behav Neurosci* 107:3-22.
- McDonald RJ, White NM. 1994. Parallel information processing in the water maze: evidence for independent memory systems involving dorsal striatum and hippocampus. *Behav Neural Biol* 61:260-270.
- McHugh TJ, Blum KI, Tsien JZ, Tonegawa S, Wilson MA. 1996. Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. *Cell* 87:1339-1349.
- McLamb RL, Williams LR, Nanry KP, Wilson WA, Tilson HA. 1990. MK-801 impedes the acquisition of a spatial memory task in rats. *Pharmacol Biochem Behav* 37:41-45.
- Morris RG. 1989. Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. *J Neurosci* 9:3040-3057.
- Morris RG. 1990. Toward a representational hypothesis of the role of hippocampal synaptic plasticity in spatial and other forms of learning. *Cold Spring Harbor Symp Quant Biol* 55:161-173.
- Morris RG, Anderson E, Lynch GS, Baudry M. 1986. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* 319:774-776.
- Myers SJ, Dingledine R, Borges K. 1999. Genetic regulation of glutamate receptor ion channels. *Annu Rev Pharmacol Toxicol* 39:221-241.
- Parent A, Rowe W, Meaney MJ, Quirion R. 1995. Increased production of inositol phosphates and diacylglycerol in aged cognitively impaired rats after stimulation of muscarinic, metabotropic-glutamate and endothelin receptors. *J Pharmacol Exp Ther* 272:1110-1116.
- Pavlidis P, Montgomery J, Madison DV. 2000. Presynaptic protein kinase activity supports long-term potentiation at synapses between individual hippocampal neurons. *J Neurosci* 20:4497-4505.
- Pitkanen M, Sirvio J, MacDonald E, Niemi S, Ekonsalo T, Riekkinen PS. 1995. The effects of D-cycloserine and MK-801 on the performance of rats in two spatial learning and memory tasks. *Eur Neuropsychopharmacol* 5:457-463.
- Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, Parent A, White N, Meaney MJ. 1995. Facilitation of acetylcholine release and cognitive performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. *J Neurosci* 15:1455-1462.
- Richter-Levin G, Canevari L, Bliss TV. 1995. Long-term potentiation and glutamate release in the dentate gyrus: links to spatial learning. *Behav Brain Res* 66:37-40.
- Rowe WB, Spreekmeester E, Meaney MJ, Quirion R, Rochford J. 1998. Reactivity to novelty in cognitively-impaired and cognitively-unimpaired aged rats and young rats. *Neuroscience* 83:669-680.
- Schuman EM, Madison DV. 1991. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254:1503-1506.
- Seeburg PH, Single F, Kuner T, Higuchi M, Sprengel R. 2001. Genetic manipulation of key determinants of ion flow in glutamate receptor channels in the mouse. *Brain Res* 907:233-243.
- Selcher JC, Atkins CM, Trzaskos JM, Paylor R, Sweatt JD. 1999. A necessity for MAP kinase activation in mammalian spatial learning. *Learn Mem* 6:478-490.
- Shen J, Barnes CA, McNaughton BL, Skaggs WE, Weaver KL. 1997. The effect of aging on experience-dependent plasticity of hippocampal place cells. *J Neurosci* 17:6769-6782.
- Stemmelin J, Lazarus C, Cassel S, Kelche C, Cassel JC. 2000. Immunohistochemical and neurochemical correlates of learning deficits in aged rats. *Neuroscience* 96:275-289.
- Van der Zee EA, Luiten PG. 1999. Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. *Prog Neurobiol* 58:409-471.
- Whishaw IQ, Auer RN. 1989. Immediate and long-lasting effects of MK-801 on motor activity, spatial navigation in a swimming pool and EEG in the rat. *Psychopharmacology (Berl)* 98:500-507.
- Yokoi M, Kobayashi K, Manabe T, Takahashi T, Sakaguchi I, Katsura G, Shigemoto R, Ohishi H, Nomura S, Nakamura K, Nakao K, Katsuki M, Nakanishi S. 1996. Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science* 273:645-647.

Appendix IV

Ethics approval certificates



McGill

University Animal Care Committee

McGill University
James Administration Building
845 Sherbrooke Street West
Room 429
Montreal, Quebec, Canada
H3A 2T5

Comité universitaire de protection des animaux

Université McGill
Pavillon James de l'administration
845, rue Sherbrooke Ouest
Bureau 429
Montréal, Québec, Canada
H3A 2T5

Tel.: (514) 398-2837
Fax: (514) 398-4853
www.mcgill.ca/rgo/animal

January 11, 2005

The McGill University Animal Care Committee certifies that
Jonathan Brouillette has successfully completed a
Mouse Methodology Workshop on December 15, 2004

The training included the following procedures:

- ✓ Handling and restraint
- ✓ Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- ✓ Gavage (tube feeding)
- ✓ Blood collection: saphenous vein and cardiac puncture
- ✓ Determination of anaesthetic depth
- ✓ Euthanasia by cervical dislocation

** Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed*

Certification is valid for 5 years, starting on the date of the workshop.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training



McGill

University Animal Care Committee

McGill University
James Administration Building
845 Sherbrooke Street West
Room 429
Montreal, Quebec, Canada
H3A 2T5

Comité universitaire de protection des animaux

Université McGill
Pavillon James de l'administration
845, rue Sherbrooke Ouest
Bureau 429
Montréal, Québec, Canada
H3A 2T5

Tel.: (514) 398-2837
Fax: (514) 398-4853
www.mcgill.ca/rgo/animal

June 3, 2003

The McGill University Animal Care Committee certifies that
Jonathan Brouillette has successfully completed a
Rat Methodology Workshop on January 29, 2003.

The training included the following procedures:

- ☒ Handling and restraint
- ☒ Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- ☒ Gavage (tube feeding)
- ☒ Blood collection: saphenous and cardiac puncture
- ☒ Determination of anaesthetic depth
- ☒ Euthanasia by cervical dislocation
- ☒ Euthanasia by decapitation

** Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed*

Certification is valid for 5 years, starting on the date of the workshop.

Suzanne Smith
Research Ethics Officer for Animal Studies
animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

Appendix V

Manuscript Waivers

De: "Journals Rights" <JournalsRights@oxon.blackwellpublishing.com>

À: "Jonathan Brouillette" <jo_brouillette@yahoo.fr>

Le: Mon, 20 Aug 2007 09:30:38 +0100

Objet: RE: Permission to incorporate an article in my Ph.D. thesis

Dear Jonathan Brouillette,

Thank you for your email request. Permission is granted for you to use the material below for your thesis, subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis commercially.

With best wishes,
Sally

Sally Byers
Permissions Assistant
Wiley-Blackwell Publishing Ltd.
PO Box 805
9600 Garsington Road
Oxford OX4 2DQ
UK
Tel. 01865 476149
Fax. 01865 471149

From: Jonathan Brouillette [mailto:jo_brouillette@yahoo.fr]

Sent: 19 August 2007 19:12

Journals Rights

Subject: Permission to incorporate an article in my Ph.D. thesis

Hi,

It is required by McGill University to include waivers from the publishers of previously published materials. By the present email, I would like to ask for permission to incorporate the following manuscript where I am first author in my Ph.D. thesis:

Title: Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABAB receptors in scopolamine-induced amnesia.

Authors: Brouillette J, Young D, During MJ and Quirion R

Journal title: Journal of Neurochemistry

Date: Epub ahead of print

Volume: Epub ahead of print

Pages: Epub ahead of print

Thank you, Best regards,

Jonathan Brouillette

Douglas Mental Health University Institute
McGill University
5 boulevard LaSalle
Montreal, Quebec, Canada
H4H 1R3

**SPRINGER LICENSE
TERMS AND CONDITIONS**

Aug 20, 2007

This is a License Agreement between Jonathan Brouillette ("You") and Springer ("Springer"). Please note that you are liable to account for Value Added Tax (VAT). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

License Number	1773111352844
License date	Aug 20, 2007
Licensed content publisher	Springer
Licensed content publication	Neurochemical Research
Licensed content title	Neuroprotective Effects of Natural Products: Interaction with Intracellular Kinases, Amyloid Peptides and a Possible Role for Transthyretin
Licensed content author	Stéphane Bastianetto
Licensed content date	Dec 31, 1969
Volume number	
Issue number	
Pages	1 - 6
Type of Use	Thesis / Dissertation
Details of use	University intranet
Portion of the article	Full text
Title of your thesis / dissertation	Characterization of novel genes involved in learning and memory in rodent models
Expected completion date	Aug 2007
Total	0.00 USD

Terms and Conditions

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided it is password protected or on the university's intranet, destined to microfilming by UMI and University repository. For any

other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well). Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted

However figures and illustrations may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights

Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice: Disclaimer

You must include the following copyright and permission notice in connection with any reproduction of the licensed material:

"Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Indemnity

You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms

Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by the country's law in which the work was originally published.

v1.1

De : Jones, Jennifer (ELS-OXF) <J.Jones@elsevier.co.uk>
Pour : <jonathan.brouillette@douglas.mcgill.ca>

Date : Lundi, Août 20, 2007 03:14AM

Objet : RE: Obtain Permission

Dear Jonathan Brouillette

We hereby grant you permission to reprint the material detailed below at no charge in your thesis subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.
2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"This article was published in Publication title, Vol number, Author(s), Title of article, Page Nos, Copyright Elsevier (or appropriate Society name) (Year)."
3. Your thesis may be submitted to your institution in either print or electronic form.
4. Reproduction of this material is confined to the purpose for which permission is hereby given.
5. This permission is granted for non-exclusive world English rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form other than submission. Should you have a specific electronic project in mind please reapply for permission
6. This includes permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Jennifer Jones
Rights Assistant

Elsevier is pleased to announce our partnership with Copyright Clearance Center's Rightslink service. Rightslink will handle Elsevier's journal permission requests. For your future requests, you have to go through Rightslink to obtain permissions. With Rightslink (r) it's faster and easier than ever before to obtain permission to use and republish material from Elsevier. Using Rightslink is as simple as:

Simply visit: <http://www.sciencedirect.com/> and locate your desired content.

Click on Permissions within the table of contents or in the tool-box to the right of the online article to open the following page:

1. Select the way you would like to reuse the content

2. Create an account if you haven't already

3. Accept the terms and conditions and you're done

Please contact Rightslink Customer Care with any questions or comments

Concerning this service: Copyright Clearance Center Rightslink Customer

Care Tel (toll free): 877/622-5543 Tel: 978/777-9929

E-mail: customercare@copyright.com

Web: <http://www.rightslink.com>

Elsevier Limited, a company registered in England and Wales with company number 1982084, whose registered office is The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom.

-----Original Message-----

From: jonathan.brouillette@douglas.mcgill.ca

[<mailto:jonathan.brouillette@douglas.mcgill.ca>]

Sent: 19 August 2007 18:57

To: Health Permissions (ELS)

Subject: Obtain Permission

This Email was sent from the Elsevier Corporate Web Site and is related to Obtain Permission form:

Product: Customer Support
Component: Obtain Permission
Web server: <http://www.elsevier.com>
IP address: 10.10.24.148
Client: Mozilla/4.0 (compatible; MSIE 7.0; Windows NT 5.1; .NET CLR 1.1.4322)
Invoked from:
http://www.elsevier.com/wps/find/obtainpermissionform.cws_home?isSubmitted=yes&navigateXmlFileName=/store/prod_webcache_act/framework_support/obtainpermission.xml

Request From:
Permission to use an article in my Ph.D. thesis Jonathan Brouillette
Douglas Mental Health University Institute
6875 LaSalle Blvd
H4H 1R3
Montreal
Canada

Contact Details:
Telephone: 514-761-6131
Fax: 514-762-3034
Email Address: jonathan.brouillette@douglas.mcgill.ca

Use the following material:

ISSN/ISBN:

Title: Neurobiology of Aging

Author(s): Brouillette J and Quirion R

Volume: Epub ahead of print

Issue: Epub ahead of print
Year: Epub ahead of print
Pages: Epub - Epub
Article title: Transthyretin: a key gene involved in the ...

How much of the requested material is to be used:
The entire article

Are you the author: Yes
Author at institute: Yes

How/where will the requested material be used: [how_used]

Details:

It is required by McGill University to include waivers from the publishers of previously published materials. I would like to ask for permission to incorporate this manuscript which is Epub ahead of print where I am first author in my Ph.D. thesis.

Additional Info: This manuscript is Epub ahead of print

- end -

For further info regarding this automatic email, please contact:
WEB APPLICATIONS TEAM (esweb.admin@elsevier.co.uk)

This email is from Elsevier Limited, a company registered in England and Wales with company number 1982084,

whose registered office is The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom.

Appendix V

Manuscript Waivers

De: "Journals Rights" <JournalsRights@oxon.blackwellpublishing.com>

À: "Jonathan Brouillette" <jo_brouillette@yahoo.fr>

Envoyé: Mon, 20 Aug 2007 09:30:38 +0100

Objet: RE: Permission to incorporate an article in my Ph.D. thesis

Dear Jonathan Brouillette,

Thank you for your email request. Permission is granted for you to use the material below for your thesis, subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis commercially.

With best wishes,
Sally

Sally Byers
Permissions Assistant
Wiley-Blackwell Publishing Ltd.
PO Box 805
9600 Garsington Road
Oxford OX4 2DQ
UK
Tel.01865 476149
Fax. 01865 471149

From: Jonathan Brouillette [mailto:jo_brouillette@yahoo.fr]

Sent: 19 August 2007 19:12

Journals Rights

Subject: Permission to incorporate an article in my Ph.D. thesis

Hi,

It is required by McGill University to include waivers from the publishers of previously published materials. By the present email, I would like to ask for permission to incorporate the following manuscript where I am first author in my Ph.D. thesis:

Title: Hippocampal gene expression profiling reveals the possible involvement of Homer1 and GABAB receptors in scopolamine-induced amnesia.

Authors: Brouillette J, Young D, During MJ and Quirion R

Journal title: Journal of Neurochemistry

Date: Epub ahead of print

Volume: Epub ahead of print

Pages: Epub ahead of print

Thank you, Best regards,

Jonathan Brouillette

Douglas Mental Health University Institute
McGill University
3801 boulevard LaSalle
Montreal, Quebec, Canada
H4H 1R3

**SPRINGER LICENSE
TERMS AND CONDITIONS**

Aug 20, 2007

This is a License Agreement between Jonathan Brouillette ("You") and Springer ("Springer"). Please note that you are liable to account for Value Added Tax (VAT). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

License Number	1773111352844
License date	Aug 20, 2007
Licensed content publisher	Springer
Licensed content publication	Neurochemical Research
Licensed content title	Neuroprotective Effects of Natural Products: Interaction with Intracellular Kinases, Amyloid Peptides and a Possible Role for Transthyretin
Licensed content author	Stéphane Bastianetto
Licensed content date	Dec 31, 1969
Volume number	
Issue number	
Pages	1 - 6
Type of Use	Thesis / Dissertation
Details of use	University intranet
Portion of the article	Full text
Title of your thesis / dissertation	Characterization of novel genes involved in learning and memory in rodent models
Expected completion date	Aug 2007
Total	0.00 USD

Terms and Conditions

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided it is password protected or on the university's intranet, destined to microfilming by UMI and University repository. For any

other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well). Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted

However figures and illustrations may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights

Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice: Disclaimer

must include the following copyright and permission notice in connection with any reproduction of the licensed material:

"Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Indemnity

You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing

This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms

Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by the country's law in which the work was originally published.

v1.1

De : "Jones,Jennifer (ELS-OXF)" <J.Jones@elsevier.co.uk>
Pour : <jonathan.brouillette@douglas.mcgill.ca>

Date : Lundi, Août 20, 2007 03:14AM
Objet : RE: Obtain Permission

Dear Jonathan Brouillette

We hereby grant you permission to reprint the material detailed below at no charge in your thesis subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"This article was published in Publication title, Vol number, Author(s), Title of article, Page Nos, Copyright Elsevier (or appropriate Society name) (Year)."

3. Your thesis may be submitted to your institution in either print or electronic form.

Reproduction of this material is confined to the purpose for which permission is hereby given.

5. This permission is granted for non-exclusive world English rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form other than submission. Should you have a specific electronic project in mind please reapply for permission

6. This includes permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Jennifer Jones
Rights Assistant

Elsevier is pleased to announce our partnership with Copyright Clearance Center's Rightslink service. Rightslink will handle Elsevier's journal permission requests. For your future requests, you have to go through Rightslink to obtain permissions. With Rightslink (r) it's faster and easier than ever before to obtain permission to use and republish material from Elsevier. Using Rightslink is as simple as:

Simply visit: <http://www.sciencedirect.com/> and locate your desired content.

Click on Permissions within the table of contents or in the tool-box to the right of the online article to open the following page:

1. Select the way you would like to reuse the content
2. Create an account if you haven't already
3. Accept the terms and conditions and you're done

Please contact Rightslink Customer Care with any questions or comments concerning this service: Copyright Clearance Center Rightslink Customer Care Tel (toll free): 877/622-5543 Tel: 978/777-9929
E-mail: customercare@copyright.com
Web: <http://www.rightslink.com>

Elsevier Limited, a company registered in England and Wales with company number 1982084, whose registered office is The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom.

-----Original Message-----

From: jonathan.brouillette@douglas.mcgill.ca
[<mailto:jonathan.brouillette@douglas.mcgill.ca>]
Sent: 19 August 2007 18:57
To: Health Permissions (ELS)
Subject: Obtain Permission

This Email was sent from the Elsevier Corporate Web Site and is related to Obtain Permission form:

Product: Customer Support
Component: Obtain Permission
Web server: <http://www.elsevier.com>
IP address: 10.10.24.148
Client: Mozilla/4.0 (compatible; MSIE 7.0; Windows NT 5.1; .NET CLR 1.1.4322)
Invoked from:
http://www.elsevier.com/wps/find/obtainpermissionform.cws_home?isSubmitted=yes&navigateXmlFileName=/store/prod_webcache_act/framework_support/obtainpermission.xml

Request From:
Permission to use an article in my Ph.D. thesis Jonathan Brouillette
Douglas Mental Health University Institute
6875 LaSalle Blvd
H4H 1R3
Montreal
Canada

Contact Details:
Telephone: 514-761-6131
Fax: 514-762-3034
Email Address: jonathan.brouillette@douglas.mcgill.ca

use the following material:

ISSN/ISBN:
Title: Neurobiology of Aging
Author(s): Brouillette J and Quirion R
Volume: Epub ahead of print

Issue: Epub ahead of print
Year: Epub ahead of print
Pages: Epub - Epub
Article title: Transthyretin: a key gene involved in the ...

How much of the requested material is to be used:
The entire article

Are you the author: Yes
Author at institute: Yes

How/where will the requested material be used: [how_used]

Details:

It is required by McGill University to include waivers from the publishers of previously published materials. I would like to ask for permission to incorporate this manuscript which is Epub ahead of print where I am first author in my Ph.D. thesis.

Additional Info: This manuscript is Epub ahead of print

- end -

For further info regarding this automatic email, please contact:

WEB APPLICATIONS TEAM (esweb.admin@elsevier.co.uk)

This email is from Elsevier Limited, a company registered in England and Wales with company number 1982084,

whose registered office is The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom.