Experimental therapy and cognitive outcomes in a rat transgenic model of Alzheimer disease-like amyloid beta neuropathology

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This thesis is dedicated to my Parents and Grandparents

Abstract

Alzheimer's disease (AD) is the leading cause of dementia worldwide and currently no effective treatment exists. In AD, the accumulation of amyloid beta (Aβ) begins decades before cognitive symptoms and progresses from intraneuronal material to extracellular plaques. To date, however, the precise mechanism by which the early buildup of Aβ peptides leads to cognitive dysfunction remains unknown. Using a rat transgenic model of AD, this thesis aims to resolve outstanding issues surrounding the impact of early Aβ accumulation on cognition and evaluate the result of potential therapy using the multi-target drug lithium.

The disease model employed in these studies is the McGill-R-Thy-APP Alzheimer transgenic rat. These rats express a genetic mutation that leads to an aggressive early-onset form of AD in humans. The rats show intraneuronal accumulation of Aβ as early as 2-weeks postnatally, with the first Aβ plaques appearing in the hippocampus and entorhinal cortex at 6-months of age. By 15-months of age, a heavy burden of Aβ plaques is observed throughout the hippocampus and cortex. These rats display severe learning and memory deficits during the pre-plaque stages of the Aβ pathology, before plaques deposit. Coincident with the pre-plaque stages is a chronic neuroinflammatory process. Thus, this model recapitulates key aspects of the AD-like Aβ pathology, making it an ideal model both for the study of the earliest events in AD pathogenesis and for the evaluation of putative experimental therapeutics.

Chapter 1 of this thesis serves as a review of the pathophysiological characteristics and genetic risk factors associated with AD. What follows is an overview of the multiple cellular signaling cascades that are dysregulated in AD. The Chapter includes a summary of recent efforts to identify the preclinical phases of the disease. The Chapter discusses the discovery of the clinical effects of lithium, and examines some of the known mechanisms of action of this multi-target drug. Finally, the Chapter ends by summarizing the Aims and Hypotheses of this thesis.

In Chapter 2, the impact of the early Aβ accumulation on temporal and frontal lobe dysfunction is investigated. The performance of McGill-R-Thy1-APP transgenic AD rats was compared with wild-type littermate controls on a visual discrimination task using a touchscreen operant platform. The presence of intraneuronal Aβ caused a severe associative learning deficit in the AD rats that coincided with reduced nuclear translocation and genomic occupancy of the CREB co-activator, CRTC1, and decreased production of synaptic plasticity-associated transcripts *Arc*, *c-fos*, *Egr1*, and *Bdnf*. Thus, blockade of CRTC1-dependent gene expression in the early, pre-plaque phase of AD-like pathology provides a molecular basis for the cognitive deficits that figure so prominently in early AD.

Chapter 3 describes the application of a microdose lithium formulation, coded NP03, for the treatment of AD-like Aβ pathology. Lithium has been shown to reduce BACE1 activity, Aβ production, and to promote CRTC1-dependent gene expression and restore cognition. This Chapter presents data showing that NP03 treatment reversed

cognitive deficits in Alzheimer transgenic rats, as measured by standard rodent cognitive tasks. Additional data are presented showing that NP03 also significantly reduced the level of toxic Aβ and reduced BACE1 hyperactivity, and restored CRTC1 promoter occupancy in genes required for learning and memory.

The anti-oxidative stress and anti-inflammatory properties of NP03 are studied in Chapter 4, where data are presented showing that NP03 treatment significantly reduces the expression of a subset of oxidative stress and pro-inflammatory cytokines, along with reducing microglia recruitment to Aβ-neurons in the hippocampus. It is also demonstrated that NP03 restored adult hippocampal neurogenesis in the dentate gyrus in AD rats. This Chapter ends with the presentation of a hypothetical model that may account for the beneficial effects of NP03 by halting oxidative stress/neuroinflammation contributing to self-perpetuating neurotoxicity.

Chapter 5 serves as a general discussion, and as such, situates the findings of this thesis in the broader context of current literature and puts forth a theoretical model for the action of lithium in Alzheimer's disease brains. Also discussed are the experimental therapeutics currently being explored for AD, and an explanation as to why combination therapies will likely be required for the treatment of AD. Finally, the Chapter concludes by suggesting CRTC1 as a potential target for AD therapy and identifies NP03 as a therapeutic candidate for the treatment of early stage Alzheimer's disease.

Résumé

La maladie d'Alzheimer (MA) constitue la première cause de démence au monde et aucun traitement efficace n'existe à ce jour. Dans la MA, l'accumulation de peptides béta-amyloïde (Aβ) peut survenir 10 à 15 ans avant les troubles cognitifs et progresse d'un stade intracellulaire vers un stade de plaques extracellulaires. Cependant et à ce jour, le mécanisme précis par lequel l'accumulation précoce de peptides Aβ entraine les troubles cognitifs reste inconnue. En utilisant un rat transgénique modélisant la MA, ce travail de thèse a pour but de répondre aux questions en suspens ayant trait à l'impact de l'accumulation précoce d'Aβ sur la cognition ainsi que d'évaluer les résultats d'un traitement par le lithium, une molécule à cibles multiples.

Le modèle utilisé dans ces travaux est le rat transgénique Alzheimer McGill-R-Thy1-APP. Ces rats possèdent une mutation génétique qui entraine l'apparition d'une forme précoce et agressive de la maladie chez l'homme. Chez le rat, Aβ s'accumule dans les neurones dès 2 semaines après la naissance, tandis que les premières plaques d'Aβ apparaissent dans l'hippocampe et le cortex entorhinal à l'âge de 6 mois. A 15 mois, on peut observer un dépôt de plaques très marqué dans l'hippocampe et le cortex. Ces rats présentent de sévères troubles d'apprentissage et de mémoire au cours de la phase pré-plaque de la pathologie amyloïde, avant l'apparition des plaques. Un processus d'inflammation chronique coïncide avec cette phase pré-plaque. Ainsi, ce modèle récapitule des aspects clés de la pathologie amyloïde liée à la MA, ce qui en fait un modèle idéal à la fois pour étudier les évènements les plus précoces dans la genèse de la MA ainsi que pour évaluer d'éventuelles thérapies expérimentales.

Le premier Chapitre de cette thèse est une revue des caractéristiques physiopathologiques et des facteurs de risque génétique associés à la MA. S'en suit un aperçu des multiples cascades de signalisation cellulaire qui sont déréglées dans la MA. Ce Chapitre inclue un résumé des récents efforts d'identification des phases précliniques de la maladie. Ce Chapitre traite également de la découverte des effets cliniques du lithium et examine quelques-uns des mécanismes d'action connus de cette molécule à cibles multiples. Enfin, ce Chapitre s'achève sur un résumé des Buts et Hypothèse de cette Thèse.

Le Chapitre 2 traite de l'impact de l'accumulation précoce d'Aβ sur le dysfonctionnement des lobes temporaux et frontaux. La performance des rats Alzheimer McGill-R-Thy1-APP dans une tache de discrimination visuelle est comparée à celle des rats contrôles sauvages en utilisant un dispositif « touchscreen operant platform ». La présence intraneuronale d'Aβ entraine un déficit sévère d'apprentissage associé chez le rat Alzheimer, qui coïncide avec une réduction de la translocation nucléaire et de l'occupation génomique du co-activateur du facteur CREB, CRTC1, ainsi qu'avec une réduction dans la production de messagers *Arc*, *c-fos*, *Egr1* et *Bdnf* associés à la plasticité synaptique. Ainsi, le blocage de l'expression de gènes dépendant du CRTC1 au cours de la phase précoce pré-plaque de la pathologie amyloïde Alzheimer offre une base moléculaire pour expliquer les déficits cognitifs si proéminents dans la MA précoce.

Le Chapitre 3 décrit l'utilisation d'une formulation du lithium à faible dose, appelée NP03, pour le traitement de la pathologie amyloïde liée à la MA. Il a été démontré que le lithium peut réduire l'activité de BACE1 et la production d'Aβ, promouvoir l'expression de gènes dépendant du CRTC1 et rétablir la cognition. Ce Chapitre présente des résultats démontrant que le traitement par le NP03 annule les déficits cognitifs chez le rat Alzheimer, mesurés par des tests cognitifs standards. Des résultats supplémentaires montrent que le NP03 réduit de façon significative le niveau d'Aβ toxique, l'hyperactivité de BACE1 et rétablit l'occupation génomique du promoteur CRTC1 au niveau de gènes requis pour l'apprentissage et la mémoire.

Le Chapitre 4 traite des propriétés anti-stress oxidatif et anti-inflammatoires du NP03 et présente des résultats démontrant que le traitement au NP03 diminue de façon significative l'expression d'un sous-groupe de cytokines pro-inflammatoires, tout en réduisant le recrutement de microglies vers les neurones contenant Aβ dans l'hippocampe. NP03 rétablit également la neurogenèse adulte dans le gyrus denté de l'hippocampe des rats Alzheimer. Ce Chapitre s'achève par une présentation d'un modèle hypothétique pouvant expliquer les effets bénéfiques du NP03 en bloquant le stress oxidatif/la neuroinflammation qui contribuent à la neurotoxicité qui s'auto-perpétue.

Le Chapitre 5 est une discussion générale, et en tant que telle, place les résultats de cette thèse dans le contexte plus large de la littérature actuelle ; il met aussi en avant un modèle théorique d'action du lithium sur les cerveaux atteints de la MA. Les thérapies expérimentales actuellement mises en œuvre contre la MA sont également abordées, ainsi

qu'une explication des raisons pour lesquelles les thérapies combinées seront nécessaires pour traiter la MA. Enfin, ce Chapitre conclue en suggérant que CRTC1 est une cible potentielle dans le traitement de la MA et identifie le NP03 comme un candidat thérapeutique pour la phase précoce de la MA.

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List of Abbreviations

3-NT: 3-nitrotyrosine

AA: amino acids

AAV: adeno-associated virus

Aβ: amyloid beta protein

ACh: acetylcholine

AChE: actylcholinesterase

AD: Alzheimer's disease

ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10

ADAS-cog: Alzheimer's disease assessment scale, cognitive subscale

AICD: APP intracellular domain

AKT: protein kinase B (aka PKB)

aMCI: amnestic mild cognitive impairment

APP: amyloid precursor protein

Apo-E: apolipoprotein E

BACE1: beta-site amyloid precursor protein cleaving enzyme 1

BBB: blood brain barrier

BCL: B-cell lymphoma 2

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

CA1: Cornus Ammonis area 1

CA3: Cornus Ammonis area 3

CaMK: calcium/calmodulin-dependent protein kinase

cAMP: cyclic adenosine monophosphate

CANTAB: Cambridge Neuropsychological Test Automated Battery

CBP: CREB-binding protein

CC: cerebral cortex

ChIP: Chromatin immunoprecipitation

CNS: central nervous system

CRE: cAMP-responsive element

CREB: cyclic-AMP response element-binding protein

CRTC1: CREB-regulated transcription coactivator 1

CSF: cerebrospinal fluid

CT: computerized tomography

CTF: carboxy-terminal fragment

DAB: 3'3-diaminobenzidine

DAPI: 4',6-diamidino-2-phenylindole

DBS: deep brain stimulation

DCX: doublecortin

DG: dentate gyrus

EDTA: ethylenediaminetetraacetic acid

EEG: electroencephalogram

EGR1: early growth response protein 1

ERK: extracellular-regulator kinase

Fc: fragment, crystalizable region

fMRI: functional magnetic resonance imaging

FUS: focused ultrasonic

GCL: granule cell layer

GLP: good laboratory practice

GSK-3: glycogen synthase kinase-3

Hc: hippocampus

HD: Huntington disease

HNE: 4-hydroxy-2-nonenal

HRP: horseradish peroxidase

HTT: huntingtin protein

ICP-MS: inductively coupled plasma mass spectrometry

IFN-γ: interferon gamma

IgG: immunoglobulin G

IHC: immunohistochemistry

IL-1β: interleukin-1 beta

i.p. intraperitoneal

LTP: long-term potentiation

MCI: mild cognitive impairment

MEC: medial entorhinal cortex

MMSE: Mini Mental State Examination

MWM: Morris water maze task

NAD: nicotinamide adenine dinucleotide

NGS: normal goat serum

NIH: National Institutes of Health, USA

NIMH: National Institute of Mental Health, USA

NMDA: N-methyl-d-aspartate

NO: nitric oxide

NOR: novel object recognition task

NR2B: NMDA receptor 2B

NSAID: nonsteroidal anti-inflammatory drug

NT: neurotransmitter

OFC: orbitofrontal cortex

PAL: paired associate learning

PBS: phosphate-buffered saline

PD: Parkinson's disease

PET: positron emission tomography

PFC: prefrontal cortex

PKA: protein kinase A

PKB: protein kinase B (aka AKT)

PKC: protein kinase C

PSEN1: presenilin-1

PSEN2: presenilin-2

qPCR: quantitative polymerase chain reaction

RNS: reactive nitrogen species

ROS: reactive oxygen species

SCN: suprachiasmatic nucleus

SDS: sodium dodecyl sulfate

SGZ: subgranular zone

SIK: salt-inducible kinase

TNF-α: tumor necrosis factor-α

TBS: Tris-buffered saline

TG: transgenic animal

TLR: Toll-like receptor

TREM: triggering receptors expressed by myeloid cells

Wb: Western blot

Wnt: wingless signal

WT: wild type animal

Contribution of Authors

Chapters 2-4 describe works that will comprise 3 individual publications.

Dr. A. Claudio Cuello was my doctoral mentor and supervised the work. He provided intellectual guidance and edited the writing of the manuscripts.

Adriana Ducatenzeiler offered technical assistance on the immunohistological and biochemical assays and was responsible for some of the rat breeding and genotyping.

CHAPTER 2

"*Intraneuronal amyloid beta accumulation disrupts hippocampal CRTC1-dependent gene expression and cognitive function in a rat model of Alzheimer disease*"

Published online in "Cerebral Cortex, on January 11, 2016 By Wilson, E.N., Abela, A.R., Do Carmo, S., Allard, S., Marks, A.R., Welikovitch, L.A., Ducatenzeiler, A., Chudasama, Y., and Cuello, A.C.

Dr. Andrew Abela: provided assistance with programing the Whisker Software and with certain behavioural analyses.

Dr. Sonia Do Carmo: performed the ChIP and qPCR analysis.

Dr. Simon Allard: contributed to the design the ImageJ macros used for CRTC1 quantification by confocal microscopy.

Adam Marks: provided technical assistance for IHC experiments.

Lindsay Welikovitch: provided technical assistance for Wb experiments.

Dr. Yogita Chudasama: directed the touchscreen operant platform experiments and provided intellectual guidance and edited the manuscript.

CHAPTER 3

"*Microdose lithium formulation NP03 reverts neuropathology and memory loss at the earliest stages of the A β pathology in an Alzheimer disease rat model*"

In Preparation for submission: By Wilson, E.N., Do Carmo, S., Iulita, M.F., Ducatenzeiler, A., Allard, S., Marks, A.R., Windheim, J., and Cuello, A.C.

Dr. Sonia Do Carmo: performed the ChIP analysis.

Dr. M. Florencia Iulita: performed the fear conditioning tests, the Abeta ELISA.

Dr. Simon Allard: aided in the NOR and MWM testing.

Adam Marks: provided technical assistance for IHC experiments.

Joseph Windheim: provided technical assistance for Wb experiments.

CHAPTER 4

"*NP03 Reverses Pre-Plaque Oxidative Stress and Neuroinflammation in a Rat Model Alzheimer's Disease*"

In Preparation for submission. By Wilson, E.N., Iulita, M.F., Do Carmo, S., Jia, D.T., Austin, G., Butterfield, D.A., and Cuello, A.C.

Dr. Sonia Do Carmo: provided technical support for the qPCR experiments.

Dr. M. Florencia Iulita: provided technical support for the proinflammatory panel.

Dan Tong Jia: provided technical support for the microglia recruitment analysis.

Grant Austin: provided technical support with the oxidative stress markers.

Dr. Allan Butterfield: directed the oxidative stress markers experiments.

Table of Contents

CHAPTER 1: General Introduction

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Frau Auguste Deter: Alzheimer's Patient No. 1

On November 25, 1901, a 51-year-old German woman was admitted to the Municipal Mental Asylum of Frankfurt am Main with complaints of memory loss and mental disturbances (Maurer et al. 1997). *Frau* Auguste Deter was examined by the Senior Physician in Psychiatry and Neuropathology, who described "as one of her first disease symptoms, a strong feeling of jealousy towards her husband. Soon she showed rapidly increasing memory impairments; she was disoriented carrying objects to and fro in her flat and hid them. Sometimes she felt that someone wanted to kill her and began to scream loudly… After $4^{1/2}$ years of sickness she died" (Alzheimer 1907). When Frau Deter died in 1906, the physician performed an autopsy, and using advanced staining techniques for the time, he described brain atrophy and specific lesions, a "paucity of cells in the cerebral cortex and clumps of filaments between the nerve cells" (Alzheimer, 1907). The physician in charge was Dr. Alois Alzheimer, and more than a century after this discovery, there is no cure for the disease that carries his name.

Dr. Alois Alzheimer

Alois Alzheimer was born in the Bavarian town of Marktbreit am Main, in Southern Germany, on June 14th, 1864 (Maurer *et al*. 1997). As the son of a wealthy family, Alzheimer received his primary education at Catholic boarding school in the neighbouring town of Aschaffenburg. He attended medical school in Berlin, where he took an anatomy class with famous pathologist Wilhelm von Waldeyer (1836-1921), who

coined the terms “chromosome” and “neurone” (Winkelmann 2007). Alzheimer continued to study at Tübingen and Würzburg, before graduating in 1887 (Kircher and Wormstall, 1996). That same year, Alzheimer defended his doctoral thesis on the wax-producing ceruminous glands of the ear. His thesis was based on work by Swiss physiologist and histologist Albert von Kölliker (1817-1905), who had perfected Camillo Golgi’s (1843-1926) silver staining method.

In December 1888, Alzheimer was appointed clinical assistant at the Municipal Mental Asylum, at Frankfurt am Main, by director Emil Sioli (1852-1922). The Asylum was a relatively progressive institute; Sioli had just taken over the hospital, and his first acts were to order the removal of physical restraints and to institute hydrotherapy and occupational therapy programs (Goedert and Ghetti 2007). Alzheimer worked in psychiatry and neuropathology and would eventually be promoted to senior physician. The engagements during his medical and doctoral training were formative for his philosophy. It was in Frankfurt that Alzheimer worked along side Franz Nissl (1860-1919), who began at the asylum in 1889 (Terry et al. 1993). It was reported that Alzheimer and Nissl worked closely, often late into the night, while discussing histopathological preparations after a day of clinical practice, and thus becoming close friends (Klunemann et al. 2002).

This was an important time for the development for the neurosciences, and Alzheimer was interacting with some of the most influential individuals in the fields of medicine, anatomy and histology. The *zeitgeist* had been that the brain was central to

pathology for mental and neurological conditions, and neurology and psychiatry were considered new medical specialties, that were typically undifferentiated by profession (Goedert and Ghetti 2007).

It must be noted, however, that this was a dark period for the medical establishment in Germany. It had become enraptured with ideas of social Darwinism, theories of monism, and theories of degeneration and racial hygiene (Roelcke 2004). Many of these ideas were based on writings of the French psychiatrist Bénédict Augustin Morel (1809-1873) in his 1857 book *Treatise of the physical, intellectual and moral degeneration of the human species* (Morel 1857). Morel argued that mental disorders were inherited and that degeneracy was cumulative so that a worsening of symptoms occurred in subsequent generations (Nuland 1995). As a member of the German Racial Hygiene Society in 1913, it is possible that Alzheimer shared some of these same views (Weber 1997). On January 9, 1912 Alzheimer lectured to an audience of 300 at the Society's Munich chapter (Weindling 1989). Many years after Alzheimer's death, the ideas of the Racial Hygiene Society served as a basis for Nazi frameworks, contributing to sterilization of the mentally ill, before being replaced by the wartime "euthanasia" program (Weindling 2004).

Alzheimer reported *Frau* Deter's case as a precocious form of early-onset dementia, now referred to as familial, and due to a mutation in the presenilin gene (see below). When she died in 1906, Alzheimer performed an autopsy and using staining techniques he had acquired from Nissl and others, he processed the brain tissue. He

presented his findings at the Southwest German Psychiatric Physician's Association meeting in Tübingen and the proceedings from his speech were published in a short paper the next year (Alzheimer 1907) and more extensively in 1911 (Alzheimer 1911). In 1910, Emil Kraepelin (1956-1926) controversially proposed naming the disease condition after Alzheimer in his influential *Textbook of Psychiatry* (Berchtold and Cotman 1998). Alzheimer was not the first to describe plaques and dementia, but he was first in describing the enlarged neurofibrillary tangles, and the early-onset form of the disease (Graeber et al. 1997). There was competition between groups of Munich and Prague, and in the end, the German camp won, and the condition was to be called Alzheimer's disease.

Neuropathology of Alzheimer's Disease

A consistent pathological feature of AD involves the deposit of extracellular plaques comprised of aggregated amyloid beta (Aβ) protein and neurofibrillary tangles constituted of hyperphosphorylated tau (Iqbal et al. 1978; Goedert et al. 1989; Beyreuther and Masters 1991; Hardy and Allsop 1991; Selkoe 1991; Hardy and Higgins 1992; Selkoe 2001). Lesions are accompanied by neuron loss, reduction in cortical thickness, and an expansion of the ventricular space. The hippocampus, a brain region central for learning and memory and behavioural control, experiences a loss of up to 80% of neurons over the course of AD (Morris and Kopelman 1986).

Aβ Pathology in Alzheimer's Disease

Amyloid plaques around neurons and glia are one of the key pathological hallmarks in AD (Alzheimer 1907). These plaques are insoluble, quasi-crystalline deposits, centrally comprised of Aβ (usually 40-42 amino acids in length) – a peptide created through the regulated proteolysis of the transmembrane amyloid precursor protein (APP) (Hardy and Higgins 1992; Lesne et al. 2006; Citron 2010).

Neurofibrillary Tangles in Alzheimer's Disease

The second key pathological hallmark of AD is the intraneuronal deposition of NFTs (Alzheimer 1907). NFTs are comprised of paired helical filaments (PHFs) of hyperphosphorylated microtubule-associated protein tau (Weingarten et al. 1975; Grundke-Iqbal, Iqbal, Quinlan, et al. 1986; Grundke-Iqbal, Iqbal, Tung, et al. 1986). The human tau gene encodes 6 tau isoforms through alternative splicing of its mRNA (Goedert *et al*. 1989). The phosphorylation of tau at 5 epitopes is important under normal conditions to maintain cytoskeletal structure, with microtubules of the cytoskeleton serving as an intracellular transport system (Goedert 1993; Goedert et al. 2006). In AD, imbalanced phosphorylation promotes microtubule instability and cell death (Noble et al. 2003). The glycogen synthase kinase-3β (GSK-3β), protein phosphatase 2A (PP2A), and cell division protein kinase 5 (CDK5) have been shown to contribute to abnormal tau hyperphosphorylation (Gong et al. 1995; Patrick et al. 1999; Gong and Iqbal 2008). As a result, these kinases and phosphatases have become targets for reversing abnormal hyperphosphorylation of tau.

The Amyloid Hypothesis of Alzheimer's Disease

At the beginning of the 1980's, George Glenner and Caine Wong made the initiatory arguments that Aβ could be the causative component of AD (Glenner and Wong 1984). Preclinical and clinical data supporting the causative role of Aβ continued to accumulate. In 1996, it was shown that genetic mutations in *APP*, *PSEN1*, and *PSEN2* led to increased Aβ protein, similar to that found in senile plaques (Scheuner et al. 1996). In Down's syndrome, triplication of chromosome 21, which is the chromosome carrying the *APP* gene, leads to increased Aβ and early-onset dementia (Olson and Shaw 1969; Glenner and Wong 1984). Apo-E4, a major risk factor occurring in 40% of individuals with AD has been found to impair Aβ clearance from the brain (Corder et al. 1993). Despite continuing debate the amyloid hypothesis has taken its place as the prevailing model of AD pathogenesis and holds that anomalous Aβ accumulation triggers AD (Beyreuther and Masters 1991; Hardy and Allsop 1991; Selkoe 1991; Hardy and Higgins 1992). The Aβ hypothesis was immediately contentious, though what motivated this enduring controversy remains unclear (Selkoe 2011). The most frequently voiced objection had been that the number of amyloid deposits in the brain did not correlate well with the degree of cognitive impairment the patient experienced in life (Hardy and Selkoe 2002). This criticism was eventually put to bed with the discovery that soluble Aβ, though invisible in histological examination, when assayed biochemically correlates well with cognitive decline (Lue et al. 1999; McLean et al. 1999; Wang et al. 1999; Naslund et al. 2000). Others have taken issue with the appearance of tau pathology seemingly predating amyloid and the absence of neuronal loss in APP animal models as perceived weaknesses of the amyloid hypothesis. At this point, an understanding of the processes that give rise

to an imbalance between production and clearance of Aβ peptides is required before discussing compelling therapeutic targets for AD.

Aβ Homeostasis: Balancing Production and Clearance

The gene encoding amyloid precursor protein (APP) is located on chromosome 21 (Goate et al. 1991) and was cloned in 1987 (Kang et al. 1987). APP is a transmembrane protein that is cleaved by a number of cellular kinases. APP cleavage by the α-secretase A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) yields the α carboxy-terminal fragment, α-CTF, (C83), which remains membrane bound and the soluble APPα fragment (Sisodia 1992; Skovronsky et al. 2000). Subsequent cleavage of α-CTF by the gamma (γ)-secretase yields the APP intracellular domain (AICD) and soluble p3 peptide. Proteolysis initiated by the α-secretase results in the production of no Aβ, and thus, it is referred to as the non-amyloidogenic pathway. Furthermore, the product sAPPα, is actually synaptogenic (Mucke et al. 1994; Bell et al. 2008). Conversely, cleavage of APP at position 671 by the β-secretase, BACE1, yields one membrane bound β-CTF (C99) fragment and another, soluble APPβ fragment (Vassar et al. 1999). The membrane-bound β-CTF is subsequently cleaved by the γ-secretase to produce Aβ and AICD (Gervais et al. 1999). Aβ forms oligomeric species, which then aggregate into Aβ plaques, the hallmark feature of AD [reviewed in (Selkoe 1998)]. Proteolysis initiated by the β-secretase results in the production of Aβ after subsequent activity of the γ-secretase, and as a result, it is referred to as the amyloidogenic pathway. Therefore Aβ is generated through the regulated proteolysis of the APP protein, and as a result, the balance of α-and β-secretase activity has impact on the production of toxic Aβ peptide production.

Mounting evidence supports the role of low-density lipoprotein receptor-related protein (LRP) in receptor-mediated flux of Aβ across the BBB (Zlokovic 2004). Aβ can be transported across the BBB and be cleared from the brain after binding to LRP (Deane et al. 2004). In the same study, it was shown that $A\beta_{40}$ binds preferentially over $A\beta_{42}$, the much more amyloidogenic species of the peptide, pointing to impeded efflux out of the brain. LRP-ligand complex are also internalized to late endosomes to be delivered to lysosomes for degradation of for targeted transcytosis across the BBB into the plasma (Herz 2003). Conversely, Aβ can bind to the multifunction receptor for advanced glycation end products (RAGE), and this has been shown to increase its transfer from the periphery into the CNS (Zlokovic 2004). Down-regulation of RAGE has been shown to reduce influx of Aβ from the periphery into the CNS (Deane et al. 2003).

For peptidolytic removal of Aβ, two major endopeptidases have been implicated. 1) zinc metalloendopeptidases, referred to as insulin degrading enzyme (IDE) or insulysin, and 2) neprilysin. The role of IDE in Aβ degradation (reviewed in (Selkoe 2001)) is clear in IDE knockout mice, which show increased levels of Aβ and AICD. Furthermore, overexpression of IDE is related to reduction in Aβ (Leissring et al. 2003). For its part, neprilysin has been implicated as a rate-limiting Aβ-degrading enzyme (Hama et al. 2001; Iwata et al. 2001). Unilateral intracellular injection of vector expressing human neprilysin in a transgenic mouse model of cerebral amyloidosis showed a 50% decrease in ipsilateral Aβ compared to the contralateral cortex (Marr et al. 2003).

Current Alzheimer's Disease Therapeutics

There currently exists no cure for Alzheimer's disease and treatments (cholinesterase inhibitors, NMDA receptor antagonists) offer only short-lasting, symptomatic relief. In addition to these medicines, drugs targeting the psychological symptoms — including aggression, hallucination, and agitation – are also administered. Finally, in late stage, palliative AD, sedatives are also prescribed.

Cholinesterase Inhibitors and Alzheimer's Disease

The cholinergic system is a main target for Alzheimer therapy given the observation that severe loss of cortical cholinergic innervation invariably occurs in late stage AD (Bowen et al. 1976; Davies and Maloney 1976; Geula and Mesulam 1989). Cholinergic neurons cell bodies are located in the basal forebrain nuclei and their axons project long distances to innervate the cerebral cortex, hippocampus, and amygdala (Mesulam et al. 1983). This innervation is important for maintaining aspects of attention (Everitt and Robbins 1997), memory (Drachman and Leavitt 1974), and synaptic plasticity (Bear and Singer 1986). Cholinergic depletion is most severe in the temporal lobe, which includes the entorhinal cortex, where up to 80% of cholinergic axons are lost (Geula and Mesulam 1996). Associated cell loss in the nucleus basalis is heaviest in the posterior sector, where neurons that preferentially innervate the temporal lobes tend to be located (Mesulam and Geula 1988). It is not known exactly what features of cholinergic neurons specifically predisposes to degeneration, but it is hypothesized that it might be due to the long axons of the cells relating to their increased need for axonal trafficking, increased vulnerability

to Aβ, and increased energetic requirements (Wu et al. 1997; Sendera et al. 2000; Mufson et al. 2002).

Acetylcholinesterase (AChE) enzyme is responsible for the degradation of acetylcholine and mediates its activity (Perry et al. 1978). The theory behind reversible AChE-inhibitor efficacy is that preventing the breakdown of acetylcholine should boost available transmitter and cholinergic function in a neural system that is degrading. The theory held true and the first cholinesterase inhibitor (ChEI) to show success against AD was physostigmine (Davis et al. 1979). This compound however was short lasting, and associated with severe side effects including nausea and vomiting. In 1986, Summers and colleagues developed tacrine (tetrahydroaminoacridine, THA), which yielded symptomatic improvement in a small group of moderate to severe AD patients (Summers et al. 1986). In 1993, tacrine became the first drug approved in the USA for AD. Another early ChEI was metrifonate (Becker and Giacobini 1988), although it was discontinued because of a muscle weakness presented at high doses. Second generation ChEIs approved for AD include donepezil (Rogers et al. 1998), rivastigmine (Rosler et al. 1999), and galantamine (Rainer et al. 1989). However, a corollary of the cholinergic hypothesis (Whitehouse et al. 1982) is that as cholinergic function continues to decline (Davies and Maloney 1976), ChEIs become less effective. Nevertheless, ChEIs currently represent the symptomatic therapy of choice for AD.

N-methyl-D-aspartate (NMDA) receptor antagonists

The adamantine derivative, memantine (MEM, 1-amino-3,5-dimethyl-adamantane), is a low-affinity voltage-dependent uncompetitive antagonist of *N*-methyl-D-aspartate (NMDA)-type glutamatergic receptors (Fleischhacker et al. 1986; Bormann 1989; Kornhuber et al. 1989; Chen et al. 1992; Chen and Lipton 2005). Amantadine, first synthesized by Eli Lilly and Company, had been administered for influenza, and was discovered to have dopaminergic or possible anti-cholinergic properties when taken by a patient with comorbid Parkinson's disease. The drug functions in part by inhibiting excessive calcium influx that occurs with overstimulation of the NMDA receptor. Memantine is currently approved for the treatment of patients with moderate to severe dementia of the AD type. In these cases, meta-analyses indicate beneficial effects on global status and cognition (Reisberg et al. 2003; Tariot et al. 2004). Nitromemantines are second generation NMDA receptor antagonists that use the memantine moiety as a homing signal for the targeted delivery of nitric oxide (NO) to a second modulatory site on the NMDAR [for review see (Chen and Lipton 2006)]

Alzheimer's Disease Genetic Causes

There are two major forms of Alzheimer's disease broadly classified according to their etiology: 1) dominantly inherited and 2) non-dominant forms of AD (including "sporadic" AD) (Bateman et al. 2011). The following section will describe mutations in *APP*, *PSEN1*, *PSEN2* and other genetic risk factors associated with AD,

APP Mutations Cause Alzheimer's Disease

The human amyloid precursor protein (APP) gene encodes a transmembrane protein whose proteolysis gives rise to Aβ peptides (Kang *et al*. 1987). Mutations in *APP* are associated with Alzheimer's disease and Cerebral Amyloid Angiopathy. The first genetic mutations causing AD were discovered in the *APP* gene, and included the London V717I (Goate *et al*. 1991), Indiana V717F (Murrell et al. 1991) and V717G (Chartier-Harlin et al. 1991) mutations. These mutations are likely to contribute toward Aβ fibril formation by changing the charge distribution in the APP protein sequence (Miravalle et al. 2000; Baumketner et al. 2008). Other mutations affect the structure of the molecule, which contains a turn in the V24-K28 region required for folding of the monomer, thereby promoting oligomer formation (Lazo et al. 2005; Grant et al. 2007). Furthermore, these mutations may impede Aβ degradation, for example, with the Dutch mutation showing resistance to IDE (Morelli et al. 2003). The Swedish KM670/671NL mutation functions by altering the APP sequence into a better substrate for BACE1 (Vassar *et al*. 1999; Lin et al. 2000). Still other mutations in the *APP* gene accumulate distal to the γ-secretase cleavage site, including V717 mutations (Bergman et al. 2003; Hecimovic et al. 2004), and tend to elevate the $A\beta_{42}/A\beta_{40}$ ratio. In sum, mutations in *APP* either shift the generation of Aβ towards the amyloidogenic $A\beta_{42}$ isoform, or enhance the aggregation propensity of the Aβ peptides.

PSEN1 Mutations Cause Alzheimer's Disease

The Presenilin 1 A246E mutation was originally reported in conjunction with the cloning of the *PSEN1* gene (Sherrington et al. 1995). The *PSEN* A246E mutation was detected in

a Canadian family of Anglo Saxon-Celtic origin and the kindred was designated FAD1. The FAD1 pedigree was remarkable for its size and detail, describing 531 individuals over eight generation, including 52 affected family members. By investigating 51 family members at the National Institute of Mental Health in Bethesda, MD, scientists were able to create cell lines and successfully cloned the *PSEN1* gene. A detailed analysis of the family pedigree found the mode of transmission to be compatible with autosomal dominant (Nee et al. 1983). The mean age of onset in this kindred is 47 years ± 1.21 and age of death is 55 years ± 2.79, making the mean duration of illness 9 ± 1.43 years. In contrast to other studies, there was no preponderance of affected females.

The clinical characteristics of the disease in this family are indistinguishable from those in sporadic cases of Alzheimer's disease. This is clear when one considers the case report:

"**Case 2 (Subject VII-34)** – A 54-year-old, right-handed woman, the sister of patient 1, had been in good health until age 46, when she became gradually withdrawn and disagreeable. Subsequently, she began to ignore household chores, and at age 48 her husband noted a problem with her memory. Agitation became paramount at age 51. She was admitted to the NIMH at age 52 for neurological evaluation. Six months before admission, her personal hygiene deteriorated, she became disoriented to place and time, and she was only able to communicate in phrases. Neurological examination showed disorientation to time and place, poor memory, echolalia, reduced motor speech, which was limited to phrases, and poor cooperation during the examination. Other than a positive palmomental reflex, the remainder of the findings were normal. The EEG showed extremely poor organization and a continuous mixture of theta and delta activity compatible with a diffuse encephalopathy. Examination of the CSF showed normal pressure with clear, colorless fluid, and the CSF protein level was 29 mg/dL. Cultures of the CSF were negative, and serological studies gave normal results. A CT scan showed dilated lateral ventricles and prominent subarachnoid space at the base of the brain and the sylvian fissures, and over the cerebral convexities. Shortly after her NIMH admission, she was institutionalized and remains hospitalized eight years after the onset of symptoms" (Nee *et al*. 1983).

Postmortem data are available for several members of the FAD1 family. Generalized atrophy was present, but most prominent in the frontal lobes and hippocampus (Nee *et al*. 1983). Neuronal loss was observed, as well as gliosis, neurofibrillary tangles, and plaques. Pick bodies were not seen. Autopsy of a presymptomatic carrier of the A246E *PSEN1* mutation who had died of cardiac causes reported accumulation of progranulin (a growth factor expressed by microglia and neurons) plaque-like structures, in medial and frontal regions in addition to amyloid plaques (Gliebus et al. 2009).

In vitro, this, and other subsequently identified *PSEN1* mutations (Hutton et al. 1996), are associated with an increase in the $A\beta_{42}/A\beta_{40}$ ratio (Murayama et al. 1999), however, the level of Aβ from each mutation does not correlate with age of onset of FAD, suggesting there are other factors involved that determine the age of onset in FAD caused by *PSEN1* mutations. For example, studying *PSEN1* transfected COS-7 cells helped researchers link *PSEN1* mutations to GSK-3β, and its substrates tau and β-catenin, affecting tau phosphorylation and β-catenin-regulated T cell factor transcription activity (Murayama et al. 1998; Takashima et al. 1998). The authors raise the possibility that this association may influence the age of onset associated with each *PSEN1* mutation (Murayama *et al*. 1999). The *PSEN1* A246E gene mutation has been introduced into mice, combined with *APP* mutations, which are now commercially available. The *APP*Swe/*PSEN1*(A246E) mouse was developed first and shows Aβ plaques in a region

specific manner by nine to 12 months of age (Borchelt et al. 1996). Subsequent experiments showed cognitive deficits on the Morris water maze test of spatial learning and memory at 11-12 months of age (Puolivali et al. 2002). The *APP*(V7171)x *PSEN1* (A246E) mice show increased brain $A\beta_{42}/A\beta_{40}$ levels and develop Aβ plaques earlier at 6-9 months (Dewachter et al. 2000). Thus, with the establishment of cell and rodent lines, the contribution of FAD1 kindred to Alzheimer's research continues.

PS2 Mutations Cause Alzheimer's Disease

Rare coding variants in *PSEN2* have also been associated with familial AD, with the first described in 1995 (Levy-Lahad et al. 1995; Rogaev et al. 1995). This was the N141I mutation, found in a kindred being German from Russia, and to date the mutation has been found in 11 families all originating from three small adjacent villages southwest of Saratov, Russia (Bird et al. 1988). As *PSEN2* mutations are much more rare compared to *PSEN1*, there have not been many evaluation of them, nor has the phenotype been extensively described. In culture studies, *PSEN2* mutations have been shown to produce excessive Aβ peptide (Walker et al. 2005). The clinical phenotype of the N141I mutation includes memory problems, psychosis, personality changes, depression, hallucinations, delusions, and seizures (Jayadev et al. 2010). Neuroimaging of Aβ in a 52-year-old carrier of *PSEN2* N141I mutation showed a similar pattern of retention as someone with late onset AD (Klunk et al. 2004) and preclinical *PSEN1* carriers (Klunk et al. 2007).

Tau Mutations in Alzheimer's Disease?

While tau mutations do exist they generally do not generate Alzheimer's disease Aβ plaques, are not found in to be mutant in familial AD, and instead cause a form of frontotemporal dementia (Hong et al. 1998; Hutton et al. 1998; Spillantini et al. 1998). This is counterintuitive, given the prominence of tau accumulation in neurofibrillary tangles and dystrophic neurites. It does show that the primacy of AD rests with Aβ, since mutations in *APP* and presenilin can produce profound alterations in wild-type tau in AD, but not *vice versa* (Selkoe 2011). The short life span of laboratory animals makes it difficult to replicate the tau pathology *in vivo*, so experimenters rely on the addition of mutant tau to *APP* or *PSEN* mutations to form bigenic animals to approximate the Alzheimer pathology.

Genetic Risk Factors for Alzheimer's Disease

ApoE Polymorphisms and Alzheimer's Disease

Apolipoprotein E (Apo-E) is a major cholesterol carrier that supports lipid transport and injury repair in the brain (Liu et al. 2013). The apolipoprotein E 4 allele (Apo-E4) polymorphism was discovered to greatly increase the risk of developing the sporadic, late-onset AD (LOAD) (Poirier et al. 1993; Strittmatter et al. 1993). Carriers of Apo-E2 have less chance, compared to Apo-E3, and Apo-E4 accelerates risk, and each allele has a worldwide frequency of 8.4%, 77.9%, and 13.7%, respectively (Poirier *et al*. 1993; Farrer et al. 1997), with the frequency of the Apo-E4 allele increased approximately 40% in AD patients. Apo-E is predominantly produced by astrocytes, and transports

cholesterol to neurons through low-density lipoprotein receptors (LDLR) (Bu 2009). APO-E4 affects brain network activity, memory, and rate of cognitive decline. Mechanistically, this is achieved through Aβ-dependent and independent manners. Apo-E isoforms differentially regulate Aβ production, aggregation and clearance (Schmechel et al. 1993; Polvikoski et al. 1995; Kok et al. 2009; Kim et al. 2011; Bien-Ly et al. 2012). Independently of Aβ, Apo-E4 might be less efficient in delivering cholesterol and essential lipids required for maintenance of synaptic integrity and plasticity (Ji et al. 2003; Wang et al. 2005; Herz and Chen 2006). Apo-E4 has also been shown to be a crucial regulator of innate immunology, and may therefore promote inflammation that could exacerbate AD pathogenesis (Szekely et al. 2008; Ringman et al. 2012). Finally, Apo-E isoforms have differential roles in maintenance of vascular health (Yin et al. 2012), and may contribute to AD in this axis as well.

TREMs and Alzheimer's Disease

Triggering receptors expressed by myeloid cells (TREMs) are a family of receptors that include excitatory and inhibitory isoforms encoded by a gene cluster linked to the immunoglobulin gene family (Bouchon et al. 2000). TREM1 and TREM2 activate myeloid cells by signaling through the adaptor protein DAP12 (Bouchon et al. 2001). Rare TREM2 gene variants have been shown to contribute as much to AD risk as the ApoE4 genotype, with two groups simultaneously reporting an increase in risk of as much as 3-fold (Guerreiro et al. 2013; Jonsson et al. 2013; Neumann and Daly 2013). TREM2 is an innate immune receptor expressed on microglia cells. In the periphery, TREM2 acts as a negative regulator of inflammatory cytokine and Toll-like receptor

(TLR) responses and phagocytosis (Colonna 2003). In the brain, TREM2 limits Aβ-induced neuritic damage by enabling microglia to surround and alter Aβ plaque structure (Ulrich and Holtzman 2016; Wang et al. 2016). *Trem2* mRNA is up regulated in the brains of *APP*, *PSEN* and *APP*/*PSEN* transgenic mice (Matarin et al. 2015), and cells surrounding Aβ plaques in Alzheimer transgenic mice express high levels of TREM2 (Frank et al, 2008). $A\beta_{42}$ peptides have been shown to activate the TREM2 receptor to produce phagocytosis, production of ROS, and induction of NF-kB (Jones BM et al, 2014). Thus it is possible that TREM2 serves as a gate for microglial responses initiated by Aβ (Guerreiro and Hardy 2013; Guerreiro *et al.* 2013).

Diagnosis of Alzheimer's Disease

The pathological processes underlying AD begin decades before cognitive symptoms become obvious (Jack et al. 2010). Cerebrospinal fluid, magnetic resonance imaging (MRI), and positron emission tomography (PET) biomarkers can identify preclinical AD, where brain pathology is established but cognition remains unaffected. For neuroimaging biomarkers, hippocampal volume measurements made using high-resolution structural MRI serve as an indicator of preclinical AD (Jack et al. 2012; Knopman et al. 2012; Jack et al. 2014). CSF is produced by the choroid plexus and bathes the brain, filling the ventricles of the brain and spinal cord, and has a high turnover rate (Bering 1955). By sampling CSF, one may assess expression of factors in the brain. In the clinic CSF is collected via lumbar puncture. Established CSF biomarkers include $A\beta_{1\text{-}42}$, total tau (t-tau), and phosphorylated tau_{181} (p-tau), and new biomarkers are continuously being reported (Vos et al. 2013). In PET, binding potentials of a ligand to a certain target are

calculated and serve as a measure of that species. Pittsburg compound B (PiB) may be used as the imaging biomarker for Aβ (Klunk *et al*. 2004). Tau-PET imaging has recently been developed and it too looks promising (Villemagne et al. 2015).

Preclinical Alzheimer's Disease Biomarkers

Early diagnosis of Alzheimer's disease is now possible, as is the staging of disease development using positron emission tomography (PET) scans. For instance, tau and amyloid build-up can be seen in PET scans, showing the disease's developments and offering clues about how it progresses. Recently, in a project led by Prof. William Jagust at the University of California Berkeley's School of Public Health, 5 young adults (between the ages of 20-26), 33 cognitively healthy adults between 64 and 90, and 15 Alzheimer's disease patients between 53 and 77 were examined (Scholl et al. 2016). It was found that a rank generated based on tau PET agent ^{18}F-AV-1451 and Aβ PiB PET tracer uptake was accurately reflected by the cognitive ability and diagnosis of the participants. Researchers found that tau was present in the medial temporal lobe, where the hippocampus is located, in most aging brains and related to episodic memory decline in aging. However, more telling is the co-occurrence of tau and Aβ, which was associated with different patterns of tau tracer retention. Thus, the authors conclude that AV-1451 PET imaging allows *in vivo* Braak tau staging (see below) based on the tracer uptake. While issues of sensitivity remain, and although new ligands will need to be developed, visualizing the development of Alzheimer's disease in the brains of living people will aid in diagnosis and treatment.

Braak Staging of Alzheimer's Disease

Braak staging is a classification system of disease severity based on location and amount of tau pathology, aspects which reflect more closely cognitive symptoms and disease severity compared to Aβ pathology alone (Braak and Braak 1991, 1995). Braak staging is delineated as the following: Stages I-II) characterized by a mild or severe alteration of the transentorhinal layer Pre-alpha (referred to as the transentorhinal stages I-II); Stages III-IV) marked by a conspicuous affection of layer Pre-alpha in both transentorhinal region and proper entorhinal cortex and mild involvement of the first Ammon's horn sector (referred to as the limbic stage); Stages V-VI) hallmark is the destruction of virtually all isocortical association areas (referred to as the isocortical stages). Conveying AD pathology according to Braak staging represents a stable scale against which to measure neurodegeneration. The scale is not affected by education level, cognitive reserve, sex, medication history or other confounding factors that drive heterogeneity in disease populations. As a result discovery of the disease-related processes can be made more straightforward.

The practicality of Braak staging is most clearly illustrated by the following example. A group in the Netherlands performed liquid chromatography-tandem mass spectrometry on isolated hippocampus CA1 and subiculum tissue from 40 AD cases at various Braak stages (Hondius et al. 2016). Females were overrepresented slightly in this heterogeneous population, and individuals had varying cognitive ability, varying diagnostic positions, varying Aβ pathology, but when patients were classified only according to Braak staging, then researchers were able to discern significant shifts in

protein expression during AD pathogenesis and found clusters that were modulated according to Braak stage. One could then consider the level of Braak staging in the context of education, neural reserve, and medical history in order to predict AD progression.

NIA-AA Preclinical AD Classification

In 2011, the National Institute of Aging and Alzheimer's Association (NIA-AA) working group proposed new criteria for staging preclinical AD, such that Stage 1 is characterized by the presence of β-amyloidosis alone, and Stages 2 and 3 by the presence of both β-amyloidosis and neuronal injury (Sperling et al. 2011). However, it has just recently been reported that there is low concordance between CSF and imaging neuronal injury biomarkers that may lead to different NIA-AA staging classification (Vos et al. 2016). This disparity likely reflects different pathophysiological processes, and thus groups that are defined using different neuronal injury markers may have different responses to therapeutic interventions. To explain the lower concordance between neuronal injury markers, the authors provide the following example: CSF levels of tau likely reflect diffuse neuronal injury, while hippocampal volume measures focal changes. Further, a loss of gray matter observed in MRI could be due to both a loss of dendritic branching and cell death. Complicating matters further, each neuronal injury marker may become abnormal as a different stage of the AD process. Very recently, the International Working Group (IWG) has presented an updated definition of the preclinical stage of AD, and now advocate for "preclinical AD" when the risk is particularly high (positive Aβ and tau markers) and that of "asymptomatic at risk for clinical AD (AR-AD)" when the evolution

to a clinical AD is likely or still needs to be determined (for example, when only one pathophysiological marker considered abnormal) (Dubois et al. 2016).

Resting State fMRI

In observing brain physiology, functional magnetic resonance imaging (fMRI) exploits changes in energy signals stemming from differences in blood oxygenation levels (Ekstrom 2010). This blood oxygen level-dependent (BOLD) signal serves as an indicator of activity within a brain region, and for this reason, *event-related* fMRI has been used to investigate connectivity and functional mapping of the brain at work. Spontaneous low frequency (0.01-0.08 Hz) fluctuations were discovered in the motor cortex of healthy subjects using so-called *resting-state* fMRI. These low frequency fluctuations are physiologically meaningful and reflect spontaneous neural activity (Biswal et al. 1995; Shmuel and Leopold 2008). Resting-state fMRI connection studies with temporal analysis have led to the identification of connectivity maps across the brain in healthy subjects and in those with brain disorders. More recently, the amplitude of the low frequency fluctuations has been found to be more useful in differentiating physiological states of the brain. Furthermore, these resting-state evaluations show less between-subject variance and are better controlled when compared to event-related fMRI studies (Zuo et al. 2010).

Alzheimer Disease Detectable Using Resting-State fMRI

Emerging evidence has demonstrated how resting-state fMRI may be used to diagnose Alzheimer disease in clinical populations. The default mode network is a neural network

comprised of posterior cingulate/precuneus, medial prefrontal, and bilateral lateral parietal cortex that shows synchronous, low frequency BOLD signal changes during rest (Binder et al. 1999). Behaviorally, the default mode network is thought to be involved in episodic memory including reviewing past knowledge or preparing future actions, associative tasks that are difficult for those suffering from Alzheimer disease. In 2004, Greicius and colleagues published results showing degeneration in certain brain regions affected by Alzheimer disease, namely the posterior cingulate cortex and the hippocampus. In addition, these authors used resting-state fMRI to identify significant disruptions in default mode network co-activation in patients with Alzheimer disease (Greicius et al. 2004). Though diagnostic power was somewhat low (85% sensitivity and 77% specificity in distinguishing Alzheimer patients from healthy elderly adults), this study provided proof in principle that resting state fMRI could identify those in the group with incipient Alzheimer disease.

Diagnostic power was increased in a study showing if time-course data are explored during the resting state (specifically activation patterns anastomosing between the identified nuclei), more subtle deficits are observed in Alzheimer disease patients (Koch et al. 2012). The power is demonstrated in the following example: the group found that the best single parameter (independent component analysis) for distinguishing Alzheimer's patients from healthy controls was activity of the default mode network in the anterior cingulate cortex (sensitivity 53%, specificity 71%). The authors also performed time course correlation analyses, and in this case, connectivity between the posterior cingulate cortex and superior frontal cortex was most telling in detecting

Alzheimer disease in patients (sensitivity 73%, specificity 71%). A combined model of analysis integrating independent components analysis and time-course analyses achieved full sensitivity (100%) and higher specificity (95%) in diagnosing the Alzheimer disease individuals in the scanner. Future investigations will evaluate the ability of the combined method to predict whether patients with mild cognitive impairment will convert to Alzheimer disease. Thus, resting-state fMRI stands as a powerful diagnostic tool in for the early detection, diagnosis, and tracking the progression of AD.

Alzheimer's Disease and Neuroinflammation

It has been proposed that risks for late onset AD, which include genetic, biological and environmental factors, can contribute to neuroinflammation leading to neurodegeneration at later stages of AD (McNaull et al. 2010). It has also been suggested that neuroinflammation plays a beneficial role in the late stages of AD, through microglia-mediated phagocytosis of Aβ plaque material (Akiyama et al. 2000; Agostinho et al. 2010). However, there is now evidence that inflammation at the developing stages of AD is may not beneficial, and rather, may be disease aggravating. Thus, AD is characterized by a state of persistent neuroinflammation (Akiyama *et al*. 2000). Dysregulation in brain immunity and homeostasis is closely linked to Aβ pathology, so much so that it has given rise to the conceptualization of the inflammatory cascade hypothesis (Karran et al. 2011). This hypothesis holds that a positive feedback loop is established wherein Aβ and inflammatory components are positive regulators of one another and are co-aggravating. Signals stemming from peripheral inflammation can also stimulate Aβ production. A

central tenet of the inflammatory cascade hypothesis is that it is difficult to exit the cycle, either by resolving neuroinflammation or stymying the production of toxic Aβ.

Consequently, there is increasing evidence indicating that proinflammatory mechanisms contribute to AD neuropathology (Itagaki et al. 1989; Sastre et al. 2006; Lee et al. 2009) and this is supported by a growing body of evidence showing inflammation as an early event in the progression of AD. For example, microglial activation is detected in patients with mild cognitive impairment (MCI) (Parachikova et al. 2007)—a stage that often represents a prodromal phase to overt AD. Further, prolonged treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with reduced risk of developing AD (Breitner 1996; in t' Veld et al. 2001; Weggen et al. 2001; Sastre et al. 2003; Klegeris and McGeer 2005). Data from mouse and rat transgenic animal models show that inflammatory processes precede amyloid plaque deposition and are concurrent with the progressive accumulation of Aβ oligomeric material (Heneka et al. 2005; Maezawa et al. 2011; Ferretti, Bruno, et al. 2012; Wright et al. 2013; Hanzel 2014). Interventions that inhibit this initiating proinflammatory process have a positive outcome, by diminishing Aβ synthesis and improving cognition (Lim et al. 2011; Ferretti, Allard, et al. 2012; Lim et al. 2012; Vom Berg et al. 2012).

The modulation of immune molecules, including complement proteins C1q and C3, have emerged as critical mediators of synaptic refinement and plasticity through C3-dependent microglia phagocytosis of synapses (Stevens et al. 2007). Transforming growth factor (TGF)-β is a key regulator of neuronal C1q expression to initiate

complement-dependent and microglia mediated synaptic pruning in the developing visual system (Bialas and Stevens 2013). The consequence is that complement-mediated synapse elimination may become aberrantly reactivate in neurodegenerative disease. In support of this, recent work in mouse models showed that complement and microglia mediate early synapse loss in AD (Hong et al. 2016).

Microglia serve as the immune cells of the brain, and like in the periphery, they are responsible for surveillance and debris removal. Microglia function can be broadly separated into 3 main roles: 1) microglia mobilize to sites of insult and injury and remove intruders and stimulate inflammation; 2) they act as resident phagocytes, engulfing dying cells and pathogens; and 3) they have surface receptors for complement proteins, immune molecules that tag pathogens and cellular debris with a message that tells microglia to remove the intruders. Microglia normally act as sentinel cells, attacking and removing pathogens and cell debris, but become reactive in AD. Microglia are involved in Aβ plaque reduction during sustained neuroinflammation (Cherry et al. 2015). Interestingly, TREM2 limits Aβ-induced neuritic damage by enabling microglia to surround and alter Aβ plaque structure (Ulrich and Holtzman 2016; Wang *et al.* 2016).

Oxidative Stress and Alzheimer's Disease

Reactive oxygen species (ROS) are produced as a byproduct of metabolism and oxidative stress occurs in tissues where the antioxidant processes are insufficient to the level of ROS produced. Increased levels of ROS including lipid peroxidation and products of

ROS damage have been observed in mouse models of AD and in AD patients (Sultana et al. 2013; Matsumura et al. 2015; Porcellotti et al. 2015; Swomley and Butterfield 2015). For example, 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) protein modifications represent key makers of oxidative stress. HNE is one of the major products of lipid peroxidation and HNE-protein adducts are detected in the brain of patients with AD (Lauderback et al. 2001; Perluigi et al. 2009). Protein nitration stems from nitric oxide synthase production of nitric oxide, which reacts with superoxide anion to form peroxynitrite ($ONOO^-$). Peroxynitrite can react with carbon monoxide (CO_2) to produce nitrite radicals that can react with a tyrosine residue to form 3-NT. Importantly, protein nitration has been documented in MCI and in early and late AD (Tohgi et al. 1999; Butterfield, Reed, Perluigi, et al. 2007; Reed et al. 2009). Accumulating oxidatively modified proteins disrupts cellular functions through loss of catalytic function ability or by interruption of regulatory pathways (Sultana et al. 2010). Because protein oxidation generally leads to its dysfunction, the identification of oxidatively modified proteins provides insight into some of the biochemical and pathological alterations involved in AD pathogenesis (Perluigi *et al.* 2009). For example, a redox proteomic approach used to identify specific HNE-bound proteins in the inferior parietal lobule and hippocampus of AD subjects and age-matched controls revealed that HNE was bound to energy metabolism, antioxidant system, and structural proteins (Perluigi *et al.* 2009). The same approach revealed that these changes occur before the presentation of full AD, as HNE damage was observed in the brain of subjects at MCI stages (Reed et al. 2008). It is now accepted that ROS are capable of provoking neuroinflammation, which in turn produce

ROS (Block et al. 2007). What remains unclear is which process begins this self-perpetuating neurotoxic cycle.

CREB and CRTC1 Signaling in Alzheimer's Disease

Cyclic-AMP response element-binding protein (CREB) activates transcription across a diverse range of ontological categories, including synaptic/endocrine, metabolic/structural, transcription, signaling, cell cycle/proliferation and survival/stress (Impey et al. 2004), and has a central role in regulating many forms of neuroplasticity (Lonze and Ginty 2002; Benito and Barco 2010). CREB is widely inducible, with multiple parallel pathways converging to signal gene transcription. CREB binds to a cAMP-responsive element (CRE) sites consisting of the consensus sequence 5'-TGACGTCA-3' (Richards et al. 1996). This site was first identified in the somatostatin gene (Montminy et al. 1986), but there are now believed to be 10,000 CRE sites scattered throughout the mammalian genome (Cha-Molstad et al. 2004; Zhang et al. 2005). Despite being so promiscuous, context-specific activation of CREB is achieved through modulation of co-regulators.

CREB is regulated by phosphorylation on Ser129, Ser133, Ser142 and Ser143 and others (Kornhauser et al. 2002; Lonze and Ginty 2002). This is accomplished through various kinases including extracellular-regulated kinases 1 and 2 (ERK1/2) (Riccio et al. 1999; Lee et al. 2005; Socodato et al. 2009), calcium/calmodulin-dependent protein kinases II and IV (CaMKII, CaMKIV) (Dash et al. 1991; Enslen et al. 1994; Matthews et al. 1994; Sun et al. 1994; Parker et al. 1998; Kornhauser *et al*. 2002), protein kinase A

(PKA) (Radhakrishnan et al. 1997; Shaywitz and Greenberg 1999), Akt/protein kinase B (PKB) (Bonni et al. 1995; Meier et al. 1997; Du and Montminy 1998), and glycogen synthase kinase-3 (GSK-3) (Bullock and Habener 1998; Grimes and Jope 2001). Thus, CREB becomes phosphorylated through the increase in cytosolic calcium, either through L-type voltage-gated calcium channels, N-methyl-d-aspartate (NMDA) receptors, and/or release from internal stores. Nuclear CREB associates with the basal transcription apparatus including CREB-binding protein (CBP), TAFII130, which serves to recruit the RNA polymerase II holoenzyme and RNA helicase A to initiate transcription (Shaywitz and Greenberg 1999; Cardinaux et al. 2000).

CREB is downregulated in AD (Pugazhenthi et al. 2011). Brain-derived neurotrophic factor (BDNF), a key CREB target gene, is essential for proper brain development and function and learning and memory. BDNF levels are dramatically reduced in mild cognitive impairment and early in Alzheimer's disease (Peng et al. 2005; Rosa and Fahnestock 2015). The CREB regulated transcription co-regulator 1 (CRTC1, previously known as TORC1, MECT1) is a rate-limiting CREB activator (Conkright, Guzman, et al. 2003; Iourgenko et al. 2003) that is highly expressed in the brain (Altarejos et al. 2008; Watts et al. 2011). CRTC1 is sequestered to the cytoplasm in its inactive form, bound to the cytoplasmic membrane to 14-3-3 proteins (Screaton et al. 2004; Ch'ng et al. 2012). Upon dephosphorylation of Ser151 by the calcium-dependent phosphatase calcineurin, CRTC1 translocates to the nucleus. Here, CRTC1 tetramerizes, binds to the CREB bZIP domain, and stabilizes the basal transcription apparatus

(Ravnskjaer et al. 2007). This process is rapid and transient; the salt-inducible kinase 1 phosphorylates CRTC1 *de novo*, and it is shuttled back to the cytoplasm.

CRTC1 activation conveys activity-dependent neuroplasticity required for learning and memory. The group of Dr. Sheena Josselyn at the University of Toronto has been studying CREB, CRTC1, and memory for more than a decade [see retrospective: (Josselyn et al. 2015)]. The team has generated tools to increase or inhibit CRTC1, in order to more understand its role, particularly in memory (Josselyn et al. 2001; Vetere et al. 2011). For example, by applying an adeno-associated virus (AAV), they increased CRTC1 in the dentate gyrus of the hippocampus in laboratory mice (Sekeres et al. 2012). They then compared learning in the mice using a fear-conditioning paradigm. They found that by increasing CRTC1 at the time of memory formation, the memory was more stable over time, without affecting its quality (i.e., the memory remained context-specific). Similarly, if they increased DG CRTC1 during reactivation, the period when the memory is recalled, the memory was again stronger when tested later. These results indicate the specificity of targeting CRTC1 to enhance memory.

A first study by a laboratory at the Universitat Autònoma de Barcelona in Spain led by Dr. Carlos Saura demonstrated *in vitro* that CRTC1 is impaired by Aβ accumulation (Espana et al. 2010; Saura 2012). Subsequently, an *in vivo* project demonstrated that CRTC1 translocation is reduced in the CA1, CA3, and DG regions of the hippocampus following learning (Parra-Damas et al. 2014). The team showed that when this reduction was restored using AAV, the learning deficits were corrected. They

also show reduced CRTC1 levels in human hippocampus at intermediate Braak III/IV pathological stages, along with reduced transcription of CRTC1-dependent genes. These results, together with those of the Josselyn lab, serve as proof of principle that increasing CRTC1 transcription activity might be therapeutic in AD. Nevertheless, the invasiveness of AAV delivery of CRTC1 currently precludes it from being a serious therapeutic option for AD. Taken together, CREB and CRTC1 are dually inhibited in AD, and preliminary experiments attempting to correct this dysfunction have shown promise, validating these transcription factors as a possible therapeutic target in AD.

Lithium for the Treatment of Alzheimer's Disease

Emerging evidence now shows that lithium, a drug used for more than 60 years in the treatment of bipolar disorder, has disease-modifying properties in patients at risk for developing AD (Kessing et al. 2008; Forlenza et al. 2011) and in AD animal models overexpressing APP (Zhang et al. 2011). However, the mechanism of action of lithium-mediated neuroprotection is poorly understood. If properly comprehended, an understanding of the molecular events conveying the protective effect of lithium could lead to the identification of possible avenues for therapeutic agents.

Historical Uses of Lithium

Johan August Aftwedson is credited with discovering lithium (from Greek *lithos*, meaning "stone") in 1817. Lithium is a soft, silver-white metal with the symbol Li and the atomic number 3. Like all alkali metals, lithium is highly reactive and flammable.

When exposed to moist air, it corrodes quickly to a dull silvery gray, then to a black tarnish. Historical uses of lithium in medicine were described in 1944, by Sir David Kennedy Henderson (aside: interestingly, Henderson studied with Emil Kraepelin in Germany) and Robert Dick Gillespie, in their seminal book "A Text-Book of Psychiatry". In it they describe how certain wells were thought to have "special virtue" in the treatment of mental illness (Henderson and Gillespie 1944). They mention some of the more famous waters in the British Isles, and that the efficacy is likely proportional to the lithium content of the waters.

Clinical Discovery of Lithium

Soon after, John Cade, who was born in the small country town of Murtoa, Australia in 1912, reported the sedative effect of lithium. Cade's father worked with the Australian Mental Hygiene Department, and Cade spent many of his formative years living on the grounds of major mental hospitals, which had a great impact on his empathy toward the mentally ill (Cade 1999). Australian "lunatic asylums" at the turn of the 20th century acted primarily as custodial institutions rather than hospitals offering treatment. In general, no effective therapy was available for the major mental illnesses.

However, Cade was interested in the excitable states of some of the institution's manic patients, and believed that the cause of such states had to be the presence of some unknown toxin infecting their systems. He believed that this toxin could also be present in the urine (Cade 1949), and ventured to prove this hypothesis by injecting urine from manic patients into healthy animals, observing their resultant behavioural states. Cade

carried out his experiments in laboratories he had set up at the hospital and in a room off of the family kitchen; the family recalls jars of urine kept in the refrigerator for these experiments (Cade 1999). His first set of experiments failed. After injecting an aqueous solution of 8% urea intraperitoneally into guinea pigs, he observed irritation and swelling around the site of injection, killing five of ten guinea pigs (Cade 1949). Cade then wondered whether uric acid would enhance this toxicity, and in his next round of experiments he used the most soluble urate, the lithium salt. He reported a transient sedative effect on guinea pigs given 0.5% lithium carbonate in an 8% urea solution, and noted that all of the guinea pigs survived. He replicated the finding, and through additional control experiments, he was able to deduce that the sedative effect on guinea pigs was owing to lithium salt.

Cade hypothesized that the sedative effect could offer relief to the disorder of mania. However, before testing the lithium salt on his patients, he first ingested it himself, at great personal risk, to be sure that is was safe to do so (Cade 1999). He reported experiencing no adverse effects. In the coming weeks and months, he prescribed lithium openly to his patients and recorded mood improvement as clinical outcome (Cade 1949). He published his findings in 1949, detailing the recovery of ten manic patients. He concluded that lithium should be a treatment for those with "restless impulses and ungovernable tempers" which was much preferred to the medical convention at the time, the pre-frontal leucotomy. As described in the next section, it remains unknown what precisely is lithium's therapeutic mechanism of action.

Lithium Toxicity

Lithium clearance is primary through excretion in the kidneys and the major issue with the application of lithium is its severe side effect profile (Gelenberg and Jefferson 1995; Hampel et al. 2009; Azab et al. 2015). Lithium has a narrow therapeutic window, and these severe side effects preclude its long-term use in elderly patients. These adverse effects include tremor, nausea, polyuria, polydipsia, and weight gain (Azab *et al*. 2015). Because of these side effects, lower plasma concentrations are intentionally targeted for long-term maintenance treatment.

Lithium Mechanisms of Action

A number of enzymes have been identified as targets of lithium action, as reviewed in (Phiel and Klein 2001). For instance, lithium directly inhibits inositol monophosphatase (Berridge et al. 1989), the large family of related phosphomonoesterases (York et al. 1995), as well as the ubiquitous kinase GSK-3 β (Klein and Melton 1996). Lithium also reduces BACE1 activity, oxidative stress and inflammation, promotes CRTC1 association with co-factors, and restores neurogenesis. These broad effects are described in expanded detail below.

Lithium Inhibition of GSK-3

In 1996, papers (Klein and Melton 1996; Stambolic et al. 1996) simultaneously released by two separate research teams reported a direct ability of lithium to inhibit GSK-3 *in vitro* and in cells. The initial findings were difficult to extend to *in vivo* settings, as the concentration of lithium used was so far above that which could be achieved in living

systems. However, these results were eventually reproduced *in vivo* (Beaulieu et al. 2008). Li^+ ions act as an uncompetitive inhibitor of Mg^{2+}, resulting in the potential interference of lithium with magnesium-dependent cellular processes, including the binding to GSK-3 (Ryves and Harwood 2001). Indirectly, lithium activates Akt/PKB, which is a kinase that promotes inhibitory phosphorylation on GSK-3β at serine 9. Thus, lithium has been shown to be an inhibitor of GSK-3.

Lithium Inhibition of BACE1

BACE1 activity has been shown to be elevated in AD and in models of Alzheimer amyloidosis (O'Brien and Wong 2011; Ferretti, Allard*, et al.* 2012). Similarly, hyperactive BACE1 promotes Aβ accumulation in other conditions, including in mouse models of traumatic brain injury (Yu, Zhang, et al. 2012). BACE1 cleaves APP to produce toxic $A\beta_{42}$ peptides (Citron et al. 1992; Das et al. 2015). Accordingly, BACE1 inhibitors have been explored as putative therapeutic agents for the treatment of AD (Yan and Vassar 2014). Recently, Parr and colleagues showed that activation of Wnt/β-catenin signaling pathway promotes binding of T-cell factor-4, a *Bace1* gene repressor (Parr et al. 2015), and multiple lines of evidence point to a role of lithium in mimicking Wnt/β-catenin signaling (Stambolic *et al.* 1996; Hedgepeth et al. 1997; De Ferrari and Inestrosa 2000; Toledo and Inestrosa 2010). Therefore, it is also possible that modulation of Wnt/β-catenin signaling by lithium contributed to a reduction of *Bace1* gene expression.

Lithium Potentiation of CRTC1

CRTC1 binds to CREB to stabilize transcriptional machinery and provoke mRNA synthesis (Conkright, Canettieri, et al. 2003). The activity of CRTC1 in the nucleus is rapid, and rephosphorylation of CRTC1 by SIK1 returns CRTC1 to the cytoplasm (Screaton *et al*. 2004). Thus, the presence of CRTC1 in the nucleus is tightly regulated to synaptic activity (Ch'ng *et al*. 2012). Nuclear translocation of CRTC1 in response to glutamatergic neurotransmitter signaling is reduced by intracellular Aβ (Espana *et al*. 2010; Saura 2012; Parra-Damas *et al*. 2014).

Since Aβ restricts the amount of CRTC1 in the nucleus, one therapeutic strategy is to increase the association of CRTC1 with CREB in order to engage what small amount of CRTC1 is translocated to the nucleus. One group boosted CRCT1 in the nucleus simply by knocking-down SIK1 in a model of circadian rhythm phase shift (Jagannath et al. 2013). This leads to an increase in nuclear CRTC1, although there was no indication whether this resulted in gene-specific CRE transcription. Similarly, increasing the amount of time CRTC1 spends bound to the CRE promoter is another mechanism by which to increase CRTC1 output, and might be more specific, as it requires CRTC1 be bound correctly in the first place. Lithium promotes oligomerization of CRTC1 and its association with co-factors CREB and CBP (Boer et al. 2007; Heinrich et al. 2009; Heinrich et al. 2013). Brain-derived neurotrophic factor (BDNF) is essential for proper brain development and function and is particularly critical for learning and memory. Its levels are dramatically reduced in mild cognitive impairment and early in Alzheimer's disease, correlating with loss of memory, while raising BDNF levels in the

brain rescues cognitive function (Holsinger et al. 2000; Garzon and Fahnestock 2007; Rosa and Fahnestock 2015). Lithium increases neuroprotection and BDNF levels in plasma in humans (de Sousa et al. 2011; Forlenza *et al.* 2011). Thus, one of the Aims of this thesis is to evaluate the hypothesis that treatment with lithium will increase the level of CRTC1 association with plasticity gene promoters, including BDNF, in an AD model of amyloidosis. Chapter 3 will demonstrate that a novel, microdose formulation of lithium restores CRTC1 genetic occupancy in promoters of synaptic plasticity genes.

Lithium's Anti-Inflammatory Effects

Many of the anti-inflammatory properties of lithium are mediated through GSK-3, which influences pro-inflammatory cascades through NF-kB and the anti-inflammatory effects of lithium are increasingly reported (Basselin et al. 2007; Li et al. 2011; Yu, Wang, et al. 2012). Lithium lowers the production of IL-1β and TNF-α in rat primary glial cells (Nahman et al. 2012), and reduces microglia activation in ischemic mouse models (Li *et al.* 2011).. Chapter 4 will demonstrate the anti-inflammatory effects of a novel formulation of lithium in a transgenic rat model of Alzheimer-like amyloidosis.

Lithium's Anti-Oxidative Effects

Oxidative stress occurs in cells where the antioxidant capacity is outpaced by the production of oxidants, resulting in the increased production of damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Butterfield, Reed, Newman, et al. 2007). Accumulating oxidatively modified proteins disrupts cellular functions through loss of catalytic function ability or by interruption of regulatory pathways

(Sultana *et al.* 2010). Lithium lowers oxidative stress in healthy subjects and in early stage bipolar disorder by reducing free-radical formation, lipid peroxidation, and levels of hydrogen peroxide (Machado-Vieira et al. 2009; Khairova et al. 2012; de Sousa et al. 2014).

Lithium's Neurogenic Properties

Adult neurogenesis occurs almost exclusively at two sites in the rodent brain: at the subventricular zone in the walls of the lateral ventricles and at the subgranular zone in the dentate gyrus (DG) of the hippocampus (Altman and Das 1965, 1965; Kaplan and Hinds 1977). In these regions, thousands of cells are born with many becoming fully integrated into established cortical networks (Doetsch et al. 1997; Tashiro et al. 2007). Interestingly, the developmental course of adult neurogenesis includes the same milestones as embryonic and post-natal neurogenesis (Duan et al. 2008). While the function of neurogenesis at these two sites has been linked to learning and memory, it remains unclear why only these regions show such neurogenesis in adulthood, nor is it known what exact role adult neurogenesis plays in behaviour (Aimone et al. 2011; Sahay et al. 2011). Impairments in hippocampal neurogenesis were associated with amyloid deposition (Haughey et al. 2002; Zhang et al. 2007). Lithium enhances adult hippocampal neurogenesis (Chen et al. 2000; Fiorentini et al. 2010), which is inhibited by Aβ and neuroinflammation (Ito et al. 1998; Haughey *et al.* 2002; Monje et al. 2003; Mu and Gage 2011; Fuster-Matanzo et al. 2013).

Lithium Treatment of Alzheimer's Disease

There have been reports of clinical trials using lithium for the treatment of Alzheimer's disease in human populations, although with conflicting conclusions. The first clinical trial with lithium in AD was an open label study, where lithium carbonate (serum levels 0.3-0.8 mmol/L) was administered to 22 AD patients for up to one year (Macdonald et al. 2008). There was no change in Mini-Mental State Examination (MMSE) observed among participants who completed the study. The MMSE consists of an 11-question measure that tests five areas of cognitive function: orientation, registration, attention and calculation, recall, and language, and has a maximum score of 30, such that a score of 20 to 24 suggests mild dementia, 13 to 20 suggests moderate dementia, and less than 12 indicates severe dementia (Kurlowicz and Wallace 1999).

The second report was a randomized, single-blind placebo-controlled, multicenter, 10-week study that was published in 2009 (Hampel *et al*. 2009). In this study, 71 patients with mild AD, as defined by displaying a MMSE score greater than 21 and less than 26, were randomly assigned a placebo (n = 38) or lithium treatment (n = 33). The treatment was 10-weeks and included a 6-week titration phase. They found no significant treatment effect on GSK-3 or other biomarkers. Nor did they find a change in global cognitive performance, assessed using the Alzheimer's disease assessment scale (ADAS)-Cognitive subscale. The ADAS-cog consists of 11 tasks measuring disturbances of memory, language, praxis, attention and other cognitive abilities that are often referred to as the core symptoms of AD. One major concern of this report, however, is with the

application of such short treatment duration; it is unlikely that any drug would be effective if given only for a period of 10-weeks with a 6-week titration phase.

A subsequent study published in 2011 allayed some of these concerns, perhaps adding a clearer picture. In this case, a randomized, placebo-controlled, single centre, 12-month study design was employed (Forlenza *et al*. 2011). This study recruited 45 patients with mild AD (MMSA > 21 and < 26) and randomly assigned placebo (N = 24) or lithium treatment (N = 21). The 12-month treatment also included a 6-week titration phase. In contrast to the Hampel *et al*. study, these authors reported significant decrease in CSF concentrations of p-tau and improved performance on the ADAS-Cog subscale. Thus, it raises the possibility that a treatment duration longer than 10 weeks might be required for lithium to be effective in mild AD.

Finally, a trial published in 2013 demonstrated positive results using a microdose formulation of lithium. This study evaluated the administration of 300 μg of lithium given once daily for 15 months (Nunes et al. 2013). In an attempt to correct for differences in education level, Alzheimer patients were selected if they scored > 9 and < 21 on MMSE if they had 4 years or less of schooling, and > 12 and < 24 if they were at school for longer than four years. The authors found that while the placebo group (n = 45) deteriorated in MMSE scores over the 15-months, the lithium-treated group (n = 59) remained stable. These results do reinforce the therapeutic potential of lithium in the treatment of AD. However, this study was limited by the application of only a single cognitive measure, with no measure of serum lithium level performed, and no follow-up

on biomarkers. Taken together, these studies reveal the need preclinical research into the beneficial effects of lithium in the treatment of AD.

Thesis Objectives and Rationale

Alzheimer disease (AD) is characterized by the presence of Aβ plaques, intracellular neurofibrillary tangles, and a progressive decline in cognitive function (Selkoe 2001). Brain regions most affected by AD pathology are the hippocampus, entorhinal cortex and prefrontal cortex, and consequently, late-stage AD is associated with difficulties in decision-making, working memory, spatial navigation, and the acquisition and storage of new information. Aberrant cleavage of the Aβ precursor protein (APP) is a central feature in triggering pathology (Hardy and Selkoe 2002; Oddo et al. 2006). While the exact cause of AD remains unknown, biomarker evidence suggests that pathological processes underlying AD begin decades prior to overt manifestation of cognitive symptoms (Jack *et al*. 2010). This, along with the promise of disease-modifying therapies, has made the characterization of early AD a major topic of research (Nestor et al. 2004; Donohue et al. 2014).

Modeling Preclinical Alzheimer's Disease - The McGill-R-Thy1-APP Transgenic rat

A progressive and predictable evolution of pathology in transgenic animal models facilitates study of early AD. Our lab has developed a successful Alzheimer transgenic rat model that incorporates Swedish and Indiana mutations in the *APP* gene (coded McGill-R-Thy1-APP) (Leon et al. 2010). In McGill-R-Thy1-APP Alzheimer transgenic rats, a

single transgene copy per allele produces the rich AD pathological phenotype. Homozygous animals display the full AD-like pathology with the appearance of Aβ plaques matching the progression of plaque appearance in human patients with AD. These rats develop the first Aβ plaques as early as 6-9 months of age, and display intraneuronal Aβ accumulation of the toxic oligomeric soluble form before plaque formation (Leon *et al.* 2010; Iulita et al. 2014), that is coincident with a pre-plaque proinflammatory process (Hanzel et al. 2014). These rats also develop cognitive deficits at 3 months of age, before the appearance of plaques, that progress with pathology, as detected by learning and memory tasks such as the Morris water maze (MWM), fear conditioning (FC), and novel object recognition (NOR) (Leon *et al.* 2010; Iulita *et al.* 2014).

Lithium for the treatment of Alzheimer's disease

Lithium potentiates CRTC1 activity by stimulating its interaction with CREB (Boer *et al*. 2007; Heinrich *et al*. 2009; Heinrich *et al*. 2013). Accordingly, there are disease-modifying properties of lithium in patients at risk for developing AD (Kessing *et al.* 2008; Forlenza *et al.* 2011) and in AD animal models (Zhang *et al.* 2011; Sofola-Adesakin et al. 2014). Most of these studies indicate lithium neuroprotection to be mediated at least in part by the inhibition of a central cellular kinase, GSK-3β. On the other hand, some have shown the protective effect of lithium to be independent of GSK-3β inactivation. Hence, the mechanism of action of lithium-mediated neural repair is poorly understood.

Lithium is associated with many toxic side effects and therefore, is not recommended for long-term treatment. In addition, this toxic profile makes it difficult to identify the underlying mechanism of therapeutic action. This thesis will investigate the CRTC1 transcription program as a possible mechanism with which lithium interacts to be therapeutic for AD. This thesis will evaluate the therapeutic efficacy of a recently developed transmucosal formulation of lithium whose lower dose achieves therapeutic effects in the absence of associated side effects (Pouladi et al. 2012).

An Early Inflammatory Component to AD

There is abundant evidence indicating that pro-inflammatory mechanisms within the CNS contributes to cognitive impairment via cytokine-mediated interactions between neurons and glial cells (Lee *et al.* 2009). This AD-related inflammatory process is classically regarded as being an Aβ plaque-induced inflammatory process with full activation of microglia (Akiyama *et al.* 2000; Agostinho *et al.* 2010). More recently, it has been proposed that risks for late onset AD, which include genetic, biological and environmental factors, can contribute to neuroinflammation leading to neurodegeneration at later stages of AD (McNaull *et al.* 2010). Further to this, it should be assumed that in the silent stages of AD, there is an ongoing inflammatory process as it has been well documented that the lifelong use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the prevalence of AD (Klegeris and McGeer 2005). A growing body of evidence also supports the concept that inflammation is an early event in the progression of AD. Microglial activation could be detected in patients with mild cognitive impairment (MCI) (Parachikova *et al.* 2007)—a stage that often represents a prodromal phase to overt AD. It

has been recently shown in mouse and rat transgenic animal models that a pro-inflammatory process precedes amyloid plaque deposition and is concurrent with the progressive accumulation of Aβ oligomeric material (Ferretti, Bruno*, et al.* 2012; Hanzel 2014). It was also demonstrated that drugs capable of inhibiting the initial pro-inflammatory process have a positive outcome, including diminished Aβ synthesis and improved cognition (Ferretti, Allard*, et al.* 2012). The anti-inflammatory effects of lithium have been well documented (Basselin *et al.* 2007; Li *et al.* 2011; Yu, Wang*, et al.* 2012). Therefore, in addition to investigating modulation of the CRTC1 transcriptional program, this thesis will investigate the influence of the microdose lithium formulation on CNS inflammation.

Based on previous evidence and the body of available scientific information, I have adopted the following Aims and Hypotheses:

Aim 1: Characterization of executive function deficits in Alzheimer transgenic rats

Hypothesis 1: McGill-R-Thy1-APP Alzheimer transgenic rats will be impaired in novel touchscreen cognitive task at pathological phases before the deposition of Aβ as extracellular plaque.

Aim 2: Characterizing the CRTC1 transcription program required for learning and memory in Alzheimer transgenic rats

Hypothesis 2: CRTC1 transcription program will be compromised in pre-plaque Alzheimer transgenic rats.

Aim 3: Behavioural characterization after microdose lithium treatment.

Hypothesis 3: Microdose lithium treatment will prevent early, and rescue established, deficits in MWM, FC, and NORL in pre-plaque Alzheimer transgenic rats.

Aim 4: Reduction of oxidative stress and inflammatory processes after microdose lithium treatment.

Hypothesis 4: Microdose lithium treatment will reverse markers of oxidative stress in pre-plaque Alzheimer transgenic rats.

Hypothesis 5: Microdose lithium treatment will lead to a reduction in the level of pro-inflammatory markers, including TNF-α, IFN-γ, TREM2, and a reduction in recruited microglia in pre-plaque Alzheimer transgenic rats.

Aim 5: Neurogenic and neuroprotective induction after microdose lithium treatment.

Hypothesis 6: Microdose lithium treatment will rescue neurogenesis in the dentate gyrus (DG) of the hippocampus in pre-plaque Alzheimer transgenic rats.

Hypothesis 7: Microdose lithium treatment will lead to an increase in CRTC1 genetic promoter occupancy levels in genes required for learning and memory in pre-plaque Alzheimer transgenic rats.

Connecting text between Chapter 1 and Chapter 2

Chapter 2 will describe the amyloid neuropathology in pre-plaque McGill-R-Thy1 APP Alzheimer transgenic rats. Because we are interested in the earliest phases of the disease in order to gain some understanding of the possible etiology, we tested rats between the ages of 3 months to 6 months of age. We used an advanced cognitive testing system, back-translated from use in humans, and adapted so that it may be used for rats. In a task sensitive to executive function, we found that we were able to detect severe cognitive impairments in the AD rats. We also investigated the status of the CREB transcriptional co-regulator, CRTC1, as it is known to be a key regulator of neuroplasticity gene expression.

CHAPTER 2: Intraneuronal amyloid beta accumulation disrupts hippocampal CRTC1-dependent gene expression and cognitive function in a rat model of Alzheimer disease

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Abstract

In Alzheimer disease (AD) the accumulation of amyloid beta (Aβ) begins decades before cognitive symptoms and progresses from intraneuronal material to extracellular plaques. To date, however, the precise mechanism by which the early buildup of Aβ peptides leads to cognitive dysfunction remains unknown. Here we investigate the impact of the early Aβ accumulation on temporal and frontal lobe dysfunction. We compared the performance of McGill-R-Thy1-APP transgenic AD rats with wild type littermate controls on a visual discrimination task using a touchscreen operant platform. Subsequently, we conducted studies to establish the biochemical and molecular basis for the behavioral alterations. It was found that the presence of intraneuronal Aβ caused a severe associative learning deficit in the AD rats. This coincided with reduced nuclear translocation and genomic occupancy of the CREB co-activator, CRTC1, and decreased production of synaptic plasticity-associated transcripts *Arc*, *c-fos*, *Egr1*, and *Bdnf*. Thus, blockade of CRTC1-dependent gene expression in the early, pre-plaque phase of AD-like pathology provides a molecular basis for the cognitive deficits that figure so prominently in early AD.

Introduction

Alzheimer disease (AD) is the leading cause of dementia worldwide and is characterized by a progressive decline in cognitive function (Selkoe 2001). A consistent pathological feature of this neurodegenerative disease is the accumulation of extracellular amyloid beta (Aβ) plaques in brain regions important for learning and memory. Emerging evidence from transgenic animal models and human patients indicates that Aβ also accumulates intraneuronally, and contributes to disease progression (LaFerla et al. 2007; Cuello et al. 2012).

In this regard, the progressive and predictable evolution of pathology in transgenic animal models facilitates the study of early AD. Previously, we have shown that McGill-R-Thy1-APP transgenic rats display full AD-like amyloid pathology, with appearance of Aβ plaques matching progression of plaque appearance in human patients (Leon *et al*. 2010). These rats develop the first Aβ plaques at 6-9 months of age in the subiculum, with subsequent plaques appearing in the hippocampus and entorhinal cortex. As is the case with AD patients, these rats show an intraneuronal Aβ accumulation of the toxic oligomeric soluble form occurring in the hippocampus and neocortex, which precedes plaque formation (Iulita *et al*. 2014). This early accumulation of Aβ alters synaptic plasticity through persistent inhibition of long-term potentiation in the CA1 area of the hippocampus (Qi et al. 2014).

While memory deficits are a consistent feature of AD, they overlap with cognitive impairments associated with frontal dysfunction, which can be difficult to differentiate in humans, especially in the early phases of the disease. Since the rat brain matures postnatally, similar to humans (Whishaw et al. 2001), and because rats perform well in tests of prefrontal-executive function (Chudasama and Robbins 2006; Bussey et al. 2012), the McGill transgenic rat model provides a unique opportunity to identify the precise molecular mechanisms associated with prefrontal cognitive decline. Using an automated touchscreen platform, we examined performance of transgenic AD rats on a complex task that assessed visual discrimination, associative learning, and behavioral control (Schoenbaum et al. 2002; Chudasama and Robbins 2003). Similar touchscreen platforms are used in identifying cognitive decline in clinical AD patients (Blackwell et al. 2004). Here, we show that transgenic AD rats were severely impaired in learning the visual stimulus-reward association, as they committed many errors, and showed long reaction times. Importantly, this impairment was evident during the early stages of the disease, when the Aβ is predominantly intraneuronal.

Changes in gene expression affect cognition during pathological aging and, in AD, altered cAMP/Ca^{2+} response element-binding protein (CREB)-mediated transcription is associated with memory decline (Pugazhenthi *et al*. 2011). Gene transcription mediated by CREB requires the CREB-regulated transcription coactivator 1 (CRTC1) (Conkright, Canettieri, *et al*. 2003; Iourgenko *et al*. 2003), which is widely expressed in the hippocampus (Zhou et al. 2006; Watts *et al*. 2011) where it contributes to control of gene expression necessary for memory (Sekeres *et al*. 2012). In this study,

we show that transgenic AD rats experience a blockade in nuclear CRTC1 translocation in the hippocampus and a related reduction in the expression of memory function gene transcripts including *Arc*, *c-fos*, *Egr1*, and *Bdnf*. Together, these results expand our understanding as to the manner in which intraneuronal Aβ accumulation contributes to disease progression in the early stages of the AD-like amyloid pathology, before the appearance of Aβ plaques.

Materials and Methods

Animals

McGill-R-Thy1-APP transgenic rats express the Swedish double and Indiana genetic mutations in the human amyloid precursor protein gene, *hAβPP* (Figure 2-1*A*). Male and female McGill-R-Thy1-APP transgenic rats homozygous for the mutated *hAβPP* transgene and their wild type littermates were obtained from our animal colony. The animals were 4 months of age at the beginning of the experiments and were sacrificed before the appearance of amyloid plaques in the transgenic animals, at 6 months of age. Animals were housed in humidity and temperature controlled rooms under 12-hour light cycle and had access to water *ad libitum*. Food pellets were restricted to maintain animals at ~90% of their free-food weight during behavioral testing. All experiments were carried out with approval from institutional Animal Care Committee and under strict adherence to the guidelines set out by the Canadian Council on Animal Care.

Touchscreen Operant Platform

Behavior testing was conducted in automated, operant touchscreen chambers (Lafayette Instruments). Each chamber was equipped with: 1) a houselight, 2) a food magazine fitted with a light-emitting diode and photocells to detect food collection entries, 3) a pellet dispenser that delivered 45 mg dustless precision sucrose pellets (Ren's Pet Depot), and 4) a 12″x12″ touch sensitive monitor (Elo Touch Solutions). Two computer graphic stimuli were presented on the left and right side of the touchscreen (Figure 2-2*A*,*B*). A black Plexiglas mask was attached to the front of the screen approximately 0.6″ from the surface of the display to restrict the rats' access to the visual stimuli through a left and right response window (2.05″x 2.05″). The apparatus and online data collection for each chamber were controlled using the Whisker control system (Cardinal and Aitken 2010).

Behavioral Procedure

Pretraining: Rats were first habituated to the apparatus and then trained to make a nosepoke touch response to a white square (2″x2″) that was presented on the left or right side of the screen. A nosepoke touch response to the white square was rewarded with a single sucrose pellet. Animals transitioned to the acquisition phase of the visual discrimination task when their performance reached 50 reward pellets delivered within a 20-minute session.

Visual discrimination task: Each session began with the illumination of the houselight and the food magazine light. After a 5 s intertrial interval, the rat initiated the trial by making a food magazine entry. This resulted in simultaneous presentation of two stimuli

on the screen; one was associated with a sucrose pellet (the A+ stimulus) and the second was not (the B- stimulus). The rewarded stimulus was counterbalanced across subjects. The same pair of stimuli was presented on every trial and the left-right positions of the stimuli (i.e., which stimulus was on the left and which was on the right) were determined pseudorandomly. The rat was required to make a nosepoke touch response to one of the stimuli. The stimuli remained on the screen until the rat made a nosepoke touch response to either stimulus. A correct response to A+ was followed by the disappearance of the stimuli and the delivery of a sucrose pellet concomitant with illumination of the food magazine. The next trial was initiated by a food magazine entry after a 5 s intertrial interval. An incorrect response to B- resulted in the disappearance of the stimuli from the screen and a 5 s timeout period during which all of the lights were extinguished. Consequently, rats were presented with correction trials such that after an incorrect response, the same stimulus configuration (i.e., the A+ and B- stimuli remained in the same left/right positions) was presented over successive trials until the rat responded correctly. Each session comprised a maximum of 60 non-correction trials, with up to an infinite number of correction trials. Rats were given up to one hour to complete the session. Rats were required to learn to respond to the correct, reinforced stimulus to an average criterion of 70% accuracy for two consecutive days, for non-correction trials only. Rats were given 50 sessions to acquire the visual discrimination task.

Locomotor Activity

After behavioral testing, locomotor activity was assessed using four standard home cage activity frames. Each home cage was a clear polycarbonate tub (61 cm wide x 37 cm long x 20.5 cm high) lined with sawdust, and covered with a barrier filter lid (Ancare, Bellmore, USA). Each home cage was placed within a Cage Rack SmartFrame™ (58 cm wide x 60.33 cm long x 2.11 cm high) equipped with infrared photobeams located on the interior perimeter of the frame (Lafayette, Indiana, USA). The rat was placed in the activity cage for 2 hours. The total number of horizontal beam breaks was recorded using MotorMonitor™ software, version 5.05 (Lafayette, Indiana, USA), and transmitted to a Dell Optiplex 745 computer (MCS, Montreal, CA).

Immunoenzymatic Reactions

Preparation of tissue was as previously described (Hanzel *et al*. 2014). To reveal amyloid neuropathology, tissue sections were incubated overnight in anti-McSA1 (MediMabs, Montreal), a mouse monoclonal antibody raised against synthetic peptide of Aβ corresponding to amino acids 1-12 of human *APP* (Grant et al. 2000). Tissue sections were then incubated in goat anti-mouse antibody (MP Biochemicals), followed by a mouse anti-peroxidase monoclonal antibody complex (MAP/HRP complex, MediMabs) and developed using DAB as the chromogen (Vector Laboratories, Inc.). Sections were then processed through a graded alcohol series, defatted and cleared in xylenes, and coverslipped using Permount mounting medium (Fisher Scientific). Aβ immunostained sections were imaged using a Zeiss Microimaging desk scanner (Zeiss Microimaging).

Immunofluorescence

Briefly, sections were blocked with 10% normal serum and incubated at 4ºC for 48 hours in a PBST with 5% serum and rabbit monoclonal anti-CRTC1 (Cell Signaling Technology, 2587) antibody solution. Sections were washed and incubated with cross-preabsorbed secondary antibodies, which minimizes the potential for cross reactivity, conjugated to Alexa 594 (Jackson Immunoresearch Laboratories, Inc., 112-585-167). Finally, sections were washed with PBS, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and coverslipped with Aqua Polymount (Polysciences).

Image Analysis

For CRTC1 nuclear translocation analysis, three distinct cell populations from the anterior aspect of the dorsal hippocampus were sampled: 1) the granule cell layer of the lateral blade of the dentate gyrus (DG); 2) the superficial pyramidal layer of CA1; and 3) the superficial pyramidal layer of CA3. Five regions on up to three sections were sampled under guidance of the DAPI channel for each CA1, CA3, and DG using a Zeiss LSM 510 confocal microscope (Carl Zeiss Canada). Confocal images were saved with 16-bit depth and were processed using NIH ImageJ software (U.S. National Institutes of Health). For the analysis of signals, an *ad hoc*, automated macro using NIH ImageJ software was applied. Five fields spanning 73.12 μm X 73.12 μm centered over the appropriate cellular layer were imaged on up to three sections per animal. Sampling in this manner yielded counts of approximately 275 neurons in CA1, 180 neurons in CA3, and 500 neurons in

the DG for each animal. Nuclear CRTC1 was defined as that signal which overlapped with the DAPI staining for nuclear heterochromatin. The optical slice of the confocal microscopy was less than 0.9 μm, giving sufficient resolution to show overlapping signals. The macro was programmed to automatically select the DAPI images and apply background removal, Gaussian blur, thresholding according to mean filter, and finally watershedding to separate touching nuclei. This macro was programmed to then automatically define the extent of the DAPI-labeled nuclei and to use those defined regions to measure, in the second channel, CRTC1 signal. Nuclear CRTC1 signal was measured as the mean signal intensity of CRTC1 overlapping DAPI signal, while total CRTC1 was represented as the mean signal intensity of the field. This analysis is shown in Figure 2-1.

Figure 2-1: Depicting automated image analysis of CRTC1 using ImageJ.

For the analysis of signals, an *ad hoc*, automated macro using NIH ImageJ software was applied. Total CRTC1 was represented as the mean signal intensity of the field. Nuclear CRTC1 was defined as the mean signal intensity overlapping the DAPI staining for nuclear heterochromatin. The macro was programmed to automatically select the DAPI images and apply background removal, Gaussian blur, thresholding according to mean filter, and finally watershedding to separate touching nuclei. This macro was programmed to then automatically define the extent of the DAPI-labeled nuclei (outlined in yellow) and to use those defined regions to measure, in the second channel, CRTC1 signal.

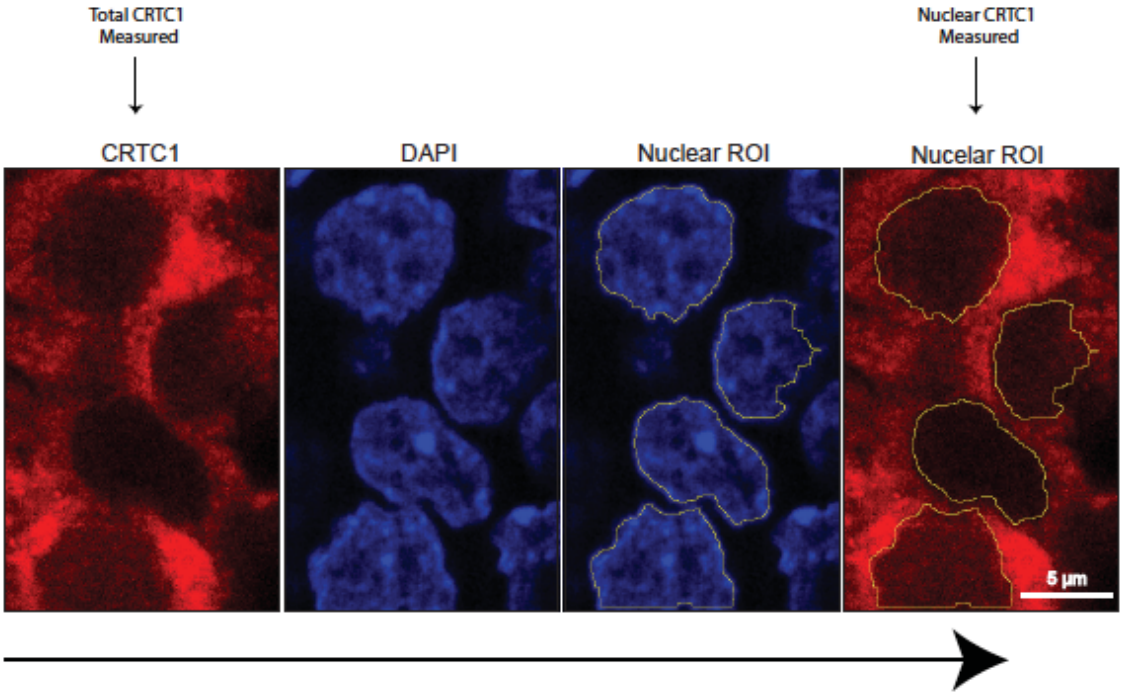

Figure 2-1: Depicting automated image analysis of CRTC1 using ImageJ.

Subcellular Fractionation

Subcellular fractionation of frozen hippocampal tissue was completed using the Biovision Inc. Nuclear/Cytosol Fractionation Kit (Cat: K266) according to manufacturers instructions. Protein concentrations were determined according to the Lowry method. Normalized loads of each extract were analyzed by Western blotting. Fraction purity was confirmed using antibodies toward nuclear specific histone H3 (Cell Signaling Technology, 9715) and cytosolic cyclophilin A (Abcam, ab41684). Nuclear and cytosolic levels of CRTC1 were revealed using the rabbit monoclonal anti-CRTC1 antibody (Cell Signaling Technology). Primary antibodies were detected using HRP-conjugated goat-anti rabbit secondary antibody (Jackson Immunoresearch Laboratories, Inc., 111-035-144). Membranes were processed according to directions using the Enhanced Chemiluminescent System (GE Healthcare) and were exposed to Hyblot CL films (Denville). Relative integrated optical density was determined using the CLIQS 1D gel analysis software (Totallab Ltd).

Chromatin Immunoprecipitation (ChIP) Assay

Cortical tissue was cross-linked in formaldehyde solution (50 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% formaldehyde, pH 8.0). After quenching the formaldehyde reaction by adding 2.5M glycine solution, tissue was homogenized in lysis buffer (10 mM Tris-HCl (pH 8.0), 0.1% sodium deoxycholate, 0.5% N-laurylsarcosine), supplemented with Complete™ protease inhibitors (Roche). DNA was sheared to 100-500 bp fragments using a Bioruptor Plus UCD-300 sonicator (Diagenode). An agarose gel electrophoresis of DNA fragments following ChIP

sonification protocol is shown in **Figure 2-2**. After sonication, 10% Triton X-100 was added to the tissue lysate and the mix was cleared by centrifugation at 15,000 x *g* for 15 min (4°C). Protein concentration was determined using the Bradford assay (BioRad).

Figure 2-2: Agarose gel electrophoresis of DNA fragments following ChIP sonification protocol.

Left to right: 100 bp ladder, Cross-linked DNA after 10 cycles of sonication, after 20 cycles, after 30 cycles (as was used for ChIP). Ideal fragment size is 100-300 bp, as shown.

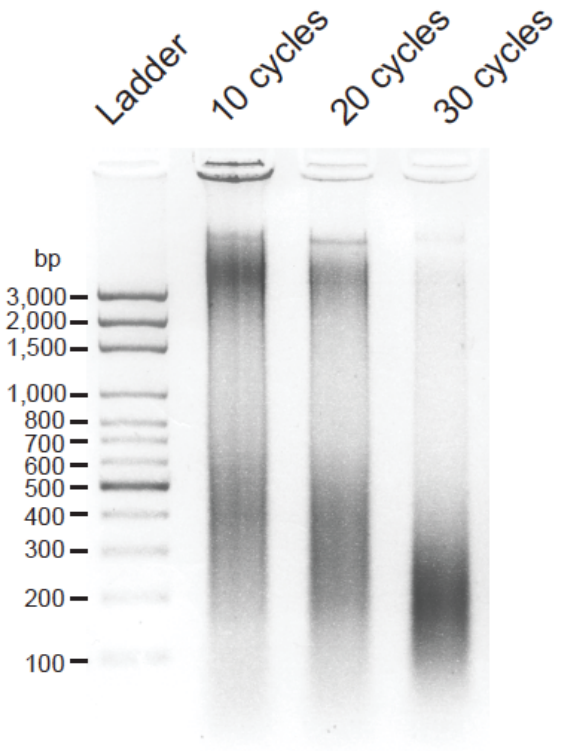

Figure 2-2: Agarose gel electrophoresis of DNA fragments following ChIP sonification protocol.

Tissue lysate was first precleared by incubation with PureProteome protein A magnetic beads (EMD Millipore). Cleared tissue lysate was then added to PureProteome protein A magnetic beads previously saturated with antibodies, and immunoprecipitation was carried overnight at 4°C. The antibodies used were CREB, CRTC1 rabbit monoclonal antibodies (1:250, Cell Signaling Technology) and anti-rat IgG rabbit polyclonal antibody as a negative control (1:250, Sigma-Aldrich). Beads were washed with RIPA wash buffer (50 mM HEPES–KOH (pH 7.6), 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 and 0.7% Na-deoxycholate) followed by one wash with TE buffer supplemented with 50 mM NaCl. DNA-protein complexes were eluted from the beads using elution buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1 % SDS) and by heating the samples at 65°C for 15 min with frequent vortexing. Contaminating RNAs were digested using RNaseA (Sigma-Aldrich) followed by a proteinase K (Roche) treatment to digest remaining proteins. The immunoprecipitated DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen) and were eluted in 30 µl of 10 mM Tris–HCl (pH 8.0). Quantification of immunoprecipitated DNA was assessed by qPCR with EvaGreen® (MBI EVOlution EvaGreen qPCR Mix, Montreal Biotech Inc.) using the Illumina Eco Instrument and Software (Illumina Inc). For a list of primers used see Table 2-1. ChIP data were normalized to input DNA from each sample.

Table 2-1. Nucleotide sequences of the primers used for ChIP-qPCR experiments

Gene	5'-3' Forward sequence	5'-3' Reverse sequence	Amplicon (bp)
BDNF IV	ATGCAATGCCCTGGAAC	GTGAATGGGAAAGTGGGTG	182
c-Fos	CTCAGTTGCTAGCTGCAATCG	CCCCCTCCAGTTTCTCTGTT	113
EGR-1	CTTGGATGGGAGGTCTTCAC	CGAATCGGCCTCTATTTCAA	145
Arc	ACCCAGCATGGGGACTCAAGGGCCT	GCTGCTCACCTGGGGGTGACTGCTC	149

Gene Expression Analysis

Total RNA was extracted from rat cortex using the RNeasy Mini Kit (Qiagen), following manufacturer instructions. Residual DNA was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). To generate cDNA, total RNA was retro-transcribed using an oligo-dT primer with the Omniscript RT Kit (Qiagen). Quantification of transcript expression was assessed by qRT-PCR with EvaGreen® (MBI EVOlution EvaGreen qPCR Mix, Montreal Biotech Inc.) using the Illumina Eco Instrument and Software (Illumina Inc). Expression of each gene was normalized to the housekeeping gene for β-actin. For the list of primers used, see Table 2-2.

Table 2-2. Nucleotide sequences of the primers used for Q-RT-PCR gene expression experiments

Gene	5'-3' Forward sequence	5'-3' Reverse sequence	Amplicon (bp)
Arc	GTTGACCGAAGTGTCCAAGC	CCGTCCAAGTTGTTCTCCAG	84
BDNF IV	CGCCATGCAATTTCCACTATCAATAATTTAAC	CTTTTTCAGTCACTACTTGTCAAAGTAAAC	200
c-Fos	GGGAGCTGACAGATACGCTC	AGGACCCTGACCCCATAGTC	97
EGR-1	AATCCTCAAGGGGAGCCGA	CACCAGCGCCTTCTCGTTAT	93
Il1β	CAGCTTTCGACAGTGAGGAGA	TCTGGACAGCCCAAGTCAAG	97
IL6	CATTCTGTCTCGAGCCCACC	GCTGGAAGTCTCTTGCGGAG	91
TGFβ	CTGCTGACCCCCACTGATAC	AGCCCTGTATTCCGTCTCCT	94
β-actin	AGGCATCCTGACCCTGAAG	GCTCATTGTAGAAAGTGTGG	95

Data Analysis

For the behavioral results, data for each variable were subjected to a one-tail independent samples *t* test or repeated measures ANOVA using SPSS statistical software, version 20 (SPSS Inc.). Statistical significance was set at $p < 0.05$. Mann Whitney non-parametric tests were performed when normal distribution data could not be assumed. The brains of all Alzheimer transgenic and wild type rats were analyzed to make direct comparisons

with CRTC1 expression. Subsequent tissue processing for Western blot, ChIP, and qPCR was performed on a randomly selected subset of behaviorally tested animals. This number secured sufficient statistical power and is it specified in the results section for each application, as it corresponds.

Results

Intraneuronal Aβ Accumulation Precedes Plaque Deposition

McGill-R-Thy1-APP transgenic rats express the human *APP* gene with Swedish double and Indiana mutations under control of the neuron specific murine Thy1.2 promoter (Figure 2-3*A*). These mutations are associated with a rapidly progressing early-onset form of Alzheimer disease. Using a highly specific antibody that targets amino acids 1-12 of human Aβ (Grant *et al*. 2000), immunohistochemical analysis revealed that extracellular Aβ plaques were present across the hippocampus and cerebral cortex in 15-month-old Alzheimer transgenic rats (Figure 2-3*B*). Importantly, although extracellular Aβ plaques had yet to deposit, the pre-plaque phase of the Aβ pathology was characterized by heavy burden of intraneuronal Aβ in the hippocampus and neocortex of 6-month-old rats that underwent behavioral testing.

Figure 2-3: Progression of amyloid pathology in McGill-R-Thy1-APP rats

The McGill-R-Thy1-APP rat transgenic model of the Alzheimer amyloid pathology exhibits a pre-plaque phase preceding the appearance of extracellular plaques where Aβ is primarily accumulated intraneuronally in the cerebral cortex and hippocampus. (*A*) DNA construct used for expression of transgenic human APP with Swedish double (Swe**) and Indiana (Ind*) mutations. Thy1: the neuron-specific murine thy1.2 promoter. (*B*) Expression of Aβ peptides following transgenesis. Aβ-burdened neurons (arrows, lower left panel) were present in the hippocampus and cerebral cortex (CC) of 6-month-old Alzheimer transgenic rats. Extracellular Aβ plaques were widespread across the hippocampus and in the cerebral cortex in 15-month-old Alzheimer transgenic rats. Higher magnification micrographs illustrate Aβ-burdened neurons of lamina V of the cerebral cortex at the pre-plaque stages and the occurrence of Aβ-immunoreactive plaques at the post-plaque stages, respectively.

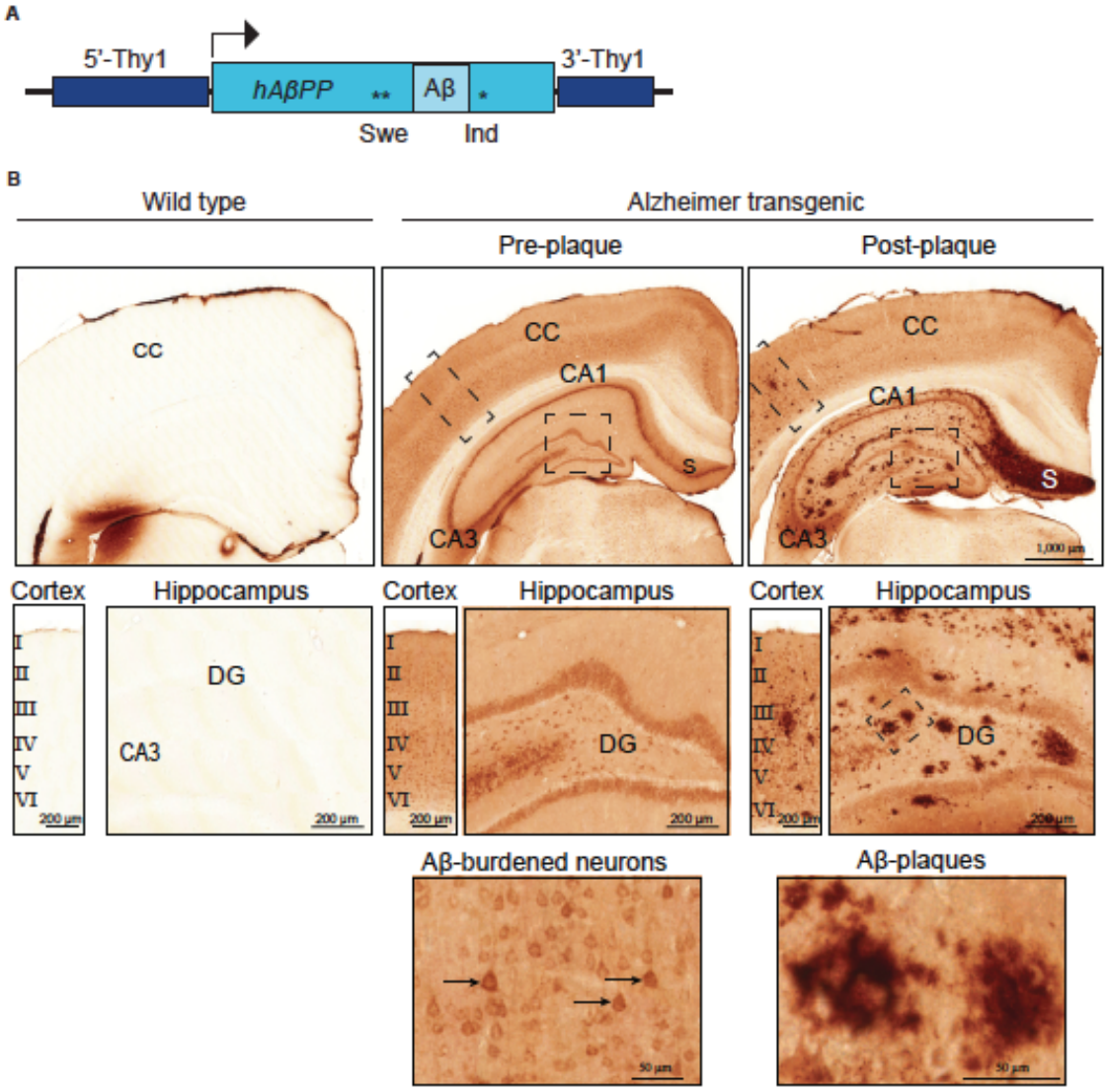

Figure 2-3: Progression of amyloid pathology in McGill-R-Thy1-APP rats

Intraneuronal Aβ Impairs Associative Learning in AD Rats

We tested pre-plaque transgenic Alzheimer rats and their wild type controls because we were interested in determining the effect of intraneuronal Aβ on associative learning. Rats were presented with a pair of visual stimuli on a touchscreen (Figure 2-4*A*,*B*) and a nosepoke touch response to the correct stimulus was rewarded with a sucrose pellet. For each trial, the left/right position of the correct stimulus was pseudorandom. When the rat made an incorrect response, the same trial with the same left/right stimulus configuration was repeated (i.e., correction trials) until the rat responded correctly. Therefore, correction trial errors indicate the animal was repeating incorrect responses to the same side in successive trials whereas non-correction trial errors indicate the animal was repeating errors to the same stimulus.

The learning curves for individual wild type (n = 5) and Alzheimer transgenic rats (n = 10) are shown in Figure 2-4*C* and *D*, respectively. Rats were trained until they reached a criterion of 70% accuracy for non-correction trials on two consecutive sessions. Rats were given a maximum of 50 sessions to acquire the task. All wild type rats quickly learned that only one stimulus was positively associated with reward, and they reached criterion performance in an average of 15 sessions, committing few errors, displaying high motivation and fast decision times in this task. In contrast, only one rat from the Alzheimer transgenic group learned the stimulus-reward relationship after 31 sessions, *i.e.*, twice as many sessions as the control wild type. The remaining 9 rats failed to reach criterion, even with 50 sessions of training (mean sessions ± S.E.M.: WT, 14.60 ± 2.42; TG, 48.80 ± 2.04; $t_{(13)}$=10.141; $p < 0.001$). We plotted the proportion of total trials within

a session that were non-correction or correction trials to gauge some sense of how the trial types were distributed (Figure 2-4*E*). This analysis confirmed that the wild type rats made a higher proportion of non-correction trials as training progressed while reducing the need for repeat trials to correct their errors. In contrast, the Alzheimer rats performed a lower proportion of non-correction trials and an almost equivalent proportion of correction trials within a session, reflecting no obvious pattern in learning. In fact, when we look at the error types alone, the Alzheimer rats made many non-correction trial errors: ($t_{(13)}$=5.652; $p < 0.001$; Figure 2-4*F*) as well as correction errors ($t_{(13)}$=4.508; $p < 0.001$; Figure 2-4*G*) as if they were choosing randomly.

Another notable impairment was in speed of response. The Alzheimer rats took over 10 seconds to make their response suggesting that the animals were severely compromised in their capacity to make a choice ($p < 0.001$, Mann-Whitney *U* test; Figure 2-4*H*), and they were slower than the wild types in collecting their reward ($t_{(13)}$=9.191; $p < 0.001$; Figure 2-4*I*). However, their long latencies cannot be attributed entirely to a motor deficit. First, although the Alzheimer rats were less active than the wild types in their locomotor behavior ($F_{(1,12)}$=25.74; $p < 0.0001$), both groups showed a general decline in activity with time ($F_{(7,84)}$=35.40; $p < 0.0001$) and were no different from each other for the last 45 minutes of locomotor testing. Second, while Alzheimer rats took over 10 seconds to make their choice, they took only three seconds to collect their food suggesting they are not slow in all aspects of behavior. Moreover, the rats consumed all their reward pellets indicating that they were not demotivated. Thus, the pre-plaque

transgenic Alzheimer rats had a significant cognitive impairment in this early phase of the amyloid pathology, where Aβ peptides are primarily accumulated intraneuronally.

Figure 2-4: Intraneuronal Aβ impairs associative learning in AD rats

Alzheimer transgenic rats at the pre-plaque stage were already severely impaired in associative learning. (*A*) Photograph showing touchscreen operant platform and rat making nose-poke touch response to visual stimulus. (*B*) Schematic illustration of stimulus pair used in the visual discrimination task and the reward contingencies. (*C, D*) Individual learning curves are shown for wild type (n = 5) and Alzheimer transgenic rats (n = 10). The blue line represents criterion performance at 70% accuracy on non-correction trials over two consecutive training sessions. (*E*) Proportion of total correction and non-correction trials for the first 35 sessions only. Wild type rats commit more non-correction trials and fewer correction trials with time, whereas the Alzheimer rats fail to show such improvement. (*F*) Pre-plaque Alzheimer transgenic rats made many non-correction trial errors and (*G*), correction trial errors, while learning the stimulus-reward association and never reached criterion performance, indicating that they never learned the stimulus-reward association. (*H*) Alzheimer transgenic rats were very slow in making a response. (*I)* The Alzheimer transgenic rats were also slow in collecting food rewards when they made a correct response. Data represent mean ± SEM. ***$p < 0.001$.

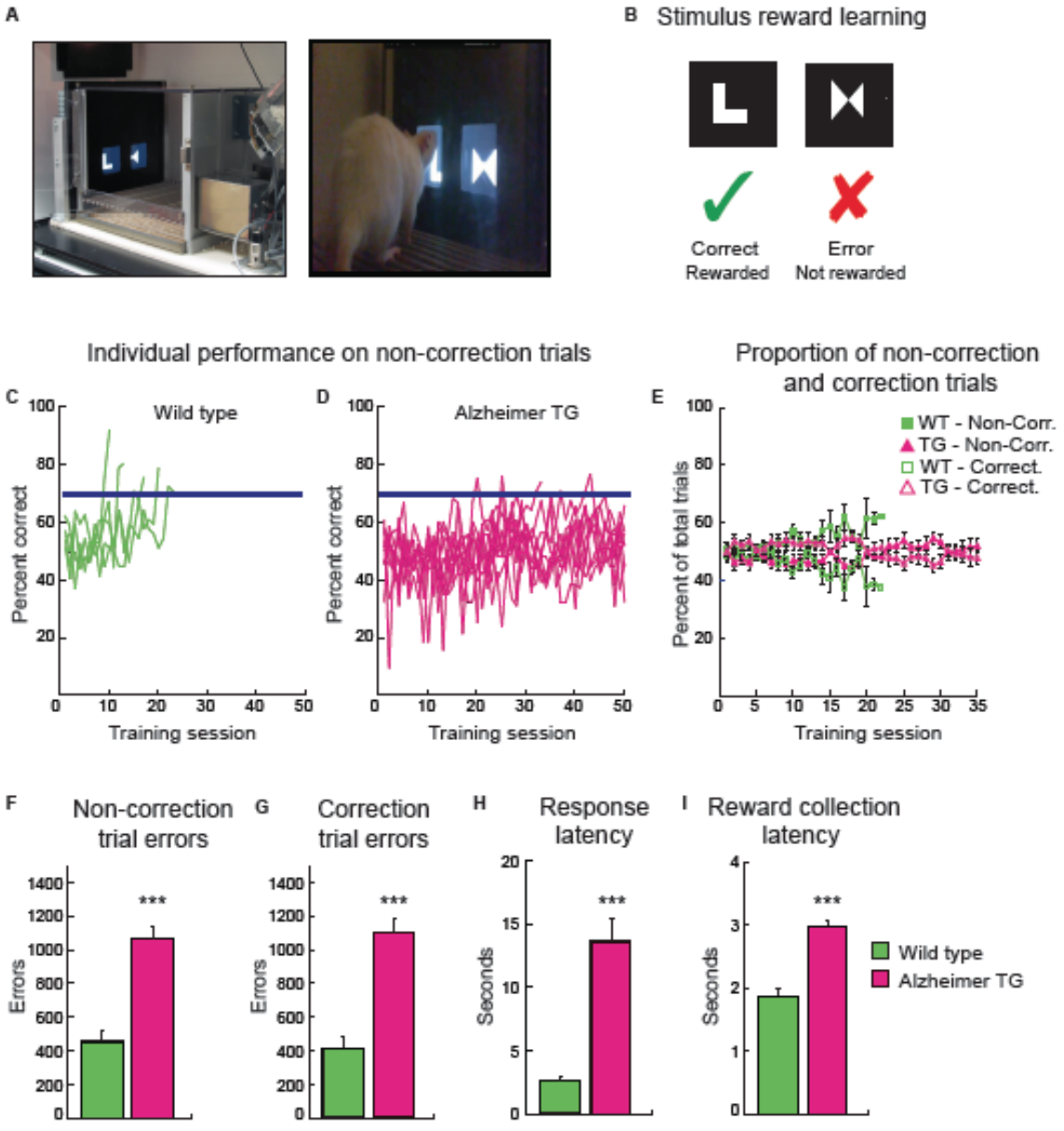

Figure 2-4: Intraneuronal Aβ impairs associative learning in AD rats

Intraneuronal Aβ Blocks CRTC1 Nuclear Translocation

To investigate molecular changes associated with the earliest stages of the AD-like amyloid pathology (akin to the human preclinical AD), we analyzed the brains of the young transgenic rats at the pre-plaque stage after behavioral testing. For instance, it is well accepted that glutamatergic synaptic transmission promotes nuclear translocation of CRTC1 in a calcium- and calcineurin-dependent manner (Screaton *et al*. 2004; Li et al. 2009; Ch'ng *et al*. 2012), leading to a transient stabilization of the CREB transcription complex, and activation of CRE-regulated gene expression (Bittinger et al. 2004). Upon phosphorylation by salt-inducible kinase (SIK), CRTC1 is shuttled back to the cytoplasm (Takemori and Okamoto 2008). Therefore, CRTC1-dependent gene expression is tightly regulated by its subcellular localization and Aβ has been shown to negatively affect CRTC1-dependent gene transcription (Espana *et al*. 2010). This led us to investigate whether the accumulation of intraneuronal Aβ is sufficient to impede CRTC1 nuclear translocation in this rat model.

The brains of wild type (n = 5) and AD transgenic rats (n = 10) were immunohistochemically processed (Figure 2-5*A*-*C*) and nuclear CRTC1 was defined as that signal which overlapped with DAPI staining for heterochromatin. We found that AD rats showed a significant reduction in nuclear CRTC1 in the dentate gyrus (DG) ($t_{(13)}$=2.824; $p < 0.01$), and in the pyramidal neurons of CA1 ($t_{(13)}$=4.572; $p < 0.001$). Not surprisingly, the most striking nuclear accumulation occurred in CA3 ($t_{(13)}$=3.002; $p < 0.01$), a region known to have the most excitatory connections. A reduction in total CRTC1 expression (cytoplasmic and nuclear) was observed only in area CA1 ($t_{(13)}$=1.841;

$p < 0.05$; Figure 2-5*D*) indicating that reduced nuclear CRTC1 was primarily due to reduced cytoplasmic-nuclear translocation, rather than to a global reduction of available CRTC1.

Figure 2-5. Confocal microscopy reveals impaired hippocampal CRTC1 nuclear signaling

Confocal imaging reveals reduced nuclear accumulation of CRTC1 in neurons of the hippocampal formation before Aβ plaque deposition. (*A*) Brain diagram showing the hippocampal region and method of confocal image analysis (*B-D*) Immunofluorescent staining revealed CRTC1 expression in CA1, the dentate gyrus (DG), and CA3 regions of the hippocampus in wild type (n = 5) and Alzheimer transgenic rats (n = 10). A significant reduction in nuclear CRTC1 was observed in CA1, the DG, and CA3 regions of the hippocampus in Alzheimer transgenic compared with wild type rats when examined at 6-months of age. Immunofluorescent staining revealed a significant reduction in total CRTC1 in AD transgenic rats only in the CA1 region, indicating that the reduction of nuclear CRTC1 was not due to an overall reduction of available CRTC1. These results indicate that reduced nuclear CRTC1 in neurons across the hippocampal formation is an early event in the Aβ pathology preceding the appearance of amyloid plaques. Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale bar A = 5 μm; B-D = 20 μm.

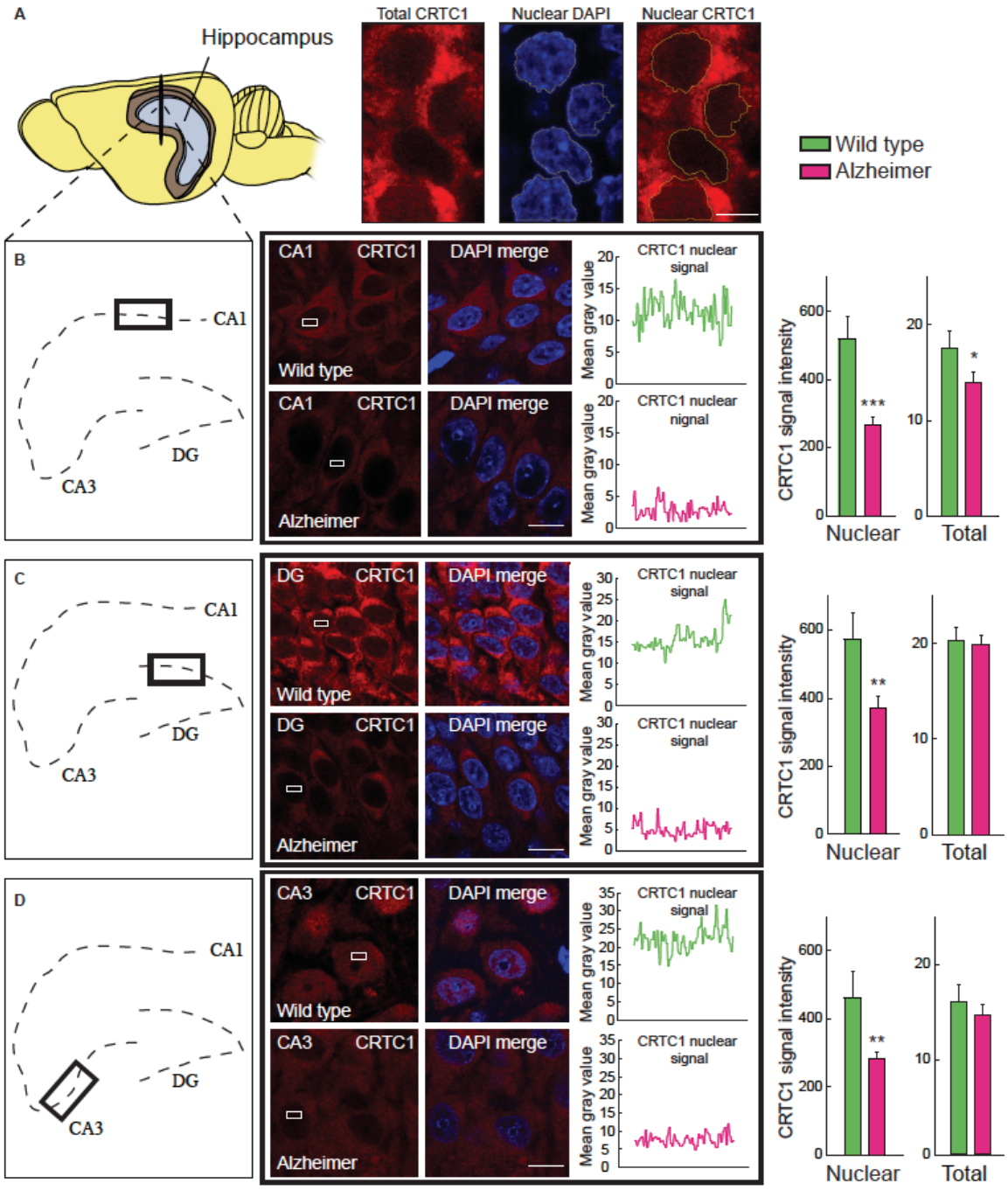

Figure 2-5. Confocal microscopy reveals impaired hippocampal CRTC1 nuclear signaling

We next prepared cytosolic and nuclear subcellular fractions to confirm reduced nuclear CRTC1 in Alzheimer rats. Hippocampal tissue from a randomly selected subset of behaviorally tested wild type (n = 4) and Alzheimer transgenic (n = 4) rats was used for this analysis. We found that CRTC1 was highly expressed in the cytosolic fraction and to a lesser extent in the nuclear fraction (Figure 2-6*A*). We found no significant difference in CRTC1 expression in the cytosolic fractions between wild type and Alzheimer transgenic rats (Figure 2-6*B*). Consistent with the confocal imaging results (Figure 2-5*B-D*), nuclear CRTC1 was significantly reduced in Alzheimer transgenic rats (Figure 2-6*C;* $t_{(6)}$=2.174; $p < 0.05$). Together, these results indicate that intraneuronal Aβ impaired CRTC1 nuclear translocation at the early stages of AD-like amyloid pathology.

Figure 2-6. Subcellular fractionation confirms impaired CRTC1 nuclear translocation in hippocampus at pre-plaque AD stages.

(*A*) CRTC1-immunoreactive bands were detected in cytosolic and nuclear fractions of hippocampal tissue from wild type (n = 4) and Alzheimer transgenic rats (n = 4). (*B, C)* While no significant difference in CRTC1 levels was observed in the cytosolic fractions, a significant reduction in nuclear CRTC1 occurred in the Alzheimer transgenic rats. Cyclophilin A and histone H3 were used as cytosolic and nuclear markers, respectively. Data represent the mean ± SEM. $*p < 0.05$.

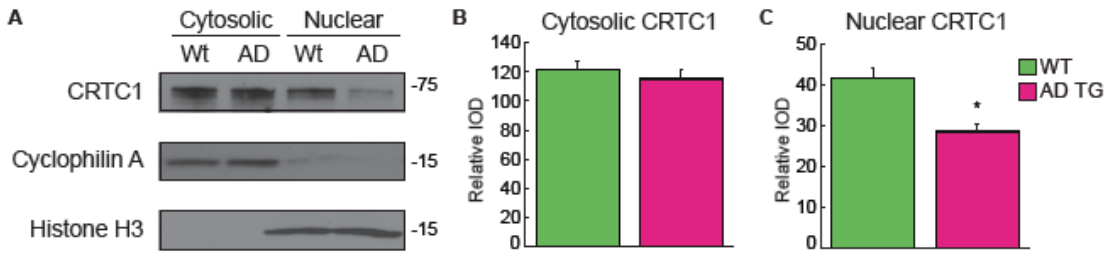

Figure 2-6. Subcellular fractionation confirms impaired CRTC1 nuclear translocation in hippocampus at pre-plaque AD stages

Intraneuronal Aβ Accumulation Results in Reduced CRTC1 Genomic Occupancy and Neuroplasticity-Related Gene Expression

Learning involves Hebbian and homeostatic forms of synaptic plasticity that require production of key plasticity-related proteins, and is highly dependent on CRTC1 for gene transcription. To test whether CRTC1 promoter occupancy is altered during the early stages of the Aβ pathology, we performed a chromatin co-immunoprecipitation (ChIP) assay. The results revealed decreased binding of CRTC1 to genetic regions examined in the Alzheimer transgenic rats. Figure 2-7*A* shows a two-fold reduction of the immediate early genes *Arc* ($t_{(8)}$=3.790; $p < 0.01$), *c-fos* ($t_{(8)}$=2.054; $p < 0.05$), and *Egr1* ($t_{(8)}$=2.314; $p < 0.05$), as well as the growth factor *Bdnf* ($t_{(8)}$=2.354; $p < 0.05$) in the Alzheimer transgenic rats ($n = 6$) compared with wild-type rats ($n = 4$). Previous studies have shown that inactive CREB binds to gene promoters and co-factors such as CRTC1 are required for gene expression (Mayr and Montminy 2001; Kornhauser *et al*. 2002; Conkright, Canettieri, *et al*. 2003). Accordingly, ChIP analyses did not reveal significant differences in CREB promoter binding between the wild type and Alzheimer transgenic rats (Figure 2-7*B*). A decrease in CRTC1 binding to promoter elements is expected to result in a concomitant reduction in CRTC1-dependent gene expression. To test whether the lower binding of CRTC1 in AD rats was consistent with this scenario, we assessed the expression of those genes under CRTC1 regulatory control. We found that the Aβ-induced decreased binding of CRTC1 to gene promoters was directly reflected by diminished transcript production for *Arc* ($t_{(8)}$=2.857; $p < 0.05$), *c-fos* ($t_{(8)}$=2.219; $p < 0.05$), *Egr1* ($t_{(8)}$=3.621; $p < 0.01$), and *Bdnf* ($t_{(8)}$=2.063; $p < 0.05$) in AD rats compared to wild type (Figure 2-7*C*).

Figure 2-7. CRTC1 promoter occupancy and CRTC1-dependent gene expression are reduced at pre-plaque AD stages.

(*A*) ChIP assays demonstrate recruitment of CRTC1 to CRE-responsive *Arc*, *c-fos*, *Egr1*, and *Bdnf* promoters. For CRTC1, we observed a two-fold reduction in AD transgenic rats (n = 6) of recruitment to *Arc, c-fos, Egr1,* and *Bdnf* promoters relative to the same promoters in wild type rats (n = 4). (*B*) Recruitment of CREB to CRE-responsive promoters was not different in Alzheimer transgenic rats. IgG indicates immunoprecipitation with an irrelevant antibody and confirms specific binding of CRTC1 and CREB antibodies. (*C*) Gene expression analysis of CREB-dependent genes in Alzheimer transgenic rats compared to wild-type animals revealed reduced production of transcripts for *Arc*, *c-fos*, *Egr1*, and *Bdnf*. Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$.

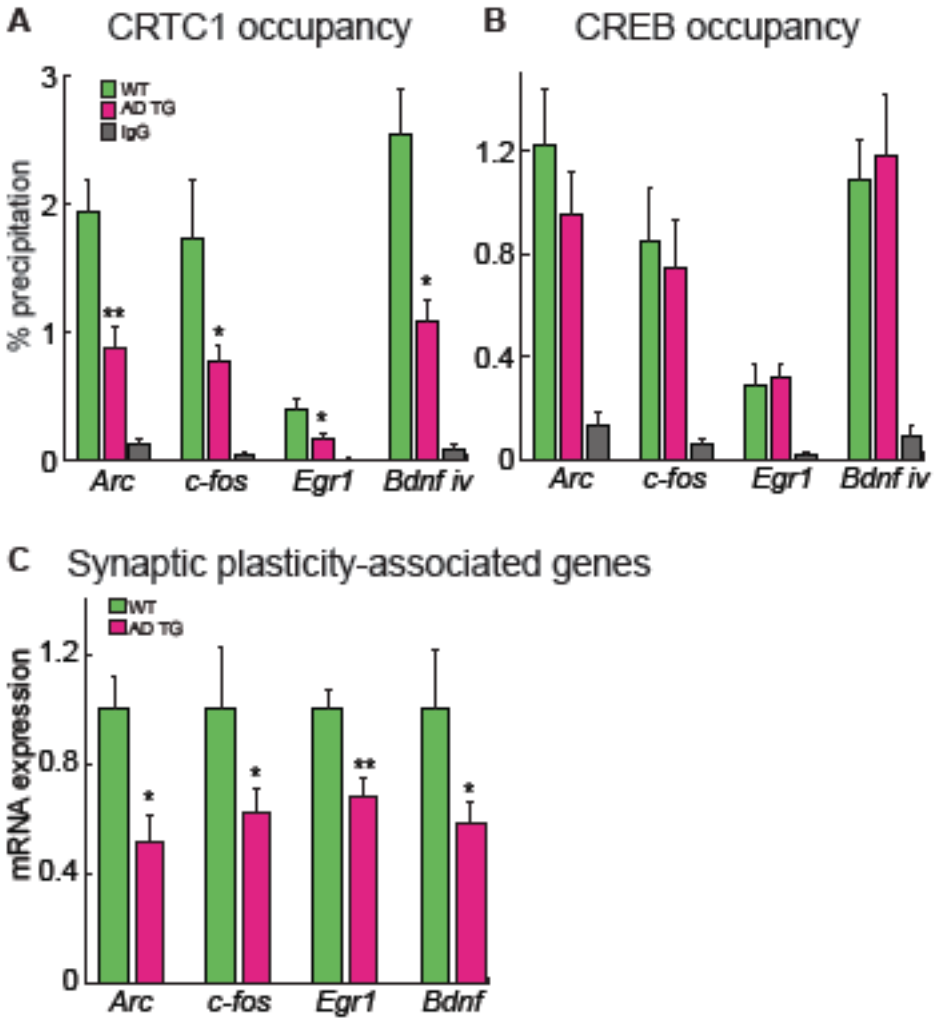

Figure 2-7. CRTC1 promoter occupancy and CRTC1-dependent gene expression are reduced at pre-plaque AD stages

Discussion

Biomarker evidence suggests that pathological processes underlying AD begin decades prior to the overt manifestation of cognitive symptoms (Jack *et al*. 2010). To intervene at preclinical stages of the disease, however, further investigation is needed to into the mechanisms by which an early buildup of Aβ peptides contributes to disease progression (Donohue *et al*. 2014). In this study, we found that abnormal levels of intraneuronal Aβ caused a severe associative learning deficit in AD transgenic rats. Impaired cognition coincided with reduced nuclear translocation and genomic occupancy of the CREB co-activator, CRTC1, and decreased production of synaptic plasticity-associated transcripts *Arc*, *c-fos*, *Egr1*, and *Bdnf*.

We provide the first evidence of an impairment in associative learning in Alzheimer transgenic rats using an automated touchscreen behavior platform. The operant touchscreen platform for behavioral testing holds translational value given its use in humans (Chudasama and Robbins 2006). Just like patients at early stages of AD, our pre-plaque Alzheimer transgenic rats were slow learners, unable to correct their errors (Blackwell *et al*. 2004). While a previous study using a mouse Alzheimer model failed to demonstrate an acquisition or learning impairment on the visual discrimination task (Romberg et al. 2013), acquisition deficits are known to be among the major contributors of cognitive decline in AD patients (Becker et al. 1987; Germano and Kinsella 2005). Pasquier and colleagues showed an acquisition deficit in early AD (Pasquier et al. 2001); Alzheimer disease patients (Mini Mental State Examination score of 23) acquired less information in a list-learning test compared with healthy counterparts. It is feasible that a

difficulty to learn in early AD can lead to incomplete storage and retention of information, leading to poor recall or retrieval of information at a later stage. This might explain why the pre-plaque transgenic AD rats needed several repeat correction trials; they never learned the stimulus-reward association. In support of this, when differences in initial learning were controlled, Alzheimer patients showed the same rate of forgetting on a picture recognition test administered at intervals over the course of a week when compared to healthy controls. Thus, it is feasible that the anterograde amnesic deficit observed in AD might be related to an initial learning deficit (Kopelman 1985).

The Alzheimer transgenic rats demonstrated rigidity and inflexibility in their behavior, as shown by the increased number of errors to the incorrect stimulus, and the need for repeat trials to help them correct their errors. The errors resulted in time-out and no reward but these animals were unable to use this negative feedback to guide their response and continued responding incorrectly. This type of inflexibility is typically associated with prefrontal dysfunction, and can be experimentally produced through lesions to the orbitofrontal cortex in rats (Chudasama and Robbins 2003) and monkeys (Dias et al. 1996). It is also observed in patients with frontotemporal dementia, who have a more selective ventral orbital prefrontal pathology (Rahman et al. 1999). It is possible, therefore, that the executive impairment of inflexibility and disinhibition shown by Alzheimer rats was due to pathology extending beyond the temporal lobe into the orbitofrontal cortex, and was compounded by pathology to other prefrontal regions (Bussey et al. 1997; Chudasama and Robbins 2003). However, the learning difficulty observed in the pre-plaque Alzheimer transgenic rats cannot be attributed to prefrontal

dysfunction alone, because rats with selective prefrontal or orbitofrontal lesions do not show an acquisition deficit when tested on the same task (Bussey *et al*. 1997; Chudasama et al. 2001; Chudasama and Robbins 2003).

Notably, the Alzheimer rats took over 10 seconds to make a decision to respond to the touchscreen and showed very long reward collection latencies. Since the rats collected all their reward pellets, we can rule out demotivation as the primary cause of the learning impairment. Likewise, we can reject the possibility that AD rats were visually impaired since their wild type littermates improved their accuracy for non-correction trials and were therefore able to track the correct stimulus to some extent. Although the Alzheimer rats were less active than the wild types, it was not the case that they were motorically incapacitated; the Alzheimer rats were substantially faster to collect their rewards (i.e., within 3 seconds on average) than they were to make a choice response. One possibility is that the Alzheimer rats were easily distracted or became disengaged while in the process of making a response. This might hinder the animals' ability to actively monitor or sequence their actions, or effectively use working memory to guide their responses. Thus, their long reaction times may reflect a general difficulty in scheduling goal directed actions. This hypothesis needs to be tested directly, but is in keeping with human case reports of AD patients showing that cognitive decline is accompanied by slow decision-making and motor actions (Hebert et al. 2010; Buchman and Bennett 2011; Bennett et al. 2012).

CRTC1 is implicated in dendrite arborization of developing cortical neurons (Li *et al*. 2009; Finsterwald et al. 2010), neuronal survival in response to ischemia (Sasaki et al. 2011), addiction mediated through brain reward circuits (Hollander et al. 2010; Dietrich et al. 2011), circadian clock entrainment (Jagannath *et al*. 2013; Sakamoto et al. 2013), mood regulation (Breuillaud et al. 2012), as well as hippocampal L-LTP and memory (Zhou *et al*. 2006; Kovacs et al. 2007; Sekeres *et al*. 2012). Our study provides new insight into how CRTC1-dependent gene expression is coincident with dysfunction on associative learning tasks at the early stages of Aβ pathology. It is likely that intraneuronal Aβ disrupts the tracking of synaptic glutamatergic activity that is achieved through CRTC1 nuclear translocation (Ch'ng *et al*. 2012). Consistent with this idea, we observed decreased gene transcription of *Bdnf, Arc, Egr1,* and *c-fos*. Our data build on the work of others that has shown a similar hippocampal reduction in nuclear CRTC1 in a mouse model of AD, and decreased CRTC1 levels in human brain at intermediate Braak III–VI pathological stages (Parra-Damas *et al*. 2014). We extend these results by documenting the behavioral impact of dysregulated CRTC1 in a robust rat model of Aβ pathology, using an advanced cognitive testing platform.

Certain benefits exist in using rats as a model for Alzheimer disease. For example, the rat is physiologically, genetically and morphologically closer to humans than mice, has a complex CNS, and, like humans, has postnatal brain development (Whishaw *et al*. 2001). In addition, rats are behaviorally well characterized and demonstrate complex cognitive behaviors (Whishaw *et al*. 2001; Chudasama and Robbins 2006). Rats are increasingly used to mimic key pathological hallmarks of neurodegenerative diseases

including Alzheimer disease, and it has been reported that some transgenic rat models offer more accurate representations of the human disease compared to mice bearing the same transgene (Do Carmo and Cuello 2013). Finally, a key benefit of using a rat model in studies of cognition is that dysfunction can be more readily detected as rats have fewer available mechanisms for neural compensation. Conceptually, cognitive reserve results from underlying neural mechanisms including neural reserve, the resilience of pre-existing cognitive networks, and neural compensation, which allows the use of compensatory neural resources (Stern et al. 2005). BDNF, which we found to be reduced at the transcript level in Alzheimer rats, enhances neural reserve in humans by increasing efficiency in cognitive networks underlying executive control (van Praag et al. 2000). Cognitive reserve is enhanced in individuals with greater educational and occupational attainment and through factors such as aerobic physical exercise and life-long learning (reviewed in (Stern 2012)). The consequence is that individuals with higher cognitive reserve will present Alzheimer's disease symptoms at a later stage of the pathology (Hanyu et al. 2008). In this regard, the sensitivity of the touchscreen platform could prove useful in detecting subtle cognitive decline in individuals at early stages of AD, and in individuals with high cognitive reserve. For instance, it is worth noting that a paired associate learning (PAL) touchscreen task in humans has been used to detect early cognitive impairments, which were correlated with alterations in biomarkers of CNS synaptic plasticity (Kiddle et al. 2015).

In summary, intraneuronal Aβ accumulation is sufficient to disrupt CRTC1-dependent gene expression and cognitive function at the early stages of the AD-like

amyloid pathology, before Aβ plaques appear. These results provide greater understanding relating to the early progression of Aβ pathology, and identify CRTC1-dependent gene transcription as target for therapeutic intervention at the early AD stages.

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Connecting text between Chapter 2 and Chapter 3

Chapter 1 described severe cognitive impairment in McGill-R-Thy1-APP Alzheimer transgenic rats at an early, pre-plaque stage of the AD-like amyloid pathology. Therefore, with the CRTC1 transcription program representing a possible therapeutic target for the early stages of AD neuropathology, we evaluated mechanisms that may restore its function. Chapter 3 evaluates exactly that possibility, by experimentally increasing CRTC1-dependent gene expression using a novel microdose lithium formulation. We also evaluated other known mechanisms of action of lithium with respect to the Alzheimer's disease pathology.

CHAPTER 3: Microdose lithium formulation NP03 reverts neuropathology and memory loss at the earliest stages of the Aβ pathology in an Alzheimer disease rat model

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Microdose lithium formulation NP03 reverts neuropathology and memory loss at the earliest stages of the Aβ pathology in an Alzheimer disease rat model

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Abstract

Accumulating evidence reveals a possible protective effect of the multi-target drug lithium in those at risk for developing Alzheimer's disease (AD) and in AD animal models. For example, lithium has been shown to diminish key aspects of AD, including activity of GSK-3β, amyloid precursor protein (APP) cleavage enzyme BACE1, Aβ pathology, and memory loss. However, conventional lithium treatment has a severe side effect profile, making it inappropriate for long-term treatment. Here, we test whether a novel microdose lithium formulation, coded NP03, shows comparable ability as conventional lithium to rescue neuropathology and behavioral impairments in a rat model of Alzheimer amyloidosis. NP03 treatment for eight weeks restored novel object recognition, hippocampal-dependent spatial learning and fear conditioning in Alzheimer transgenic rats. These changes coincided with reduced toxic $A\beta_{42}$ peptides and BACE1 hyperactivity and restored promoter binding of the CREB transcriptional co-regulator CRTC1 in genes required for synaptic plasticity. Together these data confirm the ability of NP03 to reverse many of the key AD pathologies, raising the possibility that it be of therapeutic value in the early stages of the disease.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the deposit of extracellular amyloid beta (Aβ) plaques, intracellular neurofibrillary tangles, and cognitive impairment (Selkoe 2001). A causative role of Aβ in AD pathogenesis is well established (Citron 2010), such that early intracellular Aβ accumulation initiates a synaptic failure (Hardy and Selkoe 2002; Oddo *et al.* 2006; Qi *et al.* 2014). Lithium, a drug used for more than 60 years in the management of bipolar disorder, has disease-modifying properties in patients at risk for developing AD (Kessing *et al*. 2008; Forlenza *et al*. 2011; Nunes *et al*. 2013; Mauer et al. 2014) and in AD animal models (Zhang *et al*. 2011). Although there have been many shifts in our understanding of the mechanism of action of lithium (Belmaker 2004), the precise manner in which lithium confers its protective effects in AD remains poorly understood. Early reports linked its function to the reversible inactivation of glycogen synthase kinase-3β (GSK-3β) – an Aβ-induced kinase that leads to the hyperphosphorylation of tau protein and microtubular dysfunction – and to the potentiation of the neuroprotective Wnt/β-catenin signaling pathway (Klein and Melton 1996; Stambolic *et al.* 1996; Alvarez et al. 1999). Other reports showed that lithium reduces the amyloid precursor protein (APP) cleavage enzyme BACE1 and diminishes Aβ pathology (Zhang *et al.* 2011; Yu, Zhang*, et al.* 2012; Sofola-Adesakin *et al.* 2014). Finally, more recent studies have shown lithium to stimulate CRTC1 oligomerization and the interaction between CRTC1 and CREB, a prerequisite for cAMP-induced CREB-dependent gene expression that is required for synaptic plasticity and memory (Kandel 2001; Boer *et al.* 2007; Heinrich *et al.* 2009; Heinrich *et al.* 2013). Furthermore, clinical trials with lithium support its multi-target

function, and have shown it to modulate Aβ, tau, GSK-3β and to stabilize cognition (Forlenza *et al.* 2011; Nunes *et al.* 2013). However, while lithium appears as an attractive therapeutic candidate for the treatment of AD, conventional lithium has a narrow therapeutic window and a severe side effect profile, making it inappropriate for long-term treatment, especially in the elderly (Gelenberg and Jefferson 1995; Azab *et al*. 2015).

NP03 is a novel microdose lithium formulation, where lithium is encapsulated in a lipidic micelle, allowing enhanced uptake and facilitating passage across the blood-brain barrier (BBB). Thus, the micelle formulation of lithium (NP03) allows for significantly lower amounts of lithium to be delivered, thereby avoiding adverse side effects. We evaluated this formulation (40 µg Li/kg) in pre-plaque McGill-R-Thy1-APP Alzheimer transgenic rats, which, in previous studies, showed *in vivo* disruption of intrinsic excitability in Aβ-burdened neurons (Leon *et al.* 2010; Qi *et al.* 2014). In further studies, we found that intraneuronal Aβ accumulation blocks CRTC1 synaptonuclear transport, resulting in impaired promoter occupancy, and subsequent impairment in neuroplasticity gene expression (Wilson et al. 2016). Because these rats show severe cognitive impairment during the pre-plaque stages of the Aβ pathology (Leon *et al.* 2010; Galeano et al. 2014; Iulita *et al.* 2014; Martino Adami et al. 2015; Wilson *et al.* 2016), they serve as an ideal model to evaluate therapeutic efficacy of potential therapeutics at early AD-related disease stages.

In this study, we tested whether microdose lithium formulation NP03 is similarly capable of rescuing a subset of AD associated pathologies modulated by conventional

lithium. Thus, we hypothesized that NP03 treatment would reduce BACE1 mRNA and its activity, reduce brain Aβ peptides, and restore CRTC1 promoter occupancy in pre-plaque Alzheimer transgenic rats. Further, we hypothesized that NP03 would rescue cognitive impairments at the pre-plaque stage of the AD-like Aβ pathology.

We found that NP03 reversed deficits in novel object recognition (NOR), hippocampal-dependent spatial learning, and amygdalar-dependent auditory fear conditioning in Alzheimer transgenic rats. We also found that NP03 treatment significantly reduced BACE1 gene expression and reduced BACE1 activity. Furthermore, NP03 reduced the level of toxic $A\beta_{42}$ peptides. Finally, NP03 restored CRTC1 promoter occupancy in genes required for learning and memory. Current AD therapeutics offer limited, short-lasting relief for the symptoms of AD and no disease-modifying drug exists. We reveal that NP03 shows therapeutic benefit in a transgenic Alzheimer rat model, raising the possibility that it be of therapeutic value in the early stages of AD.

MATERIALS AND METHODS

Animals

McGill-R-Thy1-APP transgenic rats express the human amyloid precursor protein gene *APP* with Swedish and Indiana mutations (Leon *et al.* 2010). Rats were socially housed and maintained under 12-hr light-dark schedule at 21^{o}C, with free access to food and water. Male and female homozygous transgenic rats and their wild type littermates were

used for this study. All procedures were carried out in accordance with the guidelines set out by the Canadian Council of Animal Care and were approved by the Animal Care Committee of McGill University.

Drug treatment

Drug administration was performed daily, 5 days per week. Animals were treated starting at 3 months of age for two months with vehicle (1 mL/kg) or NP03 (40 µg Li/kg; 1 mL/kg) by deposit on the rectal mucosa. Experimental groups included wild-type littermates treated with vehicle (n = 6), wild-type littermates treated with NP03 (n = 7), homozygous McGill-R-Thy1-APP Alzheimer transgenic rats treated with vehicle (n = 8), and homozygous McGill-R-Thy1-APP Alzheimer transgenic rats treated with NP03 (n = 14). To determine the amount of lithium in brain tissue following NP03 treatment, a separate group of 250 – 300 g wild-type rats were treated with increasing doses 0 µg Li/kg (n = 4), 40 µg Li/kg (n =6), 160 µg Li/kg (n = 6), and 600 µg Li/kg (n =6) in the form of NP03. NP03 and empty vehicle were kindly provided by Medesis Pharma, Montpellier, France.

Behavioral tests

Novel object recognition

A black box with the dimensions of 220 cm x 300 cm x 500 cm served as an arena for this task. One day prior to testing, animals were habituated to the open field for 5 minutes. In the Familiarization phase, rats were placed inside the box for 2 minutes to explore five objects arranged in a rectangle. In order to mask odor cues, the objects were

rubbed in soiled cage bedding to saturate scents. Exploration was defined as any time the snout was in contact or within 2.5 cm of an object with clear whisking motions of the whiskers. Time spent exploring each of the 5 objects was recorded to rule out any spontaneous object preference. No animals showed a spontaneous preference for any of the objects. In the novel object recognition phase of the task 30 minutes after the Familiarization phase, a new object replaced one object. Rats were given 3 minutes to freely explore the objects. Time spent exploring the familiar (TF) and novel object (TN) was recorded and a recognition index (RI) for the displaced object was calculated according to $RI_{novel} = TN/ TF+TN$.

Morris water maze

A circular pool of 1.75 m diameter filled to 1 m with water made opaque by the addition of non-toxic white paint. Water was maintained at a temperature of 22-23°C. Distal visual cues were positioned around the room outside the pool. Animals were tracked using HVS Image software (HVS Image, Mountain View, CA) connected to a camera positioned over the pool. During pretraining habituation, rats were placed in the pool to swim freely for 120 seconds. After swimming, rats were towel-dried, and returned to a heated home cage. During spatial training, each training block included two training sessions separated by an intertrial interval of 1 hour. The release position for each training session was randomized. Rats were towel-dried and returned to a heated home cage immediately after each swim. For the probe trial, a retention test was administered one day following 5 days of spatial training trials. The training platform was removed and rats were placed in the pool for 60 seconds. To verify visual acuity of rats, 24 hours

after the last testing phase, the platform was moved to a new position and made visible by raising it 1-2 cm above the water. Rats were placed in the opposite quadrant from the visible platform and were required to swim to it within 60 seconds. None of the rats tested displayed a visual deficit.

Fear conditioning

The fear conditioning protocol was designed following previously reported procedures (Nader et al. 2000; Iulita *et al.* 2014). Rats were tested in chambers containing a sound generator, located within a ventilated sound-attenuating box (Panlab, Barcelona, Spain). The floor of the chamber consisted of steel rods connected to an electrical shock source and a weight transducer system that allowed the tracking of movement. The system was connected to a computer that recorded the amount of freezing in each session (Freezing v1.3.01 software, Panlab). The software recorded all freezing episodes in each of the phases, considering a freezing episode as immobility for at least 2 sec. Rats were handled and habituated to the behavior room for 2 days before testing (day 1-2). During the habituation phase, conditioning day and contextual fear conditioning test, the chamber was scented with coconut extract and cleaned with 70% ethanol between rats. In order to simulate a different environment, on the day of the cued fear conditioning test, the metallic grid inside the chamber was removed, its walls were covered with visual cues, scented with mint extract and cleaned with 1% acetic acid between rats. On day 3, rats were allowed to explore the chamber for 5 min and returned to their home cages. These responses were used as an indicator of basal exploratory and locomotor activity. The next day, after an initial 90 sec-phase of exploration (baseline), rats were presented with a 30

sec tone (75 dB, 5 kHz), which co-terminated with a 2 sec footshock (0.75 mA). Animals were allowed to recover (120 sec) and were then returned to their home cages (post-shock phase). For the evaluation of contextual fear conditioning memory (24 h after auditory conditioning), rats were placed in the chamber in identical conditions and their freezing behavior was recorded during 8 min. Finally, 48 h after auditory conditioning, animals were allowed to explore the new, modified chamber for 120 sec (baseline). This was followed by three consecutive tone presentations (30 sec, 75dB, 5 kHz), each separated by a 30 sec pause.

Tissue preparation

Brain tissue was harvested one hour following reactivation of the auditory fear memory to coincide with peak gene expression required for synaptic stabilization and consolidation. Briefly, rats were deeply anesthetized by i.p. administration of Equithesin (0.3 mL/100 g) and transcardially perfused with ice-cold perfusion buffer. The intact left hemisphere was post-fixed for 48 hours in a 4% formalin solution in 0.1 M phosphate buffer, pH 7.4, then moved to 30% sucrose at 4^{o}C until cryosectioning, while the remaining right hemisphere was dissected and tissue was frozen at -80^{o}C until biochemical analysis.

Determination of blood serum lithium levels

Blood samples were drawn after 3 weeks of treatment by sephanous vein and from the ascending aorta at the time of perfusion. Sera were separated by centrifuging at 4°C for

ten minutes at 5000 x g and were frozen at -20°C until analysis. Lithium concentration was determined using a lithium sensitive electrode (minimum detection limit of 0.06 mmol/L) and was carried out by the Clinical Laboratories of Diagnostic and Therapeutic Services, at the Royal Victoria Hospital, Montreal, Canada.

Determination of brain lithium levels

A separate group of rats were used for these experiments (see *Animals*, above). Three to five hours following NP03 or vehicle administration, animals were euthanized by an i.p. overdose of pentobarbital and the brain was dissected and immediately frozen at -20°C. Each brain was washed in cold physiological serum, weighed (mean value 1.7 g), and dried on a heating block at 100°C for 48 hours. For mineralization of the tissue, 65% nitric acid was added and brains were heated for 24 hours at 100°C. The residual liquid volume was measured and a portion corresponding to 0.2 g brain tissue was diluted with QSP water. This solution was analyzed by inductively coupled plasma mass spectrometry (ICP-MS; ICAP-Q, ThermoScientific). For each brain, the lithium concentration was calculated in ng/g of fresh tissue.

Immunohistochemistry

Free-floating tissue sections were washed in PBS, treated with 0.3% hydrogen peroxide in PBS-T to quench endogenous peroxidase activity, and blocked for 1 hour in 10% normal goat serum (NGS). Sections were incubated overnight at 4°C in a solution containing mouse monoclonal anti-Aβ amyloid peptide (McSa1, 1:2000, MediMabs, Montreal, QC) in 10% NGS with PBST. McSa1 was raised against a synthetic peptide of

Aβ recognizing amino acids 1-12 of human Aβ (Grant *et al.* 2000). Tissue sections were washed and then incubated with goat anti-mouse IgG (1:100, MP Biochemicals, Solon, OH) in 5% NGS with PBST for 1 hour and subsequent mouse anti-peroxidase monoclonal antibody complex (1:30, MAP/HRP complex, Medimabs) for 1 hour. DAB was used as the chromogen (Vector Laboratories, Burlington, ON). Sections were mounted on glass slides and allowed to air-dry overnight. Sections were then defatted and cleared in a graded alcohol series with terminal xylenes and coverslipped with Permount mounting medium (Fisher Scientific, Ottawa, ON). Slides were imaged using an Axio Imager 2 microscope (Carl Zeiss Canada, North York, ON) running Zen Blue software (Carl Zeiss).

Chromatin immunoprecipitation (ChIP)

Cortical tissue was cross-linked in formaldehyde solution [50 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% formaldehyde, pH 8.0]. The formaldehyde reaction was quenched with a 2.5 M glycine solution, and tissue was homogenized in lysis buffer [10 mM Tris-HCl (pH 8.0), 0.1% sodium deoxycholate, 0.5% N-laurylsarcosine], supplemented with Complete™ protease inhibitors (Roche). DNA was sheared to 100-500 bp fragments using a Bioruptor Plus UCD-300 sonicator (Diagenode). After sonication, 10% Triton X-100 was added to the tissue lysate and the mix was cleared by centrifugation at 15,000 x *g* for 15 min (4°C). Protein concentration was determined using the Bradford assay (BioRad, Mississauga, ON).

Tissue lysate was first precleared by incubation with PureProteome protein A magnetic beads (EMD Millipore). Cleared tissue lysate was then added to PureProteome protein A magnetic beads previously saturated with antibodies, and immunoprecipitation was carried overnight at 4°C. The antibody used was an anti-CRTC1 rabbit monoclonal antibody (1:250, Cell Signaling Technology) and anti-rat IgG rabbit polyclonal antibody as a negative control (1:250, Sigma-Aldrich). Beads were washed with RIPA wash buffer [50 mM HEPES–KOH (pH 7.6), 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 and 0.7% Na-deoxycholate] followed by one wash with TE buffer supplemented with 50 mM NaCl. DNA-protein complexes were eluted from the beads using elution buffer [50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1 % SDS] and by heating the samples at 65°C for 15 min with frequent vortexing. Contaminating RNAs were digested using RNaseA (Sigma-Aldrich) followed by a proteinase K (Roche) treatment to digest remaining proteins. The immunoprecipitated DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen) and were eluted in 10 mM Tris–HCl (pH 8.0). Quantification of immunoprecipitated DNA was assessed by qPCR with the Sso Advanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Instrument and software (Bio-Rad). For a list of primers used see **Table 3-1**. ChIP data were normalized to input DNA from each sample.

Table 3-1. Nucleotide sequences of the primers used for ChIP-qPCR experiments

Gene	5'-3' Forward sequence	5'-3' Reverse sequence	Amplicon (bp)
Bdnf iv	ATGCAATGCCCTGGAAC	GTGAATGGGAAAGTGGGTG	182
c-fos	CTCAGTTGCTAGCTGCAATCG	CCCCCTCCAGTTTCTCTGTT	113
Arc	ACCCAGCATGGGGACTCAAGGGCCT	GCTGCTCACCTGGGGGTGACTGCTC	149

Gene expression analysis

Total RNA was extracted from rat hippocampal tissue using the RNeasy Mini Kit (Qiagen), following manufacturer instructions. Residual DNA was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). To generate cDNA, total RNA was retro-transcribed using the iScript Reverse Transcription Supermix (Bio-Rad). Quantification of transcript expression was assessed by qRT-PCR with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Instrument and software. Expression of *Bace1* and *mhAPP* were normalized against the average of two housekeeping genes (*Hprt and Gapdh*), and interpreted using the comparative ΔΔCt method. For the list of primers used, see **Table 3-2**.

Table 3-2. Nucleotide sequences of the primers used for Q-RT-PCR gene expression experiments

Gene	5'-3' Forward sequence	5'-3' Reverse sequence	Amplicon (bp)
Bace1	CCCACAGACGCTCAACATCC	AGACTTTCGGAGGTCTCGGT	130
mhAPP	AGGACTGACCACTCGACCAG	CGGGGGTCTAGTTCTGCAT	393
Gapdh	TGATGGGTGTGAACCACGAG	TCATGAGCCCTTCCACGATG	132
Hprt	TTCCTCCTCAGACCGCTTTTC	CATCACTAATCACGACGCTGG	80

BACE1 activity detection assay

BACE1 activity in cortical tissue was assessed using the fluorescence resonance energy transfer (FRET)-based β-secretase (BACE1) Activity Detection Kit (Sigma-Aldrich, St. Louis, MO; CS0010). 200 µg of protein from tissue lysates was incubated in the presence

of BACE1 substrate and assay buffer for 2 hours at 37°C. The fluorescent signal generated was measured using a fluorescent plate reader (excitation: 320 nm; emission: 405 nm. Fluorescent units (FU) from a blank were subtracted from all signal readings and a standard curve was used to estimate pmol present in each sample.

TBS soluble and insoluble human $A\beta_{40}$ and $A\beta_{42}$ ELISA

Approximately 20-25 mg of cortical tissue was homogenized in ice-cold 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA (pH 7.6) homogenization buffer supplemented with Complete Mini protease inhibitors (Roche Diagnostics) and PhosStop phosphatase inhibitor cocktail tablets (Roche Diagnostics). Samples were ultracentrifuged at 4°C for 1 hour at 100,000 x g using an Optima Max XP Ultracentrifuge (Beckman Coutler). The supernatant was removed and stored at -20°C and this represented the soluble fraction. The pellet was resuspended in 5 M guanidine HCl, 50 mM Tris-HCl (pH 8.0). Samples were sonicated 2 x 2 seconds (5 second pause) at 40% amplitude, incubated on ice for 30 minutes, and then centrifuged at 4°C for 1 hour at 100,000 x g. Finally, the supernatant representing the insoluble fraction was removed and stored at -20°C. The total protein in the homogenates was determined by the method of Lowry. Levels of soluble and insoluble human $A\beta_{40}$ and $A\beta_{42}$ were assessed according to directions provided in ELISA kits from Invitrogen (Cat. KHB3482 and KHB3442).

Statistical analysis

Data were analyzed using 2-way analysis of variance (ANOVA) with Tukey's post-hoc tests using GraphPad Prism software, (GraphPad Software Inc., La Jolla, CA). Means of

two groups were compared using a two-tailed unpaired Student's *t*-tests (GraphPad Prism). Statistical significance was defined as $p < 0.05$.

RESULTS

McGill-R-Thy1-APP Alzheimer transgenic rats express the mutated human Aβ precursor protein gene (*mhAβPP*) and show intraneuronal Aβ as early as two weeks postnatally, and extracellular plaques appear at 6 months of age, spreading from the hippocampus to the cortex. Rats at the pre-plaque state already show impaired cellular signaling, impaired long-term potentiation, and severe cognitive deficits (Leon *et al.* 2010; Hanzel *et al.* 2014; Iulita et al. 2014; Qi *et al.* 2014; Wilson *et al.* 2016). Thus, we started drug treatment at this pre-plaque time point to assess the ability of NP03 to revert established Aβ-driven pathology and related cognitive impairment.

NP03 Treatment

We evaluated a microdose formulation of lithium, NP03, in the treatment of AD-like amyloid pathology in an Alzheimer transgenic rat model at the early, pre-plaque stage of the disease. NP03 contains the lithium citrate salt (**Figure 3-1a**) deposited in a water-in-oil microemulsion and its enhanced delivery beyond the BBB allows significantly lower levels of lithium to be delivered, avoiding the off target effects associated with conventional lithium. Alzheimer transgenic rats and their wild type littermates received either NP03 (40 µg Li/kg; 1 ml/kg) or vehicle (1 ml/kg) 5 days per week for a period of 8 weeks **(Figure 3-1b)**. In a separate pilot experiment, we found that rats treated with

0.024% lithium carbonate developed polydipsia and polyuria, significant weight loss, and as a result, treatment was discontinued after 4 weeks (**Appendix 1**). Importantly, no weight loss was observed following NP03 treatment (2-way ANOVA, $p > 0.05$; **Figure 3-1c**). Serum lithium levels were below detection limit (o.o6 mmol/L), however, the level of lithium in the brain of rats treated with 40 µg Li/kg NP03 was shown to be 3.36 ng/g of brain tissue as measured by ICP-MS analysis. Of note, the concentration of lithium in the brain of rats increased with increasing doses of lithium, suggesting the saturation is not reached at the 40 µg Li/kg NP03 dose (**Figure 3-1d**). Finally, we compared transcript production of the Thy1 promoter-driven *mhAβPP* transgene using qPCR and confirm that NP03 treatment did not affect its expression ($t_{(13)} = 0.3601$; $p = 0.7246$; **Figure 3-1e**).

Figure 3-1: Details on NP03 Formulation and Experimental Design

a) Skeletal formula of lithium tricitrate tetrahydrate salt, delivered as a water-in-oil microemulsion through transmucosal uptake. The formulation (40 μg Li/kg body weight) was delivered to the rectal mucosa daily, 5 days per week for 8 weeks.

b) Prior to the onset of Aβ plaque deposition in the Alzheimer transgenic rats, all rats were treated and assessed on a battery of cognitive tasks (beginning at 3-months of age and ending at 5-months of age).

c) There was no significant change in rat body weight in response to NP03 treatment. Rat weight was recorded daily at the time of drug or vehicle administration. WT Veh n = 6; WT NP03 n = 6; AD TG Veh n = 8; AD TG NP03 n = 16. Data represent mean ± SEM. 2-way ANOVA, $p > 0.05$.

d) Brain levels of lithium attained with increasing doses 0 μg Li/kg (n = 4), 40 μg Li/kg (n = 6), 160 μg Li/kg (n = 6), and 600 μg Li/kg (n = 6) in the form of NP03. Treatment with 40 μg Li/kg body weight yielded a brain concentration of 3.36 ng Li/g of brain tissue. Data represent mean ± SEM.

e) Gene expression analysis of hippocampal tissue by qRT-PCR confirmed that NP03 did not affect expression of the *mhAβPP* transgene (AD Veh n = 6; AD NP03 n = 9). Data represent mean ± SEM. Means of two groups analyzed using two-tailed unpaired *t* test, $p > 0.05$.

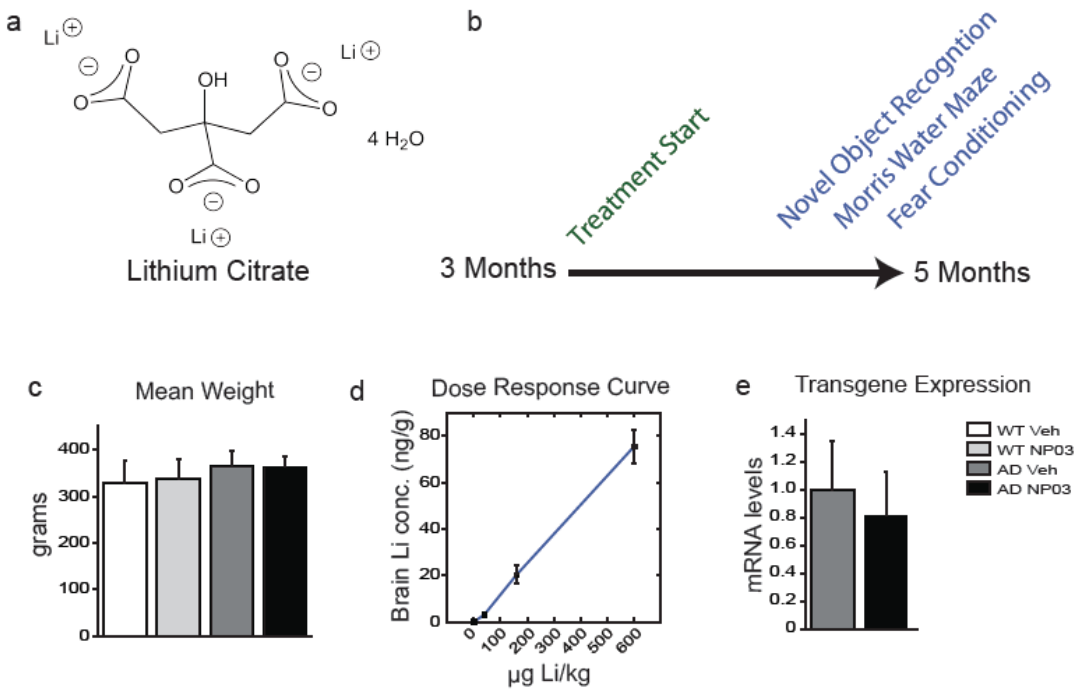

Figure 3-1: Details on NP03 Formulation and Experimental Design

NP03 Restored Novel Object Recognition in Alzheimer Transgenic Rats

We first evaluated the efficacy of NP03 treatment on cognition using a battery of tests sensitive to various aspects of learning and memory. Rats were tested during treatment weeks 5-8. The first test, the NOR task, involves online working memory and requires articulation between the hippocampus and the entorhinal cortex (Murray and Mishkin 1986). The NOR task exploits a natural tendency for rats to find novel object interesting, as presumably novel objects could represent a new source of food, water, or could hold a mate or a threat (Ennaceur and Delacour 1988). When exposed to multiple objects, therefore, rats will typically spend more time exploring a novel object compared to one that is familiar.

During the 3-minute acquisition phase, rats demonstrate a high level of exploratory behavior, spending approximately half of the allotted time exploring objects (**Figure 3-2a**). There was no effect of Transgene ($F_{(1,26)} = 0.01$; $p = 0.9622$) or Treatment ($F_{(1,26)} = 0.37$; $p = 0.5509$) on exploratory behavior, as all rats spent the same amount of time exploring objects in the area and there was no spontaneous preference to any of the objects. Before the test phase, one familiar object was replaced with a novel object. After a 30-minute intertrial interval, rats were placed in the arena (**Figure 3-2b**) We observed a significant Treatment x Transgene Interaction ($F_{(1,26)} = 5.65$; $p = 0.0251$). *Post hoc* analyses revealed a significant reduction in the novel object preference for the vehicle-

treated Alzheimer transgenic rats compared to the wild-type control ($p < 0.05$). This indicated that the Alzheimer transgenic rats were unable to recognize the previously encountered objects. NP03 completely reversed this deficit in Alzheimer transgenic rats ($p < 0.05$).

Figure 3-2: NP03 Restored Novel Object Recognition in Alzheimer Transgenic Rats

a) The acquistion phase revealed that Alzheimer transgenic rats were unimpaired in exploratory behavior. All rats spent approximately half of their time in the area actively exploring objects. WT Veh n = 5; WT NP03 n = 6; AD TG Veh n = 5; AD TG NP03 n = 14. Data represent mean ± SEM. $p > 0.05$, 2-way ANOVA.

b) Chance preference for the novel object (1/5 objects) is equal to 0.2 (dashed green line). As indicated by the reduced preference ratio for the novel object, Alzheimer transgenic rats showed impaired novel object recognition. NP03 treatment completely restored novel object recognition performance in Alzheimer transgenic rats. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

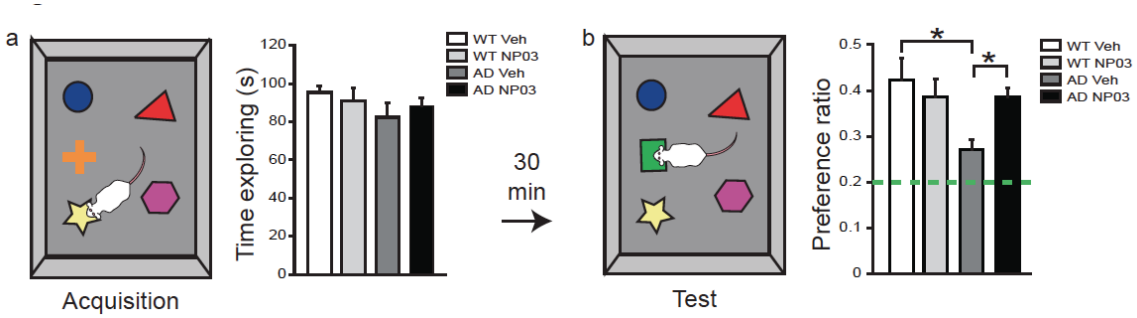

Figure 3-2: NP03 Restored Novel Object Recognition in Alzheimer Transgenic Rats

NP03 Restored Hippocampal-Dependent Spatial Learning in Alzheimer Transgenic Rats

To analyze the consequences of intraneuronal Aβ and NP03 treatment on spatial learning and memory, rats were tested in the Morris water maze (MWM) task (Morris 1981; Morris 1984). This task involves the specific and disproportional involvement of the hippocampal formation in the spatial aspects of MWM learning [reviewed in (D'Hooge and De Deyn 2001)]. In the task, rats are placed in a circular pool of opaque water and are required to learn the location of a submerged platform. Upon successive training trials, rats are expected to use visual cues to navigate to the submerged platform.

At the start of training on Day 1, mean platform latencies were not significantly different between the four groups tested, ranging from 67.00 to 85.75 seconds (**Figure 3-3a,b**). All rats showed improved performance with successive training, as indicated by reduced escape latency over the course of training from Day 1 - Day 5 (Repeated Measures Two-way ANOVA: Time Effect $F_{(4,135)} = 12.28$; $p < 0.0001$). However, during the acquisition phase, *post hoc* analysis revealed that Alzheimer transgenic rats were impaired on Day 2 ($p < 0.01$) and 3 ($p < 0.05$) when compared to the wild-type group, as it took significantly longer for Alzheimer transgenic rats to locate the hidden platform. NP03 prevented this acquisition deficit in Alzheimer transgenic rats, and Alzheimer transgenic rats treated with NP03 performed at the same level as wild-type rats ($p > 0.05$). A Probe Trial was administered 24-hours after the last training trial wherein the platform was removed. The number of crosses made by rats over the previous platform location was measured during a one-minute period. On the Probe Trial we found that

Alzheimer transgenic rats made as many platform crosses as wild-type rats (**Figure 3-3c**). This indicated that while the Alzheimer transgenic rats took longer over training Days 1-5 in learning to navigate to the hidden platform, they were eventually able to do so, and, as a result, showed no deficit when tested on the subsequent Probe Trial.

Figure 3-3: NP03 Restored Hippocampal-Dependent Spatial Learning in Alzheimer Transgenic Rats

a) Schematic showing experimental procedure for the Morris water maze. During Days 1 through 5 (D1-D5) of the acquisition phase, rats were tested on their latency to find a submerged platform located in the target quadrant. The platform was removed from the pool and on Day 6, rats were subjected to the Probe Trial.

b) During the acquisition phase, Alzheimer transgenic rats showed longer escape latencies on Day 2 and Day 3, compared to the wild-type rats that received vehicle, although all rats showed improved performance after 5 days of training, as indicated by reduced escape latency. WT Veh n = 6; WT NP03 n = 7; AD TG Veh n = 5; AD TG NP03 n = 13. Data represent mean ± SEM. * $p < 0.05$ compared to WT Veh, ** $p < 0.01$ compared to wild-type vehicle; Tukey's multiple comparisons *post hoc* test.

c) There was no difference in percentage time spent by each group in the target quadrant during the Probe Trial. Chance performance of 25% indicated by red dashed line. Data represent mean ± SEM. $p > 0.05$, 2-way ANOVA.

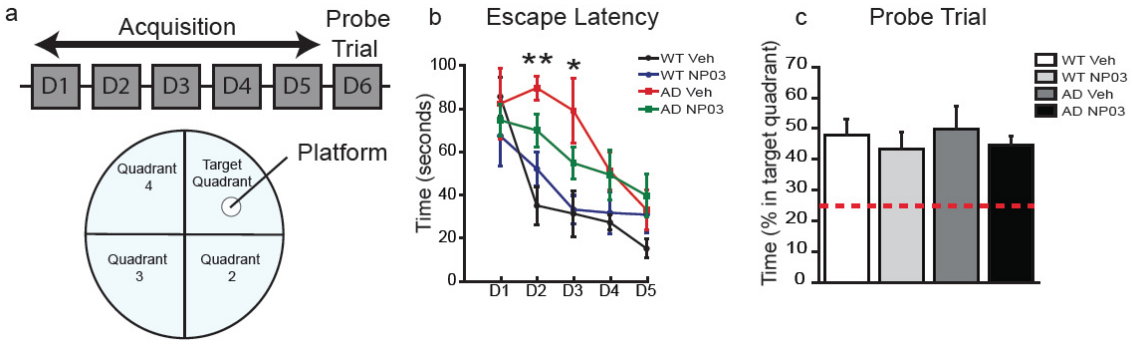

Figure 3-3: NP03 Restored Hippocampal-Dependent Spatial Learning in Alzheimer Transgenic Rats

NP03 Restored Auditory Fear Conditioning in Alzheimer Transgenic Rats

In the auditory fear-conditioning task, all rats displayed baseline-freezing behavior of approximately 20% when placed into the training context (CXT-A), regardless of genotype and treatment (**Figure 3-4a**). The presentation of a 5 kHz tone elicited no change from baseline freezing levels, confirming that 5 kHz tone was initially innocuous. The addition of a mild footshock coterminating with the tone led to an elevation in freezing for all rats. We tested contextual memory 24 hours later by returning rats to the training context (CXT-A). It is expected that the rats would associate this CXT-A with the footshock, and should therefore display elevated freezing when returned to that context. Consistent with previous reports (Iulita *et al.* 2014), all of the rats showed high levels of freezing when returned to CXT-A, indicating Alzheimer transgenic rats were unimpaired in contextual fear memory (**Figure 3-4b**). To verify the contextual specificity of the fear response, we tested rats in a novel context (Context B, CXT-B) 24 hours later and all groups showed baseline freezing (**Figure 3-4c**). Upon presentation of the 5 kHz tone in CXT-B, however, wild-type rats demonstrated a high level of freezing. In contrast, the Alzheimer transgenic rats showed similar low levels of freezing, indicating a failure to form the fear memory. Alzheimer transgenic rats treated with NP03 showed high levels of freezing, indicating that cued recall was rescued by NP03 treatment. These observations were supported by the results of ANOVA, which revealed a significant Transgene X Treatment interaction ($F_{(1,24)} = 9.19$; $p = 0.0058$), as well as a significant main effect for Treatment ($F_{(1,24)} = 4.66$; $p = 0.0411$), but not for Transgene ($F_{(1,24)} = 0.07$; $p = 0.7939$). *Post hoc* analyses revealed that vehicle-treated Alzheimer transgenic rats had lower levels of freezing compared to vehicle-treated wild type rats ($p < 0.05$),

while there was no difference between control animals and NP03-treated Alzheimer transgenic animals ($p > 0.05$). Tissue was collected 1 hour following the reactivation of the auditory fear memory to allow measurement of CRTC1 promoter occupancy coincident with peak neural plasticity gene expression (Guzowski et al. 2001).

Together, these behavioral results indicate that NP03 reversed severe Aβ-driven cognitive deficits in pre-plaque Alzheimer transgenic rats, including in novel object recognition, spatial learning, and auditory fear conditioning.

Figure 3-4: NP03 Restored Auditory Fear Conditioning in Alzheimer Transgenic Rats

a) Fear behavior was assessed by measuring % freezing. All rats demonstrated baseline freezing of approximately 20% in Context A. The presentation of a 5 kHz tone elicited no change in freezing percentage, however, post-shock freezing was elevated compared to baseline. WT Veh n = 6; WT NP03 n = 5; AD TG Veh n = 6; AD TG NP03 n = 11. Data represent mean ± SEM.

b) 24-hours following auditory conditioning, rats were returned to Context A and demonstrated an elevated freezing response, indicative of formation of contextual fear memory. Baseline freezing from the habituation phase is indicated by the red dashed line.

c) This memory was specific to Context A since freezing behavior returned to baseline levels when rats were placed into a new Context B, 24-hours later. After the sounding of the 5 kHz tone, wild-type rats demonstrated the fear memory by freezing. The vehicle-treated Alzheimer transgenic animals were significantly impaired on the cued memory recall, and did not freeze above baseline, indicating a failure to associate the tone with the foot shock. Importantly, freezing was restored to wild type levels in the NP03-treated Alzheimer transgenic rats. Data represent mean ± SEM. ** $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

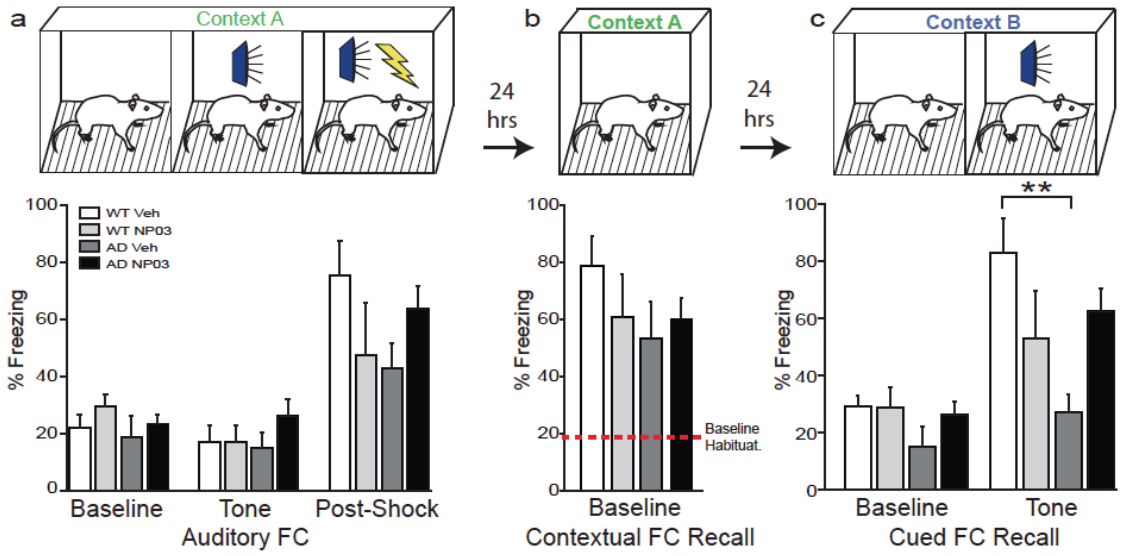

Figure 3-4: NP03 Restored Auditory Fear Conditioning in Alzheimer Transgenic Rats

NP03 Reduced BACE1 Activity and the Ratio of $A\beta_{42}/A\beta_{40}$

After assessing the impact of NP03 treatment on behavioral deficits, we next delved into the biochemical changes that might have occurred to give rise to the observed behavioral restoration. We observed a 5-fold increase in *BACE1* mRNA in the hippocampus of Alzheimer transgenic rats relative to the wild-type rats (**Figure 3-5a**) that was restored to normal levels with NP03 treatment (Treatment x Transgene Interaction: $F_{(1,23)} = 6.91$; $p = 0.0150$; WT:Veh vs. TG:Veh, $p < 0.05$; TG:Veh vs. TG:NP03, $p < 0.01$). In addition, using a FRET-based activity assay in which fluorescent signal increases with substrate cleavage by BACE1, we observed increased activity in BACE1 in Alzheimer transgenic rats compared to the wild type (**Figure 3-5b**). NP03-treatment reduced BACE1 activity to baseline levels, a reduction of 12.96%. These observations were supported by the results of ANOVA, which revealed a significant Transgene X Treatment interaction ($F_{(1,25)} = 9.547$; $p = 0.0050$), as well as a significant main effect for Treatment ($F_{(1,25)} = 6.65$; $p = 0.0162$), but not for Transgene ($F_{(1,25)} = 3.28$; $p = 0.0822$). *Post hoc* analyses revealed that vehicle-treated Alzheimer transgenic rats had significantly elevated BACE1 activity compared to vehicle- and NP03-treated wild-type rats ($p < 0.05$). NP03 reduced BACE1 activity in Alzheimer transgenic rats compared to the vehicle-treated Alzheimer transgenic rats ($p < 0.01$) to a level not significantly different from the wild type ($p > 0.05$).

Given the increase in BACE1 gene expression and activity in Alzheimer transgenic rats, we subsequently analyzed the levels of TBS- and guanidine-soluble

human $A\beta_{40}$ and $A\beta_{42}$ peptides. In the TBS-soluble fraction, $A\beta_{42}$ peptides (**Figure 3-5c**) and $A\beta_{40}$ peptides (**Figure 3-5d**) were highly expressed in Alzheimer transgenic rats. Importantly, treatment with NP03 led to a 4.5-fold reduction in soluble $A\beta_{42}$ ($t_{(12)}$ = 2.028; p = 0.0327), while levels of soluble $A\beta_{40}$ were unchanged ($t_{(12)}$ = 6.197; $p > 0.05$). The reduction in $A\beta_{42}$ led to a significant decrease in the soluble $A\beta_{42}/A\beta_{40}$ ratio ($t_{(12)}$ = 2.187; p = 0.0247; **Figure 3-5e**). Insoluble $A\beta_{42}$ and $A\beta_{40}$ were not detected at this early, 6-month time point (data not shown). Together, these results indicate that NP03 treatment led to a significant reduction in BACE1 mRNA levels, BACE1 activity, and levels of soluble $A\beta_{42}$ in Alzheimer transgenic rats.

Figure 3-5: BACE1 Activity and $A\beta_{42}/A\beta_{40}$ Ratio Reduced by NP03

a) Gene expression analysis of hippocampal tissue revealed a 5-fold elevation in *Bace1* mRNA in vehicle-treated Alzheimer transgenic rats compared to the wild-type groups. This increase was significantly reduced in NP03-treated Alzheimer transgenic rats. WT Veh n = 4; WT NP03 n = 6; AD Veh n = 5; AD NP03 n = 12. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Tukey's multiple comparisons *post hoc* test.

b) FRET-based activity assay for BACE1. Fluorescent signal enhancement is observed as the substrate is cleaved by BACE1, and shows increased activity of BACE1 of approximately 13% in vehicle-treated Alzheimer transgenic rats. NP03 treatment led to a significant reduction in BACE1 activity in NP03-treated Alzheimer transgenic rats. WT Veh n = 7; WT NP03 n = 6; AD Veh n = 5; AD NP03 n = 11. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Tukey's multiple comparisons *post hoc* test.

c) Level of human $A\beta_{42}$ was assessed in both soluble and insoluble fractions. Insoluble $A\beta_{42}$ peptides were not detected in these conditions and at this early time point of the Aβ pathology. NP03 treatment significantly reduced the level of $A\beta_{42}$ in Alzheimer transgenic rats. Data represent mean ± SEM. * $p < 0.05$, unpaired *t* test.

d) Level of human $A\beta_{40}$ was similarly assessed in both soluble and insoluble fractions. Insoluble $A\beta_{40}$ peptides were not detected in these conditions and at this early time point of the Aβ pathology. In contrast to its effects on soluble $A\beta_{42}$, NP03 treatment had no effect on the level of $A\beta_{40}$ in Alzheimer transgenic rats. Data represent mean ± SEM. Unpaired *t* test.

e) Reduction in $A\beta_{42}$ but not $A\beta_{40}$ led to a significant reduction in the $A\beta_{42}/A\beta_{40}$ ratio in response to NP03 treatment in Alzheimer transgenic rats. Data represent mean ± SEM. * $p < 0.05$, unpaired *t* test.

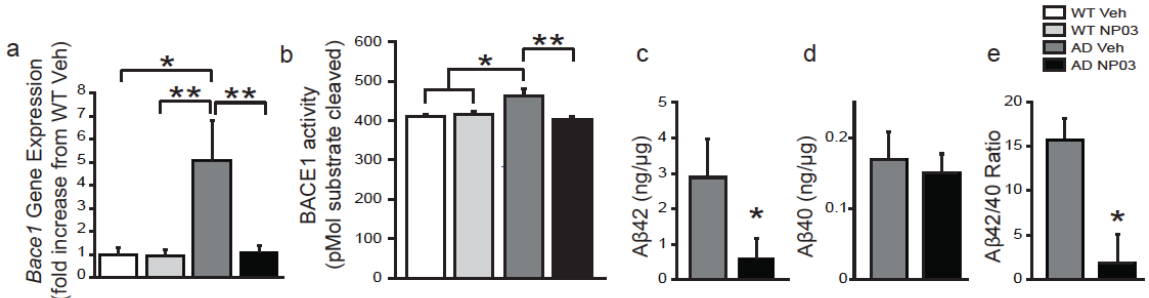

Figure 3-5: BACE1 Activity and $A\beta_{42}/A\beta_{40}$ Ratio Reduced by NP03

NP03 Restored CRTC1 Promoter Binding in Synaptic Plasticity-Associated Genes

Because lithium has been shown to increase CRTC1 oligomerization, and CREB, CBP binding (Boer *et al.* 2007; Heinrich *et al.* 2009; Heinrich *et al.* 2013), we next tested whether NP03 could reverse the Aβ-driven impairment in CRTC1 promoter occupancy observed in Alzheimer transgenic rats (Wilson *et al.* 2016).

Tissue was extracted 1 hour following reactivation of the auditory fear memory, to coincide with the peak activity-dependent gene expression. We found that in Alzheimer transgenic rats, genomic promoter occupancy was significantly reduced for *Bdnf iv* (**Figure 3-6a**). These observations were supported by the results of ANOVA, which revealed a significant main effect for Transgene ($F_{(1,30)} = 9.50$; $p = 0.0044$). *Post hoc* analyses revealed that vehicle-treated Alzheimer transgenic rats had significantly reduced CRTC1 promoter occupancy at *Bdnf iv* compared to vehicle- and NP03-treated wild type ($p < 0.05$). NP03 restored CRTC1 *Bdnf iv* promoter occupancy to a level not significantly different from the wild type ($p > 0.05$). CRTC1 promoter occupancy was also reduced at *c-fos* in Alzheimer transgenic rats (Transgene X Treatment interaction ($F_{(1,29)} = 5.77$; $p = 0.0229$; **Figure 3-6b**). *Post hoc* analyses revealed that vehicle-treated Alzheimer transgenic rats had significantly lower CRTC1 occupancy of *c-fos* promoters compared to the wild-type vehicle group ($p < 0.05$). NP03 significantly increased CRTC1 occupancy in Alzheimer transgenic rats compared to the vehicle-treated Alzheimer transgenic rats ($p < 0.05$) to a level not significantly different from the wild type ($p > 0.05$). Finally, there was a trend towards reduced CRTC1 binding to the *Arc* promoter in

Alzheimer transgenic rats compared to the wild type (**Figure 3-6c**), although this trend failed to reach statistical significance ($p > 0.05$).

Figure 3-6: NP03 Restored CRTC1 Promoter Occupancy in Alzheimer Transgenic Rats

a) CRTC1 genomic occupancy at *Bdnf iv* promoters was significantly reduced in vehicle-treated Alzheimer transgenic rats compared to the wild type. NP03 treatment restored CRTC1 occupancy to *Bdnf iv* promoters to a level not significantly different from the wild type. WT Veh n = 7; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 14. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

b) CRTC1 promoter occupancy at *c-fos* was significantly reduced in vehicle-treated Alzheimer transgenic rats compared to the wild-type vehicle group. NP03 treatment significantly restored CRTC1 occupancy to *c-fos* promoters in Alzheimer transgenic rats. WT Veh n = 6; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 14. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

c) We observed a trend towards reduced CRTC1 occupancy to *Arc* in the vehicle treated Alzheimer transgenic group, although this reduction failed to reach significance (2-way ANOVA, $p > 0.05$). This trend was not observed in the Alzheimer transgenic rats treated with NP03. WT Veh n = 7; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 15. Data represent mean ± SEM.

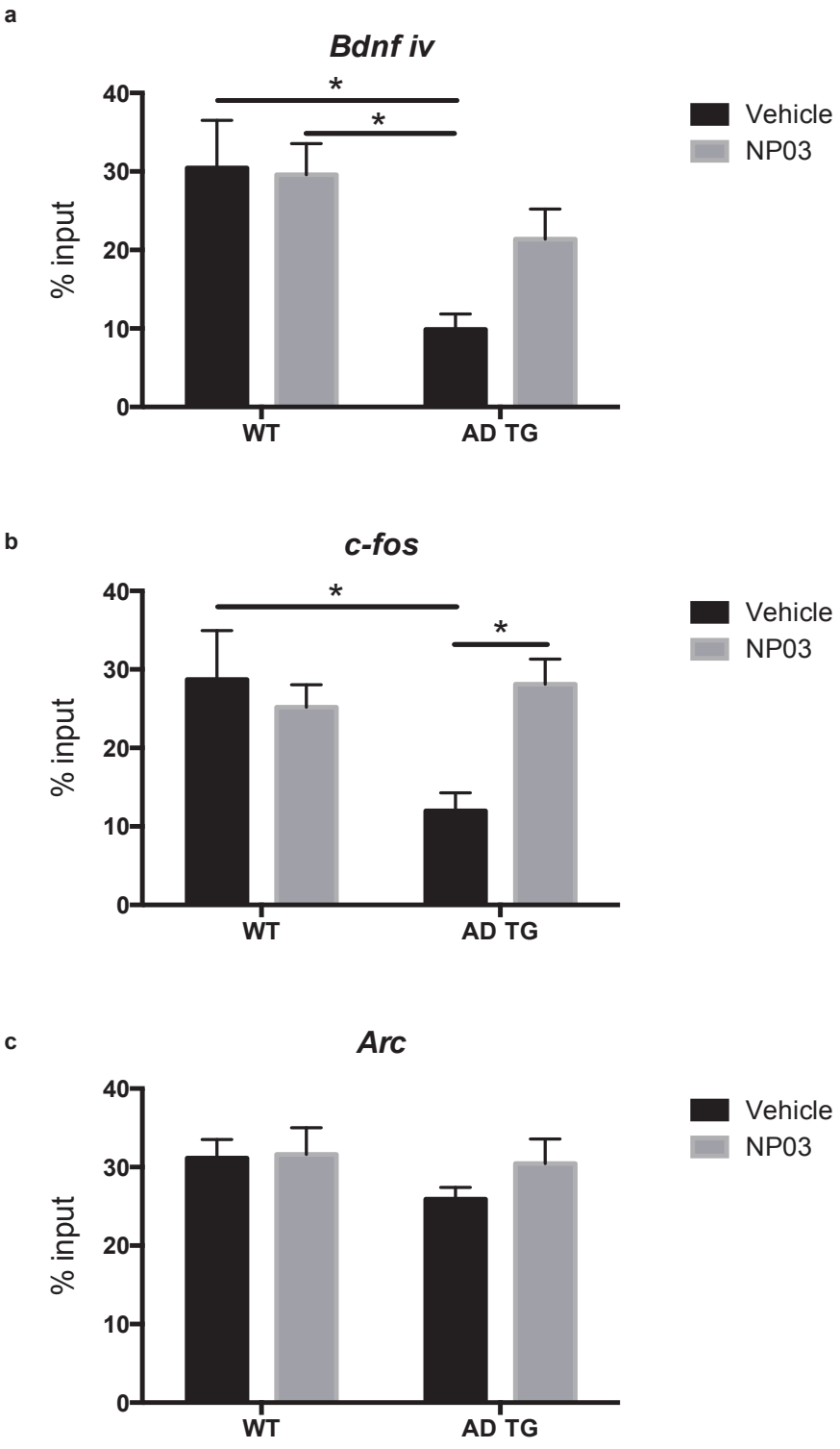

Figure 3-6: NP03 Restored CRTC1 Promoter Occupancy in Alzheimer Transgenic Rats

DISCUSSION

Current Alzheimer therapies are not disease modifying and offer only mild, short-lasting relief from disease symptoms. Here, we show that a novel, microdose formulation of lithium, coded NP03, reversed deficits in novel object recognition, hippocampal-dependent spatial learning, and fear conditioning in Alzheimer transgenic rats. Importantly, NP03 reduced the level of $A\beta_{42}$ peptides and reduced BACE1 hyperactivity. NP03 restored CRTC1 *Bdnf* and *c-fos* promoter occupancy – aspects of the CRTC1 transcription program required for learning and memory. The effects were observed in the absence of any adverse events relating to lithium toxicity.

NP03 Reversed AD-Related Memory Loss in Alzheimer rats

The pathological processes underlying AD are thought to begin decades prior to cognitive decline and dementia diagnosis (Jack *et al*. 2010; Sperling *et al*. 2011). However, emerging data have indicated that subtle cognitive deficits associated with AD might be masked through cognitive reserve mechanisms, including neural compensation and neural reserve (Stern *et al*. 2005). For this reason, rodent studies are useful in that they generally have fewer available resources for compensation (Wilson *et al*. 2016). In McGill-R-Thy1-APP Alzheimer transgenic rats, cognitive impairment and memory loss begin prior to the appearance of extracellular Aβ plaques (Iulita *et al*. 2014; Wilson *et al*. 2016), making them an ideal tool for investigating the early (preclinical) events related to Aβ pathology.

Spatial learning is severely impaired in AD, contributing to disorientation, confusion, and wandering (Rolland et al. 2007). On the Morris water maze, Alzheimer transgenic rats demonstrated normal performance on training days 4-5 and on the probe trial, but were slow during the acquisition phase of the task, as demonstrated by significantly longer platform latencies on Days 2 and 3. This indicates that the Alzheimer transgenic rats were eventually able to acquire the task, but required more training than the wild-type rats to do so. When Alzheimer transgenic rats were treated with NP03, spatial learning was restored.

Auditory fear conditioning is a Pavlovian conditioning paradigm that requires rats to learn that a tone signals an impending mild footshock. It has been shown that a single training session wherein an innocuous tone is paired with a mildly aversive footshock is sufficient to trigger freezing in rats whenever the tone is subsequently presented. This behavior requires articulation between neurons of the lateral amygdala and the hippocampus (Gore et al. 2015) and deals with the formation of a fear memory engram – a specific subpopulation of lateral amygdala neurons (Josselyn 2010). The neurons that are incorporated into the engram outcompete their neighbors, depending on their intrinsic excitability. It has previously been shown that CRTC1 mediates auditory fear conditioning in mice (Nonaka et al. 2014). We have previously demonstrated that while not affecting the passive membrane properties of the cell, the accumulation of intraneuronal Aβ leads to decreased intrinsic excitability of neurons (Qi *et al.* 2014) and to impaired CRTC1 function in Alzheimer transgenic rats (Wilson *et al.* 2016). Our results here confirm that NP03 reverses the Aβ-induced impairments in amygdalar-

dependent fear memory, likely through the reduction of intraneuronal Aβ, and perhaps by restoring CRTC1 function.

These deficits are similar to those of our previous report showing that pre-plaque Alzheimer transgenic rats were impaired during the acquisition of a one-pair visual discrimination task (Wilson *et al*. 2016). Such impairments in acquisition are common during the early and later stages of AD (Pasquier *et al*. 2001). Reports indicate that the memory deficits associated with AD are not due to forgetting *per se*, but rather to a difficulty in encoding new information (Kopelman 1985). This is supported by data showing that the rates of forgetting are equal in AD patients and healthy control subjects (Becker *et al*. 1987). Taken together, our data appear to indicate a global impairment in acquisition, which impacted performance on novel object recognition, spatial learning, and fear conditioning tasks.

BACE1 Activity and the $A\beta_{42}/A\beta_{40}$ Ratio

BACE1 activity has been shown to be elevated in AD and in models of Alzheimer amyloidosis (O'Brien and Wong 2011; Ferretti, Allard, *et al.* 2012). Similarly, hyperactive BACE1 promotes Aβ accumulation in other conditions, including in mouse models of traumatic brain injury (Yu, Zhang, *et al.* 2012). BACE1 cleaves APP to produce toxic $A\beta_{42}$ peptides (Citron *et al.* 1992; Das *et al.* 2015). Accordingly, BACE1 inhibitors have been explored as putative therapeutic agents for the treatment of AD (Yan and Vassar 2014). We observed increased *Bace1* mRNA expression that coincided with increased BACE1 activity in Alzheimer transgenic rats. NP03-treatment reduced the level

of BACE1 activity and mRNA and we propose that it is by this mechanism that the soluble levels of $A\beta_{42}$ peptides were reduced. This reduction in soluble $A\beta_{42}$ is significant, because the severity of dementia has been found to correlate with the presence of soluble Aβ peptides rather than with the final fibrillar Aβ deposits in the brain (McLean *et al.* 1999). Similarly, neuroimaging studies in preclinical AD report that higher cortical Aβ load is associated with greater rates of cognitive decline (Lim et al. 2014). However, two main Aβ fragments, $A\beta_{42}$ and $A\beta_{40}$, are produced through cleavage of APP (Suzuki et al. 1994; Duff et al. 1996; Scheuner *et al.* 1996), and an increase in the $A\beta_{42}/A\beta_{4o}$ ratio coincides with more aggressive forms of the disease (Hellstrom-Lindahl et al. 2009). Thus, it has been demonstrated in CSF that the ratio of $A\beta_{42}/A\beta_{40}$ is more predictive of Aβ and tau, in identifying incipient AD in MCI, and for correctly stratifying patients and controls than $A\beta_{42}$ alone, as $A\beta_{40}$ can serve as an internal reference (Lewczuk et al. 2004; Hansson et al. 2007; Wiltfang et al. 2007). We found that NP03 treatment reduced the brain $A\beta_{42}/A\beta_{40}$ ratio in Alzheimer transgenic rats. Given that a higher ratio of soluble $A\beta_{42}/A\beta_{40}$ has been shown to predict cognitive decline, it is logical to assume that the reduction of this ratio by NP03 contributed towards the cognitive recovery observed in Alzheimer transgenic rats treated with the drug. Recently, Parr and colleagues showed that activation of Wnt/β-catenin signaling pathway promotes binding of T-cell factor-4, a *Bace1* gene repressor (Parr *et al.* 2015), and multiple lines of evidence point to a role of lithium in mimicking Wnt/β-catenin signaling (Stambolic *et al.* 1996; Hedgepeth *et al.* 1997; De Ferrari and Inestrosa 2000; Toledo and Inestrosa 2010). Therefore, it is also possible that modulation of Wnt/β-catenin signaling by lithium

contributed to the reduction of *Bace1* observed here. However, additional experiments are required to test this hypothesis.

NP03 Restored CRTC1 Promoter Occupancy

While the multi-target nature of lithium is well accepted, few reports have investigated the impact of its action mediated through CRTC1. Current hypotheses concerning CRTC1 function are based upon its cellular localization and its putative interaction as a macromolecular complex with the plasticity/signaling CREB transcription factor and the CREB response element. Gene expression mediated by CRTC1 function in hippocampus is critical to both memory consolidation and reconsolidation (Sekeres *et al.* 2012). In rodent models of AD, CRTC1 function becomes dysregulated early, when Aβ peptides are still intraneuronally confined (Parra-Damas *et al.* 2014; Matarin *et al.* 2015; Wilson *et al.* 2016). For example, in a previous study, we showed an Aβ-mediated impairment in CRTC1-dependent gene transcription that was reflected by reduced CRTC1 nuclear translocation, and reduced CRTC1 occupancy at synaptic plasticity-associated gene promoters correlated with reduced transcription of plasticity-associated genes (Wilson *et al.* 2016). Lithium enhances CRTC1 oligomer formation and the interaction between CREB co-activating factors CRTC1 and CREB binding protein (Boer *et al.* 2007; Heinrich *et al.* 2009; Heinrich *et al.* 2013) to promote gene expression. We have demonstrated that the application of NP03 enhances CRTC1 interaction with *Bdnf iv* and *c-fos* gene promoters in Alzheimer transgenic rats. As shown in Chapter 2, a reduction in CRTC1 promoter occupancy was related to a concomitant reduction in transcript production of these genes. Thus, it is likely that the NP03-driven restoration in CRTC1

genomic occupancy contributed to the behavioral recovery observed in Alzheimer transgenic rats treated with NP03.

On the other hand, it remains important to note that other aspects surrounding lithium's mechanism of action may have contributed to its positive effects in Alzheimer transgenic rats beyond the modulation of BACE1 and CRTC1 promoter occupancy. For example, lithium inhibits activity of GSK-3β – a central kinase with wide a number of actions including the hyperphosphorylation and destabilization of the cytoskeletal protein tau (Stambolic *et al*. 1996; Takashima *et al*. 1998; Jope and Johnson 2004). We have not yet observed overt tau pathology in our rat model of Alzheimer amyloidosis, and have not assessed GSK-3β status here; however, NP03 has been shown to inhibit GSK-3β in a mouse model of Huntington's disease (Pouladi *et al.* 2012). Similarly, in drosophila models, lithium has been shown to inhibit mRNA translation, causing a reduction in protein synthesis, including in Aβ (Sofola-Adesakin *et al.* 2014). Finally, lithium has been shown to have anti-inflammatory (Li *et al.* 2011; Yu, Wang*, et al.* 2012) and anti-oxidative properties (Khairova *et al.* 2012; de Sousa *et al.* 2014), both of which are prominent features in early AD neuropathology. The anti-oxidative and anti-inflammatory effects of NP03 will be the topic of investigation in Chapter 4.

Lithium for the Treatment of AD

There have been reports of the effect of lithium on AD in human populations, although with conflicting conclusions (Kessing *et al*. 2008; Mauer *et al*. 2014). Epidemiological studies suggest that while individuals with bipolar disorder have an increased risk of

developing AD compared to the general population, lithium treatment reduces this risk to that of the general population (Nunes et al. 2007).

The first clinical trial with lithium in AD was an open label study, where lithium carbonate (serum levels 0.3-0.8 mmol/L) was administered to 22 AD patients for up to one year (Macdonald *et al*. 2008). The study had a high discontinuation rate; two patients died while receiving lithium, although in neither case was the treatment felt to be related to the cause of death. There was no change in Mini-Mental State Examination (MMSE) observed among participants who completed the study. In a second, randomized, single-blind, placebo-controlled, multicenter, 10-week study (Hampel *et al*. 2009), 71 patients with mild AD, as defined by a MMSE score greater than 21 and less than 26, were randomly assigned a placebo (n = 38) or lithium sulfate treatment (n = 33). The treatment duration was 10-weeks and included a 6-week titration phase where the lithium dose was adjusted to target the serum lithium concentration of 0.5-0.8 mmol/L. The starting dose of lithium sulfate was 42 mg (6 mmol Li^+) as 1 tablet in the morning and 1 tablet in the evening (approximately 12 hours apart). Dosages were escalated at weekly intervals until the target serum concentration was achieved, with 4 tablets taken in the morning and another 4 taken in the evening during the maintenance phase. In cases where dose-limiting toxicity occurred, the dose was lowered to the maximum tolerated dose. The authors found no significant treatment effect on GSK-3 activity or on the levels of p-tau_{181} or p-tau_{231}. Similarly, levels of $A\beta_{1-42}$ in CSF and plasma were not significantly different within the lithium-treated group compared to the placebo group. Nor did they find a change in global cognitive performance, assessed using the Alzheimer's disease

assessment scale (ADAS)-Cognitive subscale. 45.5% of the patients in the lithium group showed at least 1 adverse event, and drug-related adverse events were significantly greater in the lithium group than the placebo group.

A subsequent study published in 2011 was a randomized, placebo-controlled, single centre, 12-month study design (Forlenza *et al*. 2011). These authors hypothesized that the previous negative results were because of investigation of lithium in people with clinically manifest AD, whereas the protective effects of lithium would be better assessed at earlier stages of the pathology. Thus, their study evaluated 45 patients with aMCI (MMSE > 21 and < 26) and randomly assigned placebo (N = 24) or lithium (0.25-.05 mm/L) treatment (N = 21). The 12-month treatment also included a 6-week titration phase. In contrast to the previous study, lithium treatment in aMCI was associated with a significant decrease in cerebrospinal fluid concentrations of phosphorylated-tau and improved performance on the ADAS-Cog subscale. Thus, these data indicate that an earlier intervention and extended treatment duration may be required for lithium to be effective in AD.

Finally, a trial published in 2013 demonstrated positive results using a microdose formulation of lithium. This study evaluated the administration of 300 μg of lithium given once daily for 15 months (Nunes *et al*. 2013). In an attempt to correct for differences in education level, Alzheimer patients were selected if they scored > 9 and < 21 on MMSE if they had 4 years or less of schooling, and > 12 and < 24 if they were at school for longer than four years. While the placebo group (n = 45) deteriorated in

MMSE scores over the 15-month treatment phase, the lithium-treated group (n = 59) remained stable. However, these findings were limited by the application of a single cognitive measure, the absence of serum lithium level measures, and by the absence of any disease-relevant biomarkers. Taken together, these studies reveal that there is still much work required before determining the precise benefit of lithium in the treatment of AD. Proper studies will need to investigate well-defined clinical populations, and should include biomarker measures, along with cognitive assessment, to determine treatment efficacy.

NP03 Treatment a Significant Improvement Over Conventional Lithium

Conventional lithium is associated with a significant adverse side-effect profile including nausea, dizziness, nephrotoxicity, polyuria, polydipsia, and tremor (Gelenberg and Jefferson 1995; Azab *et al.* 2015). Therefore, the justification of NP03 as a less-toxic alternative to conventional lithium stems from the microdose nature of the formulation. This is especially important given the long-term treatment required for AD, and its application in elderly populations (Hampel *et al.* 2009). In addition to the positive modulatory effects reported here, a previous study using a Huntington mouse model revealed that NP03 reduced aggregation of huntingtin protein, reduced GSK-3β levels and the levels of p-tau, and restored behavioral deficits (Pouladi *et al.* 2012). Furthermore, a 26-week toxicology study of NP03 in rats (GLP) revealed no observation of the main toxicology parameters, even up to a dose of 0.6 mg Li/kg. Thus, the safety profile of NP03 makes it an ideal candidate for long-term treatment. Finally, our results support the hypotheses that NP03 performs as well as conventional lithium in restoring

cognition and in reducing key aspects of the AD-like pathology in Alzheimer transgenic rats.

Conclusion

NP03 completely restores spatial learning and memory, recognition memory, and associative learning in a rat model of AD. NP03 reduces toxic brain $A\beta_{42}$, and is a potent inhibitor of the BACE1 enzyme. Finally, NP03 restores CRTC1 promoter occupancy in genes associated with synaptic plasticity. Importantly, the present preparation, dosage, and administration route does not provoke adverse effects. Together, these observations support the investigation of NP03 as a therapeutic candidate in mild-moderate and prodromal AD.

ACKNOWLEDGMENTS

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Connecting text between Chapter 3 and Chapter 4

Chapter 3 demonstrated that treatment of pre-plaque MyGill-R-Thy1-APP Alzheimer transgenic rats with a microdose lithium formulation reduces BACE1 activity and Aβ pathology while restoring CRTC1 promoter occupancy and cognitive function. Chapter 4 will evaluate the ability of this formulation to modulate CNS oxidative stress and inflammatory mechanisms, which are known to be disease aggravating at such early stages of the Aβ pathology.

CHAPTER 4: NP03 Reverses Pre-Plaque Oxidative Stress and Neuroinflammation in a Rat Model Alzheimer's Disease

NP03 Reverses Pre-Plaque Oxidative Stress and Neuroinflammation in a Rat Model Alzheimer's Disease

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ABSTRACT

Late stage Alzheimer's disease is associated with an inflammatory response, with proinflammatory cytokines activating microglia-mediated phagocytosis of amyloid beta (Aβ) plaques and cellular debris. However, accumulating evidence now point to a disease-aggravating role of inflammation, initiated at the earliest stages of the Aβ pathology, and contributing to neuritic damage. The escalating proinflammatory response has been shown to be a consequence of Aβ-induced oxidative stress. In a previous report, we demonstrated that a low dose formulation of lithium, NP03, was effective in diminishing Aβ, and restoring cognition in Alzheimer transgenic rats. Lithium, a multi-target drug widely used for the management of bipolar disorder, has shown anti-oxidative and anti-inflammatory properties. Therefore, in this study we evaluated the possibility that the positive effects of NP03 were due in part to its action modulating oxidative stress and inflammation in the AD brain. Here we report that in Alzheimer transgenic rats, intraneuronal Aβ accumulation leads to an increase in oxidative stress markers 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT). Furthermore, pre-plaque Alzheimer rats displayed increased inflammatory cytokine production, including in TNF-α, IFN-γ, IL-5, and TREM2. These changes were accompanied with increased microglia recruitment towards Aβ-burdened neurons in the hippocampus, and impaired hippocampal neurogenesis. These effects were reversed in Alzheimer transgenic rats treated with NP03. Together, these results suggest that if activated at early stages, lithium-mediated anti-oxidant and anti-inflammatory properties may be beneficial in delaying the pathological processes underlying AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of extracellular amyloid beta (Aβ) plaques, neurofibrillary tangles composed of aggregated hyperphosphorylated tau, and synaptic loss (Selkoe 2001). It has been proposed that risks for late onset AD, which include genetic, biological and environmental factors, can contribute to neuroinflammation leading to neurodegeneration at later stages of AD (McNaull *et al.* 2010). It has also been suggested that neuroinflammation plays a beneficial role in the late stages of AD, through microglia-mediated phagocytosis of Aβ plaque material, however, there is now evidence that inflammation at the earliest stages of AD may not beneficial, and rather, may be disease aggravating (Akiyama *et al.* 2000; Agostinho *et al.* 2010).

AD is associated with increased production of reactive oxygen and nitrogen species (ROS and RNS) and loss of function of antioxidant defenses (Butterfield 1997; Markesbery 1997; Aksenov et al. 1998; Munch et al. 1998; Butterfield et al. 1999; Koppal et al. 1999; Markesbery and Carney 1999; Varadarajan et al. 1999; Butterfield and Kanski 2002; Pratico 2008, 2008; Sultana et al. 2008; Mosconi et al. 2010; Di Domenico, Pupo, Giraldo, Badia, et al. 2016; Di Domenico, Pupo, Giraldo, Lloret, et al. 2016), and emerging evidence has linked sustained oxidative stress to consequent neuroinflammation and AD pathogenesis (Blalock et al. 2004; Butterfield et al. 2013; Cobb and Cole 2015). For example, oxidative stress leads to the release of tumor necrosis factor alpha (TNF-α) (Munhoz et al. 2004; Tangpong et al. 2006; Aluise et al. 2011; Grinberg et al. 2013), interferon gamma (IFN-γ) (Baranano and Snyder 2001;

Blalock *et al.* 2004) and members of the interleukin family (Baranano and Snyder 2001; Schmuck et al. 2002), and antioxidant treatment has been shown to reduce the level of proinflammatory markers (Hayslip et al. 2015). Consequently, there is amassing evidence indicating that proinflammatory mechanisms contribute to AD neuropathology (Itagaki *et al.* 1989; Sastre *et al.* 2006; Lee *et al.* 2009) and this is supported by a growing body of evidence showing inflammation as an early event in the progression of AD. For example, microglial activation is detected in patients with mild cognitive impairment (MCI) (Parachikova *et al.* 2007)—a stage that often represents a prodromal phase to overt AD.

Epidemiological data strongly support the notion that prolonged treatment with anti-inflammatory drugs (NSAIDs) in individuals without clinically diagnosed Alzheimer's diminishes the prevalence of this neurodegenerative disease [for review see (McGeer and McGeer 2013)]. Preclinical and clinical studies would indicate that the anti-inflammatory consequences reduce the risk of developing AD (Breitner 1996; in t' Veld *et al.* 2001; Klegeris and McGeer 2005) or the AD pathology in experimental models (Weggen *et al.* 2001; Sastre *et al.* 2003). Data from mouse and rat transgenic animal models show that inflammatory processes precede amyloid plaque deposition and are concurrent with the progressive accumulation of Aβ oligomeric material (Heneka *et al.* 2005; Maezawa *et al.* 2011; Ferretti, Bruno*, et al.* 2012; Wright *et al.* 2013; Hanzel 2014). Furthermore, interventions that inhibit this initiating proinflammatory process have a positive outcome, by diminishing Aβ synthesis and improving cognition (Lim *et al.* 2011; Ferretti, Allard*, et al.* 2012; Lim *et al.* 2012; Vom Berg *et al.* 2012; Pimentel et al. 2015).

The multi-target drug lithium lowers oxidative stress in healthy subjects and in early stage bipolar disorder by reducing free-radical formation, lipid peroxidation, and levels of hydrogen peroxide (Machado-Vieira *et al.* 2009; Khairova *et al.* 2012; de Sousa *et al.* 2014). In addition to anti-oxidant properties, the anti-inflammatory effects of lithium are increasingly reported (Basselin *et al.* 2007; Li *et al.* 2011; Yu, Wang*, et al.* 2012). Lithium lowers the production of IL-1β and TNF-α in rat primary glial cells (Nahman *et al.* 2012), and reduces microglia activation in ischemic mouse models (Li *et al.* 2011). Similarly, lithium enhances adult hippocampal neurogenesis (Chen *et al.* 2000; Fiorentini *et al.* 2010), which is inhibited by Aβ and neuroinflammation (Ito *et al.* 1998; Haughey *et al.* 2002; Monje *et al.* 2003; Mu and Gage 2011; Fuster-Matanzo *et al.* 2013).

Despite these positive influences, the serious side-effect profile of lithium – including nephrotoxicity, polyuria, nausea, and tremor – make it inappropriate for long-term treatment, especially in the elderly (Gelenberg and Jefferson 1995; Hampel *et al.* 2009; Azab *et al.* 2015). NP03, a microemulsion formulation of lithium, allows for therapeutic efficacy while significantly reducing the amount of lithium to be administered, thereby avoiding the damaging side effects typically associated with conventional lithium (Pouladi *et al.* 2012; Wilson et al. To Be Submitted). We previously demonstrated that NP03 restored learning and memory and reduced soluble Aβ in a rat Alzheimer transgenic model (Wilson *et al.* To Be Submitted). We are now interested in determining whether these changes were due in part to a positive modulatory effect of

NP03 on oxidative stress and neuroinflammatory processes occurring during the development of Aβ neuropathology.

To test this hypothesis, we treated McGill-R-Thy1-APP Alzheimer transgenic rats with NP03 and evaluated markers of oxidative stress and neuroinflammation. McGill-R-Thy1-APP Alzheimer transgenic rats carry a mutation in the *APP* gene known to cause early-onset AD. These rats are cognitively impaired at 3 months of age, during the pre-plaque phase of the Aβ pathology (Leon *et al.* 2010). Additionally, we have shown that these rats exhibit an early, neuron-driven proinflammatory response (Hanzel *et al.* 2014). To date, however, the oxidative stress component has yet to be investigated in this model.

Here, we report that oxidative stress markers 4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT), and inflammatory markers TNF-α and IFN-γ were elevated during the initializing stages of the Aβ pathology in the cortex of Alzheimer transgenic rats. In the hippocampus, we observed increased *Trem2* mRNA expression, microglia recruitment to Aβ-burdened neurons, and reduced neurogenesis. NP03 treatment of Alzheimer rats reduced oxidative stress, cytokine secretion, *Trem2* expression, microglia recruitment, and restored hippocampal neurogenesis. Together, these results indicate that NP03 is effective in modulating the Aβ-mediated oxidative stress and neuroinflammation during the initializing stages of Aβ pathology.

METHODS

Animals

McGill-R-Thy-APP transgenic rats carry the mutated human amyloid precursor protein (APP; Swedish double and Indiana mutations) present in aggressive, early-onset forms of Alzheimer's disease in humans. Rats were socially housed and received food and water *ad libitum*. All procedures were carried out in accordance with the guidelines set out by the Canadian Council of Animal Care and were approved by the Animal Care Committee of McGill University.

NP03 Drug Treatment

Adult male and female homozygous McGill-R-Thy-App transgenic rats and their wild type littermates were treated for 8 weeks beginning at 3 months of age. Rats were randomly distributed to either treated or vehicle group. Experimental groups included wild-type littermates treated with vehicle (n = 6), wild-type littermates treated with NP03 (n = 7), homozygous McGill-R-Thy1-APP Alzheimer transgenic rats treated with vehicle (n = 8), and homozygous McGill-R-Thy1-APP Alzheimer transgenic rats treated with NP03 (n = 14). Drug administration was performed daily, 5 days per week. Vehicle (1 mL/kg) or NP03 (40 µg Li/kg; 1 mL/kg) were deposited on the rectal mucosa (NP03 and vehicle provided by Medesis Pharma, France).

Analysis of Oxidative Stress Markers

Approximately 100 mg of cortical tissue was homogenized in isolation buffer (0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 20 µg/mL trypsin inhibitor, 0.2

mM PMSF, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 5 μg/mL aprotinin, pH 7.4). Protein concentrations were determined using the bicinchoninic acid biochemical assay (Smith et al. 1985). Oxidative stress in each sample was measured using the slot blot technique (Barone et al. 2015). HNE and 3-NT samples were prepared in Lamelli buffer (4% SDS, 20% glycerol, 120 mM Tris-Cl, pH 6.8) and diluted to 100 μL with PBS. The BCA results were then used to make 1000 μL solutions with equal concentrations of protein from the 100 μL solutions. Briefly, 250 μL of protein were blotted onto nitrocellulose membrane (162-0112, BioRad, Germany), blocked in 3% BSA (9048-46-8, Sigma-Aldrich, St. Louis, MO) in TBS (24.23 g/L Tris, 80.06 g/L NaCl, pH 7.6) with Tween 1% for 1 hour. The membranes were incubated in primary antibodies for either HNE (1:6,250, HNE11-S, Alpha Diagnostic Intl. Inc., San Antonio, TX) or 3-NT (1:3,125, N0409, Sigma-Aldrich, St. Louis, MO) in blocking solution for 2 hours at room temperature. Membranes were washed with TBS-T and incubated 1 hour in anti-rabbit IgG alkaline phosphatase secondary antibody solution (1:10,000, A3812, Sigma). Chromogen was detected using the BCIP/NBT Alkaline phosphatase detection kit (99 μL BCIP, 198 μL NBT, 30 mL ALP buffer; BCIP: 34040, Thermo Scientific, Rockford, IL; NBT: 34035, Thermo Scientific; ALP buffer: 8.498g/700 mL Tris, 4.091g/700mL NaCl, 0.71155g/700mL $MgCl_2$, pH 9.5). Membranes were photographed using a BioRad imager (1708265, BioRad, Hercules, CA) and analyzed using ImageLab software (Version 4.1, BioRad, Hercules, CA).

Multiplex Analysis of Inflammatory Cytokines

Cortical tissue was homogenized in RIPA buffer (50 mM Tris pH 8.0, 0.5% NP-40, 0.1% SDS, 0.5% deoxicholic acid, 1mM EDTA, 300 mM NaCl). The lysates were analyzed by electrochemoluminescence-linked sandwich immunoassays V-Plex^{TM} Multi-Spot Assay System (K15059D; Meso Scale Discovery, Rockville, MD) and samples were processed according to manufacturer's directions. Levels of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-1β, IL-4, IL-5, IL-6, IL-10, and IL-13 were read on a Sector 6000 Imager instrument (Meso Scale Discovery). Cytokine concentrations were calculated in reference to calibrators for each individual analyte, normalized to total protein input (determined according to the Lowry method), and expressed as analyte amount in pg cytokine/mg protein.

Trem2 Gene Expression Analysis

Total RNA was extracted from rat hippocampus using the RNeasy Mini Kit (Qiagen), following manufacturer instructions. Residual DNA was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). To generate cDNA, total RNA was retro-transcribed using the iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON). Quantification of transcript expression was assessed by qRT-PCR with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Instrument and Software (Bio-Rad). Expression of *Trem2* was normalized against two housekeeping genes (*Hprt and Gapdh*), and interpreted using the comparative ΔΔCt method. For the list of primers used, see **Table 1**.

Table 1. Nucleotide sequences of the primers used for Q-RT-PCR gene expression experiments

Gene	5'-3' Forward sequence	5'-3' Reverse sequence	Amplicon (bp)
Trem2	AACTTCAGATCCTCACTGGACCC	TGGAGGGGAGAGTATGCCAAT	111
Gapdh	TGATGGGTGTGAACCACGAG	TCATGAGCCCTTCCACGATG	132
Hprt	TTCCTCCTCAGACCGCTTTTC	CATCACTAATCACGACGCTGG	80

Aβ Immunohistochemistry

Free-floating tissue sections were washed in PBS, rendered permeable and quenched of endogenous peroxidase activity with 0.3% hydrogen peroxide in PBS-T, and blocked for 1 hour in 10% normal goat serum (NGS). Sections were incubated overnight in a solution containing mouse monoclonal anti-Aβ (McSa1, MediMabs, Montreal, QC) in 10% NGS with PBST. McSa1 is raised against a synthetic peptide of Aβ related to amino acids 1-12 of human APP (Grant *et al.* 2000). Tissue sections were washed and then incubated with goat anti-mouse IgG (MP Biochemicals, Solon, OH) in 5% NGS with PBST for 1 hour and subsequent mouse anti-peroxidase monoclonal antibody complex (MAP/HRP complex, Medimabs) for 1 hour. DAB was used as the chromogen (Vector Laboratories, Burlington, ON). Sections were mounted on glass slides and allowed to air-dry overnight. Sections were then defatted and cleared in a graded alcohol series with terminal xylenes and coverslipped with Permount mounting medium (Fisher Scientific, Ottawa, ON). Slides were imaged using an Axio Imager 2 microscope (Carl Zeiss Canada, North York, ON) running Zen Blue software (Carl Zeiss).

Microglia Recruitment

Tissue sections were treated with 0.3% H_20_2 in PBS for 20 minutes and permeabilised by incubating in PBS with 1% Tween-20 (PBST) for 10 minutes, blocked for 1 hour in 10% normal goat serum (NGS) and incubated overnight with rabbit Iba1 antibody (1:500, Wako Laboratory Chemicals, 019-19741) to reveal microglia and mouse monoclonal NeuN antibody (1:1000, Chemicon, MAB377) to reveal neurons. Tissue sections were then washed, incubated in biotinylated anti-rabbit (1:200, MP Biochemicals) in 5% NGS in PBST for 1 hour. After washing, sections were incubated for 1 hour in Vectastain ABC kit (Vector Laboratories, Inc.) prepared following supplier directions, washed and then revealed using 3' 3–diaminobenzidine (DAB; Vector Laboratories) as a chromogen. Sections were then washed and incubated 1 hour in goat anti-mouse (1:100, MP Biochemicals) in 5% NGS for 1 hour, followed by a 1 hour incubation in mouse anti-peroxidase monoclonal antibody complex (MAP/HRP complex, MediMabs, QC, Canada). Tissues were then developed using the Vector SG kit (Vector Laboratories), following manufacturer's directions. Sections were mounted on slides, air-dried overnight, dehydrated and cleared through a graded alcohol series with xylenes and then finally coverslipped. A minimum of three sections spanning the anterior portion of the dorsal hippocampus was stained for each animal. Using ImageJ software (National Institutes of Health, Bethesda, MD), a 100 X 200 μm grid was centered over CA1, and an experimenter blinded to conditions counted the number of microglia per field. Three images were captured from distinct CA1 regions for each section, with two sampling grids placed in each image, to give a total number of 12 sampling grids/animal.

Doublecortin Analysis

Tissue sections containing the anterior portion of the dorsal hippocampus were washed with PBS then incubated for 20 minutes in 50% ethanol followed by 15 minutes in PBST. Tissue was blocked using 10% normal rabbit serum in PBST for 1 hour at room temperature while shaking. Primary incubation was performed using a goat polyclonal antibody to doublecortin (C-18; 1:600, Santa Cruz, sc-8066) and a mouse monoclonal antibody to NeuN (1:1000, Chemicon, MAB377) in 5% rabbit serum in PBST for 48 hours. Sections were washed with PBS and incubated overnight in PBS with 5% rabbit serum and Alexa Fluor 594 donkey anti-goat (1:400, Jackson ImmunoResearch Laboratories, 705-585-147) and goat anti-mouse 488 (1:400, Jackson ImmunoResearch, A11037). Finally, sections were washed with PBS, counterstained with DAPI and mounted using Aqua Polymount (Polysciences, Inc.). Doublecortin-positive cells were counted by an experimenter blind to conditions and expressed as a mean number of cells per section for each condition.

Statistical Analysis

Data for each variable were subjected to two-way ANOVA followed by Tukey's multiple comparisons tests using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Progressive Aβ Pathology in McGill-R-Thy1-APP Alzheimer Transgenic Rats

McGill-R-Thy1 APP transgenic rats express the mutated human Aβ precursor protein gene (*mhAβPP*) and show intraneuronal Aβ as early as two weeks postnatal, and extracellular plaques appear at 6 months of age, spreading from the hippocampus to the cortex. Stained tissue sections from Alzheimer rats using an antibody specific to human Aβ are shown in **Figure 4-1a-c**. Rats at the pre-plaque state have pronounced neuroinflammation, impaired cellular signaling, impaired long-term potentiation, and severe cognitive impairment (Leon *et al.* 2010; Hanzel *et al.* 2014; Iulita *et al.* 2014; Qi *et al.* 2014; Wilson *et al.* 2016).

Figure 4-1: Aβ neuropathology in McGill-R-Thy1 APP Alzheimer Transgenic Rats

a) At 5 months of age, Alzheimer transgenic rats show intraneuronal accumulation of human Aβ peptides in the hippocampus and cortex. This represents the pre-plaque stage of the pathology. This phase has been associated with a pronounced Aβ-driven neuroinflammation, impaired intracellular signaling, impaired neuronal physiology, and severe cognitive impairment.

b) At 15 months of age Aβ plaques have deposited and are observed in the hippocampus, with few appearing in the cortex.

c) At 21 months of age, extensive Aβ plaque deposition is observed throughout the hippocampus and cortex. Scale bar = 1 mm.

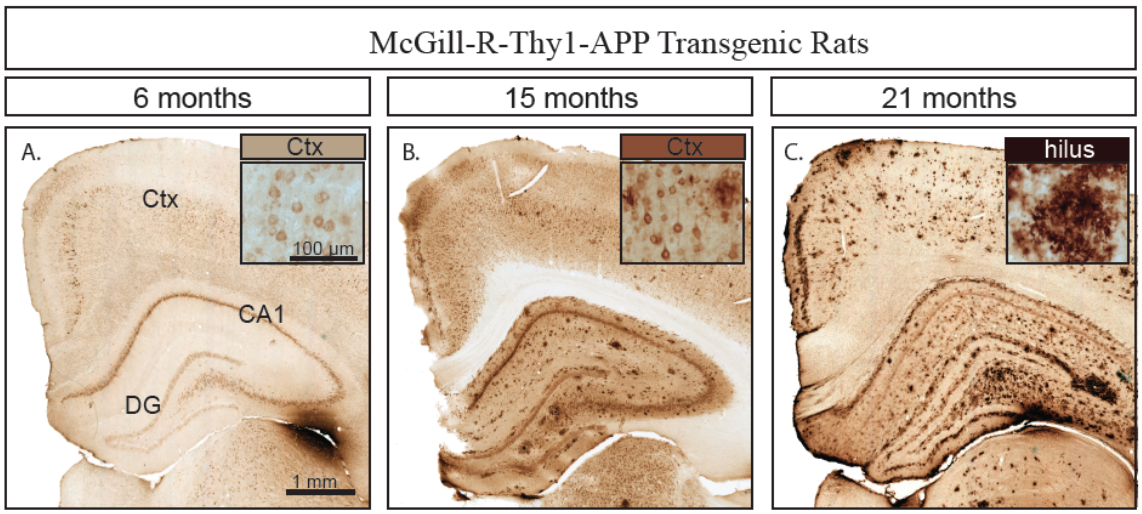

Figure 4-1: Aβ neuropathology in McGill-R-Thy1 APP Alzheimer Transgenic Rats

NP03 Reverses Oxidative Stress in Early Stages of AD-Like Amyloid Neuropathology

4-hydroxynonenal (HNE) and 3-nitrotyrosine (3-NT) protein modifications represent makers of oxidative stress. HNE is one of the major products of lipid peroxidation and HNE-protein adducts are detected in the brain of patients with AD (Lauderback *et al.* 2001; Perluigi *et al.* 2009). Protein nitration stems from nitric oxide synthase production of nitric oxide, which reacts with superoxide anion to form peroxynitrite ($ONOO^-$). Peroxynitrite can react with carbon monoxide (CO_2) to produce nitrite radicals that can react with a tyrosine residue to form 3-NT. Importantly, protein nitration has been documented in MCI and in early and late AD (Tohgi *et al.* 1999; Butterfield, Reed, Perluigi*, et al.* 2007; Reed *et al.* 2009).

We found that at this early stage of the Alzheimer-like amyloid pathology, there was a significant increase in HNE-bound protein in cortical tissue of Alzheimer transgenic rats (2-way ANOVA: Treatment x Transgene interaction: $F_{(1,25)} = 10.23$; $p = 0.0037$; **Figure 4-2a**). *Post hoc* analysis revealed a 60% increase in the Alzheimer transgenic rats over the wild-type rats receiving vehicle (Tukey's multiple comparisons test, WT:Veh vs. TG:Veh, $p < 0.01$). This difference was abolished when Alzheimer transgenic rats were treated with NP03 (TG:Veh vs. TG:NP03, $p < 0.05$). Similarly, 3-NT levels were 50% higher in AD the cortex of Alzheimer transgenic rats compared to the wild type (main effect of Treatment: $F_{(1,25)} = 5.78$; $p = 0.0239$; main effect of Transgene $F_{(1,25)} = 11.91$; $p = 0.0020$; WT:Veh vs. TG:Veh, $p < 0.05$; WT:NP03 vs. TG:Veh, $p < 0.01$; **Figure 4-2b**). As was the case with HNE, the elevation of 3-NT was abolished when Alzheimer transgenic rats were treated with NP03 (TG:Veh vs. TG:NP03, $p <$

0.05). Together, these findings indicate that Aβ-driven changes in HNE and 3-NT precede protein carbonylation at the early stage of the Aβ pathology, and that NP03 can prevent Aβ-induced oxidative stress *in vivo*.

Figure 4-2. NP03 reverses oxidative stress in pre-plaque AD rats.

a) Cortical levels of 4-hydroxy-2-nonenal (HNE) were analyzed using the slot blot technique. Levels were elevated in the Alzheimer transgenic vehicle group 60% above the wild type. NP03 significantly reduced HNE expression, restoring it to wild-type levels. WT Veh n = 6; WT NP03 n = 6; AD Veh n = 6; AD NP03 n = 11. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Tukey's multiple comparisons *post hoc* test.

b) Cortical expression of 3-nitrotyrosine (3-NT) was analyzed using the slot blot technique. Levels were elevated 50% in the Alzheimer transgenic vehicle group compared to the wild-type vehicle treated rats. NP03 significantly reduced levels of 3-NT to baseline levels. WT Veh n = 6; WT NP03 n = 6; AD Veh n = 6; AD NP03 n = 11. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Tukey's multiple comparisons *post hoc* test.

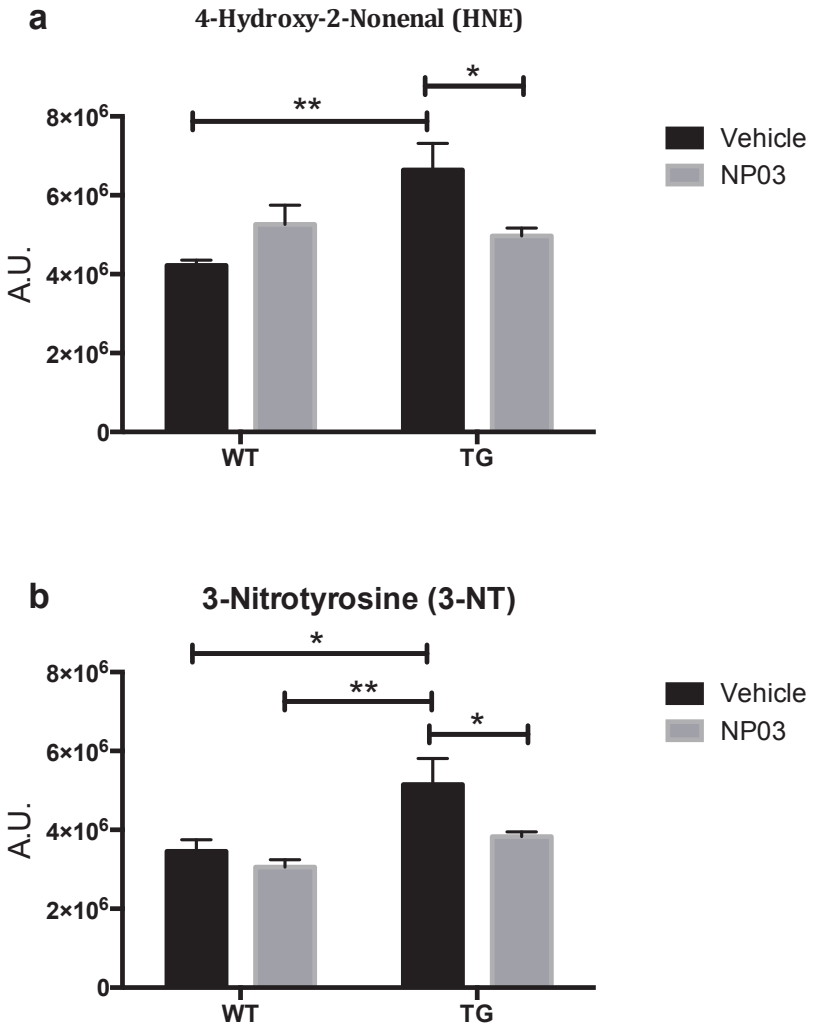

Figure 4-2. NP03 reverses oxidative stress in pre-plaque AD rats

NP03 Reduced Expression of Inflammatory Factors

We next investigated the expression of proinflammatory cytokines in Alzheimer transgenic and wild-type rats, treated with either NP03 or vehicle. Multiplex analysis revealed that cortical samples from pre-plaque Alzheimer transgenic brains expressed higher levels of tumor necrosis factor-α (TNF-α) compared to the wild-type rats (Treatment x Transgene interaction $F_{(1,26)} = 8.91$; $p = 0.0061$; **Figure 4-3a**). *Post hoc* analyses revealed a significant increase in the Alzheimer transgenic rats over the wild-type rats receiving either vehicle or NP03 (WT:Veh vs. TG:Veh, and WT:NP03 vs. TG:Veh, $p < 0.05$), and NP03 significantly reduced expression of TNF-α in Alzheimer transgenic rats (TG:Veh vs. TG:NP03, $p < 0.001$). Similarly, expression of interferon-γ (IFN-γ) was elevated in the cortex of Alzheimer transgenic rats (Treatment x Transgene interaction $F_{(1,27)} = 18.84$; $p = 0.0002$; **Figure 4-3b**). *Post hoc* analysis confirmed a significant increase in the Alzheimer transgenic rats over the wild-type rats receiving either vehicle (WT:Veh vs. TG:Veh, $p < 0.01$) or NP03 (WT:NP03 vs. TG:Veh, $p < 0.05$). Importantly, NP03 significantly reduced IFN-γ expression in Alzheimer transgenic rats (TG:Veh vs. TG:NP03, $p < 0.001$).

Figure 4-2. NP03 reduced TNF-α and IFN-γ expression in pre-plaque AD rats.

a) Cortical expression of TNF-α was analyzed using multiplex analysis. Levels were significantly elevated in the Alzheimer transgenic vehicle group. NP03 significantly reduced TNF-α overexpression, restoring it to wild-type levels. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 13. Data represent mean ± SEM. * $p < 0.05$, *** $p < 0.001$, Tukey's multiple comparisons *post hoc* test.

b) Cortical expression of IFN-γ was analyzed using multiplex analysis. Levels were significantly elevated in the Alzheimer transgenic vehicle group compared to the wild-type vehicle rats. NP03 significantly reduced levels of IFN-γ to baseline wild-type levels. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 7; AD NP03 n = 13. Data represent mean ± SEM. ** $p < 0.01$, *** $p < 0.001$, Tukey's multiple comparisons *post hoc* test.

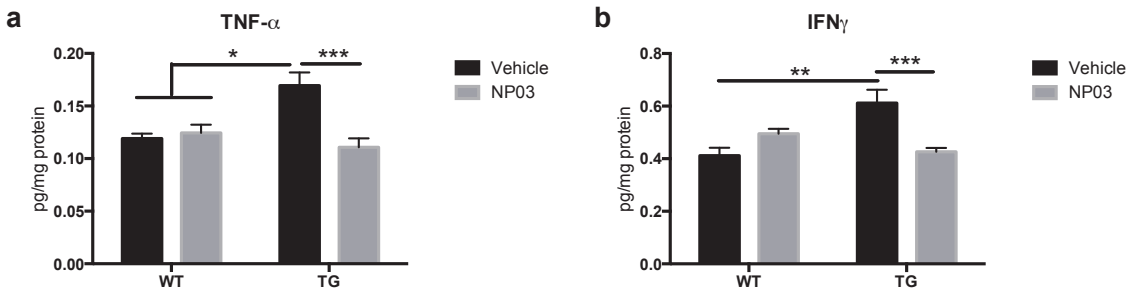

Figure 4-3. NP03 reduced TNF-α and IFN-γ expression in pre-plaque AD rats

Members of the interleukin (IL) family of cytokines are secreted extracellularly in response to elevated TNF-α and IFN-γ. Our results indicate specificity in modulation such that not all interleukins are provoked simultaneously, but rather that a certain subset are induced at this early pathological stage. For instance, there was a non-significant trend towards elevated IL-1β in cortical extracts of Alzheimer transgenic rats at this pre-plaque time point ($p > 0.05$; **Figure 4-4a**). At the same time, a significant interaction was detected for IL-4 (Treatment x Transgene interaction $F_{(1,25)} = 4.31$; $p = 0.0482$; **Figure 4-4b**), although p*ost hoc* analysis failed to detect a significant difference between any of the groups ($p > 0.05$). On the other hand, there was a significant elevation in IL-5 in Alzheimer transgenic rats (main effect of Treatment: $F_{(1,26)} = 4.50$; $p = 0.0436$; **Figure 4-4c**). In this case, *post hoc* analysis confirmed a significant increase in the Alzheimer transgenic rats over the wild-type rats receiving NP03 (WT:NP03 vs. TG:Veh, $p < 0.05$), which was reduced with NP03 (TG:Veh vs. TG:NP03, $p < 0.05$). IL-6 expression was not different in any of the groups assessed ($p > 0.05$; **Figure 4-4d**), raising the possibility that at this early time point, IL-6 is not yet modulated. For IL-10 there was a significant Treatment x Transgene interaction: $F_{(1,26)} = 5.69$; $p = 0.0247$; **Figure 4-4e**). *Post hoc* analysis revealed that while the elevation in IL-10 in Alzheimer transgenic rats compared to controls was non-significant, the results did reveal that NP03 significantly reduced IL-10 expression in Alzheimer transgenic rats (TG:Veh vs. TG:NP03, $p < 0.05$). Finally, IL-13 showed a slight trend towards being elevated in Alzheimer transgenic rats (**Figure 4-4f)**, although this trend failed to reach statistical significance ($p > 0.05$). Taken together, Alzheimer transgenic rats showed a significant elevation in TNF-α, IFN-γ, IL-4, IL-5, and a trend towards elevated IL-1β, IL-10 and IL-13 compared to the wild-type rats.

NP03 reduced expression of these elevated inflammatory markers in Alzheimer transgenic rats to control levels. These results confirm that occurrence of a proinflammatory process preceding amyloid plaque deposition is blocked with the application of NP03.

Figure 4-4. NP03 reduced elevated interleukins to baseline levels in pre-plaque Alzheimer transgenic rats.

a) Multiplex analysis of IL-1β shows a very minor trend towards elevated levels in Alzheimer transgenic vehicle rats, indicating that at this early stage of the pathology, protein level of IL-1β are not yet elevated. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 13. Data represent mean ± SEM. Data analyzed with 2-way ANOVA, $p > 0.05$.

b) IL-4 shows a non-significant trend towards elevation in levels in Alzheimer transgenic vehicle rats compared to the wild-type vehicle group. WT Veh n = 3; WT NP03 n = 6; AD Veh n = 6; AD NP03 n = 11. Data represent mean ± SEM. Data analyzed with 2-way ANOVA, $p > 0.05$.

c) IL-5 was significantly elevated in Alzheimer transgenic vehicle rats compared to the wild-type NP03 group. This increase was significantly reduced with NP03 treatment. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 13. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

d) Expression of IL-6 was not different in any of the groups tested, indicating that at this early stage of the pathology, IL-6 is not yet modulated. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 7; AD NP03 n = 13. Data represent mean ± SEM. Data analyzed with 2-way ANOVA, $p > 0.05$.

e) There was a non-significant trend toward elevated IL-10 in the Alzheimer transgenic vehicle group compared to the wild-type vehicle control group. However, there was a significant reduction in the Alzheimer transgenic NP03 group compared to the Alzheimer transgenic vehicle group. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 13. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

f) There was a slight trend towards elevation of IL-13 in the Alzheimer transgenic vehicle group over all other groups. WT Veh n = 4; WT NP03 n = 7; AD Veh n = 6; AD NP03 n = 12. Data represent mean ± SEM. Data analyzed with 2-way ANOVA, $p > 0.05$.

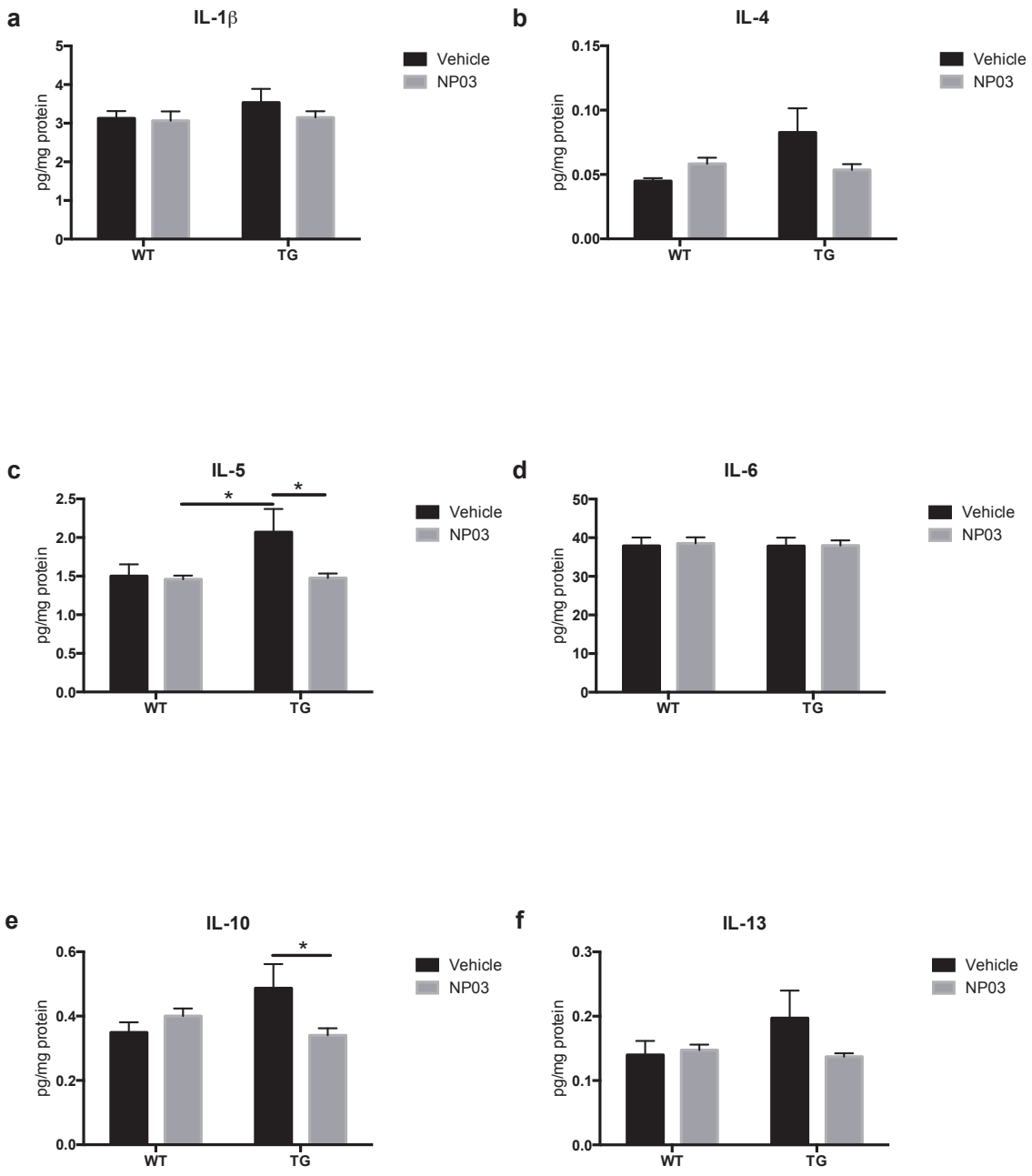

Figure 4-4. NP03 restored elevated interleukin cytokine expression to baseline levels

NP03 Reduced *Trem2* mRNA expression

TREM2 limits Aβ-induced neuritic damage by enabling microglia to surround and alter Aβ plaque structure (Ulrich and Holtzman 2016; Wang *et al.* 2016). *Trem2* mRNA is up-regulated in the brains of *APP*, *PSEN* and *APP/PSEN* transgenic mice (Matarin *et al.* 2015), and cells surrounding Aβ plaques in Alzheimer transgenic mice express high levels of TREM2 (Frank et al, 2008). $A\beta_{42}$ peptides have been shown to activate the TREM2 receptor to produce phagocytosis, production of ROS, and induction of NF-kB (Jones BM et al, 2014). Although less is known about the role of TREM2 in early, pre-plaque stages of the Aβ pathology, expression has been shown to rise with the increase in cortical Aβ (Guerreiro and Hardy 2013; Guerreiro *et al.* 2013; Jonsson *et al.* 2013). Importantly, microglia migration might be defective in TREM2 heterozygotes, as TREM2 has been previously linked via network analysis to genes involved in cytoskeletal rearrangements – the basis of phagocytosis and migration (Forabosco et al. 2013). Thus it is possible that TREM2 serves as a gate for microglial responses initiated by Aβ.

We found a 2-fold increase in *Trem2* mRNA in the hippocampus of Alzheimer transgenic rats compared to the wild-type rats (Treatment Effect: $F_{(1,22)} = 3.92$; $p = 0.0603$; Transgene Effect: $F_{(1,22)} = 6.34$; $p = 0.0196$). *Post hoc* analysis revealed that vehicle-treated Alzheimer transgenic rats had increased expression of *Trem2* compared to vehicle and NP03-treated wild-type rats (**Figure 4-5**; $p < 0.05$). Further, NP03 significantly reduced *Trem2* mRNA expression in Alzheimer transgenic rats ($p < 0.05$), restoring it to baseline levels. Taken together, these results indicate that NP03 reduced

the early oxidative stress and inflammatory processes that occur in this transgenic rat model of Alzheimer's disease.

Figure 4-5: NP03 Reduced *Trem2* mRNA expression

We observed a 2-fold increase in *Trem2* mRNA in the hippocampus of Alzheimer transgenic rats compared to the wild-type NP03 group. NP03 reduced the expression in Alzheimer transgenic rat, restoring it to baseline levels. WT Veh n = 4; WT NP03 n = 5; AD Veh n = 6; AD NP03 n = 11. Data represent mean ± SEM. * $p < 0.05$, Tukey's multiple comparisons *post hoc* test.

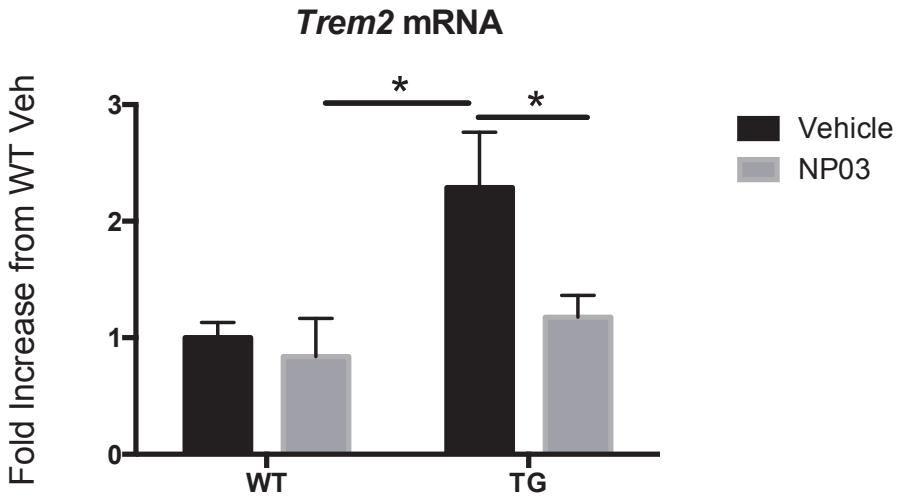

Figure 4-5: NP03 Reduced *Trem2* mRNA expression in the hippocampus

Microglia Recruitment to Aβ-Burdened Neurons is Reduced by NP03

Previous studies in our model have shown microglia recruitment towards Aβ-burdened neurons in the hippocampus and cortex of Alzheimer transgenic rats (Hanzel *et al.* 2014). Therefore, to evaluate the possible modulation of microglia in response to increased hippocampal *Trem2* expression, we analyzed microglia recruitment towards Aβ-burdened CA1 neurons.

We revealed microglia by targeting Iba1, a microglia-specific calcium binding protein (Ito *et al.* 1998). We found that the total number of Iba1-positive microglia in the CA1 region of the hippocampus in treated and untreated, wild-type and Alzheimer transgenic rats was not significantly different in any of the groups tested. However, when we measured the number of microglia within close proximity (within 100 μm) of Aβ-burdened CA1 neurons (**Figure 4-6a**), we found a significant Treatment x Transgene Interaction ($F_{(1,22)} = 4.40$; $p = 0.0476$). *Post hoc* analysis revealed that vehicle-treated Alzheimer transgenic rats had higher number of microglia recruited to CA1 pyramidal neurons compared to vehicle and NP03-treated wild-type rats (**Figure 4-6b**; $p < 0.01$). Further, NP03 significantly reduced the number of recruited microglia in Alzheimer transgenic rats ($p < 0.05$) to a number that was not significantly different to the wild types ($p > 0.05$). Taken together, these results indicate that the recruitment of microglia to Aβ-burdened neurons in Alzheimer transgenic rats at the pre-plaque stage of the AD-like pathology can be prevented with the application of NP03.

Figure 4-6: NP03 Reduced Microglia Recruitment in the Hippocampus.

a) Representative micrographs illustrating a double-labeling for NeuN (blue signal) and microglia (Iba1, brown signal) in the hippocampus of 5 month-old (pre-plaque) wild-type and Alzheimer transgenic, vehicle- and NP03-treated rats.

b) Graph showing recruitment of microglia towards pyramidal neurons of the hippocampus CA1 region in Alzheimer transgenic animals was reduced with NP03 treatment. WT Veh n = 5; WT NP03 n = 5; AD Veh n = 7; AD NP03 n = 9. Data represent mean ± SEM. * $p < 0.05$; ** $p < 0.01$; Tukey's multiple comparisons *post hoc* test.

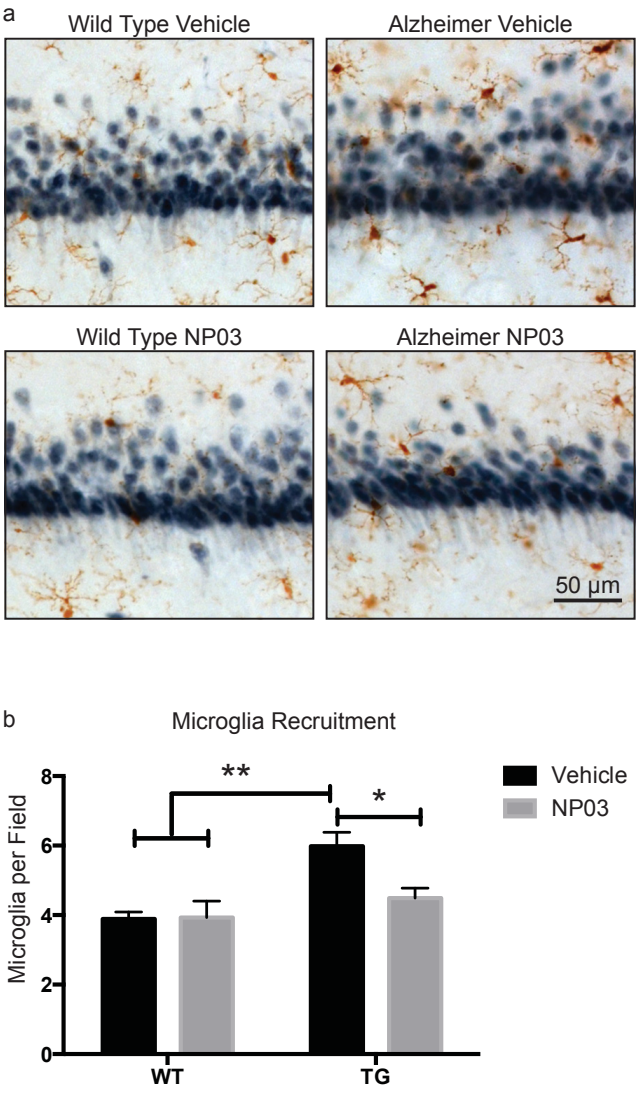

Figure 4-6: NP03 reduced microglia recruitment in the hippocampus

NP03 Restored Adult Neurogenesis in the SGZ of the Hippocampus

Impairments in hippocampal neurogenesis are associated with amyloid deposition (Haughey *et al.* 2002; Zhang *et al.* 2007). Lithium was shown to enhance hippocampal neurogenesis (Chen *et al.* 2000; Fiorentini *et al.* 2010). We processed tissue sections containing the SGZ using an antibody specific for the protein doublecortin (**Figure 4-7a,b**), a reliable and specific marker of adult neurogenesis (Rao and Shetty 2004). Alzheimer transgenic rats showed impaired neurogenesis in the subgranular zone of the dentate gyrus compared to the wild-type groups (**Figure 4-7c**; Treatment x Transgene Interaction $F_{(1,12)} = 8.65$; $p = 0.0124$; (WT:Veh and WT:NP03 vs. TG:Veh, $p < 0.05$). NP03 prevented this loss in Alzheimer transgenic rats compared to vehicle-treated Alzheimer transgenic rats (TG:Veh vs. TG:NP03, $p < 0.01$).

Figure 4-7: NP03 restored adult hippocampal neurogenesis

a) DCX-expressing cells (green) in subgranular zone (sgz) of dentate gyrus. Counterstained with a marker of mature neurons, NeuN (red). GCL, granule cell layer. Scale bar = 25 μm.

b) Micrographs showing DCX-expressing cells (green) in subgranular zone (sgz) of dentate gyrus. Counterstained with the nuclear marker DAPI. Scale bar = 25 μm.

c) Quantification reveals a significant reduction in DCX+ cells in Alzheimer transgenic rats compared to wild-type rats. NP03 treatment significantly restored hippocampal neurogenesis in Alzheimer transgenic rats. WT Veh n = 4; WT NP03 n = 4; AD Veh n = 4; AD NP03 n = 4. Data represent mean ± SEM. * $p < 0.05$; ** $p < 0.01$, Tukey's multiple comparisons *post hoc* test.

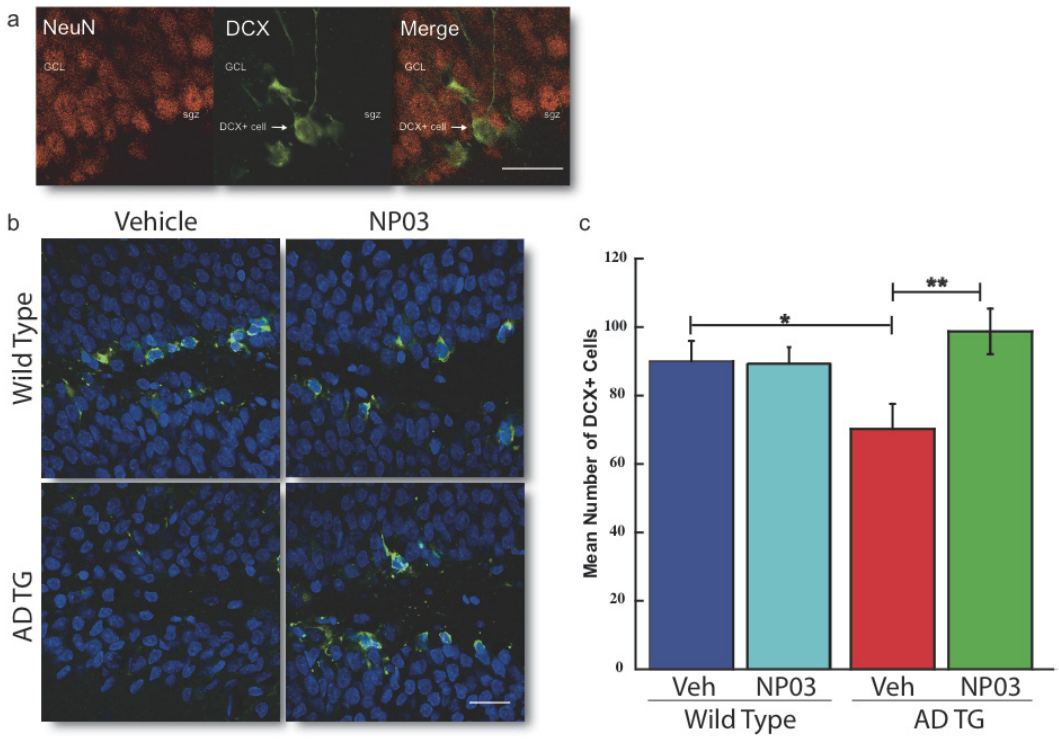

Figure 4-7: NP03 restored adult hippocampal neurogenesis

DISCUSSION

The latent, preclinical stages of AD are associated with the presence of oxidative stress and a proinflammatory response. Accordingly, efforts that reverse the early, disease-aggravating CNS oxidative stress and inflammation may slow the progression of AD. As a result, there is increasing interest in identifying compounds that may slow or prevent these processes during the silent stages of AD. Our results show that at the intraneuronal stages of the AD-like amyloid pathology, Alzheimer transgenic rats have significant oxidative damage, as demonstrated by elevated HNE and 3-NT. In addition, at the early Aβ pathological stages there are already signs of an progressing inflammatory response, as indicated by increased expression of TNF-α, IFN-γ, and IL-4, and IL-5. Together these changes were associated with microglia recruitment to Aβ-burdened neurons (as previously reported (Ferretti, Bruno*, et al.* 2012; Hanzel *et al.* 2014) and impaired neurogenesis in the hippocampus. Our results indicated that the novel microdose lithium formulation, NP03, significantly reduced disease-aggravating oxidative stress, and a similar reduction in inflammatory markers, microglia recruitment, and restored dentate neurogenesis.

Early Oxidative Stress in Alzheimer Transgenic Rats

Oxidative stress occurs in cells where the antioxidant capacity is outpaced by the production of oxidants, resulting in the increased production of damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Butterfield, Reed, Newman*, et al.* 2007). Accumulating oxidatively modified proteins disrupts cellular functions through loss of catalytic function ability or by interruption of regulatory pathways

(Sultana *et al.* 2010). Because protein oxidation generally leads to its dysfunction, the identification of oxidatively modified proteins provides insight into the biochemical and pathological alterations involved in AD pathogenesis (Perluigi *et al.* 2009). For example, a redox proteomic approach used to identify specific HNE-bound proteins in the inferior parietal lobule and hippocampus of AD subjects and age-matched controls revealed that HNE was bound to energy metabolism, antioxidant system, and structural proteins (Perluigi *et al.* 2009). The same approach revealed that these changes occur before the presentation of full AD, as HNE damage was observed in the brain of subjects at MCI stages (Reed *et al.* 2008). In an attempt to determine the earliest Aβ-driven oxidative responses, a time study of neuronal cultures treated with Aβ showed that lipid peroxidation occurred first and rapidly, followed by protein carbonylation (D. Alan Butterfield, personal communications). Therefore, our confirmation of increased HNE- and 3-NT-modified proteins in pre-plaque Alzheimer transgenic rats is in accordance with existing reports. We found that NP03 significantly reduced oxidative modifications in Alzheimer transgenic rats, reducing HNE and 3-NT to wild-type levels. This raises the possibility that NP03 be useful in preventing oxidative stress damage in early AD and in other conditions of oxidative stress.

Early Neuroinflammation in Alzheimer Transgenic Rats

In AD, an inflammatory trigger, such as Aβ-driven oxidative stress, prompts microglial activation, and neuronal damage, leading to increased expression of microglia activators, in effect generating a self-perpetuating neurotoxicity (Block *et al.* 2007). We have previously demonstrated neuroinflammation preceding the appearance of Aβ plaques in

Alzheimer transgenic mice (Ferretti, Allard*, et al.* 2012; Ferretti, Bruno*, et al.* 2012). In addition, we found that the inflammatory signals at this early phase originate from Aβ-burdened neurons in APP rats (Hanzel *et al.* 2014).

Previous work has shown that fibrillar Aβ elicits secretion of proinflammatory cytokines IL-1, IL-6, and TNF-α to promote microglia and astrocyte engulfment of Aβ plaques. For instance, TNF-α has been found to be elevated circulating in CSF or in neuritic plaque regions in brains (Dickson et al. 1993). However, there is now abundant evidence that an early glial response to secreted cytokines and Aβ contributes to AD pathogenesis. At the early stages of the Aβ pathology, we observed a trend towards elevated IL-1β, IL-4, IL-10 and IL-13, along with significant increases in IL-5, TNF-α, and IFN-γ. Cytokines TNF-α (Fillit et al. 1991; Tobinick et al. 2006; Park and Bowers 2010) and IFN-γ (Bate et al. 2006) have been previously implicated in neuronal death and AD pathogenesis. For example, Wood and colleagues performed a screen that correlated cytokine expression with quantified pathologic disease state and then tested whether cytokines up-regulated in AD tissues could affect neuronal viability. They found that TNF-α, IL-4, and IL-5 were up-regulated in AD tissue and that TNF-α reduced viability in neuron culture when applied alone (Wood et al. 2015). Further, there exists a direct relationship between oxidative stress and IL-4 and IL-5 gene expression and cytokine secretion (Frossi et al. 2003; Nakamaru et al. 2015). Interestingly, IL-5 has been shown to promote progenitor differentiation into neurons. This has prompted the hypothesis that IL-5 up-regulation in human AD tissues may represent an attempt to compensate for

neuronal death (Mehler et al. 1993; Wood *et al.* 2015), although this remains under investigation.

The cytokine IL-10 is thought to suppress neuroinflammation, however, concurrent reports in 2015 showed that viral injection to overexpress IL-10 in Alzheimer transgenic mice led to the overexpression of Aβ and plaques and impaired learning and memory (Chakrabarty et al. 2015), while genetically knocking out *Il10* in mice showed decreased plaques and improved memory (Guillot-Sestier et al. 2015). These contradictory results underscore the complexity of immune signaling in evolving pathological states such as in AD.

There is some evidence for the involvement of IL-13 in the modulation of neuronal integrity and synaptic function in patients with multiple sclerosis (Rossi et al. 2011), raising the possibility of a potential role of IL-13 in neuroprotection. Consequently, intracerebral injection of a mixture of IL-4 and IL-13 showed efficacy in reducing Aβ and improving cognitive deficits in an APP mouse model (Kawahara et al. 2012).

Microglia are pivotal for the immune response in AD and other neurological conditions and normally act as sentinel cells, attacking and removing pathogens and cell debris, although they become reactive in AD (Simard and Rivest 2004; Richard et al. 2008; Boissonneault et al. 2009). Microglia are involved in Aβ plaque reduction during sustained, late, neuroinflammation (Cherry *et al.* 2015). We observed recruitment of

microglia to Aβ-burdened neurons in the hippocampus of Alzheimer transgenic rats, which was reduced alongside the NP03-driven reduction in TNF-α secretion. Thus, it is likely that the reduction in microglia signaling factors, including markers of oxidative stress and proinflammatory cytokine TNF-α, contributed to the reduction of recruited microglia in NP03-treated Alzheimer transgenic rats. Whole-exome and whole-genome sequencing strategies identified mutations in *Trem2* (triggering receptor expressed in myeloid cells 2), which confer increased risk of AD (Guerreiro *et al.* 2013; Jonsson *et al.* 2013). We found that *Trem2* mRNA was significantly increased in Alzheimer transgenic rats. Our results are fitting with other recent reports showing that cerebrospinal fluid-soluble TREM2 is increased in AD (Piccio et al. 2016), and *Trem2* mRNA is up-regulated in the brains of Alzheimer transgenic mice (Matarin *et al.* 2015). Cells surrounding Aβ plaques in Alzheimer transgenic mice express high levels of TREM2 (Frank et al, 2008), and TREM2 has been shown to limit neuritic damage in AD by enabling microglia to surround and alter Aβ plaque structure (Ulrich and Holtzman 2016; Wang *et al.* 2016). $A\beta_{42}$ peptides have been shown to activate the TREM2 receptor to produce phagocytosis, production of ROS, and induction of NF-kβ (Jones BM et al, 2014). Expression of TREM2 increases with level of cortical Aβ, and *Trem2* heterozygotes show impaired microglia recruitment (Guerreiro *et al.* 2013; Jonsson *et al.* 2013). Together, these lines of evidence suggest a role of TREM2 in microglial recruitment. In support of this, we found that microglial recruitment to Aβ-burdened neurons was higher in rats expressing higher levels of *Trem2*.

Which Comes First? Oxidative Stress vs. Neuroinflammation

We propose a scenario wherein the production of toxic $A\beta_{42}$ oligomers induces mitochondrial toxicity and oxidative stress. This oxidative stress consequently triggers inflammatory reactions within neurons. At the establishment of this circuit, neuroinflammatory cascades trigger additional oxidative damage, thereby producing a self-perpetuating neurotoxic cycle. Emerging evidence has linked sustained oxidative stress to consequent neuroinflammation and AD pathogenesis (Blalock *et al.* 2004; Butterfield *et al.* 2013; Cobb and Cole 2015). Among the cellular changes that occur in response to the initiation of the ROS/inflammation feedback loop is the recruitment of microglia (**Figure 8a**). We have shown these to be early events in the development of Alzheimer's-like pathology. In early, pre-plaque stages of the AD pathology, increases in intraneuronal Aβ promote oxidative stress, as indicated by increases in HNE and 3-NT. We propose that these factors act as a so-called "inflammatory fifth column" – apparently beneficial agents that actually lend a harmful effect on the brain through increased secretion of proinflammatory cytokines IFN-γ, TNF-α, and members of the interleukin family. These signaling factors undermine neural repair to favor neurotoxicity by contributing toward increased secretion of proinflammatory cytokines, microglia recruitment towards Aβ-burdened neurons, further increased Aβ and ROS production. In a previous report, we found that NP03 reduces production of toxic Aβ in Alzheimer transgenic rats (Wilson *et al.* To Be Submitted), therefore it is possible that this mechanism leads to an attenuation in the self-perpetuating neurotoxic cycle initiated by ROS (**Figure 8b**). This was supported by our observation of reduced proinflammatory cytokine production, reduced *Trem2* mRNA, and reduced numbers of recruited microglia

in the hippocampus. Thus by reducing Aβ production, NP03 reduces activity of the inflammatory fifth column in early stages of Alzheimer's disease.

Figure 4-8: Inflammation as the "the Fifth Column" in Early Alzheimer's Disease

a) In early, pre-plaque stages of the AD pathology, increases in intraneuronal Aβ promote oxidative stress, as indicated by increases in HNE and 3-NT. These factors represent the so-called "inflammatory fifth column" – where supposed beneficial agents actually lend deleterious effects through increased secretion of proinflammatory cytokines IFN-γ, TNF-α, and members of the interleukin family, and *Trem2* gene expression. The inflammatory fifth column undermines neural repair mechanisms by contributing toward increased secretion of proinflammatory cytokines, microglia recruitment towards Aβ-burdened neurons, and a further increase in Aβ and ROS production in favor of neurotoxicity.

b) In Alzheimer transgenic rats treated with NP03, reduced production of toxic Aβ prevents the self-perpetuating neurotoxic cycle initiated by ROS. This was supported by our observation of reduced proinflammatory cytokine production, reduced *Trem2* mRNA, and reduced numbers of recruited microglia in the hippocampus. Thus NP03 reduces activity of the inflammatory fifth column in early stages of Alzheimer's disease.

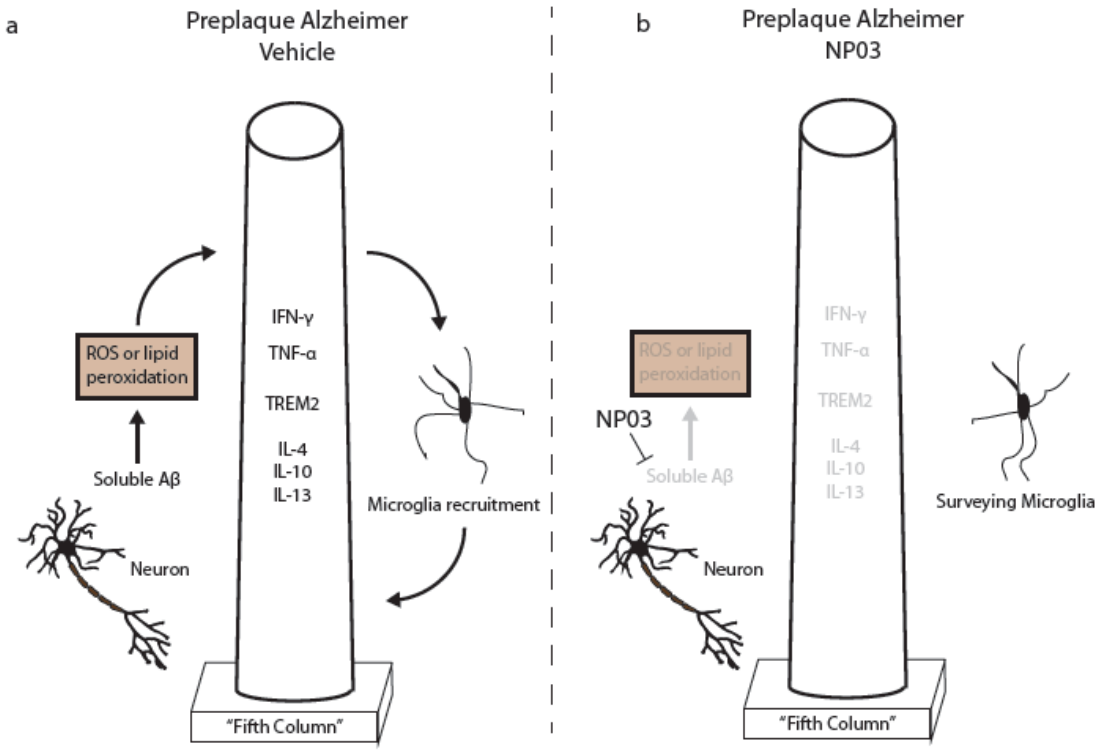

Figure 4-8: Inflammation as the "the Fifth Column" in Early Alzheimer's Disease

NP03 Restores Adult Hippocampal Neurogenesis

Finally, neuroinflammation has been demonstrated to negatively affect adult neurogenesis (Mu and Gage 2011; Fuster-Matanzo *et al.* 2013). For example, neuroinflammation alone inhibits neurogenesis while inflammatory blockade with NSAIDs restores neurogenesis (Monje *et al.* 2003). In addition, there are also data showing impairments in hippocampal neurogenesis associated with Aβ deposition (Haughey *et al.* 2002; Zhang *et al.* 2007). We found that NP03 can prevent this loss at early stages of neuropathology. This is consistent with earlier reports showing that lithium improves hippocampal neurogenesis in APP mutant mice (Fiorentini *et al.* 2010). It is not known what exact role adult neurogenesis plays in behavior (Aimone *et al.* 2011; Sahay *et al.* 2011). Recent data indicate that incorporation of newly generated neurons in the hippocampus subgranular zone is important for inhibitory modulation of mature granule cells, whose influence is dynamically regulated: declining naturally with age, and increasing by environmental enrichment (Drew et al. 2015). Furthermore, neurogenesis is important for pattern separation and the normal processing of information in the hippocampal circuit (Akers et al. 2014). Thus, it is likely that this early impairment in hippocampal neurogenesis contributes to the cognitive impairments that figure so prominently in the Alzheimer transgenic rats (Leon *et al.* 2010; Iulita *et al.* 2014; Wilson *et al.* 2016). In addition, it is possible that the effect of NP03 on restoring hippocampal neurogenesis contributed to the procognitive effects of the drug (Wilson *et al.* To Be Submitted).

It is important to note that NP03 may have a different pharmacology compared to conventional lithium, since a basic principle of pharmacology is that different doses and formulation of the same drug may act on different targets (Mauer *et al.* 2014). This is the first report to confirm low dose lithium action on oxidative stress and inflammation in the brain. Additional mechanistic studies are required to determine the exact action of this microdose lithium formulation. Efforts to stem the early, oxidative stress-induced CNS inflammation may be beneficial in slowing the progression of AD. Oxidative stress, proinflammatory cytokine expression, *Trem2* mRNA, and microglia recruitment were found to be elevated in Alzheimer transgenic rats at the pre-plaque phase of the AD-like Aβ pathology. We demonstrated here that NP03 could reduce Aβ-induced oxidative stress, proinflammatory cytokine secretion, microglia recruitment, and restore adult hippocampal neurogenesis *in vivo*. These results confirm a possible use of NP03 in reducing oxidative stress and neuroinflammation in CNS conditions, including in Alzheimer's disease.

ACKNOWLEDGEMENTS

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CHAPTER 5: General Discussion

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After more than a century since patient Auguste Deter was admitted to the clinic of Dr. Alois Alzheimer tomes of data have amassed contributing to the understanding of the disease that was given his name. With his first reports, Alzheimer initiated the medical literature on AD: he called it a precocious form of senile dementia, that, when examined histologically, had frank abnormal features referred to today as plaque and tangle pathology (Alzheimer 1907, 1911). The plaque component was later identified as consisting of Aβ (Masters et al. 1985), and the amyloid hypothesis was born (Beyreuther and Masters 1991; Hardy and Allsop 1991; Selkoe 1991; Hardy and Higgins 1992). Despite this progress, there currently exists no cure for AD. Given the latent silent stage preceding disease symptoms, studies on the earliest phases of the pathology could yield valuable information regarding the pathophysiological causes of AD. Finally, these pathophysiological causes might be targeted via therapeutic intervention.

By using a robust rodent model that recapitulates many key aspects of the Alzheimer-like Aβ pathology (Leon *et al*. 2010), this thesis has shown:

1. That abnormal intraneuronal accumulation of Aβ is sufficient to cause severe associative learning deficits in pre-plaque Alzheimer transgenic rats (Chapters 2 and 3).
2. The CRTC1 transcription program in the hippocampus is dysregulated early as a result of intraneuronal Aβ accumulation (Chapters 2 and 3).

3. The novel microdose formulation of lithium, NP03, protects against early cognitive deficits in pre-plaque Alzheimer transgenic rats, by reducing BACE1 activity and by diminishing Aβ (Chapter 3).
4. NP03 restores CRTC1 genomic occupancy in synaptic plasticity genes directly under CRTC1 transcriptional control (Chapter 3).
5. NP03 reduces markers of oxidative stress including HNE and 3-NT (Chapter 4).
6. NP03 reduces pro-inflammatory profile in Alzheimer rats, including cytokine production, TREM2 expression, microglia recruitment, and restores hippocampal neurogenesis (Chapter 4).

These findings underscore 1) the important role of intraneuronal Aβ in provoking severe biochemical disruptions in the brain at early stages of the AD-like pathology; and 2) serve as preclinical proof-of-principle of the use of a microdose lithium formulation NP03 as a therapeutic candidate for early stages of Alzheimer's disease.

This chapter will serve as a general discussion of these results and will begin by summarizing many of the rapidly expanding experimental therapeutics currently under evaluation for the treatment of AD. This will lead to a discussion of an emergent theme of this thesis: why multi-targeted strategy will likely be required to effectively treat AD. What follows will be a review of the proof-of-principle generated by this thesis on the ability of NP03 to treat the early stages of AD. This section will include a theoretical model of lithium's mechanism of action in AD. Finally, the chapter concludes by

recommending that NP03 be tested clinically in early AD patients and at-risk populations.

Experimental Treatments for Alzheimer's Disease

In very general terms, a review of the therapeutic strategies in AD reveals that the main current approaches generally follow two main tacks: 1) blocking effects of Aβ on tau and other factors via diminishing its production or enhancing its clearance; and 2) increasing neuroplasticity via increased trophism and/or artificial stimulation. The former is seemingly the most likely to be effective, since the capacity of neuroplasticity to be effective is limited in the face of a continual insult by accumulating Aβ, and subsequent death of neurons. The following section describes some general approaches in experimental treatments for AD. Information was in part obtained from trial listings at www.clinicaltrials.gov.

GSK-3 Inhibitors

Simultaneous publications in 1996 (Klein and Melton 1996; Stambolic *et al*. 1996) reported a direct ability of lithium to inhibit GSK-3 *in vitro* and in cells. Additional studies revealed that Li^{+} ions act as an uncompetitive inhibitor of Mg^{2+} (Ryves and Harwood 2001). Indirectly, lithium activates Akt/PKB, a kinase that promotes inhibitory phosphorylation on GSK-3β at serine 9. Additional GSK-3 inhibitors have since been explored for the treatment of AD. This includes a group of ATP-competitive inhibitors of GSK-3, such as indirubin derivatives (Leclerc et al. 2001), paullone derivatives (Leost et al. 2000), SB415286 and SB216763 (Coghlan et al. 2000), and AR-A014418 (Bhat et al. 2003). Other compounds include L803-mts, an 11-residue peptide that is a substrate-

competitive specific inhibitor of GSK-3 (Plotkin et al. 2003), highly selective ATP non-competitive inhibitor TDZD-8 (Martinez et al. 2002), and VP0.7, an allosteric selective GSK-3 inhibitor that binds to the C-terminal lobe of the enzyme (Palomo et al. 2011). In addition to AD, inhibition of GSK-3 has restored impaired cognition in multiple conditions including Down's syndrome, Fragile X syndrome, Parkinson's disease, stroke, chemotherapy, traumatic brain injury, diabetes, irradiation, AIDS [reviewed in (King et al. 2014)]. Thus, positive outcomes from behavioural studies with these compounds in various models suggest that GSK-3 inhibitors should be more widely considered as interventions in conditions of neurological disease.

BACE1 Inhibitors

In Chapter 3, it was demonstrated that by inhibiting gene expression of BACE1 using microdoses of lithium, there was a reduction in the overall activity levels of BACE1. This corresponded with reduced levels of amyloidogenic processing and reduced $A\beta_{42}$. A shift toward the non-amyloidogenic pathway, favouring the production of the less toxic $A\beta_{40}$, is a promising strategy for AD therapy. This might be accomplished by reducing the activity of BACE1. As such, BACE1 inhibitors have been designed and are under evaluation by Janssen Research and Development in Belgium. Furthermore, BACE inhibitor LY3314814 from Eli Lily and Company has recently been investigated. Meanwhile, Eisai and Biogen have partnered on a Phase 1/2 study that finished in 2016. Merck has tested their compound verubecestat (MK-8931) in their EPOCH study, which will continue in mild to moderate AD patients until mid-2019. In their APECS study, the

compound is being tested in prodromal patients, paired with PET scan. The APECS study will be completed in 2021.

Aβ Vaccines and Antibodies

An extraordinary effort to use antibodies to provoke Aβ clearance has been ongoing for nearly two decades. As the Aβ hypothesis holds that Aβ is a triggering and aggravating factor in AD, the rationale behind Aβ immunotherapy is to use the body's immune system for its immunoneutralization and removal. The first of these studies showed that active immunization against Aβ reduced pathology in an Alzheimer mouse model (Schenk et al. 1999). Subsequently, it was shown that peripherally administered antibodies against Aβ peptide enter the CNS and were also able to reduce pathology (Bard et al. 2000). There is controversy over the exact mechanism leading to Aβ immunotherapy, however. Some propose that the mechanism of Aβ immunotherapy is by Fc receptor-mediated phagocytosis of amyloid (Bard *et al*. 2000), while others have shown it to be through non-Fc-mediated mechanisms, and instead showing it results from reduced Aβ aggregation and increased clearance (Bacskai et al. 2002; McLaurin et al. 2002). The idea of two separate pools of Aβ, one in the brain, the other in the periphery, represented an important shift in our understanding of Aβ toxicology in the brain. It meant that the periphery represented an "Aβ sink", wherein CNS Aβ could be deposited and sequestered to reduce any toxic impact on the brain. This raised the possibility that reducing peripheral levels of Aβ would allow for CNS Aβ to be removed. Accordingly, peripheral anti-Aβ antibody alters Aβ clearance in the CNS and plasma and decreases Aβ brain burden through peripheral sequestration (DeMattos et al. 2001). Unfortunately,

there were side effects with these early immunotherapy animal studies: Aβ immunization also targeted vascular amyloid (Schroeter et al. 2008), and led to cerebral microhemorrhages, as reported in a number of animal models (Pfeifer et al. 2002; Wilcock et al. 2004; Racke et al. 2005; Wilcock et al. 2007).

AD patients immunized with aggregated $A\beta_{1-42}$ and the adjuvant QS21 showed evidence of a reduction in amyloid load (Nicoll et al. 2003; Ferrer et al. 2004; Masliah et al. 2005; Holmes et al. 2008), although neurofibrillary tangles were not reduced. There were mixed clinical effects with improvement observed on some memory tests, although others remained unchanged following 1 year of treatment (Gilman et al. 2005). These early clinical trials involving immunization with $A\beta_{1-42}$ were halted because of the development of aseptic meningoencephalitis in 6% of treated patients (Orgogozo et al. 2003). Trials were also associated with cerebral amyloid angiopathy and microhemorrhage (Boche et al. 2008). Upon autopsy, it was noted that the brains of treated patients with meningoencephalitis showed increased T-cell-mediated autoimmune response (Nicoll *et al*. 2003; Ferrer *et al*. 2004). This was likely due to the use of the full-length Aβ, which contains T-cell epitopes in the C-terminal region (Monsonego et al. 2003). As a result of these side effects, new antibodies target only the N-terminal region of Aβ.

Eli Lily is investigating solanezumab in their EXPEDITION 3 study, which has a primary completion date of 2016 and a study completion in 2018. Eisai and Biogen have partnered with their monoclonal antibody, Aducanumab (BIIB037), with one trial

finishing in 2016, and with a Phase 3 trial currently underway with primary completion in 2020. Similarly, Hoffman-La Roche has developed a fully human anti-Aβ monoclonal antibody that binds with sub-nanomolar affinity to Aβ fibrils (Ostrowitzki et al. 2012). It was shown that this antibody, gantenerumab, induced cellular phagocytosis of human Aβ deposits of AD brain slices when co-cultured with primary human macrophages. Likewise, in APP751(Swedish)xPS2(N141l) transgenic mice, gantenerumab significantly reduced small Aβ plaques by recruiting microglia. Interestingly, this was accomplished without reducing the plasma levels of Aβ, suggesting undisturbed systemic clearance of soluble Aβ (Bohrmann et al. 2012). Roche is currently testing gantenerumab in a study of patients with mild AD, with primary completion in 2019. Previous safety studies have noted that amyloid-related imaging abnormalities (ARIA) are a concern (Ostrowitzki *et al*. 2012). Roche reported no efficacy on primary or secondary endpoints in this early study, and halted the trial. *Post hoc* analysis did however reveal a trend toward a benefit in the fastest progressors – those that show more rapid decline. This led them to begin a Phase 3 clinical trial with 1000 patients with mild AD. They are also participating in the Dominantly Inherited Alzheimer Network (DIAN) study, which is in parallel evaluating solanezumab from Eli Lily. This trial is directed by Dr. Randall J. Bateman at the Washington University School of Medicine (Bateman et al. 2012; Morris et al. 2012). What remains promising about gantenerumab is that appears to be lowering Aβ by eliciting effector cell-mediated clearance, rather than promoting plasma Aβ clearance through antibody-mediated sequestration of peripheral Aβ, which represents a limited therapeutic option (Bohrmann *et al*. 2012).

Focused Ultrasound

An Australian group is exploring a non-invasive focused ultrasonic (FUS) technology that purportedly clears the brain of neurotoxic Aβ plaques in mice. The team claims that the oscillating sound waves permeablize the blood brain barrier and stimulate microglia lysosomal internalization of Aβ, as imaged using spinning disk confocal microscopy and high-resolution three-dimensional reconstruction (Leinenga and Gotz 2015). The treated AD mice displayed improved performance on a battery of rodent cognitive tasks. Interestingly, this work corroborates that already completed by a group at Sunnybrook Health Sciences Centre in Toronto, that applied focused ultrasound to a single brain region, combined with microbubbles – small gas bubbles injected into the bloodstream— to open the blood brain barrier, to reduce Aβ plaque burden and improve memory (Jordao et al. 2013). Leinenga and Gotz are planning to test the FUS procedure on sheep prior to beginning human clinical trials in 2017.

Stem Cell Therapy

Stem cell therapy is of promise in Parkinson's disease, where intrastriatal stem cell transplantation has been found to re-innervate the striatum, restore dopamine releases, and can induce long-lasting improvement of motor function (Lindvall 2016). The potential to produce inducible pluripotent stem cells (iPSCs) has been widely investigated in neurodegenerative diseases as they can be cultured from all germ layers (Pen and Jensen 2016). However, issues remain pertaining to the use of stem cells in treating Alzheimer's disease, including the transplantation location (there is massive cell loss throughout the brain), and stem cell tracking (where do transplanted cells end up?). For

the latter, new MRI technology now allows researchers to trace the movement of injected stem cells labeled with contrast agents, making it clearer where they end up (Janowski et al. 2014). As for the former question, some hints have come from animal studies where stem cell therapy has had pro-cognitive effects that shed light on the fate on injected cells. It is postulated that these new cells could be beneficial by maturing into neurons and replacing those that are lost. Another alternative proposal, however, is that these cells mature into glia and help by reducing the level of amyloid burden. In a mouse study by the team of Dr. Frank LaFerla, it was found that injected cells did neither. Instead, they promote the formation of CA1 synapses in part through their release of the neurotrophic factor BDNF (Blurton-Jones et al. 2009). This was an important discovery, since BDNF expression had recently been shown to be reduced in AD because of Aβ (Peng et al. 2009). What is interesting is that the pro-cognitive changes occurred without any reduction in plaques or tangles. These results highlight the importance in considering the "bystander" effects of stem cells, which have been demonstrated in Parkinson's and other conditions (Lee et al. 2007). Thus in AD, stem cells may not necessarily function as a replacement mechanism *per se*, but rather, would add trophic support to the molecular milieu in the diseased CNS by so-called "nursing", or neurotrophic effects.

In preclinical research undertaken by Swiss biotechnology company Stemedica International, weekly injection of human mesenchymal stem cells into the bloodstream for 10 weeks was shown to reduce amyloid level by one-third in APP/PS1 mice. In addition, the researchers, Dr. Alexei Lukashev and Dr. Tristan Bolmont, showed a significant reduction of pro-inflammatory microglia (AAT Springfield/Athens

Symposium, Athens, Greece, March 8-13, 2016). The company has recently received research board approval from Emory University in Atlanta, Georgia, to initiate a clinical study enrolling 40 subjects with mild to moderate dementia due to AD, to assess the safety and preliminary efficacy of a single intravenous dose of allogeneic human mesenchymal stem cells. There is much remaining to be uncovered regarding the mechanism of stem cell therapy for AD. Some argue that additional mouse studies should be completed prior to beginning human clinical trials, while others hold that the questions that remain can only be answered with human trials. In either case, it is likely that the benefit of such a treatment will be shown to be of limited benefit in patients, given the presence of an ongoing neuropathological disease process, provoking continuous neuron loss.

Deep Brain Stimulation

Deep brain stimulation (DBS), a useful therapy in Parkinson's disease (Pouratian et al. 2012), is increasingly investigated in other neurological disorders (Kim and Pouratian 2014). For instance, electrical stimulation of memory nodes has shown promise in animal studies, inducing neuroplasticity through hippocampal neurogenesis, and cognitive restoration (Hamani et al. 2011; Stone et al. 2011).

Researchers from United States and Canada, led by Dr. Andres Lozano, of the University Health Network, Toronto, are assessing the therapeutic benefit of DBS in a 12-month double-blind randomized controlled feasibility study to evaluate the safety, efficacy and tolerability of deep brain stimulation of the fornix (DBS-f) in patients with

mild, probable AD. Their rationale is that the electrical stimulation of established memory circuits provokes neuroplasticity and promotes protection and repair (Mirzadeh et al. 2015). The fornix is a major fibre bundle within the memory circuit of Papez that interconnects the subiculum and hippocampus to the mammillary nuclei and septal area (Tsivilis et al. 2008). The group, known as the ADvance Research Group, has already completed a phase I trial of DBS of memory circuits in AD, with promising results in clinical assessment of cognitive function, glucose metabolism, and with neuroanatomical volumetric measurements showing reduced rates of atrophy (Laxton et al. 2010). Recently, the Advance Research Group reported on the surgical safety on a larger study involving 42 patients with mild, probable AD treated with bilateral DBS-f (Ponce et al. 2015). Adverse events (AE) were reported, with four patients (9.5%) requiring return to surgery for explantation due to infection (n = 2), lead repositioning (n = 1), or chronic subdural hematoma (n = 1). Aside from these AEs, no patients experienced neurological deficits as a result of the study. 90 days after surgery, bilateral fornix DBS was well tolerated by patients. The larger study will be completed soon, and the data will be important in determining the long-term efficacy of DBS-f and its feasibility in AD therapy. These DBS results are yet another intervention that supports the notion that the induction of neuroplasticity as a promising therapeutic avenue for the treatment of AD.

The MEND Program

Finally, a novel therapeutic program from University of California Los Angeles (UCLA) targets environmental factors in an effort to reduce the prevalence of sporadic AD, with positive results. The program is designed to achieve metabolic enhancement for

neurodegeneration and involved the case study of 10 individuals with varying severity ranging from subjective cognitive impairment (SCI), amnesic mild cognitive impairment (aMCI), and AD (Bredesen 2014). The program varied depending on the ability of the patients, but one example included 1) the elimination of simple carbohydrates; 2) the elimination of gluten and processed food, and increased vegetables, fruits, and non-farmed fish; 3) yoga; 4) meditation 20 minutes twice per day; 5) supplement with 0.5 mg melatonin orally at bedtime; 6) increased sleep to 7-8 hours per night; 7) 1 mg methylcobalamin daily; 8) 2000 IU vitamin D3 daily; 9) 2000 mg fish oil daily; 10) 200 mg co-enzyme Q_{10}; 11) optimized oral hygiene used electric tooth flosser and brush; 12) fasted a minimum of 12 hours between dinner and breakfast, and for 3 hours between dinner and bedtime 13) exercise for a minimum of 30 minutes, 4-6 days per week. The results indicate that for patients with SCI, aMCI, and early stage AD memory loss was reversed, and improvement was sustained throughout the therapeutic program. Interestingly, 6 of the patients who had reported that cognitive decline was negatively impacting their job performance were able to return to work or continue working without difficulty. These results are anecdotal and the group is currently recruiting patients for a more extensive, controlled clinical trial to evaluate the therapeutic program. Nevertheless, this program is likely only of benefit to those with mild cases of AD, not the more aggressive, genetically determined forms of the disease.

Alzheimer's Disease Requires Combinatory Therapy

Given the multifactorial nature of Alzheimer's disease, it is likely that a combinatory therapy incorporating multiple therapies such as those listed above would be necessary to

ultimately halt the disease. One difficulty, however, is that combinatory clinical trials are very difficult to perform. Each component of a combinatory therapy needs to be understood in isolation before applying it towards the multidrug treatment. Even if both compounds have been approved, trials involving multiple compounds can be very costly and take years to perform. Nevertheless, an ideal candidate for the treatment of AD would likely be a combination that stops the overproduction of Aβ, reduces early CNS inflammation, prevents the accumulation of NFTs, and shifts neurons to a state of neuroprotection.

Proof-of Principle of NP03 in Early AD

This thesis showed that the novel formulation of lithium, NP03, accessed some of the known targets of conventional lithium. However, given the diffences in distribution and penetrance of the new formulation, it should not be taken for granted that this formulation should act on all the targets of conventional lithium previously identified. The targets which we found to be modulated by treatment of microdose NP03 are summarized in **Figure 5-1.**

Figure 5-1: Summary of targets modified by NP03 in pre-plaque Alzheimer Transgenic Rats

NP03 treatment of pre-plaque, Alzheimer transgenic rats restored 1) performance on spatial learning in the Morris water maze; 2) auditory fear conditioning; 3) novel object recognition; 4) hippocampal neurogenesis; 5) CRTC1 binding to *Bdnf iv*, *c-Fos*, and *Arc* promoters. NP03 treatment reduced 6) proinflammatory cytokines IFN-γ, TNF-α, IL-5; 7) *Trem2* expression; 8) markers of ROS HNE and 3-NT; 9) BACE1 mRNA and activity; and 10) the ratio of soluble human $A\beta_{42}/A\beta_{40}$.

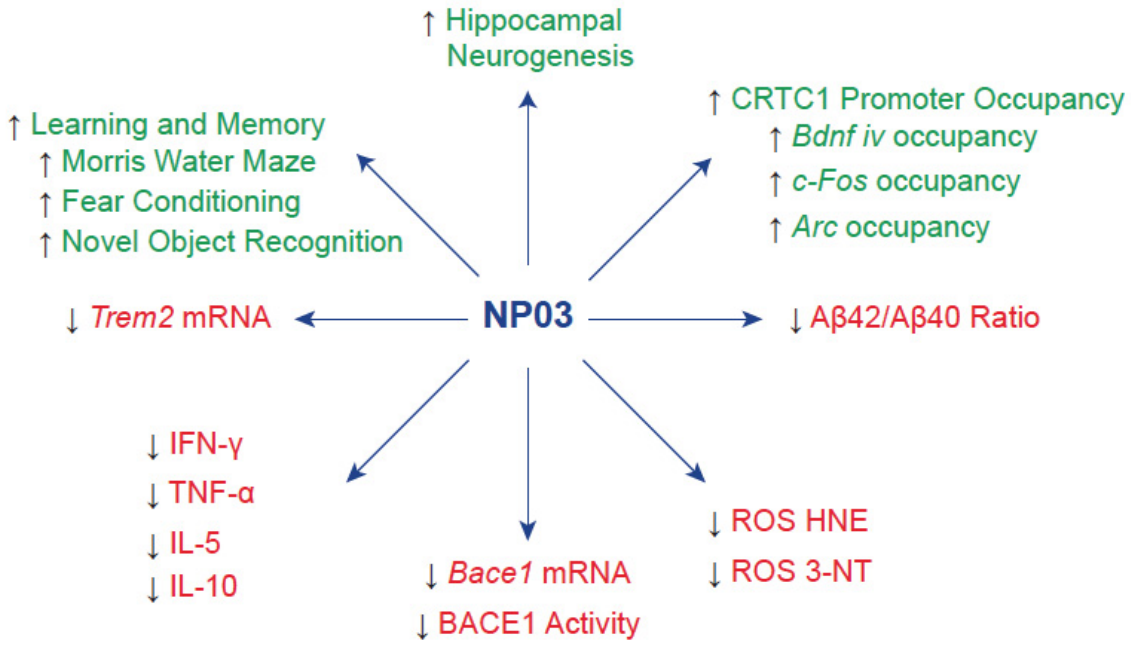

Figure 5-1: Summary of targets modified by NP03 in pre-plaque Alzheimer Transgenic Rats

Chapter 2 demonstrated that intraneuronal Aβ was sufficient to block CRTC1 nuclear translocation and impaired learning and memory. Treatment of these rats with NP03 at the pre-plaque state prevented cognitive impairment, and a reduction in the levels of soluble $A\beta_{42}$ (Chapter 3). We found that NP03 also significantly reduced the level of BACE1 activity approximately 12.5%, restoring it to baseline levels. Data from other groups indicate that lithium is capable of producing a transcriptional blockade on the *BACE1* by signalling through the Wnt/β-catenin signalling pathway (Parr *et al*. 2015). This may be achieved through modification of the kinase GSK-3β, a key inhibitor of Wnt/β-catenin signalling which has been shown to be inhibited by NP03 in mouse model of Huntington's disease (Pouladi *et al*. 2012).

However, the pleiotropic activity of lithium extends its multi-target nature, as various mechanisms may be directly or indirectly mediated. For example, it is possible the pro-cognitive effects of NP03 stemmed from an indirect effect of reducing toxic Aβ, but also through direct promotion of CRTC1 signalling to enhanced neuroprotection and neuroplasticity in support of learning and memory (Chapter 3). This might be assessed in future experiments by exogenously adding Aβ to nullify the effects of lithium along this axis. Similarly, it is difficult to discern whether the reduction in pro-inflammatory signalling arose directly from lithium action, or indirectly through lithium's action on Aβ (Chapter 4). Again, this effect could be investigated by infusing Aβ into systems treated with lithium.

NP03 in Post-Plaque Alzheimer Transgenic Rats

In Chapters 2 and 3, it was demonstrated that NP03 (40 μg Li/kg) had a beneficial impact on cognition, neuroplasticity, and neuroinflammation in 6-month-old AD transgenic rats. This time point could be considered to represent a quite early pre-symptomatic phase of the disease. During this early phase, NP03 reverted the development of the Aβ-mediated pathologies. With better diagnostic methods, successful therapeutic candidates may be administered at earlier stages of AD pathology, where they will have a greater likelihood of being effective. However, there remains an ethical responsibility to develop therapeutics for those already at later stages of AD. A therapeutic candidate would be required that shows promise in reverting a long-established Aβ neuropathology. For this reason, a second cohort of McGill-R-Thy1-APP Alzheimer transgenic rats (littermates of those investigated in Chapters 2 and 3) were allowed age to a point where extracellular plaque burden is high, in a later stage of the Alzheimer-like Aβ pathology. These rats were treated for three months with NP03 (40 μg Li/kg). The results of these experiments will be part of a paper that will be completed after this thesis examination. Preliminary data (**Appendix Figure 6-2**) indicate that while 15-month old Alzheimer transgenic rats were significantly impaired on the novel object recognition task, NP03 administration at the late stage of the Aβ pathology completely restored novel object recognition.

CRTC1 Transcription Program as an Alzheimer's Disease Target

There are numerous reports highlighting the role of CRTC1 in a wide variety of biological processes. Introduction of dominant-negative forms of CRTC1 in CA1 neurons blocks gene expression required for late phase long-term potentiation (LTP) without

affecting the early phase LTP (which is gene transcription independent) (Zhou *et al*. 2006; Kovacs *et al*. 2007). CRTC1 transcription is impaired in Huntington disease (HD) (Chaturvedi et al. 2012) and it has been suggested that mutated huntingtin protein (HTT) interferes with CRTC1 nuclear localization and transcriptional activity (Jeong et al. 2012). The NAD-dependent protein deacetylase, Sirt1, recues this deficit *in vitro* and in a mouse HD model. Neuroprotection is provided by CRTC1 in advance of ischemia, as reviewed in (Namura et al. 2013). Neuroprotection provided by CRTC1 has also been demonstrated in that the antioxidant Tanshinone IIa associates with induced nuclear translocation of CRTC1 (Liu et al. 2010). CRTC1 signaling contributes to circadian pain in mouse models of chronic constriction injury through the NMDA receptor 2B (NR2B) (Xia et al. 2015). This pain can be relieved with intrathecal treatment with CRTC1 interference adenovirus. Genetic deletion of CRTC1 in loss-of-function experiments provide supporting evidence for the role of diminished CRTC1 levels in depression. *Crtc1*$^{-/-}$ mice were shown to have many behavioral abnormalities, including pathological aggression and depression-related behaviours (Breuillaud *et al*. 2012). Activation of CRTC1 chromosomal rearrangement underlies the etiology of certain malignant tumors, identifying *Crtc* as a *bona fide* cancer gene (Komiya et al. 2010). Entrainment of the biological clock requires plasticity that is generated by neurons of the suprachiasmatic nuclei (SCN). Jagannath and colleagues demonstrated that this plasticity is achieved though signaling of CRTC1 (Jagannath *et al*. 2013). SIK1 silenced mice showed significantly enhanced phase shifts and significantly faster re-entrainment. Thus, because SIK1 acts as a negative regulator of CRTC1 is therefore represents an interesting target for CRTC1 augmentation.

The CRTC1 transcription program can be added to the multitude of cellular signaling pathways that are dysregulated in Alzheimer's disease. The CRTC1 transcription program is recruited during development and in established neural circuits where activity-dependent modification is required (Lee *et al*. 2005; Li *et al*. 2009). This is most clearly illustrated in learning and memory, when memory traces, or memory engram (for review, see (Josselyn *et al*. 2015), are modified in the process of learning (Ch'ng *et al*. 2012; Nonaka *et al*. 2014). Sekeres and colleagues demonstrated that inhibiting CRTC1 at both consolidation and reconsolidation is suffficent to destabilize memory storage, impeding future recall (Sekeres *et al*. 2012). They also demonstrated the opposite, that increasing CRTC1 in the dentate gyrus of the hippocampus increased the *strength* of a memory being encoded, with no effect on the *character* of the memory. While these reports were being prepared for publication, the group of Carlos Saura published results that validate those of Chapter 2 (Parra-Damas *et al*. 2014). The group investigated CRTC1 translocation in the same three neuronal populations as was done in this thesis, and strikingly found the same results. Added to the rodent results, the Saura group also showed important data from human AD patients, where CRTC1 was reduced in patients at late Braak stages (Parra-Damas *et al*. 2014). Experimental manipulation of CRTC1 using AAV in AD mice restored learning and memory. This serves as important proof-of-prinicple in targeting CRTC1 in human AD, as AAV is currently being explored as a treatment option for humans.

Following unsuccessful pilot experiments with conventional doses of lithium, the approach adopted for the research narrated in this thesis was to use microdose lithium NP03 as a non-invasive strategy by which to activate CRTC1, as viral vector expression in humans is not a likely translational approach. The doctoral thesis by Annette Heinrich, in Germany, showed that lithium promotes the oligomerization of CRTC1 *in vitro*, leading to increased association of CRTC1 with CREB and CREB-binding protein (CBP) (Heinrich *et al*. 2009; Heinrich *et al*. 2013). Further investigation revealed a large body of literature documenting the protective effects of lithium in humans. The mechanism by which lithium extended therapeutic action was unclear, with many papers identifying specific members of the glutamatergic ERK-CREB-CRTC1-BDNF cascade, but few integrating their findings into the wider cascade. Another disparate body of literature approaches but fails to identify CRTC1 when discussing impaired BDNF in the presence of Aβ, for example. Experiments described in Chapter 2, and the paper from the Saura group are to date the only communications to synthesize a complete picture of the dysregulation of CRTC1, as brought about by Aβ in AD.

In our hypothetical "Lithium Circuit Model in Alzheimer's Disease", presented here, it holds that a normal CNS circuit responds to natural or artificial stimulation, which leads to induction, immediate early gene (IEG) expression to yield neuroplastic change, and a sculpted, balanced circuit (Kandel 2001). In AD, Aβ-mediated impaired integration of synaptic signalling combines (Qi *et al.* 2014) with reduced induction through impaired IEG activity, leading circuit dysregulation in the form of hypotrophism (Peng *et al.* 2005; Peng *et al.* 2009). Under normal circumstances, lithium, through its sedative action, leads

to reduced stimulation (Bech et al. 1979). At the same time, lithium has been shown to potentiate induction 6-fold through the activation of CRTC1, which targets directly the expression of IEGs (Zhou *et al.* 2006; Heinrich *et al.* 2009; Heinrich *et al.* 2013). Overall, this effect should produce hypertrophism in the circuit. The result is a circuit that undergoes continuous sculpting, although still adhering to activity-dependent plasticity (Lachance and Chaudhuri 2004; Lalonde et al. 2009; Li *et al.* 2009; Ch'ng *et al.* 2012). In AD, lithium treatment produces a balanced circuit by compensating reduced stimulation by enhancing IEG induction, thereby leading to neural repair (Kempermann et al. 2000; Tan et al. 2008). This model provides a framework in which to consider the effect of lithium through mechanisms parallel to the reduction of Aβ.

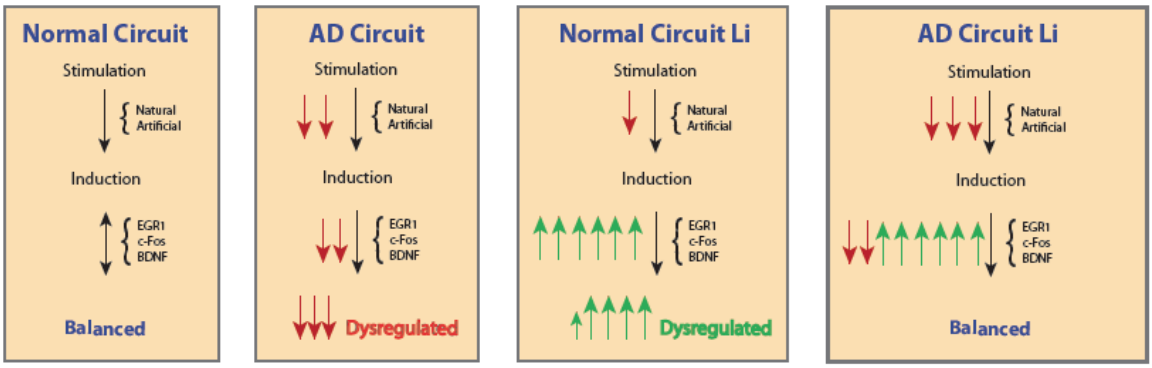

Figure 5-1. A Proposed Model for the CNS Effects of Lithium in the Alzheimer's Pathology

Conclusions

The present thesis evaluated the effect of NP03 on multiple aspects of the AD-like Aβ pathology. It was found that NP03 in pre-plaque AD transgenic rats reduced Aβ, BACE1, pro-inflammatory cytokines, microglia recruitment, and restored cognition, hippocampal neurogenesis, and CRTC1 promoter occupancy. This was achieved without side effects. Preliminary results from a pilot experiment assessed whether the same dose of NP03 (40 µg Li/kg) administered at the late, post-plaque stage of the pathology would still be of therapeutic benefit. **Appendix 2** preliminary data indicate post-plaque Alzheimer transgenic rats showed a complete recovery on novel object recognition when treated for three months with NP03. These results are encouraging since they reveal the power of NP03 to revert an entrenched, established pathology in Alzheimer transgenic rats. Experiments to investigate the neuropathological correlates of this late-stage behavioural improvement are being completed. In that regard, and notwithstanding that major Aβ toxic effects derive from soluble material, it would be interesting to evaluate the plaque burden in Alzheimer's transgenic rats following NP03 treatment.

A number of considerations must be acknowledged: First, the observations made here were made in a single rodent AD model, thus there exists a small risk that the observations are phenomenological. Therefore, it would be important to repeat these observations in another AD animal model. For instance, by evaluating NP03 in a model that includes tau pathology. A second consideration involves the assumption that NP03 be similarly efficacious when administered at late stages of the AD-like pathology. It is possible that if NP03 continues to be anti-inflammatory in this context. If so, it may be

detrimental to late-stage inflammation, and potentially disease aggravating, as the late stage AD inflammation has a component of Aβ removal and tissue resolution (Boissonneault *et al*. 2009; McGeer and McGeer 2013; Pimentel *et al*. 2015). Additional studies are required to tease out the role of late stage neuroinflammation in AD and its response to NP03 treatment. Finally, the multi-target nature of lithium has not yet been, and may never be absolutely known. Therefore, caution must be taken when ascribing the effect of one formulation of lithium (for instance NP03) to a certain mechanism of action. This problem might be partially reduced through proteomic screens and use of cell culture, but it will remain very difficult to say with any certainty that lithium is working directly though a given target. The fact that we have employed a significantly lower concentration of lithium ($1/400^{th}$ of the dose compared to conventional dosing) implies that lithium at such concentrations should have a different pharmacological profile than that known at much higher doses.

Desirability of an NP03 Trial in Early AD Patients and At-Risk Populations

There currently exists no cure for AD. NP03 acts on multiple aspects of the AD pathology and is without side effects. Therefore, it is proposed here that NP03 be evaluated in clinical populations at the early stages of AD. The initial symptoms of AD include changes in mood, including agitation, depression, and impulsivity. Therefore, these changes would be ideal to serve as primary outcome measures in an AD clinical trial evaluating NP03. Additional exploratory endpoints could include performance on cognitive tests, CSF levels of p-tau, GSK-3β, and $A\beta_{42}/A\beta_{40}$ ratio, brain levels through neuroimaging, including Aβ, inflammation, and tau. At the same time, the prophylactic

effects of NP03 should be evaluated in healthy, at-risk individuals. This could be achieved in longitudinal studies assessing earlier time points of NP03 initiation. Such populations could include those with *APP*, *PSEN1*, and *PSEN2* mutations, those with Down's syndrome, and those with family history of AD. In the mutation and DS groups, a well-defined expected age of onset of disease symptoms would allow one to determine how much of a delay is made possible by NP03 administration. Even a modest delay of AD symptoms of a few years would be a much-welcomed contribution, in the knowledge that a 5 year delay in clinical AD onset signifies a 50% reduction in AD prevalence (Quebec 2009).

APPENDIX

Appendix I: Results of Pharmacotherapy Study Using Conventional Lithium in Rats

Conventional lithium has a narrow therapeutic window and a great many known neurotoxic and significant adverse effects, especially in the elderly. These include mood disturbances, somnolence, nausea and diarrhea (Young and Newham 2006). A failed pilot rat pharmacotherapy study revealed side effects associated with lithium treatment that included loss of appetite, polydipsia, polyuria and dehydration - making treatment with standard lithium formulations inappropriate for long-term treatment. For this reason, standard lithium could not, therefore, be considered among the possibilities in the way of Alzheimer therapeutic. However, Medesis Pharma has developed a new medication vector (called NP03) that allows small molecules (in this case, lithium) to be packaged to cross the blood brain barrier at much lower doses. This novel formulation of lithium allows for the utilization of a significantly lower (400x less that of conventional lithium) dose to achieve therapeutic effects (**A)**, but most of all it avoids the side effects commonly associated with long-term lithium treatment **(B)**. Therefore this new lithium formulation (NP03) has significant potential translational value.

Figure 6-1. Serum Lithium Levels After 1-Month Treatment and Related Weight Loss. A) Serum lithium levels in rats receiving diet supplemented with 0.024% $LiCaO_3$ falls within the recommended therapeutic range (0.5-1.5 mEq/L) compared to rats fed a control diet. Lithium remains below detection limit (<0.06 mEq/L) in NP03-treated rats and vehicle-treated rats. B) Lithium-Related Weight Loss. Mean weight in percentage relative to control for lithium-treated and control rats. A substantial weight-loss is noted in rats that received lithium-carbonate diet, while NP03 rats were not significantly different in weight compared to vehicle-treated rats.

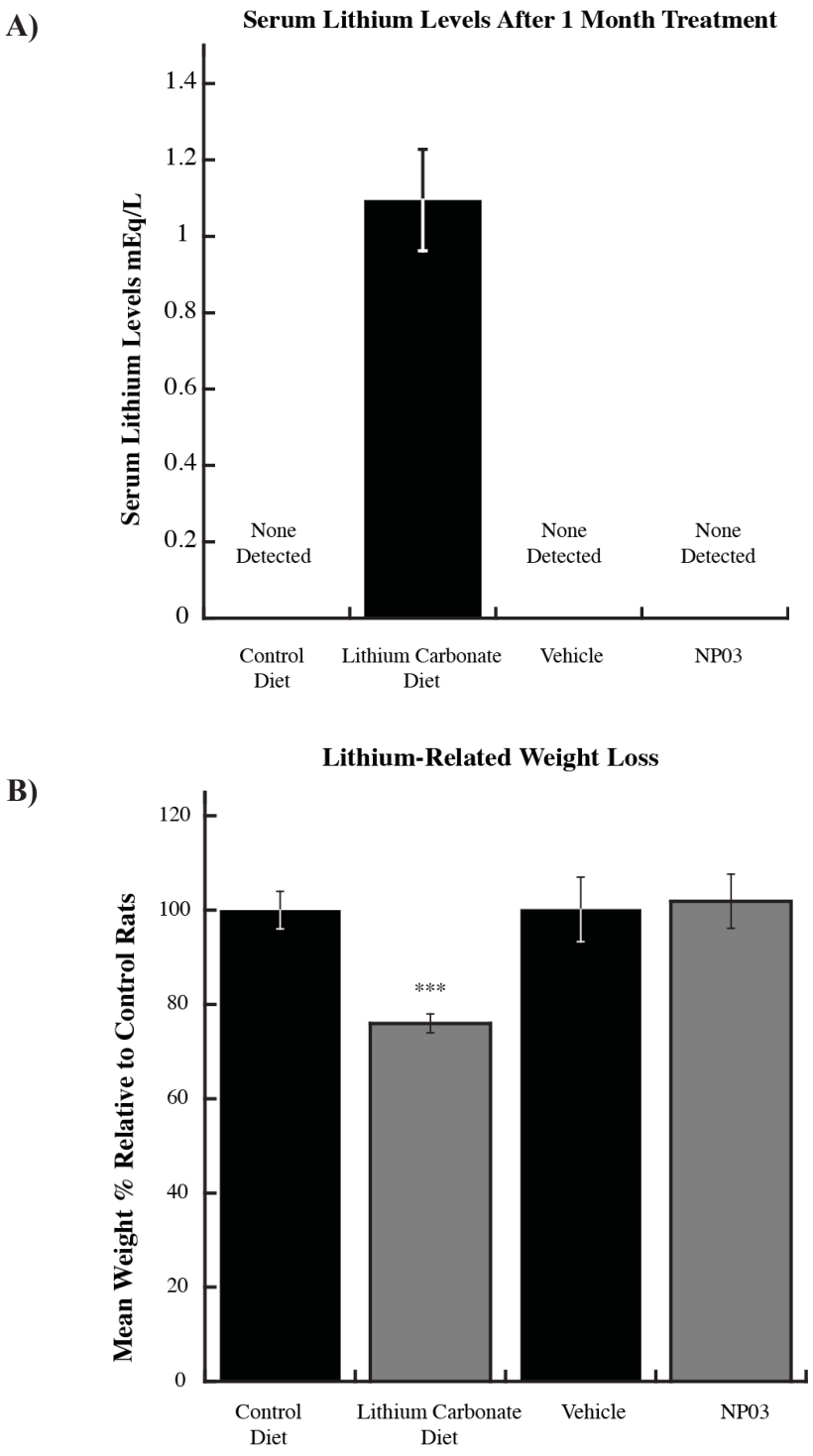

Figure 6-1. Serum lithium levels after 1-Month lithium carbonate treatment and related weight loss.

Appendix 2: NP03 Treatment of Late-Stage Alzheimer Transgenic Rats

In Chapters 2 and 3, it was demonstrated that NP03 (40 μg Li/kg) had a beneficial impact on cognition, neuroplasticity, and neuroinflammation in 6-month-old AD transgenic rats. These results could be considered to represent a pre-symptomatic phase of the disease. During this early phase, NP03 reverted the development of the Aβ-mediated pathologies. With better diagnostic methods, successful therapeutic candidates may be administered at earlier stages of AD pathology, where they will have a greater likelihood of being effective. However, there remains an ethical responsibility to develop therapeutics for those already at later stages of AD. A therapeutic candidate would be required that shows promise in reverting a long-established Aβ neuropathology. For this reason, a second cohort of McGill-R-Thy1-APP Alzheimer transgenic rats (littermates of those investigated in Chapters 2 and 3) were allowed age to a point where extracellular plaque burden is high, in a later stage of the Alzheimer-like Aβ pathology. These rats were treated for three months with NP03 (40 μg Li/kg). Preliminary data are shown in Figure 6-2 and indicate that while 15-month old Alzheimer transgenic rats were significantly impaired on the novel object recognition task, NP03 administration at the late stage of the Aβ pathology completely restored novel object recognition. On going experiments are examining the effect of NP03 on Aβ plaque load, expression of synaptic plasticity-associated gene expression, and on neuroinflammation.

Figure 6-2: Alzheimer transgenic and wild-type rats of 15-months of age were treated with either NP03 (40 μg Li/kg; 1 mL/kg) or Vehicle (1 mL/kg) for 3-months. At 18 months of age, all rats were tested on the Novel Object Recognition task. The wild-type vehicle-treated group displayed a clear preference for the novel object, with a recognition index of ~40%. In contrast, Alzheimer transgenic vehicle-treated rats were significantly impaired, and showed a chance level preference for the novel object, ~20% (yellow line). NP03 had no effect on the wild-type rats, as they were not different compared to those receiving the vehicle. NP03 treatment completely restored Novel Object Recognition in the Alzheimer transgenic rats. WT Veh n = 9; WT NP03 n = 9; AD Veh n = 8; AD NP03 n = 9. Data represent mean ± SEM. * = $p < 0.05$, Tukey's *post hoc* for multiple comparisons.

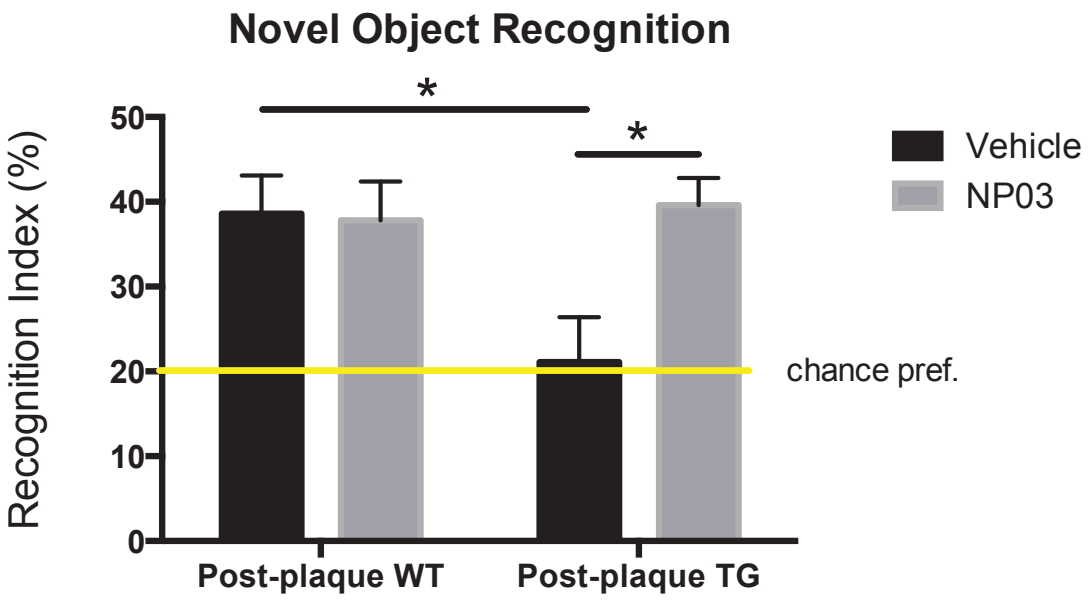

Figure 6-2: Cognitive recovery in Alzheimer transgenic rats of 15-months of age treated NP03

THESIS REFERENCES

Agostinho P, Cunha RA, Oliveira C. 2010. Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. Curr Pharm Des 16:2766-2778.

Aimone JB, Deng W, Gage FH. 2011. Resolving new memories: a critical look at the dentate gyrus, adult neurogenesis, and pattern separation. Neuron 70:589-596.

Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang HL, Wheeler AL, Guskjolen A, Niibori Y, Shoji H, Ohira K, Richards BA, Miyakawa T, Josselyn SA, Frankland PW. 2014. Hippocampal Neurogenesis Regulates Forgetting During Adulthood and Infancy. Science 344:598-602.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. 2000. Inflammation and Alzheimer's disease. Neurobiol Aging 21:383-421.

Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S, Markesbery WR. 1998. The expression of key oxidative stress-handling genes in different brain regions in Alzheimer's disease. J Mol Neurosci 11:151-164.

Altarejos JY, Goebel N, Conkright MD, Inoue H, Xie J, Arias CM, Sawchenko PE, Montminy M. 2008. The Creb1 coactivator Crtc1 is required for energy balance and fertility. Nat Med 14:1112-1117.

Altman J, Das GD. 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Altman J, Das GD. 1965. Post-natal origin of microneurones in the rat brain. Nature 207:953-956.

Aluise CD, Miriyala S, Noel T, Sultana R, Jungsuwadee P, Taylor TJ, Cai J, Pierce WM, Vore M, Moscow JA, St Clair DK, Butterfield DA. 2011. 2-Mercaptoethane sulfonate prevents doxorubicin-induced plasma protein oxidation and TNF-alpha release: implications for the reactive oxygen species-mediated mechanisms of chemobrain. Free Radic Biol Med 50:1630-1638.

Alvarez G, Munoz-Montano JR, Satrustegui J, Avila J, Bogonez E, Diaz-Nido J. 1999. Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. FEBS Lett 453:260-264.

Alzheimer A. 1907. Über eine eigenartigen Erkrankung der Hirnrinde. Allg Z Psychiat 64:146-148.

Alzheimer A. 1911. über eigenartige Krankheitsfälle des späteren Alters. Zeitschrift Gesamte Neurol Psychiatr 4:356-385.

Azab AN, Shnaider A, Osher Y, Wang D, Bersudsky Y, Belmaker RH. 2015. Lithium nephrotoxicity. Int J Bipolar Disord 3:28.

Bacskai BJ, Kajdasz ST, McLellan ME, Games D, Seubert P, Schenk D, Hyman BT. 2002. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. J Neurosci 22:7873-7878.

Baranano DE, Snyder SH. 2001. Neural roles for heme oxygenase: contrasts to nitric oxide synthase. Proc Natl Acad Sci U S A 98:10996-11002.

Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med 6:916-919.

Barone E, Cenini G, Di Domenico F, Noel T, Wang C, Perluigi M, St Clair DK, Butterfield DA. 2015. Basal brain oxidative and nitrative stress levels are finely regulated by the interplay between superoxide dismutase 2 and p53. J Neurosci Res 93:1728-1739.

Basselin M, Villacreses NE, Lee HJ, Bell JM, Rapoport SI. 2007. Chronic lithium administration attenuates up-regulated brain arachidonic acid metabolism in a rat model of neuroinflammation. J Neurochem 102:761-772.

Bate C, Kempster S, Last V, Williams A. 2006. Interferon-gamma increases neuronal death in response to amyloid-beta1-42. J Neuroinflammation 3:7.

Bateman RJ, Aisen PS, De Strooper B, Fox NC, Lemere CA, Ringman JM, Salloway S, Sperling RA, Windisch M, Xiong C. 2011. Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease. Alzheimers Res Ther 3:1.

Bateman RJ, Xiong C, Benzinger TL, Fagan AM, Goate A, Fox NC, Marcus DS, Cairns NJ, Xie X, Blazey TM, Holtzman DM, Santacruz A, Buckles V, Oliver A, Moulder K, Aisen PS, Ghetti B, Klunk WE, McDade E, Martins RN, Masters CL, Mayeux R, Ringman JM, Rossor MN, Schofield PR, Sperling RA, Salloway S, Morris JC. 2012. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. New Engl J Med 367:795-804.

Baumketner A, Krone MG, Shea JE. 2008. Role of the familial Dutch mutation E22Q in the folding and aggregation of the 15-28 fragment of the Alzheimer amyloid-beta protein. Proc Natl Acad Sci U S A 105:6027-6032.

Bear MF, Singer W. 1986. Modulation of visual cortical plasticity by acetylcholine and noradrenaline. Nature 320:172-176.

Bech P, Thomsen J, Prytz S, Vendsborg PB, Zilstorff K, Rafaelsen OJ. 1979. The profile and severity of lithium-induced side effects in mentally healthy subjects. Neuropsychobiology 5:160-166.

Becker E, Giacobini E. 1988. Mechanisms of cholinesterase inhibition in senile dementia of the Alzheimer type. Drug Dev Res 12:163-195.

Becker JT, Boller F, Saxton J, McGonigle-Gibson KL. 1987. Normal rates of forgetting of verbal and non-verbal material in Alzheimer's disease. Cortex 23:59-72.

Bell KF, Zheng L, Fahrenholz F, Cuello AC. 2008. ADAM-10 over-expression increases cortical synaptogenesis. Neurobiol Aging 29:554-565.

Belmaker RH. 2004. Bipolar disorder. New Engl J Med 351:476-486.

Benito E, Barco A. 2010. CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci.

Bennett DA, Schneider JA, Buchman AS, Barnes LL, Boyle PA, Wilson RS. 2012. Overview and findings from the rush Memory and Aging Project. Curr Alzheimer Res 9:646-663.

Berchtold NC, Cotman CW. 1998. Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s. Neurobiol Aging 19:173-189.

Bergman A, Religa D, Karlstrom H, Laudon H, Winblad B, Lannfelt L, Lundkvist J, Naslund J. 2003. APP intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations. Exp Cell Res 287:1-9.

Bering EA, Jr. 1955. Studies on the role of the choroid plexus in tracer exchanges between blood and cerebrospinal fluid. J Neurosurg 12:385-392.

Berridge MJ, Downes CP, Hanley MR. 1989. Neural and developmental actions of lithium: a unifying hypothesis. Cell 59:411-419.

Beyreuther K, Masters CL. 1991. Amyloid precursor protein (APP) and beta A4 amyloid in the etiology of Alzheimer's disease: precursor-product relationships in the derangement of neuronal function. Brain Pathol 1:241-251.

Bhat R, Xue Y, Berg S, Hellberg S, Ormo M, Nilsson Y, Radesater AC, Jerning E, Markgren PO, Borgegard T, Nylof M, Gimenez-Cassina A, Hernandez F, Lucas JJ, Diaz-Nido J, Avila J. 2003. Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. J Biol Chem 278:45937-45945.

Bialas AR, Stevens B. 2013. TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. Nat Neurosci 16:1773-1782.

Bien-Ly N, Gillespie AK, Walker D, Yoon SY, Huang Y. 2012. Reducing human apolipoprotein E levels attenuates age-dependent Abeta accumulation in mutant human amyloid precursor protein transgenic mice. J Neurosci 32:4803-4811.

Binder JR, Frost JA, Hammeke TA, Bellgowan PS, Rao SM, Cox RW. 1999. Conceptual processing during the conscious resting state. A functional MRI study. J Cogn Neurosci 11:80-95.

Bird TD, Lampe TH, Nemens EJ, Miner GW, Sumi SM, Schellenberg GD. 1988. Familial Alzheimer's disease in American descendants of the Volga Germans: probable genetic founder effect. Ann Neurol 23:25-31.

Biswal B, Yetkin FZ, Haughton VM, Hyde JS. 1995. Functional connectivity in the motor cortex of resting human brain using echo-planar MRI. Magn Reson Med 34:537-541.

Bittinger MA, McWhinnie E, Meltzer J, Iourgenko V, Latario B, Liu X, Chen CH, Song C, Garza D, Labow M. 2004. Activation of cAMP response element-mediated gene expression by regulated nuclear transport of TORC proteins. Curr Biol 14:2156-2161.

Blackwell AD, Sahakian BJ, Vesey R, Semple JM, Robbins TW, Hodges JR. 2004. Detecting dementia: novel neuropsychological markers of preclinical Alzheimer's disease. Dement Geriatr Cogn Disord 17:42-48.

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.

Block ML, Zecca L, Hong JS. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69.

Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM. 2009. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci U S A 106:13594-13599.

Boche D, Zotova E, Weller RO, Love S, Neal JW, Pickering RM, Wilkinson D, Holmes C, Nicoll JA. 2008. Consequence of Abeta immunization on the vasculature of human Alzheimer's disease brain. Brain 131:3299-3310.

Boer U, Eglins J, Krause D, Schnell S, Schofl C, Knepel W. 2007. Enhancement by lithium of cAMP-induced CRE/CREB-directed gene transcription conferred by TORC on the CREB basic leucine zipper domain. Biochem J 408:69-77.

Bohrmann B, Baumann K, Benz J, Gerber F, Huber W, Knoflach F, Messer J, Oroszlan K, Rauchenberger R, Richter WF, Rothe C, Urban M, Bardroff M, Winter M, Nordstedt

C, Loetscher H. 2012. Gantenerumab: a novel human anti-Abeta antibody demonstrates sustained cerebral amyloid-beta binding and elicits cell-mediated removal of human amyloid-beta. J Alzheimers Dis 28:49-69.

Boissonneault V, Filali M, Lessard M, Relton J, Wong G, Rivest S. 2009. Powerful beneficial effects of macrophage colony-stimulating factor on beta-amyloid deposition and cognitive impairment in Alzheimer's disease. Brain 132:1078-1092.

Bonni A, Ginty DD, Dudek H, Greenberg ME. 1995. Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. Mol Cell Neurosci 6:168-183.

Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS. 1996. Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. Neuron 17:1005-1013.

Bormann J. 1989. Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels. Eur J Pharmacol 166:591-592.

Bouchon A, Dietrich J, Colonna M. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol 164:4991-4995.

Bouchon A, Hernandez-Munain C, Cella M, Colonna M. 2001. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. J Exp Med 194:1111-1122.

Bowen DM, Smith CB, White P, Davison AN. 1976. Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. Brain 99:459-496.

Braak H, Braak E. 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 82:239-259.

Braak H, Braak E. 1995. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging 16:271-278.

Bredesen DE. 2014. Reversal of cognitive decline: a novel therapeutic program. Aging (Milano) 6:707-717.

Breitner JC. 1996. The role of anti-inflammatory drugs in the prevention and treatment of Alzheimer's disease. Annu Rev Med 47:401-411.

Breuillaud L, Rossetti C, Meylan EM, Merinat C, Halfon O, Magistretti PJ, Cardinaux JR. 2012. Deletion of CREB-regulated transcription coactivator 1 induces pathological aggression, depression-related behaviors, and neuroplasticity genes dysregulation in mice. Biol Psychiatry 72:528-536.

Bu G. 2009. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. Nat Rev Neurosci 10:333-344.

Buchman AS, Bennett DA. 2011. Loss of motor function in preclinical Alzheimer's disease. Expert Review of neurotherapeutics 11:665-676.

Bullock BP, Habener JF. 1998. Phosphorylation of the cAMP response element binding protein CREB by cAMP-dependent protein kinase A and glycogen synthase kinase-3 alters DNA-binding affinity, conformation, and increases net charge. Biochemistry (Mosc) 37:3795-3809.

Bussey TJ, Holmes A, Lyon L, Mar AC, McAllister KA, Nithianantharajah J, Oomen CA, Saksida LM. 2012. New translational assays for preclinical modelling of cognition in schizophrenia: the touchscreen testing method for mice and rats. Neuropharmacology 62:1191-1203.

Bussey TJ, Muir JL, Everitt BJ, Robbins TW. 1997. Triple dissociation of anterior cingulate, posterior cingulate, and medial frontal cortices on visual discrimination tasks using a touchscreen testing procedure for the rat. Behav Neurosci 111:920-936.

Butterfield DA. 1997. beta-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. Chem Res Toxicol 10:495-506.

Butterfield DA, Howard B, Yatin S, Koppal T, Drake J, Hensley K, Aksenov M, Aksenova M, Subramaniam R, Varadarajan S, Harris-White ME, Pedigo NW, Jr., Carney JM. 1999. Elevated oxidative stress in models of normal brain aging and Alzheimer's disease. Life Sci 65:1883-1892.

Butterfield DA, Kanski J. 2002. Methionine residue 35 is critical for the oxidative stress and neurotoxic properties of Alzheimer's amyloid beta-peptide 1-42. Peptides 23:1299-1309.

Butterfield DA, Reed T, Newman SF, Sultana R. 2007. Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. Free Radic Biol Med 43:658-677.

Butterfield DA, Reed TT, Perluigi M, De Marco C, Coccia R, Keller JN, Markesbery WR, Sultana R. 2007. Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease. Brain Res 1148:243-248.

Butterfield DA, Swomley AM, Sultana R. 2013. Amyloid beta-peptide (1-42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. Antioxid Redox Signal 19:823-835.

Cade JF. 1949. Lithium salts in the treatment of psychotic excitement. Med J Aust 2:349-352.

Cade JF. 1999. John Frederick Joseph Cade: family memories on the occasion of the 50th anniversary of his discovery of the use of lithium in mania. 1949. Aust N Z J Psychiatry 33:615-618.

Cardinal RN, Aitken MR. 2010. Whisker: a client-server high-performance multimedia research control system. Behav Res Methods 42:1059-1071.

Cardinaux JR, Notis JC, Zhang Q, Vo N, Craig JC, Fass DM, Brennan RG, Goodman RH. 2000. Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. Mol Cell Biol 20:1546-1552.

Ch'ng TH, Uzgil B, Lin P, Avliyakulov NK, O'Dell TJ, Martin KC. 2012. Activity-dependent transport of the transcriptional coactivator CRTC1 from synapse to nucleus. Cell 150:207-221.

Cha-Molstad H, Keller DM, Yochum GS, Impey S, Goodman RH. 2004. Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. Proc Natl Acad Sci U S A 101:13572-13577.

Chakrabarty P, Li A, Ceballos-Diaz C, Eddy JA, Funk CC, Moore B, DiNunno N, Rosario AM, Cruz PE, Verbeeck C, Sacino A, Nix S, Janus C, Price ND, Das P, Golde TE. 2015. IL-10 alters immunoproteostasis in APP mice, increasing plaque burden and worsening cognitive behavior. Neuron 85:519-533.

Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, et al. 1991. Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. Nature 353:844-846.

Chaturvedi RK, Hennessey T, Johri A, Tiwari SK, Mishra D, Agarwal S, Kim YS, Beal MF. 2012. Transducer of regulated CREB-binding proteins (TORCs) transcription and function is impaired in Huntington's disease. Hum Mol Genet 21:3474-3488.

Chen G, Rajkowska G, Du F, Seraji-Bozorgzad N, Manji HK. 2000. Enhancement of hippocampal neurogenesis by lithium. J Neurochem 75:1729-1734.

Chen HS, Lipton SA. 2005. Pharmacological implications of two distinct mechanisms of interaction of memantine with N-methyl-D-aspartate-gated channels. J Pharmacol Exp Ther 314:961-971.

Chen HS, Lipton SA. 2006. The chemical biology of clinically tolerated NMDA receptor antagonists. J Neurochem 97:1611-1626.

Chen HS, Pellegrini JW, Aggarwal SK, Lei SZ, Warach S, Jensen FE, Lipton SA. 1992. Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. J Neurosci 12:4427-4436.

Cherry JD, Olschowka JA, O'Banion MK. 2015. Arginase 1+ microglia reduce Abeta plaque deposition during IL-1beta-dependent neuroinflammation. J Neuroinflammation 12:203.

Chudasama Y, Bussey TJ, Muir JL. 2001. Effects of selective thalamic and prelimbic cortex lesions on two types of visual discrimination and reversal learning. Eur J Neurosci 14:1009-1020.

Chudasama Y, Robbins TW. 2003. Dissociable contributions of the orbitofrontal and infralimbic cortex to pavlovian autoshaping and discrimination reversal learning: further evidence for the functional heterogeneity of the rodent frontal cortex. J Neurosci 23:8771-8780.

Chudasama Y, Robbins TW. 2006. Functions of frontostriatal systems in cognition: comparative neuropsychopharmacological studies in rats, monkeys and humans. Biol Psychol 73:19-38.

Citron M. 2010. Alzheimer's disease: strategies for disease modification. Nat Rev Drug Discov 9:387-398.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Cobb CA, Cole MP. 2015. Oxidative and nitrative stress in neurodegeneration. Neurobiol Dis 84:4-21.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem Biol 7:793-803.

Colonna M. 2003. TREMs in the immune system and beyond. Nat Rev Immunol 3:445-453.

Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M. 2003. TORCs: transducers of regulated CREB activity. Mol Cell 12:413-423.

Conkright MD, Guzman E, Flechner L, Su AI, Hogenesch JB, Montminy M. 2003. Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness. Mol Cell 11:1101-1108.

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.

Cuello AC, Allard S, Ferretti MT. 2012. Evidence for the accumulation of Abeta immunoreactive material in the human brain and in transgenic animal models. Life Sci 91:1141-1147.

D'Hooge R, De Deyn PP. 2001. Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev 36:60-90.

Das U, Wang L, Ganguly A, Saikia JM, Wagner SL, Koo EH, Roy S. 2015. Visualizing APP and BACE-1 approximation in neurons yields insight into the amyloidogenic pathway. Nat Neurosci.

Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER. 1991. cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase. Proc Natl Acad Sci U S A 88:5061-5065.

Davies P, Maloney AJ. 1976. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2:1403.

Davis KL, Mohs RC, Tinklenberg JR. 1979. Enhancement of memory by physostigmine. New Engl J Med 301:946.

De Ferrari GV, Inestrosa NC. 2000. Wnt signaling function in Alzheimer's disease. Brain Res Rev 33:1-12.

de Sousa RT, van de Bilt MT, Diniz BS, Ladeira RB, Portela LV, Souza DO, Forlenza OV, Gattaz WF, Machado-Vieira R. 2011. Lithium increases plasma brain-derived neurotrophic factor in acute bipolar mania: a preliminary 4-week study. Neurosci Lett 494:54-56.

de Sousa RT, Zarate CA, Jr., Zanetti MV, Costa AC, Talib LL, Gattaz WF, Machado-Vieira R. 2014. Oxidative stress in early stage Bipolar Disorder and the association with response to lithium. J Psychiatr Res 50:36-41.

Deane R, Du Yan S, Submamaryan RK, LaRue B, Jovanovic S, Hogg E, Welch D, Manness L, Lin C, Yu J, Zhu H, Ghiso J, Frangione B, Stern A, Schmidt AM, Armstrong DL, Arnold B, Liliensiek B, Nawroth P, Hofman F, Kindy M, Stern D, Zlokovic B. 2003. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. Nat Med 9:907-913.

Deane R, Wu Z, Sagare A, Davis J, Du Yan S, Hamm K, Xu F, Parisi M, LaRue B, Hu HW, Spijkers P, Guo H, Song X, Lenting PJ, Van Nostrand WE, Zlokovic BV. 2004. LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. Neuron 43:333-344.

DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. 2001. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 98:8850-8855.

Dewachter I, Van Dorpe J, Smeijers L, Gilis M, Kuiperi C, Laenen I, Caluwaerts N, Moechars D, Checler F, Vanderstichele H, Van Leuven F. 2000. Aging increased amyloid peptide and caused amyloid plaques in brain of old APP/V717I transgenic mice by a different mechanism than mutant presenilin1. J Neurosci 20:6452-6458.

Di Domenico F, Pupo G, Giraldo E, Badia MC, Monllor P, Lloret A, Eugenia Schinina M, Giorgi A, Cini C, Tramutola A, Butterfield DA, Vina J, Perluigi M. 2016. Oxidative signature of cerebrospinal fluid from mild cognitive impairment and Alzheimer disease patients. Free Radic Biol Med 91:1-9.

Di Domenico F, Pupo G, Giraldo E, Lloret A, Badia MC, Schinina ME, Giorgi A, Butterfield DA, Vina J, Perluigi M. 2016. Autoantibodies Profile in Matching CSF and Serum from AD and aMCI patients: Potential Pathogenic Role and Link to Oxidative Damage. Curr Alzheimer Res 13:112-122.

Dias R, Robbins TW, Roberts AC. 1996. Dissociation in prefrontal cortex of affective and attentional shifts. Nature 380:69-72.

Dickson DW, Lee SC, Mattiace LA, Yen SH, Brosnan C. 1993. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia 7:75-83.

Dietrich JB, Takemori H, Grosch-Dirrig S, Bertorello A, Zwiller J. 2011. Cocaine induces the expression of MEF2C transcription factor in rat striatum through activation of SIK1 and phosphorylation of the histone deacetylase HDAC5. Synapse.

Do Carmo S, Cuello AC. 2013. Modeling Alzheimer's disease in transgenic rats. Mol Neurodegener 8:37.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046-5061.

Donohue MC, Sperling RA, Salmon DP, Rentz DM, Raman R, Thomas RG, Weiner M, Aisen PS. 2014. The Preclinical Alzheimer Cognitive Composite: Measuring Amyloid-Related Decline. JAMA neurology.

Drachman DA, Leavitt J. 1974. Human memory and the cholinergic system. A relationship to aging? Arch Neurol 30:113-121.

Drew LJ, Kheirbek MA, Luna VM, Denny CA, Cloidt MA, Wu MV, Jain S, Scharfman HE, Hen R. 2015. Activation of local inhibitory circuits in the dentate gyrus by adult-born neurons. Hippocampus.

Du K, Montminy M. 1998. CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 273:32377-32379.

Duan X, Kang E, Liu CY, Ming GL, Song H. 2008. Development of neural stem cell in the adult brain. Curr Opin Neurobiol 18:108-115.

Dubois B, Hampel H, Feldman HH, Scheltens P, Aisen P, Andrieu S, Bakardjian H, Benali H, Bertram L, Blennow K, Broich K, Cavedo E, Crutch S, Dartigues JF, Duyckaerts C, Epelbaum S, Frisoni GB, Gauthier S, Genthon R, Gouw AA, Habert MO, Holtzman DM, Kivipelto M, Lista S, Molinuevo JL, O'Bryant SE, Rabinovici GD, Rowe C, Salloway S, Schneider LS, Sperling R, Teichmann M, Carrillo MC, Cummings J, Jack CR, Jr. 2016. Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria. Alzheimers Dement 12:292-323.

Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D, Morgan D, Gordon MN, Holcomb L, Refolo L, Zenk B, Hardy J, Younkin S. 1996. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. Nature 383:710-713.

Ekstrom A. 2010. How and when the fMRI BOLD signal relates to underlying neural activity: the danger in dissociation. Brain Res Rev 62:233-244.

Ennaceur A, Delacour J. 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31:47-59.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR. 1994. Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J Biol Chem 269:15520-15527.

Espana J, Valero J, Minano-Molina AJ, Masgrau R, Martin E, Guardia-Laguarta C, Lleo A, Gimenez-Llort L, Rodriguez-Alvarez J, Saura CA. 2010. {beta}-Amyloid Disrupts

Activity-Dependent Gene Transcription Required for Memory through the CREB Coactivator CRTC1. J Neurosci 30:9402-9410.

Everitt BJ, Robbins TW. 1997. Central cholinergic systems and cognition. Annu Rev Psychol 48:649-684.

Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. 1997. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA 278:1349-1356.

Ferrer I, Boada Rovira M, Sanchez Guerra ML, Rey MJ, Costa-Jussa F. 2004. Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. Brain Pathol 14:11-20.

Ferretti MT, Allard S, Partridge V, Ducatenzeiler A, Cuello AC. 2012. Minocycline corrects early, pre-plaque neuroinflammation and inhibits BACE-1 in a transgenic model of Alzheimer's disease-like amyloid pathology. J Neuroinflammation 9:62.

Ferretti MT, Bruno MA, Ducatenzeiler A, Klein WL, Cuello AC. 2012. Intracellular Abeta-oligomers and early inflammation in a model of Alzheimer's disease. Neurobiol Aging 33:1329-1342.

Fillit H, Ding WH, Buee L, Kalman J, Altstiel L, Lawlor B, Wolf-Klein G. 1991. Elevated circulating tumor necrosis factor levels in Alzheimer's disease. Neurosci Lett 129:318-320.

Finsterwald C, Fiumelli H, Cardinaux JR, Martin JL. 2010. Regulation of dendritic development by BDNF requires activation of CRTC1 by glutamate. J Biol Chem 285:28587-28595.

Fiorentini A, Rosi MC, Grossi C, Luccarini I, Casamenti F. 2010. Lithium improves hippocampal neurogenesis, neuropathology and cognitive functions in APP mutant mice. PLoS One 5:e14382.

Fleischhacker WW, Buchgeher A, Schubert H. 1986. Memantine in the treatment of senile dementia of the Alzheimer type. Prog Neuropsychopharmacol Bol Psychiatry 10:87-93.

Forabosco P, Ramasamy A, Trabzuni D, Walker R, Smith C, Bras J, Levine AP, Hardy J, Pocock JM, Guerreiro R, Weale ME, Ryten M. 2013. Insights into TREM2 biology by network analysis of human brain gene expression data. Neurobiol Aging 34:2699-2714.

Forlenza OV, Diniz BS, Radanovic M, Santos FS, Talib LL, Gattaz WF. 2011. Disease-modifying properties of long-term lithium treatment for amnestic mild cognitive impairment: randomised controlled trial. Br J Psychiatry 198:351-356.

Frossi B, De Carli M, Daniel KC, Rivera J, Pucillo C. 2003. Oxidative stress stimulates IL-4 and IL-6 production in mast cells by an APE/Ref-1-dependent pathway. Eur J Immunol 33:2168-2177.

Fuster-Matanzo A, Llorens-Martin M, Hernandez F, Avila J. 2013. Role of neuroinflammation in adult neurogenesis and Alzheimer disease: therapeutic approaches. Mediators Inflamm 2013:260925.

Galeano P, Martino Adami PV, Do Carmo S, Blanco E, Rotondaro C, Capani F, Castano EM, Cuello AC, Morelli L. 2014. Longitudinal analysis of the behavioral phenotype in a novel transgenic rat model of early stages of Alzheimer's disease. Front Behav Neurosci 8:321.

Garzon DJ, Fahnestock M. 2007. Oligomeric amyloid decreases basal levels of brain-derived neurotrophic factor (BDNF) mRNA via specific downregulation of BDNF transcripts IV and V in differentiated human neuroblastoma cells. J Neurosci 27:2628-2635.

Gelenberg AJ, Jefferson JW. 1995. Lithium tremor. J Clin Psychiatry 56:283-287.

Germano C, Kinsella GJ. 2005. Working memory and learning in early Alzheimer's disease. Neuropsychol Rev 15:1-10.

Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg LH, Ruffolo SC, Thornberry NA, Xanthoudakis S, Zamboni RJ, Roy S, Nicholson DW. 1999. Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. Cell 97:395-406.

Geula C, Mesulam MM. 1989. Cortical cholinergic fibers in aging and Alzheimer's disease: a morphometric study. Neuroscience 33:469-481.

Geula C, Mesulam MM. 1996. Systematic regional variations in the loss of cortical cholinergic fibers in Alzheimer's disease. Cereb Cortex 6:165-177.

Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F, Orgogozo JM. 2005. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. Neurology 64:1553-1562.

Glenner GG, Wong CW. 1984. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem Biophys Res Commun 122:1131-1135.

Glenner GG, Wong CW. 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120:885-890.

Gliebus G, Rosso A, Lippa CF. 2009. Progranulin and beta-amyloid distribution: a case report of the brain from preclinical PS-1 mutation carrier. Am J Alzheiemrs Dis Other Demen 24:456-460.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704-706.

Goedert M. 1993. Tau protein and the neurofibrillary pathology of Alzheimer's disease. Trends Neurosci 16:460-465.

Goedert M, Ghetti B. 2007. Alois Alzheimer: his life and times. Brain Pathol 17:57-62.

Goedert M, Klug A, Crowther RA. 2006. Tau protein, the paired helical filament and Alzheimer's disease. J Alzheimers Dis 9:195-207.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA. 1989. Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

Gong CX, Iqbal K. 2008. Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease. Curr Med Chem 15:2321-2328.

Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal I, Iqbal K. 1995. Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. J Neurochem 65:732-738.

Gore F, Schwartz EC, Brangers BC, Aladi S, Stujenske JM, Likhtik E, Russo MJ, Gordon JA, Salzman CD, Axel R. 2015. Neural Representations of Unconditioned Stimuli in Basolateral Amygdala Mediate Innate and Learned Responses. Cell 162:134-145.

Graeber MB, Kosel S, Egensperger R, Banati RB, Muller U, Bise K, Hoff P, Moller HJ, Fujisawa K, Mehraein P. 1997. Rediscovery of the case described by Alois Alzheimer in 1911: historical, histological and molecular genetic analysis. Neurogenetics 1:73-80.

Grant MA, Lazo ND, Lomakin A, Condron MM, Arai H, Yamin G, Rigby AC, Teplow DB. 2007. Familial Alzheimer's disease mutations alter the stability of the amyloid beta-protein monomer folding nucleus. Proc Natl Acad Sci U S A 104:16522-16527.

Grant SM, Ducatenzeiler A, Szyf M, Cuello AC. 2000. Abeta immunoreactive material is present in several intracellular compartments in transfected, neuronally differentiated,

P19 cells expressing the human amyloid beta-protein precursor. J Alzheimers Dis 2:207-222.

Greicius MD, Srivastava G, Reiss AL, Menon V. 2004. Default-mode network activity distinguishes Alzheimer's disease from healthy aging: evidence from functional MRI. Proc Natl Acad Sci U S A 101:4637-4642.

Grimes CA, Jope RS. 2001. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. J Neurochem 78:1219-1232.

Grinberg YY, Dibbern ME, Levasseur VA, Kraig RP. 2013. Insulin-like growth factor-1 abrogates microglial oxidative stress and TNF-alpha responses to spreading depression. J Neurochem 126:662-672.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. 1986. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem 261:6084-6089.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. 1986. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 83:4913-4917.

Guerreiro R, Hardy J. 2013. TREM2 and neurodegenerative disease. New Engl J Med 369:1569-1570.

Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, Cruchaga C, Sassi C, Kauwe JS, Younkin S, Hazrati L, Collinge J, Pocock J, Lashley T, Williams J,

Lambert JC, Amouyel P, Goate A, Rademakers R, Morgan K, Powell J, St George-Hyslop P, Singleton A, Hardy J. 2013. TREM2 variants in Alzheimer's disease. New Engl J Med 368:117-127.

Guillot-Sestier MV, Doty KR, Gate D, Rodriguez J, Jr., Leung BP, Rezai-Zadeh K, Town T. 2015. Il10 deficiency rebalances innate immunity to mitigate Alzheimer-like pathology. Neuron 85:534-548.

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.

Hama E, Shirotani K, Masumoto H, Sekine-Aizawa Y, Aizawa H, Saido TC. 2001. Clearance of extracellular and cell-associated amyloid beta peptide through viral expression of neprilysin in primary neurons. J Biochem (Tokyo) 130:721-726.

Hamani C, Stone SS, Garten A, Lozano AM, Winocur G. 2011. Memory rescue and enhanced neurogenesis following electrical stimulation of the anterior thalamus in rats treated with corticosterone. Exp Neurol 232:100-104.

Hampel H, Ewers M, Burger K, Annas P, Mortberg A, Bogstedt A, Frolich L, Schroder J, Schonknecht P, Riepe MW, Kraft I, Gasser T, Leyhe T, Moller HJ, Kurz A, Basun H. 2009. Lithium trial in Alzheimer's disease: a randomized, single-blind, placebo-controlled, multicenter 10-week study. J Clin Psychiatry 70:922-931.

Hansson O, Zetterberg H, Buchhave P, Andreasson U, Londos E, Minthon L, Blennow K. 2007. Prediction of Alzheimer's disease using the CSF Abeta42/Abeta40 ratio in patients with mild cognitive impairment. Dement Geriatr Cogn Disord 23:316-320.

Hanyu H, Sato T, Shimizu S, Kanetaka H, Iwamoto T, Koizumi K. 2008. The effect of education on rCBF changes in Alzheimer's disease: a longitudinal SPECT study. Eur J Nucl Med Mol Imaging 35:2182-2190.

Hanzel CE, Pichet-Binette A, Pimentel LS, Iulita MF, Allard S, Ducatenzeiler A, Do Carmo S, Cuello AC. 2014. Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. Neurobiol Aging 35:2249–2262.

Hanzel CP-B, A; Pimentel, L.S.; Iulita, M.F.; Cuello, A.C. 2014. Neuronal driven pre-plaque inflammation in a transgenic rat model of Alzheimer's disease. Neurobiol Aging.

Hardy J, Allsop D. 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci 12:383-388.

Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hardy JA, Higgins GA. 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science 256:184-185.

Haughey NJ, Liu D, Nath A, Borchard AC, Mattson MP. 2002. Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal

precursor cells in culture, by amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease. Neuromolecular Med 1:125-135.

Hayslip J, Dressler EV, Weiss H, Taylor TJ, Chambers M, Noel T, Miriyala S, Keeney JT, Ren X, Sultana R, Vore M, Butterfield DA, St Clair D, Moscow JA. 2015. Plasma TNF-alpha and Soluble TNF Receptor Levels after Doxorubicin with or without Co-Administration of Mesna-A Randomized, Cross-Over Clinical Study. PloS One 10:e0124988.

Hebert LE, Bienias JL, McCann JJ, Scherr PA, Wilson RS, Evans DA. 2010. Upper and lower extremity motor performance and functional impairment in Alzheimer's disease. Am J Alzheimers Dis Other Demen 25:425-431.

Hecimovic S, Wang J, Dolios G, Martinez M, Wang R, Goate AM. 2004. Mutations in APP have independent effects on Abeta and CTFgamma generation. Neurobiol Dis 17:205-218.

Hedgepeth CM, Conrad LJ, Zhang J, Huang HC, Lee VM, Klein PS. 1997. Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev Biol 185:82-91.

Heinrich A, Boer U, Tzvetkov M, Oetjen E, Knepel W. 2009. Stimulation by lithium of the interaction between the transcription factor CREB and its co-activator TORC. Biosci Rep 29:77-87.

Heinrich A, von der Heyde AS, Boer U, Phu do T, Tzvetkov M, Oetjen E. 2013. Lithium enhances CRTC oligomer formation and the interaction between the CREB coactivators

CRTC and CBP--implications for CREB-dependent gene transcription. Cell Signal 25:113-125.

Hellstrom-Lindahl E, Viitanen M, Marutle A. 2009. Comparison of Abeta levels in the brain of familial and sporadic Alzheimer's disease. Neurochem Int 55:243-252.

Henderson DK, Gillespie RD. 1944. A Text-Book of Psychiatry: Oxford University Press.

Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, Van Leuven F. 2005. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. J Neuroinflammation 2:22.

Herz J. 2003. LRP: a bright beacon at the blood-brain barrier. J Clin Invest 112:1483-1485.

Herz J, Chen Y. 2006. Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci 7:850-859.

Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD, Kenny PJ. 2010. Striatal microRNA controls cocaine intake through CREB signalling. Nature 466:197-202.

Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW, Zotova E, Nicoll JA. 2008. Long-term effects of Abeta42

immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372:216-223.

Holsinger RM, Schnarr J, Henry P, Castelo VT, Fahnestock M. 2000. Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease. Mol Brain Res 76:347-354.

Hondius DC, van Nierop P, Li KW, Hoozemans JJ, van der Schors RC, van Haastert ES, van der Vies SM, Rozemuller AJ, Smit AB. 2016. Profiling the human hippocampal proteome at all pathologic stages of Alzheimer's disease. Alzheimers Dement.

Hong M, Zhukareva V, Vogelsberg-Ragaglia V, Wszolek Z, Reed L, Miller BI, Geschwind DH, Bird TD, McKeel D, Goate A, Morris JC, Wilhelmsen KC, Schellenberg GD, Trojanowski JQ, Lee VM. 1998. Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. Science 282:1914-1917.

Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, Merry KM, Shi Q, Rosenthal A, Barres BA, Lemere CA, Selkoe DJ, Stevens B. 2016. Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science 352:712-716.

Hutton M, Busfield F, Wragg M, Crook R, Perez-Tur J, Clark RF, Prihar G, Talbot C, Phillips H, Wright K, Baker M, Lendon C, Duff K, Martinez A, Houlden H, Nichols A, Karran E, Roberts G, Roques P, Rossor M, Venter JC, Adams MD, Cline RT, Phillips CA, Goate A, et al. 1996. Complete analysis of the presenilin 1 gene in early onset Alzheimer's disease. Neuroreport 7:801-805.

Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J, Adamson J, Lincoln S, Dickson D, Davies P, Petersen RC, Stevens M, de Graaff E, Wauters E, van Baren J, Hillebrand M, Joosse M, Kwon JM, Nowotny P, Che LK, Norton J, Morris JC, Reed LA, Trojanowski J, Basun H, Lannfelt L, Neystat M, Fahn S, Dark F, Tannenberg T, Dodd PR, Hayward N, Kwok JB, Schofield PR, Andreadis A, Snowden J, Craufurd D, Neary D, Owen F, Oostra BA, Hardy J, Goate A, van Swieten J, Mann D, Lynch T, Heutink P. 1998. Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393:702-705.

Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH. 2004. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119:1041-1054.

in t' Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH. 2001. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. New Engl J Med 345:1515-1521.

Iourgenko V, Zhang W, Mickanin C, Daly I, Jiang C, Hexham JM, Orth AP, Miraglia L, Meltzer J, Garza D, Chirn GW, McWhinnie E, Cohen D, Skelton J, Terry R, Yu Y, Bodian D, Buxton FP, Zhu J, Song C, Labow MA. 2003. Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. Proc Natl Acad Sci U S A 100:12147-12152.

Iqbal K, Grundke-Iqbal I, Wisniewski HM, Terry RD. 1978. Chemical relationship of the paired helical filaments of Alzheimer's dementia to normal human neurofilaments and neurotubules. Brain Res 142:321-332.

Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D. 1989. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J Neuroimmunol 24:173-182.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. 1998. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 57:1-9.

Iulita MF, Allard S, Richter L, Munter LM, Ducatenzeiler A, Weise C, Do Carmo S, Klein WL, Multhaup G, Cuello AC. 2014. Intracellular Abeta pathology and early cognitive impairments in a transgenic rat model overexpressing human amyloid precursor protein: a multidimensional study. Acta Neuropathol Commun 2:61.

Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC. 2001. Metabolic regulation of brain Abeta by neprilysin. Science 292:1550-1552.

Jack CR, Jr., Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. 2010. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol 9:119-128.

Jack CR, Jr., Knopman DS, Weigand SD, Wiste HJ, Vemuri P, Lowe V, Kantarci K, Gunter JL, Senjem ML, Ivnik RJ, Roberts RO, Rocca WA, Boeve BF, Petersen RC. 2012. An operational approach to National Institute on Aging-Alzheimer's Association criteria for preclinical Alzheimer disease. Ann Neurol 71:765-775.

Jack CR, Jr., Wiste HJ, Knopman DS, Vemuri P, Mielke MM, Weigand SD, Senjem ML, Gunter JL, Lowe V, Gregg BE, Pankratz VS, Petersen RC. 2014. Rates of beta-amyloid accumulation are independent of hippocampal neurodegeneration. Neurology 82:1605-1612.

Jagannath A, Butler R, Godinho SI, Couch Y, Brown LA, Vasudevan SR, Flanagan KC, Anthony D, Churchill GC, Wood MJ, Steiner G, Ebeling M, Hossbach M, Wettstein JG, Duffield GE, Gatti S, Hankins MW, Foster RG, Peirson SN. 2013. The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. Cell 154:1100-1111.

Janowski M, Walczak P, Kropiwnicki T, Jurkiewicz E, Domanska-Janik K, Bulte JW, Lukomska B, Roszkowski M. 2014. Long-term MRI cell tracking after intraventricular delivery in a patient with global cerebral ischemia and prospects for magnetic navigation of stem cells within the CSF. PloS One 9:e97631.

Jayadev S, Leverenz JB, Steinbart E, Stahl J, Klunk W, Yu CE, Bird TD. 2010. Alzheimer's disease phenotypes and genotypes associated with mutations in presenilin 2. Brain 133:1143-1154.

Jeong H, Cohen DE, Cui L, Supinski A, Savas JN, Mazzulli JR, Yates JR, 3rd, Bordone L, Guarente L, Krainc D. 2012. Sirt1 mediates neuroprotection from mutant huntingtin by activation of the TORC1 and CREB transcriptional pathway. Nat Med 18:159-165.

Ji Y, Gong Y, Gan W, Beach T, Holtzman DM, Wisniewski T. 2003. Apolipoprotein E isoform-specific regulation of dendritic spine morphology in apolipoprotein E transgenic mice and Alzheimer's disease patients. Neuroscience 122:305-315.

Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, Andreassen OA, Engedal K, Ulstein I, Djurovic S, Ibrahim-Verbaas C, Hofman A, Ikram MA, van Duijn CM, Thorsteinsdottir U, Kong A, Stefansson K. 2013. Variant of TREM2 associated with the risk of Alzheimer's disease. New Engl J Med 368:107-116.

Jope RS, Johnson GV. 2004. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29:95-102.

Jordao JF, Thevenot E, Markham-Coultes K, Scarcelli T, Weng YQ, Xhima K, O'Reilly M, Huang Y, McLaurin J, Hynynen K, Aubert I. 2013. Amyloid-beta plaque reduction, endogenous antibody delivery and glial activation by brain-targeted, transcranial focused ultrasound. Exp Neurol 248:16-29.

Josselyn SA. 2010. Continuing the search for the engram: examining the mechanism of fear memories. J Psychiatry Neurosci 35:221-228.

Josselyn SA, Kohler S, Frankland PW. 2015. Finding the engram. Nat Rev Neurosci 16:521-534.

Josselyn SA, Shi C, Carlezon WA, Jr., Neve RL, Nestler EJ, Davis M. 2001. Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J Neurosci 21:2404-2412.

Kandel ER. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030-1038.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325:733-736.

Kaplan MS, Hinds JW. 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094.

Karran E, Mercken M, De Strooper B. 2011. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. Nat Rev Drug Discov 10:698-712.

Kawahara K, Suenobu M, Yoshida A, Koga K, Hyodo A, Ohtsuka H, Kuniyasu A, Tamamaki N, Sugimoto Y, Nakayama H. 2012. Intracerebral microinjection of interleukin-4/interleukin-13 reduces beta-amyloid accumulation in the ipsilateral side and improves cognitive deficits in young amyloid precursor protein 23 mice. Neuroscience 207:243-260.

Kempermann G, van Praag H, Gage FH. 2000. Activity-dependent regulation of neuronal plasticity and self repair. Prog Brain Res 127:35-48.

Kessing LV, Sondergard L, Forman JL, Andersen PK. 2008. Lithium treatment and risk of dementia. Arch Gen Psychiatry 65:1331-1335.

Khairova R, Pawar R, Salvadore G, Juruena MF, de Sousa RT, Soeiro-de-Souza MG, Salvador M, Zarate CA, Gattaz WF, Machado-Vieira R. 2012. Effects of lithium on oxidative stress parameters in healthy subjects. Mol Med Rep 5:680-682.

Kiddle SJ, Steves CJ, Mehta M, Simmons A, Xu X, Newhouse S, Sattlecker M, Ashton NJ, Bazenet C, Killick R, Adnan J, Westman E, Nelson S, Soininen H, Kloszewska I, Mecocci P, Tsolaki M, Vellas B, Curtis C, Breen G, Williams SC, Lovestone S, Spector TD, Dobson RJ. 2015. Plasma protein biomarkers of Alzheimer's disease endophenotypes in asymptomatic older twins: early cognitive decline and regional brain volumes. Transl Psychiatry 5:e584.

Kim J, Jiang H, Park S, Eltorai AE, Stewart FR, Yoon H, Basak JM, Finn MB, Holtzman DM. 2011. Haploinsufficiency of human APOE reduces amyloid deposition in a mouse model of amyloid-beta amyloidosis. J Neurosci 31:18007-18012.

Kim W, Pouratian N. 2014. Deep brain stimulation for Tourette syndrome. Neurosurg Clin N Am 25:117-135.

King MK, Pardo M, Cheng Y, Downey K, Jope RS, Beurel E. 2014. Glycogen synthase kinase-3 inhibitors: Rescuers of cognitive impairments. Pharmacol Ther 141:1-12.

Klegeris A, McGeer PL. 2005. Non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents in the treatment of neurodegenerative disease. Curr Alzheimer Res 2:355-365.

Klein PS, Melton DA. 1996. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci U S A 93:8455-8459.

Klunemann HH, Fronhofer W, Wurster H, Fischer W, Ibach B, Klein HE. 2002. Alzheimer's second patient: Johann F. and his family. Ann Neurol 52:520-523.

Klunk WE, Engler H, Nordberg A, Wang Y, Blomqvist G, Holt DP, Bergstrom M, Savitcheva I, Huang GF, Estrada S, Ausen B, Debnath ML, Barletta J, Price JC, Sandell J, Lopresti BJ, Wall A, Koivisto P, Antoni G, Mathis CA, Langstrom B. 2004. Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann Neurol 55:306-319.

Klunk WE, Price JC, Mathis CA, Tsopelas ND, Lopresti BJ, Ziolko SK, Bi W, Hoge JA, Cohen AD, Ikonomovic MD, Saxton JA, Snitz BE, Pollen DA, Moonis M, Lippa CF, Swearer JM, Johnson KA, Rentz DM, Fischman AJ, Aizenstein HJ, DeKosky ST. 2007. Amyloid deposition begins in the striatum of presenilin-1 mutation carriers from two unrelated pedigrees. J Neurosci 27:6174-6184.

Knopman DS, Jack CR, Jr., Wiste HJ, Weigand SD, Vemuri P, Lowe V, Kantarci K, Gunter JL, Senjem ML, Ivnik RJ, Roberts RO, Boeve BF, Petersen RC. 2012. Short-term clinical outcomes for stages of NIA-AA preclinical Alzheimer disease. Neurology 78:1576-1582.

Koch W, Teipel S, Mueller S, Benninghoff J, Wagner M, Bokde AL, Hampel H, Coates U, Reiser M, Meindl T. 2012. Diagnostic power of default mode network resting state fMRI in the detection of Alzheimer's disease. Neurobiol Aging 33:466-478.

Kok E, Haikonen S, Luoto T, Huhtala H, Goebeler S, Haapasalo H, Karhunen PJ. 2009. Apolipoprotein E-dependent accumulation of Alzheimer disease-related lesions begins in middle age. Ann Neurol 65:650-657.

Komiya T, Coxon A, Park Y, Chen WD, Zajac-Kaye M, Meltzer P, Karpova T, Kaye FJ. 2010. Enhanced activity of the CREB co-activator Crtc1 in LKB1 null lung cancer. Oncogene 29:1672-1680.

Kopelman MD. 1985. Rates of forgetting in Alzheimer-type dementia and Korsakoff's syndrome. Neuropsychologia 23:623-638.

Koppal T, Drake J, Yatin S, Jordan B, Varadarajan S, Bettenhausen L, Butterfield DA. 1999. Peroxynitrite-induced alterations in synaptosomal membrane proteins: insight into oxidative stress in Alzheimer's disease. J Neurochem 72:310-317.

Kornhauser JM, Cowan CW, Shaywitz AJ, Dolmetsch RE, Griffith EC, Hu LS, Haddad C, Xia Z, Greenberg ME. 2002. CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. Neuron 34:221-233.

Kornhuber J, Mack-Burkhardt F, Riederer P, Hebenstreit GF, Reynolds GP, Andrews HB, Beckmann H. 1989. [3H]MK-801 binding sites in postmortem brain regions of schizophrenic patients. J Neural Transm 77:231-236.

Kovacs KA, Steullet P, Steinmann M, Do KQ, Magistretti PJ, Halfon O, Cardinaux JR. 2007. TORC1 is a calcium- and cAMP-sensitive coincidence detector involved in hippocampal long-term synaptic plasticity. Proc Natl Acad Sci U S A 104:4700-4705.

Kurlowicz L, Wallace M. 1999. The Mini-Mental State Examination (MMSE). J Gerontol Nurs 25:8-9.

Lachance PE, Chaudhuri A. 2004. Microarray analysis of developmental plasticity in monkey primary visual cortex. J Neurochem 88:1455-1469.

LaFerla FM, Green KN, Oddo S. 2007. Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8:499-509.

Lalonde J, Lachance PE, Chaudhuri A. 2009. Developmental and activity-dependent genomic occupancy profiles of CREB in monkey area V1. Genes Brain Behav 8:149-160.

Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI, Markesbery WR, Butterfield DA. 2001. The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1-42. J Neurochem 78:413-416.

Laxton AW, Tang-Wai DF, McAndrews MP, Zumsteg D, Wennberg R, Keren R, Wherrett J, Naglie G, Hamani C, Smith GS, Lozano AM. 2010. A phase I trial of deep brain stimulation of memory circuits in Alzheimer's disease. Ann Neurol 68:521-534.

Lazo ND, Grant MA, Condron MC, Rigby AC, Teplow DB. 2005. On the nucleation of amyloid beta-protein monomer folding. Protein Sci 14:1581-1596.

Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM, Eisenbrand G, Meijer L. 2001. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? J Biol Chem 276:251-260.

Lee B, Butcher GQ, Hoyt KR, Impey S, Obrietan K. 2005. Activity-dependent neuroprotection and cAMP response element-binding protein (CREB): kinase coupling, stimulus intensity, and temporal regulation of CREB phosphorylation at serine 133. J Neurosci 25:1137-1148.

Lee JP, Jeyakumar M, Gonzalez R, Takahashi H, Lee PJ, Baek RC, Clark D, Rose H, Fu G, Clarke J, McKercher S, Meerloo J, Muller FJ, Park KI, Butters TD, Dwek RA, Schwartz P, Tong G, Wenger D, Lipton SA, Seyfried TN, Platt FM, Snyder EY. 2007. Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat Med 13:439-447.

Lee KS, Chung JH, Choi TK, Suh SY, Oh BH, Hong CH. 2009. Peripheral cytokines and chemokines in Alzheimer's disease. Dement Geriatr Cogn Disord 28:281-287.

Leinenga G, Gotz J. 2015. Scanning ultrasound removes amyloid-beta and restores memory in an Alzheimer's disease mouse model. Sci Transl Med 7:278ra233.

Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP, Selkoe DJ. 2003. Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. Neuron 40:1087-1093.

Leon WC, Canneva F, Partridge V, Allard S, Ferretti MT, DeWilde A, Vercauteren F, Atifeh R, Ducatenzeiler A, Klein W, Szyf M, Alhonen L, Cuello AC. 2010. A novel transgenic rat model with a full Alzheimer's-like amyloid pathology displays pre-plaque intracellular amyloid-beta-associated cognitive impairment. J Alzheimers Dis 20:113-126.

Leost M, Schultz C, Link A, Wu YZ, Biernat J, Mandelkow EM, Bibb JA, Snyder GL, Greengard P, Zaharevitz DW, Gussio R, Senderowicz AM, Sausville EA, Kunick C, Meijer L. 2000. Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25. Eur J Biochem 267:5983-5994.

Lesne S, Koh MT, Kotilinek L, Kayed 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.

Levy-Lahad E, Wijsman EM, Nemens E, Anderson L, Goddard KA, Weber JL, Bird TD, Schellenberg GD. 1995. A familial Alzheimer's disease locus on chromosome 1. Science 269:970-973.

Lewczuk P, Esselmann H, Otto M, Maler JM, Henkel AW, Henkel MK, Eikenberg O, Antz C, Krause WR, Reulbach U, Kornhuber J, Wiltfang J. 2004. Neurochemical diagnosis of Alzheimer's dementia by CSF Abeta42, Abeta42/Abeta40 ratio and total tau. Neurobiol Aging 25:273-281.

Li H, Li Q, Du X, Sun Y, Wang X, Kroemer G, Blomgren K, Zhu C. 2011. Lithium-mediated long-term neuroprotection in neonatal rat hypoxia-ischemia is associated with antiinflammatory effects and enhanced proliferation and survival of neural stem/progenitor cells. J Cereb Blood FLow Metab 31:2106-2115.

Li S, Zhang C, Takemori H, Zhou Y, Xiong ZQ. 2009. TORC1 regulates activity-dependent CREB-target gene transcription and dendritic growth of developing cortical neurons. J Neurosci 29:2334-2343.

Lim JE, Kou J, Song M, Pattanayak A, Jin J, Lalonde R, Fukuchi K. 2011. MyD88 deficiency ameliorates beta-amyloidosis in an animal model of Alzheimer's disease. Am J Pathol 179:1095-1103.

Lim JE, Song M, Jin J, Kou J, Pattanayak A, Lalonde R, Fukuchi K. 2012. The effects of MyD88 deficiency on exploratory activity, anxiety, motor coordination, and spatial learning in C57BL/6 and APPswe/PS1dE9 mice. Behav Brain Res 227:36-42.

Lim YY, Maruff P, Pietrzak RH, Ames D, Ellis KA, Harrington K, Lautenschlager NT, Szoeke C, Martins RN, Masters CL, Villemagne VL, Rowe CC. 2014. Effect of amyloid on memory and non-memory decline from preclinical to clinical Alzheimer's disease. Brain 137:221-231.

Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J. 2000. Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. Proc Natl Acad Sci U S A 97:1456-1460.

Lindvall O. 2016. Clinical translation of stem cell transplantation in Parkinson's disease. J Intern Med 279:30-40.

Liu CC, Kanekiyo T, Xu H, Bu G. 2013. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol 9:106-118.

Liu L, Zhang X, Wang L, Yang R, Cui L, Li M, Du W, Wang S. 2010. The neuroprotective effects of Tanshinone IIA are associated with induced nuclear translocation of TORC1 and upregulated expression of TORC1, pCREB and BDNF in the acute stage of ischemic stroke. Brain Res Bull 82:228-233.

Lonze BE, Ginty DD. 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, Rogers J. 1999. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 155:853-862.

Macdonald A, Briggs K, Poppe M, Higgins A, Velayudhan L, Lovestone S. 2008. A feasibility and tolerability study of lithium in Alzheimer's disease. Int J Geriatr Psychiatry 23:704-711.

Machado-Vieira R, Manji HK, Zarate CA, Jr. 2009. The role of lithium in the treatment of bipolar disorder: convergent evidence for neurotrophic effects as a unifying hypothesis. Bipolar Disorders 11 Suppl 2:92-109.

Maezawa I, Zimin PI, Wulff H, Jin LW. 2011. Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. J Biol Chemistry 286:3693-3706.

Markesbery WR. 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 23:134-147.

Markesbery WR, Carney JM. 1999. Oxidative alterations in Alzheimer's disease. Brain Pathol 9:133-146.

Marr RA, Rockenstein E, Mukherjee A, Kindy MS, Hersh LB, Gage FH, Verma IM, Masliah E. 2003. Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. J Neurosci 23:1992-1996.

Martinez A, Alonso M, Castro A, Perez C, Moreno FJ. 2002. First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. J Med Chem 45:1292-1299.

Martino Adami PV, Quijano C, Magnani N, Galeano P, Evelson P, Cassina A, Do Carmo S, Leal MC, Castano EM, Cuello AC, Morelli L. 2015. Synaptosomal bioenergetic defects are associated with cognitive impairment in a transgenic rat model of early Alzheimer's disease. J Cereb Blood flow Metab.

Masliah E, Hansen L, Adame A, Crews L, Bard F, Lee C, Seubert P, Games D, Kirby L, Schenk D. 2005. Abeta vaccination effects on plaque pathology in the absence of encephalitis in Alzheimer disease. Neurology 64:129-131.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82:4245-4249.

Matarin M, Salih DA, Yasvoina M, Cummings DM, Guelfi S, Liu W, Nahaboo Solim MA, Moens TG, Paublete RM, Ali SS, Perona M, Desai R, Smith KJ, Latcham J, Fulleylove M, Richardson JC, Hardy J, Edwards FA. 2015. A genome-wide gene-expression analysis and database in transgenic mice during development of amyloid or tau pathology. Cell Rep 10:633-644.

Matsumura A, Emoto MC, Suzuki S, Iwahara N, Hisahara S, Kawamata J, Suzuki H, Yamauchi A, Sato-Akaba H, Fujii HG, Shimohama S. 2015. Evaluation of oxidative stress in the brain of a transgenic mouse model of Alzheimer disease by in vivo electron paramagnetic resonance imaging. Free Radic Biol Med 85:165-173.

Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS. 1994. Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14:6107-6116.

Mauer S, Vergne D, Ghaemi SN. 2014. Standard and trace-dose lithium: a systematic review of dementia prevention and other behavioral benefits. Aus N Z J Psychiatry 48:809-818.

Maurer K, Volk S, Gerbaldo H. 1997. Auguste D and Alzheimer's disease. Lancet 349:1546-1549.

Mayr B, Montminy M. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599-609.

McGeer PL, McGeer EG. 2013. The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. Acta Neuropathol (Berl) 126:479-497.

McLaurin J, Cecal R, Kierstead ME, Tian X, Phinney AL, Manea M, French JE, Lambermon MH, Darabie AA, Brown ME, Janus C, Chishti MA, Horne P, Westaway D, Fraser PE, Mount HT, Przybylski M, St George-Hyslop P. 2002. Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4-10 and inhibit cytotoxicity and fibrillogenesis. Nat Med 8:1263-1269.

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL. 1999. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol 46:860-866.

McNaull BB, Todd S, McGuinness B, Passmore AP. 2010. Inflammation and anti-inflammatory strategies for Alzheimer's disease--a mini-review. Gerontology 56:3-14.

Mehler MF, Rozental R, Dougherty M, Spray DC, Kessler JA. 1993. Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Nature 362:62-65.

Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. 1997. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. J Biol Chem 272:30491-30497.

Mesulam MM, Geula C. 1988. Nucleus basalis (Ch4) and cortical cholinergic innervation in the human brain: observations based on the distribution of acetylcholinesterase and choline acetyltransferase. J Comp Neurol 275:216-240.

Mesulam MM, Mufson EJ, Levey AI, Wainer BH. 1983. Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. J Comp Neurol 214:170-197.

Miravalle L, Tokuda T, Chiarle R, Giaccone G, Bugiani O, Tagliavini F, Frangione B, Ghiso J. 2000. Substitutions at codon 22 of Alzheimer's abeta peptide induce diverse conformational changes and apoptotic effects in human cerebral endothelial cells. J Biol Chem 275:27110-27116.

Mirzadeh Z, Bari A, Lozano AM. 2015. The rationale for deep brain stimulation in Alzheimer's disease. J Neural Transm.

Monje ML, Toda H, Palmer TD. 2003. Inflammatory blockade restores adult hippocampal neurogenesis. Science 302:1760-1765.

Monsonego A, Zota V, Karni A, Krieger JI, Bar-Or A, Bitan G, Budson AE, Sperling R, Selkoe DJ, Weiner HL. 2003. Increased T cell reactivity to amyloid beta protein in older humans and patients with Alzheimer disease. J Clin Invest 112:415-422.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci U S A 83:6682-6686.

Morel B. 1857. Traité des dégénérescences physiques, intellectuelles et morales de l'espèce humaine. Paris: Baillière

Morelli L, Llovera R, Gonzalez SA, Affranchino JL, Prelli F, Frangione B, Ghiso J, Castano EM. 2003. Differential degradation of amyloid beta genetic variants associated with hereditary dementia or stroke by insulin-degrading enzyme. J Biol Chem 278:23221-23226.

Morris JC, Aisen PS, Bateman RJ, Benzinger TL, Cairns NJ, Fagan AM, Ghetti B, Goate AM, Holtzman DM, Klunk WE, McDade E, Marcus DS, Martins RN, Masters CL, Mayeux R, Oliver A, Quaid K, Ringman JM, Rossor MN, Salloway S, Schofield PR, Selsor NJ, Sperling RA, Weiner MW, Xiong C, Moulder KL, Buckles VD. 2012.

Developing an international network for Alzheimer research: The Dominantly Inherited Alzheimer Network. J Clin Invest 2:975-984.

Morris R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47-60.

Morris RG, Kopelman MD. 1986. The memory deficits in Alzheimer-type dementia: a review. Q J Exp Psychol A 38:575-602.

Morris RGM. 1981. Spatial navigation does not require the presence of local cues. Learn Motiv 12:236-260.

Mosconi L, Glodzik L, Mistur R, McHugh P, Rich KE, Javier E, Williams S, Pirraglia E, De Santi S, Mehta PD, Zinkowski R, Blennow K, Pratico D, de Leon MJ. 2010. Oxidative stress and amyloid-beta pathology in normal individuals with a maternal history of Alzheimer's. Biol Psychiatry 68:913-921.

Mu Y, Gage FH. 2011. Adult hippocampal neurogenesis and its role in Alzheimer's disease. Mol Neurodegener 6:85.

Mucke L, Masliah E, Johnson WB, Ruppe MD, Alford M, Rockenstein EM, Forss-Petter S, Pietropaolo M, Mallory M, Abraham CR. 1994. Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. Brain Res 666:151-167.

Mufson EJ, Ma SY, Dills J, Cochran EJ, Leurgans S, Wuu J, Bennett DA, Jaffar S, Gilmor ML, Levey AI, Kordower JH. 2002. Loss of basal forebrain P75(NTR)

immunoreactivity in subjects with mild cognitive impairment and Alzheimer's disease. J Comp Neurol 443:136-153.

Munch G, Schinzel R, Loske C, Wong A, Durany N, Li JJ, Vlassara H, Smith MA, Perry G, Riederer P. 1998. Alzheimer's disease--synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. J Neural Transm 105:439-461.

Munhoz C, Madrigal JL, Garcia-Bueno B, Pradillo JM, Moro MA, Lizasoain I, Lorenzo P, Scavone C, Leza JC. 2004. TNF-alpha accounts for short-term persistence of oxidative status in rat brain after two weeks of repeated stress. Eur J Neurosci 20:1125-1130.

Murayama M, Tanaka S, Palacino J, Murayama O, Honda T, Sun X, Yasutake K, Nihonmatsu N, Wolozin B, Takashima A. 1998. Direct association of presenilin-1 with beta-catenin. FEBS Lett 433:73-77.

Murayama O, Tomita T, Nihonmatsu N, Murayama M, Sun X, Honda T, Iwatsubo T, Takashima A. 1999. Enhancement of amyloid beta 42 secretion by 28 different presenilin 1 mutations of familial Alzheimer's disease. Neurosci Lett 265:61-63.

Murray EA, Mishkin M. 1986. Visual recognition in monkeys following rhinal cortical ablations combined with either amygdalectomy or hippocampectomy. J Neurosci 6:1991-2003.

Murrell J, Farlow M, Ghetti B, Benson MD. 1991. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254:97-99.

Nader K, Schafe GE, Le Doux JE. 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature 406:722-726.

Nahman S, Belmaker RH, Azab AN. 2012. Effects of lithium on lipopolysaccharide-induced inflammation in rat primary glia cells. Innate Immun 18:447-458.

Nakamaru Y, Takagi D, Homma A, Hatakeyama S, Fukuda S. 2015. Oxidative stress regulates IL-4 gene expression in mast cells through the reduction of histone deacetylase. JAMA Otolaryngol Head Neck Surg 152:48-52.

Namura S, Ooboshi H, Liu J, Yenari MA. 2013. Neuroprotection after cerebral ischemia. Ann N Y Acad Sci 1278:25-32.

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.

Nee LE, Polinsky RJ, Eldridge R, Weingartner H, Smallberg S, Ebert M. 1983. A family with histologically confirmed Alzheimer's disease. Arch Neurol 40:203-208.

Nestor PJ, Scheltens P, Hodges JR. 2004. Advances in the early detection of Alzheimer's disease. Nat Med 10 Suppl:S34-41.

Neumann H, Daly MJ. 2013. Variant TREM2 as risk factor for Alzheimer's disease. N Engl J Med 368:182-184.

Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO. 2003. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat Med 9:448-452.

Noble W, Olm V, Takata K, Casey E, Mary O, Meyerson J, Gaynor K, LaFrancois J, Wang L, Kondo T, Davies P, Burns M, Veeranna, Nixon R, Dickson D, Matsuoka Y, Ahlijanian M, Lau LF, Duff K. 2003. Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron 38:555-565.

Nonaka M, Kim R, Fukushima H, Sasaki K, Suzuki K, Okamura M, Ishii Y, Kawashima T, Kamijo S, Takemoto-Kimura S, Okuno H, Kida S, Bito H. 2014. Region-specific activation of CRTC1-CREB signaling mediates long-term fear memory. Neuron 84:92-106.

Nuland SB. 1995. Doctors : the biography of medicine. New York: Vintage.

Nunes MA, Viel TA, Buck HS. 2013. Microdose lithium treatment stabilized cognitive impairment in patients with Alzheimer's disease. Curr Alzheimer Res 10:104-107.

Nunes PV, Forlenza OV, Gattaz WF. 2007. Lithium and risk for Alzheimer's disease in elderly patients with bipolar disorder. Br J Psychiatry 190:359-360.

O'Brien RJ, Wong PC. 2011. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci 34:185-204.

Oddo S, Caccamo A, Smith IF, Green KN, LaFerla FM. 2006. A dynamic relationship between intracellular and extracellular pools of Abeta. Am J Pathol 168:184-194.

Olson MI, Shaw CM. 1969. Presenile dementia and Alzheimer's disease in mongolism. Brain 92:147-156.

Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C. 2003. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. Neurology 61:46-54.

Ostrowitzki S, Deptula D, Thurfjell L, Barkhof F, Bohrmann B, Brooks DJ, Klunk WE, Ashford E, Yoo K, Xu ZX, Loetscher H, Santarelli L. 2012. Mechanism of amyloid removal in patients with Alzheimer disease treated with gantenerumab. Arch Neurol 69:198-207.

Palomo V, Soteras I, Perez DI, Perez C, Gil C, Campillo NE, Martinez A. 2011. Exploring the binding sites of glycogen synthase kinase 3. Identification and characterization of allosteric modulation cavities. J Med Chem 54:8461-8470.

Parachikova A, Agadjanyan MG, Cribbs DH, Blurton-Jones M, Perreau V, Rogers J, Beach TG, Cotman CW. 2007. Inflammatory changes parallel the early stages of Alzheimer disease. Neurobiol Aging 28:1821-1833.

Park KM, Bowers WJ. 2010. Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. Cell Signal 22:977-983.

Parker D, Jhala US, Radhakrishnan I, Yaffe MB, Reyes C, Shulman AI, Cantley LC, Wright PE, Montminy M. 1998. Analysis of an activator:coactivator complex reveals an essential role for secondary structure in transcriptional activation. Mol Cell 2:353-359.

Parr C, Mirzaei N, Christian M, Sastre M. 2015. Activation of the Wnt/beta-catenin pathway represses the transcription of the beta-amyloid precursor protein cleaving enzyme (BACE1) via binding of T-cell factor-4 to BACE1 promoter. FASEB J 29:623-635.

Parra-Damas A, Valero J, Chen M, Espana J, Martin E, Ferrer I, Rodriguez-Alvarez J, Saura CA. 2014. Crtc1 activates a transcriptional program deregulated at early Alzheimer's disease-related stages. J Neurosci 34:5776-5787.

Pasquier F, Grymonprez L, Lebert F, Van der Linden M. 2001. Memory impairment differs in frontotemporal dementia and Alzheimer's disease. Neurocase 7:161-171.

Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. 1999. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 402:615-622.

Pen AE, Jensen UB. 2016. Current status of treating neurodegenerative disease with induced pluripotent stem cells. Acta Neurol Scand.

Peng S, Garzon DJ, Marchese M, Klein W, Ginsberg SD, Francis BM, Mount HT, Mufson EJ, Salehi A, Fahnestock M. 2009. Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease. J Neurosci 29:9321-9329.

Peng S, Wuu J, Mufson EJ, Fahnestock M. 2005. Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. J Neurochem 93:1412-1421.

Perluigi M, Sultana R, Cenini G, Di Domenico F, Memo M, Pierce WM, Coccia R, Butterfield DA. 2009. Redox proteomics identification of 4-hydroxynonenal-modified brain proteins in Alzheimer's disease: Role of lipid peroxidation in Alzheimer's disease pathogenesis. Proteomics Clin Appl 3:682-693.

Perry EK, Perry RH, Blessed G, Tomlinson BE. 1978. Changes in brain cholinesterases in senile dementia of Alzheimer type. Neuropathol Appl Neurobiol 4:273-277.

Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M. 2002. Cerebral hemorrhage after passive anti-Abeta immunotherapy. Science 298:1379.

Phiel CJ, Klein PS. 2001. Molecular targets of lithium action. Annu Rev Pharmacol Toxicol 41:789-813.

Piccio L, Deming Y, Del-Aguila JL, Ghezzi L, Holtzman DM, Fagan AM, Fenoglio C, Galimberti D, Borroni B, Cruchaga C. 2016. Cerebrospinal fluid soluble TREM2 is higher in Alzheimer disease and associated with mutation status. Acta Neuropathol (Berl).

Pimentel LS, Allard S, Do Carmo S, Weinreb O, Danik M, Hanzel CE, Youdim MB, Cuello AC. 2015. The Multi-Target Drug M30 Shows Pro-Cognitive and Anti-Inflammatory Effects in a Rat Model of Alzheimer's Disease. J Alzheimers Dis 47:373-383.

Plotkin B, Kaidanovich O, Talior I, Eldar-Finkelman H. 2003. Insulin mimetic action of synthetic phosphorylated peptide inhibitors of glycogen synthase kinase-3. J Pharmacol Exp Ther 305:974-980.

Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. 1993. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 342:697-699.

Polvikoski T, Sulkava R, Haltia M, Kainulainen K, Vuorio A, Verkkoniemi A, Niinisto L, Halonen P, Kontula K. 1995. Apolipoprotein E, dementia, and cortical deposition of beta-amyloid protein. N Engl J Med 333:1242-1247.

Ponce FA, Asaad WF, Foote KD, Anderson WS, Rees Cosgrove G, Baltuch GH, Beasley K, Reymers DE, Oh ES, Targum SD, Smith GS, Lyketsos CG, Lozano AM. 2015. Bilateral deep brain stimulation of the fornix for Alzheimer's disease: surgical safety in the ADvance trial. J Neurosurg:1-10.

Porcellotti S, Fanelli F, Fracassi A, Sepe S, Cecconi F, Bernardi C, Cimini A, Ceru MP, Moreno S. 2015. Oxidative Stress during the Progression of beta-Amyloid Pathology in the Neocortex of the Tg2576 Mouse Model of Alzheimer's Disease. Oxid Med Cell Longev 2015:967203.

Pouladi MA, Brillaud E, Xie Y, Conforti P, Graham RK, Ehrnhoefer DE, Franciosi S, Zhang W, Poucheret P, Compte E, Maurel JC, Zuccato C, Cattaneo E, Neri C, Hayden MR. 2012. NP03, a novel low-dose lithium formulation, is neuroprotective in the YAC128 mouse model of Huntington disease. Neurobiol Dis 48:282-289.

Pouratian N, Thakkar S, Kim W, Bronstein JM. 2012. Deep brain stimulation for the treatment of Parkinson's disease: efficacy and safety. Degener Neurol Neuromuscul Dis 2012.

Pratico D. 2008. Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. Ann N Y Acad Sci 1147:70-78.

Pratico D. 2008. Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. Trends Pharmacol Sci 29:609-615.

Pugazhenthi S, Wang M, Pham S, Sze CI, Eckman CB. 2011. Downregulation of CREB expression in Alzheimer's brain and in Abeta-treated rat hippocampal neurons. Mol Neurodegener 6:60.

Puolivali J, Wang J, Heikkinen T, Heikkila M, Tapiola T, van Groen T, Tanila H. 2002. Hippocampal A beta 42 levels correlate with spatial memory deficit in APP and PS1 double transgenic mice. Neurobiol Dis 9:339-347.

Qi Y, Klyubin I, Harney SC, Hu N, Cullen WK, Grant MK, Steffen J, Wilson EN, Do Carmo S, Remy S, Fuhrmann M, Ashe KH, Cuello AC, Rowan MJ. 2014. Longitudinal testing of hippocampal plasticity reveals the onset and maintenance of endogenous human Aβ-induced synaptic dysfunction in individual freely behaving pre-plaque transgenic rats: rapid reversal by anti-Aβ agents. Acta Neuropathol Commun 2:175.

Quebec Gd. 2009. Relever le défi de la maladie d'Alzheimer et des maladies apparentées. Une vision centrée sur la personne, l'humanisme et l'excellence. Rapport du comité d'experts en vue de l'élaboration d'un plan d'action pour la maladie d'Alzheimer.

Racke MM, Boone LI, Hepburn DL, Parsadainian M, Bryan MT, Ness DK, Piroozi KS, Jordan WH, Brown DD, Hoffman WP, Holtzman DM, Bales KR, Gitter BD, May PC, Paul SM, DeMattos RB. 2005. Exacerbation of cerebral amyloid angiopathy-associated microhemorrhage in amyloid precursor protein transgenic mice by immunotherapy is dependent on antibody recognition of deposited forms of amyloid beta. J Neurosci 25:629-636.

Radhakrishnan I, Perez-Alvarado GC, Parker D, Dyson HJ, Montminy MR, Wright PE. 1997. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. Cell 91:741-752.

Rahman S, Sahakian BJ, Hodges JR, Rogers RD, Robbins TW. 1999. Specific cognitive deficits in mild frontal variant frontotemporal dementia. Brain 122 (Pt 8):1469-1493.

Rainer M, Mark TH, Haushofer A. 1989. Galantamine hydrobromide in the treatment of senile dementia of Alzheimer's type. In: Kerwitz T, Thomsen L, Bickel A, editors. Pharmacological Interventions on Central Mechanisms in Senile Dementia. Munchen.: Zuckschwerd. p 53-71.

Rao MS, Shetty AK. 2004. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. Eur J Neurosci 19:234-246.

Ravnskjaer K, Kester H, Liu Y, Zhang X, Lee D, Yates JR, 3rd, Montminy M. 2007. Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. EMBO J 26:2880-2889.

Reed T, Perluigi M, Sultana R, Pierce WM, Klein JB, Turner DM, Coccia R, Markesbery WR, Butterfield DA. 2008. Redox proteomic identification of 4-hydroxy-2-nonenal-modified brain proteins in amnestic mild cognitive impairment: insight into the role of lipid peroxidation in the progression and pathogenesis of Alzheimer's disease. Neurobiol Dis 30:107-120.

Reed TT, Pierce WM, Jr., Turner DM, Markesbery WR, Butterfield DA. 2009. Proteomic identification of nitrated brain proteins in early Alzheimer's disease inferior parietal lobule. J Cell Mol Med 13:2019-2029.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ. 2003. Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med 348:1333-1341.

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 KL, Filali M, Prefontaine P, Rivest S. 2008. Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1-42 and delay the cognitive decline in a mouse model of Alzheimer's disease. J Neurosci 28:5784-5793.

Richards JP, Bachinger HP, Goodman RH, Brennan RG. 1996. Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB. J Biol Chem 271:13716-13723.

Ringman JM, Elashoff D, Geschwind DH, Welsh BT, Gylys KH, Lee C, Cummings JL, Cole GM. 2012. Plasma signaling proteins in persons at genetic risk for Alzheimer disease: influence of APOE genotype. Arch Neurol 69:757-764.

Roelcke V. 2004. Nazi medicine and research on human beings. Lancet 364 Suppl 1:s6-7.

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, et al. 1995. Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376:775-778.

Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT. 1998. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group. Neurology 50:136-145.

Rolland Y, Andrieu S, Cantet C, Morley JE, Thomas D, Nourhashemi F, Vellas B. 2007. Wandering behavior and Alzheimer disease. The REAL.FR prospective study. Alzheimer Dis Assoc Disord 21:31-38.

Romberg C, Horner AE, Bussey TJ, Saksida LM. 2013. A touch screen-automated cognitive test battery reveals impaired attention, memory abnormalities, and increased response inhibition in the TgCRND8 mouse model of Alzheimer's disease. Neurobiol Aging 34:731-744.

Rosa E, Fahnestock M. 2015. CREB expression mediates amyloid beta-induced basal BDNF downregulation. Neurobiol Aging 36:2406-2413.

Rosler M, Anand R, Cicin-Sain A, Gauthier S, Agid Y, Dal-Bianco P, Stahelin HB, Hartman R, Gharabawi M. 1999. Efficacy and safety of rivastigmine in patients with Alzheimer's disease: international randomised controlled trial. BMJ 318:633-638.

Rossi S, Mancino R, Bergami A, Mori F, Castelli M, De Chiara V, Studer V, Mataluni G, Sancesario G, Parisi V, Kusayanagi H, Bernardi G, Nucci C, Bernardini S, Martino G, Furlan R, Centonze D. 2011. Potential role of IL-13 in neuroprotection and cortical excitability regulation in multiple sclerosis. Mult Scler 17:1301-1312.

Ryves WJ, Harwood AJ. 2001. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem Biophys Res Commun 280:720-725.

Sahay A, Wilson DA, Hen R. 2011. Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. Neuron 70:582-588.

Sakamoto K, Norona FE, Alzate-Correa D, Scarberry D, Hoyt KR, Obrietan K. 2013. Clock and light regulation of the CREB coactivator CRTC1 in the suprachiasmatic circadian clock. J Neurosci 33:9021-9027.

Sasaki T, Takemori H, Yagita Y, Terasaki Y, Uebi T, Horike N, Takagi H, Susumu T, Teraoka H, Kusano K, Hatano O, Oyama N, Sugiyama Y, Sakoda S, Kitagawa K. 2011. SIK2 Is a Key Regulator for Neuronal Survival after Ischemia via TORC1-CREB. Neuron 69:106-119.

Sastre M, Dewachter I, Landreth GE, Willson TM, Klockgether T, van Leuven F, Heneka MT. 2003. Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated

receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. J Neurosci 23:9796-9804.

Sastre M, Klockgether T, Heneka MT. 2006. Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms. Int J Dev Neurosci 24:167-176.

Saura CA. 2012. CREB-regulated transcription coactivator 1-dependent transcription in Alzheimer's disease mice. Neurodegener Dis 10:250-252.

Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400:173-177.

Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S. 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 2:864-870.

Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D, Roses AD. 1993. Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. Proc Natl Acad Sci U S A 90:9649-9653.

Schmuck G, Rohrdanz E, Tran-Thi QH, Kahl R, Schluter G. 2002. Oxidative stress in rat cortical neurons and astrocytes induced by paraquat in vitro. Neurotox Res 4:1-13.

Schoenbaum G, Nugent S, Saddoris MP, Gallagher M. 2002. Teaching old rats new tricks: age-related impairments in olfactory reversal learning. Neurobiol Aging 23:555-564.

Scholl M, Lockhart SN, Schonhaut DR, O'Neil JP, Janabi M, Ossenkoppele R, Baker SL, Vogel JW, Faria J, Schwimmer HD, Rabinovici GD, Jagust WJ. 2016. PET Imaging of Tau Deposition in the Aging Human Brain. Neuron 89:971-982.

Schroeter S, Khan K, Barbour R, Doan M, Chen M, Guido T, Gill D, Basi G, Schenk D, Seubert P, Games D. 2008. Immunotherapy reduces vascular amyloid-beta in PDAPP mice. J Neurosci 28:6787-6793.

Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates JR, 3rd, Takemori H, Okamoto M, Montminy M. 2004. The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. Cell 119:61-74.

Sekeres MJ, Mercaldo V, Richards B, Sargin D, Mahadevan V, Woodin MA, Frankland PW, Josselyn SA. 2012. Increasing CRTC1 function in the dentate gyrus during memory formation or reactivation increases memory strength without compromising memory quality. J neurosci 32:17857-17868.

Selkoe DJ. 1991. The molecular pathology of Alzheimer's disease. Neuron 6:487-498.

Selkoe DJ. 1998. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. Trends Cell Biol 8:447-453.

Selkoe DJ. 2001. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741-766.

Selkoe DJ. 2001. Clearing the brain's amyloid cobwebs. Neuron 32:177-180.

Selkoe DJ. 2011. Alzheimer's disease. Cold Spring Harbor perspectives in biology 3.

Selkoe DJ. 2011. Resolving controversies on the path to Alzheimer's therapeutics. Nat Med 17:1060-1065.

Sendera TJ, Ma SY, Jaffar S, Kozlowski PB, Kordower JH, Mawal Y, Saragovi HU, Mufson EJ. 2000. Reduction in TrkA-immunoreactive neurons is not associated with an overexpression of galaninergic fibers within the nucleus basalis in Down's syndrome. J Neurochem 74:1185-1196.

Shaywitz AJ, Greenberg ME. 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821-861.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Perkicak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St George-Hyslop PH. 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754-760.

Shmuel A, Leopold DA. 2008. Neuronal correlates of spontaneous fluctuations in fMRI signals in monkey visual cortex: Implications for functional connectivity at rest. Hum Brain Mapp 29:751-761.

Simard AR, Rivest S. 2004. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. FASEB J 18:998-1000.

Sisodia SS. 1992. Beta-amyloid precursor protein cleavage by a membrane-bound protease. Proc Natl Acad Sci U S A 89:6075-6079.

Skovronsky DM, Moore DB, Milla ME, Doms RW, Lee VM. 2000. Protein kinase C-dependent alpha-secretase competes with beta-secretase for cleavage of amyloid-beta precursor protein in the trans-golgi network. J Biol Chem 275:2568-2575.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76-85.

Socodato RE, Magalhaes CR, Paes-de-Carvalho R. 2009. Glutamate and nitric oxide modulate ERK and CREB phosphorylation in the avian retina: evidence for direct signaling from neurons to Muller glial cells. J Neurochem 108:417-429.

Sofola-Adesakin O, Castillo-Quan JI, Rallis C, Tain LS, Bjedov I, Rogers I, Li L, Martinez P, Khericha M, Cabecinha M, Bahler J, Partridge L. 2014. Lithium suppresses Abeta pathology by inhibiting translation in an adult Drosophila model of Alzheimer's disease. Front Aging Neurosci 6:190.

Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, Iwatsubo T, Jack CR, Jr., Kaye J, Montine TJ, Park DC, Reiman EM, Rowe CC, Siemers E, Stern Y, Yaffe K, Carrillo MC, Thies B, Morrison-Bogorad M, Wagster MV, Phelps CH. 2011. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:280-292.

Spillantini MG, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B. 1998. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. Proc Natl Acad Sci U S A 95:7737-7741.

Stambolic V, Ruel L, Woodgett JR. 1996. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr Biol 6:1664-1668.

Stern Y. 2012. Cognitive reserve in ageing and Alzheimer's disease. Lancet Neurol 11:1006-1012.

Stern Y, Habeck C, Moeller J, Scarmeas N, Anderson KE, Hilton HJ, Flynn J, Sackeim H, van Heertum R. 2005. Brain networks associated with cognitive reserve in healthy young and old adults. Cereb Cortex 15:394-402.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA. 2007. The classical complement cascade mediates CNS synapse elimination. Cell 131:1164-1178.

Stone SS, Teixeira CM, Devito LM, Zaslavsky K, Josselyn SA, Lozano AM, Frankland PW. 2011. Stimulation of entorhinal cortex promotes adult neurogenesis and facilitates spatial memory. J Neurosci 31:13469-13484.

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.

Sultana R, Perluigi M, Allan Butterfield D. 2013. Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain. Free Radic Biol Med 62:157-169.

Sultana R, Perluigi M, Newman SF, Pierce WM, Cini C, Coccia R, Butterfield DA. 2010. Redox proteomic analysis of carbonylated brain proteins in mild cognitive impairment and early Alzheimer's disease. Antioxid Redox Signal 12:327-336.

Sultana R, Piroddi M, Galli F, Butterfield DA. 2008. Protein levels and activity of some antioxidant enzymes in hippocampus of subjects with amnestic mild cognitive impairment. Neurochem Res 33:2540-2546.

Summers WK, Majovski LV, Marsh GM, Tachiki K, Kling A. 1986. Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer type. N Engl J Med 315:1241-1245.

Sun P, Enslen H, Myung PS, Maurer RA. 1994. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev 8:2527-2539.

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., Eckman C, Golde TE, Younkin SG. 1994. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336-1340.

Swomley AM, Butterfield DA. 2015. Oxidative stress in Alzheimer disease and mild cognitive impairment: evidence from human data provided by redox proteomics. Arch Toxicol 89:1669-1680.

Szekely CA, Breitner JC, Fitzpatrick AL, Rea TD, Psaty BM, Kuller LH, Zandi PP. 2008. NSAID use and dementia risk in the Cardiovascular Health Study: role of APOE and NSAID type. Neurology 70:17-24.

Takashima A, Murayama M, Murayama O, Kohno T, Honda T, Yasutake K, Nihonmatsu N, Mercken M, Yamaguchi H, Sugihara S, Wolozin B. 1998. Presenilin 1 associates with glycogen synthase kinase-3beta and its substrate tau. Proc Natl Acad Sci U S A 95:9637-9641.

Takemori H, Okamoto M. 2008. Regulation of CREB-mediated gene expression by salt inducible kinase. J Steroid Biochem Mol Biol 108:287-291.

Tan J, Widjaja S, Xu J, Shepherd RK. 2008. Cochlear implants stimulate activity-dependent CREB pathway in the deaf auditory cortex: implications for molecular plasticity induced by neural prosthetic devices. Cereb Cortex 18:1799-1813.

Tangpong J, Cole MP, Sultana R, Joshi G, Estus S, Vore M, St Clair W, Ratanachaiyavong S, St Clair DK, Butterfield DA. 2006. Adriamycin-induced, TNF-alpha-mediated central nervous system toxicity. Neurobiol Dis 23:127-139.

Tariot PN, Farlow MR, Grossberg GT, Graham SM, McDonald S, Gergel I. 2004. Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. JAMA 291:317-324.

Tashiro A, Makino H, Gage FH. 2007. Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. J Neurosci 27:3252-3259.

Terry RD, Katzman R, Bick KL. 1993. Alzheimer disease. New York: Raven Press.

Tobinick E, Gross H, Weinberger A, Cohen H. 2006. TNF-alpha modulation for treatment of Alzheimer's disease: a 6-month pilot study. Med Gen Med 8:25.

Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C. 1999. Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. Neurosci Lett 269:52-54.

Toledo EM, Inestrosa NC. 2010. Activation of Wnt signaling by lithium and rosiglitazone reduced spatial memory impairment and neurodegeneration in brains of an APPswe/PSEN1DeltaE9 mouse model of Alzheimer's disease. Mol Psychiatry 15:272-285, 228.

Tsivilis D, Vann SD, Denby C, Roberts N, Mayes AR, Montaldi D, Aggleton JP. 2008. A disproportionate role for the fornix and mammillary bodies in recall versus recognition memory. Nat Neurosci 11:834-842.

Ulrich JD, Holtzman DM. 2016. TREM2 Function in Alzheimer's Disease and Neurodegeneration. ACS Chem Neurosci 7:420-427.

van Praag H, Kempermann G, Gage FH. 2000. Neural consequences of environmental enrichment. Nat Rev Neurosci 1:191-198.

Varadarajan S, Yatin S, Kanski J, Jahanshahi F, Butterfield DA. 1999. Methionine residue 35 is important in amyloid beta-peptide-associated free radical oxidative stress. Brain Res Bull 50:133-141.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735-741.

Vetere G, Restivo L, Cole CJ, Ross PJ, Ammassari-Teule M, Josselyn SA, Frankland PW. 2011. Spine growth in the anterior cingulate cortex is necessary for the consolidation of contextual fear memory. Proc Natl Acad Sci U S A 108:8456-8460.

Villemagne VL, Fodero-Tavoletti MT, Masters CL, Rowe CC. 2015. Tau imaging: early progress and future directions. Lancet Neurol 14:114-124.

Vom Berg J, Prokop S, Miller KR, Obst J, Kalin RE, Lopategui-Cabezas I, Wegner A, Mair F, Schipke CG, Peters O, Winter Y, Becher B, Heppner FL. 2012. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. Nat Med 18:1812-1819.

Vos SJ, Xiong C, Visser PJ, Jasielec MS, Hassenstab J, Grant EA, Cairns NJ, Morris JC, Holtzman DM, Fagan AM. 2013. Preclinical Alzheimer's disease and its outcome: a longitudinal cohort study. Lancet Neurol 12:957-965.

Vos SJB, Gordon BA, Su Y, Visser PJ, Holtzman DM, Morris JC, Fagan AM, Benzinger TLS. 2016. NIA-AA Staging of Preclinical Alzheimer Disease: Discordance and Concordance of CSF and Imaging Biomarkers. Neurobiol Aging.

Walker ES, Martinez M, Brunkan AL, Goate A. 2005. Presenilin 2 familial Alzheimer's disease mutations result in partial loss of function and dramatic changes in Abeta 42/40 ratios. J Neurochem 92:294-301.

Wang C, Wilson WA, Moore SD, Mace BE, Maeda N, Schmechel DE, Sullivan PM. 2005. Human apoE4-targeted replacement mice display synaptic deficits in the absence of neuropathology. Neurobiol Dis 18:390-398.

Wang J, Dickson DW, Trojanowski JQ, Lee VM. 1999. The levels of soluble versus insoluble brain Abeta distinguish Alzheimer's disease from normal and pathologic aging. Exp Neurol 158:328-337.

Wang Y, Ulland TK, Ulrich JD, Song W, Tzaferis JA, Hole JT, Yuan P, Mahan TE, Shi Y, Gilfillan S, Cella M, Grutzendler J, DeMattos RB, Cirrito JR, Holtzman DM, Colonna

M. 2016. TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. J Exp Med.

Watts AG, Sanchez-Watts G, Liu Y, Aguilera G. 2011. The distribution of messenger RNAs encoding the three isoforms of the transducer of regulated cAMP responsive element binding protein activity in the rat forebrain. J Neuroendocrinol 23:754-766.

Weber MM. 1997. Aloys Alzheimer, a coworker of Emil Kraepelin. J Psychiatr Res 31:635-643.

Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH. 2001. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 414:212-216.

Weindling P. 1989. Health, race and German politics between national unification and Nazism, 1870-1945. Cambridge: Cambridge University Press.

Weindling PJ. 2004. Nazi Medicine and the Nuremberg Trials: From Medical Warcrimes to Informed Consent. New York: Palgrave Macmillan.

Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW. 1975. A protein factor essential for microtubule assembly. Proc Natl Acad Sci U S A 72:1858-1862.

Whishaw IQ, Metz GA, Kolb B, Pellis SM. 2001. Accelerated nervous system development contributes to behavioral efficiency in the laboratory mouse: a behavioral review and theoretical proposal. Dev Psychobiol 39:151-170.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR. 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237-1239.

Wilcock DM, Jantzen PT, Li Q, Morgan D, Gordon MN. 2007. Amyloid-beta vaccination, but not nitro-nonsteroidal anti-inflammatory drug treatment, increases vascular amyloid and microhemorrhage while both reduce parenchymal amyloid. Neuroscience 144:950-960.

Wilcock DM, Rojiani A, Rosenthal A, Subbarao S, Freeman MJ, Gordon MN, Morgan D. 2004. Passive immunotherapy against Abeta in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage. J Neuroinflammation 1:24.

Wilson EN, Abela AR, Do Carmo S, Allard S, Marks AR, Welikovitch LA, Ducatenzeiler A, Chudasama Y, Cuello AC. 2016. Intraneuronal Amyloid Beta Accumulation Disrupts Hippocampal CRTC1-Dependent Gene Expression and Cognitive Function in a Rat Model of Alzheimer Disease. Cereb Cortex.

Wilson EN, Do Carmo S, Iulita MF, Allard S, Ducatenzeiler A, Marks AR, Cuello AC. To Be Submitted. Novel low-dose lithium formulation NP03 prevents neurodegeneration and memory loss in an APP transgenic rat model of Alzheimer's disease.

Wiltfang J, Esselmann H, Bibl M, Hull M, Hampel H, Kessler H, Frolich L, Schroder J, Peters O, Jessen F, Luckhaus C, Perneczky R, Jahn H, Fiszer M, Maler JM, Zimmermann R, Bruckmoser R, Kornhuber J, Lewczuk P. 2007. Amyloid beta peptide ratio 42/40 but

not A beta 42 correlates with phospho-Tau in patients with low- and high-CSF A beta 40 load. J Neurochem 101:1053-1059.

Winkelmann A. 2007. Wilhelm von Waldeyer-Hartz (1836-1921): an anatomist who left his mark. Clin Anat 20:231-234.

Wood LB, Winslow AR, Proctor EA, McGuone D, Mordes DA, Frosch MP, Hyman BT, Lauffenburger DA, Haigis KM. 2015. Identification of neurotoxic cytokines by profiling Alzheimer's disease tissues and neuron culture viability screening. Sci Rep 5:16622.

Wright AL, Zinn R, Hohensinn B, Konen LM, Beynon SB, Tan RP, Clark IA, Abdipranoto A, Vissel B. 2013. Neuroinflammation and neuronal loss precede Abeta plaque deposition in the hAPP-J20 mouse model of Alzheimer's disease. PloS One 8:e59586.

Wu CK, Mesulam MM, Geula C. 1997. Age-related loss of calbindin from human basal forebrain cholinergic neurons. Neuroreport 8:2209-2213.

Xia T, Cui Y, Qian Y, Chu S, Song J, Gu X, Ma Z. 2015. Regulation of the NR2B-CREB-CRTC1 Signaling Pathway Contributes to Circadian Pain in Murine Model of Chronic Constriction Injury. Anesth Analg.

Yan R, Vassar R. 2014. Targeting the beta secretase BACE1 for Alzheimer's disease therapy. Lancet Neurol 13:319-329.

Yin YW, Li JC, Wang JZ, Li BH, Pi Y, Yang QW, Fang CQ, Gao CY, Zhang LL. 2012. Association between apolipoprotein E gene polymorphism and the risk of vascular dementia: a meta-analysis. Neurosci Lett 514:6-11.

York JD, Ponder JW, Majerus PW. 1995. Definition of a metal-dependent/Li(+)-inhibited phosphomonoesterase protein family based upon a conserved three-dimensional core structure. Proc Natl Acad Sci U S A 92:5149-5153.

Young AH, Newham JI. 2006. Lithium in maintenance therapy for bipolar disorder. J Psychopharmacol (Oxf) 20:17-22.

Yu F, Wang Z, Tchantchou F, Chiu CT, Zhang Y, Chuang DM. 2012. Lithium ameliorates neurodegeneration, suppresses neuroinflammation, and improves behavioral performance in a mouse model of traumatic brain injury. J Neurotrauma 29:362-374.

Yu F, Zhang Y, Chuang DM. 2012. Lithium reduces BACE1 overexpression, beta amyloid accumulation, and spatial learning deficits in mice with traumatic brain injury. J Neurotrauma 29:2342-2351.

Zhang C, McNeil E, Dressler L, Siman R. 2007. Long-lasting impairment in hippocampal neurogenesis associated with amyloid deposition in a knock-in mouse model of familial Alzheimer's disease. Exp Neurol 204:77-87.

Zhang X, Heng X, Li T, Li L, Yang D, Du Y, Doody RS, Le W. 2011. Long-term treatment with lithium alleviates memory deficits and reduces amyloid-beta production in an aged Alzheimer's disease transgenic mouse model. J Alz Dis 24:739-749.

Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M. 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A 102:4459-4464.

Zhou Y, Wu H, Li S, Chen Q, Cheng XW, Zheng J, Takemori H, Xiong ZQ. 2006. Requirement of TORC1 for late-phase long-term potentiation in the hippocampus. PLoS One 1:e16.

Zlokovic BV. 2004. Clearing amyloid through the blood-brain barrier. J Neurochem 89:807-811.

Zuo XN, Di Martino A, Kelly C, Shehzad ZE, Gee DG, Klein DF, Castellanos FX, Biswal BB, Milham MP. 2010. The oscillating brain: complex and reliable. Neuroimage 49:1432-1445.