Consequences of forebrain cholinergic depletion and experimental cholinergic therapeutics in a transgenic rat model of Alzheimer's disease

Chiara Orciani

Integrated Program in Neuroscience
McGill University, Montreal, Canada

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This Thesis is dedicated to my parents,

Cinzia and Enrico

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Abstract (English)

Basal forebrain cholinergic neurons (BFCNs) represent the main source of cholinergic innervation to the cortex and hippocampus. They are essential for learning, memory and attention and degenerate early in Alzheimer's disease (AD), contributing to initial cognitive deficits. Consistently with the above, acetylcholinesterase inhibitors (AChEIs), improving the remaining cholinergic signalling, provide a transient relief from cognitive decline, albeit without modifying disease progression.

Several studies have recently shown that AChEIs administration reduced the rate of atrophy of the hippocampus, cortex, and basal forebrain, while patients taking medications with anticholinergic effects have more probability of developing AD. This suggests that BFCN degeneration may play a significant role in aggravating AD pathology. Consequently, basic science research on the pathological mechanisms ensured by the loss of BFCN and the investigation of novel cholinergic therapies would be of value in preventing or delaying AD pathology.

This Thesis focused on investigations into the relationship between BFCNs and neurotrophins. The phenotypic maintenance of BFCNs depends on levels of mature nerve growth factor (mNGF) and, to a lesser extent, on the mature brain-derived neurotrophic factor (mBDNF). These trophic factors are generated in target neurons and retrogradely transported to cell bodies of BFCNs. Whether BFCNs inputs impact neurotrophin availability at BFCNS synapses was not previously investigated. Our studies revealed that following a 6 month-immunolesion in Wistar rats of the nucleus basalis (nb), a basal forebrain cholinergic nuclei projecting mainly to the cortex, produced an attentional deficit accompanied by a reduction of cortical markers of glutamatergic and GABAergic neurons. Additionally, the cholinergic nb loss produced differential effects on neurotrophin availability, decreasing BDNF expression and mBDNF levels without noticeable changes in NGF levels.

Secondly, we explored the effect of cholinergic nb loss on the cortical vasculature. BFCNs synapses are known to regulate vascular tone and cerebral blood flow establishing contacts with different cell types (such as vascular smooth muscle cells, endothelial cells, and astrocytic end-

feet), which constitute the neurovascular unit (NVU). Our investigations demonstrated that losses of cholinergic nb reduced the average diameter of cortical vessels and the expression of vascular endothelial growth factor A. In addition, in such conditions, the density of astrocytes and microglia cells was increased in the cerebral cortex, showing higher colocalization of astrocytic end-feet on arterioles. Furthermore, microglia cells in the parietal cortex displayed morphological alterations indicative of an intermediate activation state.

Finally, I investigated whether a new cholinergic therapy has the potential to prevent the AD-like amyloid pathology in a transgenic rat model at early pathological stages. For that, we tested whether the selective allosteric M1 muscarinic and sigma-1 receptor agonist AF710B, administered daily (10 µg/kg) for seven months, followed by one month of drug interruption (wash-out), attenuated AD-like pathology in transgenic rats. Our studies demonstrated that AF710B prevented cognitive decline, reduced plaque deposition, neuroinflammation and facilitated conversion from BDNF precursor to mBDNF. These results would indicate some preventative, disease-modifying, properties of this compound over disease-aggravating components of the brain's amyloid pathology.

Overall, these studies reinforce the relevance of the BFCNs system in the maintenance of cerebral cortex higher functions. Additionally, they provide a foundation for further studies regarding a preventative strategy to halt or slow down the impact of brain amyloidosis at preclinical AD stages.

Résumé (Français)

Les neurones cholinergiques du cerveau antérieur basal (NCCAB) composent principalement l'innervation du cortex et de l'hippocampe. Ils sont essentiels à l'apprentissage, à la mémoire et à l'attention, et sont parmi les premiers à dégénérer dans la maladie d'Alzheimer (MA) contribuant aux premiers déficits cognitifs. Les inhibiteurs de l'acetylcholinesterase (IAchE), qui améliorent la signalisation cholinergique, peuvent adoucir le déclin cognitif sans toutefois affecter la progression de la maladie.

Plusieurs études récentes ont démontré qu'un traitement IAchE retarde l'atrophie de l'hippocampe, du cortex et du cerveau antérieur, alors que les patients suivant un traitement anticholinergiques sont plus probables à développer la MA, indiquant que la dégénération des NCCAB jouerait un rôle important dans la progression de la pathologie de la MA. Conséquemment, la recherche fondamentale sur les mécanismes pathologiques entamés par la perte des NCCAB ainsi que le développement de thérapies cholinergiques seraient pertinents à prévenir ou retarder la pathologie de la MA.

Ici, nous examinons l'interdépendance entre les NCCAB et les neurotrophines. Le phénotype des NCCAB dépend des quantités du facteur de croissance neuronal mature (mNGF) et du facteur neurotrophique mature dérivé du cerveau (mBDNF). Ces facteurs trophiques produits dans les neurones cibles et transportés de façon rétrograde au soma des NCCAB. Il demeure inconnu si les entrées des NCCAB ont un impact sur la disponibilité des neurotrophines aux synapses des NCCAB. Nos études révèlent qu'après une lésion de 6 mois du noyau basal (NB), un noyau cholinergique du cerveau antérieur basal dont les projections atteignent principalement le cortex, les rats Wistar sont atteints d'un déficit d'attention accompagné d'une réduction de marqueurs neuronaux corticaux glutamatergiques et GABAergiques. De plus, une lésion au NB modifiait la disponibilité de neurotrophines, réduisant l'expression de BDNF (et mBDNF) sans visiblement influencer les niveaux de NGF.

Ensuite, nous avons exploré l'effet d'une lésion au NB sur la vascularisation du cortex. Les synapses des NCCAB régulent le tonus vasculaire et le débit sanguin cérébral et se connectent à divers types cellulaires, comme les cellules musculaires lisses vasculaires, les cellules endothéliales, et les astrocytes composant l'unité neurovasculaire (NVU). Nos résultats

démontrent qu'une lésion au NB réduit le diamètre moyen des vaisseaux corticaux ainsi que l'expression du facteur de croissance vasculaire endothélial. De plus, suite à cette lésion du NB, la densité des astrocytes et cellules microgliales étaient enrichie dans le cortex, avec les pieds astrocytaires ayant une plus grande colocalisation avec les artérioles. Puis, les cellules microgliales du cortex pariétal ont subi un changement morphologique indiquant un état immédiat d'activation.

Finalement, nous avons examiné le potentiel d'une nouvelle thérapie cholinergique dans un modèle animal de la MA dans des rats transgéniques au stade pathologique amyloïde précoce. Pour ce faire, nous avons testé si un agoniste allostérique au récepteur muscarinique M1 et sigma-1, AF710B, administré quotidiennement (10 μg/kg) durant sept mois, suivi d'un mois d'arrêt de traitement, réduisait la pathologie de la MA dans le modèle de rats transgéniques. Nous démontrons que le traitement AF710B réduit le déclin cognitif, en réduisant la déposition de plaque et l'inflammation et en facilitant la conversion du précurseur BDNF en mBDNF. Ces résultats indiquent que AF710B aurait des propriétés préventives et modifiantes face aux composantes aggravantes de la pathologie amyloïde du cerveau.

En somme, nos résultats supportent l'importance des NCCAB pour le maintien des fonctions corticales supérieures, et ouvrent une avenue d'étude sur une stratégie préventive pour ralentir or freiner l'impact de l'amylose du cerveau au stade préclinique de la MA.

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Contributions of Authors

Chiara Orciani (CO) was the lead investigator and first author of the manuscripts in this thesis, and of this dissertation. CO designed experiments in collaboration with A. Claudio Cuello (ACC), in addition to Sonia Do Carmo (SDC), Helene Hall (HH) and Rowan Pentz (RP) for specific manuscripts. CO troubleshooted and optimized protocols, collected data, and analyzed results. CO wrote initial drafts of all manuscripts and generated figures.

ACC was the corresponding author for the manuscripts and contributed original ideas for the academic direction of this thesis, providing intellectual guidance. ACC revised and contributed to all manuscripts.

CHAPTER 2 - Long-term nucleus basalis cholinergic depletion induces attentional deficits and impacts cortical neurons and BDNF levels without affecting the NGF synthesis.

CO, HH, RP, SDC and ACC contributed to the conception and design of the study.

CO performed behaviour, western blots, qPCR and immunohistochemistry.

CO and Morgan K Foret (MKF) contributed to the immunofluorescence.

CO, HH and MKF contributed to the macros for image data analysis.

CO drafted the initial manuscript and prepared the figures.

CO, HH, RP, MKF, SDC and ACC significantly edited the final manuscript.

All authors revised and approved the final manuscript.

CHAPTER 3 - Long-term nucleus basalis cholinergic depletion affects cortical vessels, Vascular Endothelial Growth Factor-A and glial cells in Wistar rats.

CO, SDC and ACC contributed to the conception and design of the study.

CO performed qPCR, western blots and data analysis.

CO and MKF contributed to the immunofluorescence.

CO drafted the initial manuscript and prepared the figures.

CO, SDC, MKF and ACC significantly edited the final manuscript.

All authors revised and approved the final manuscript.

CHAPTER 4 - An M1 and sigma-1 receptor agonist prevents cognitive decline and Alzheimer's disease-like hallmarks in a transgenic rat model.

CO, SDC, HH, and ACC contributed to the conception and design of the study.

Chunwei Wang (CW) performed the animal breeding and genotyping.

CO, SDC, Quentin Bonomo (QB), Agustina Lavagna (AL) and MKF delivered the drug.

CO and MKF perfused and collected the brains.

CO performed behaviour, western blots, immunofluorescence, microscope imaging and data analysis.

SDC processed the samples for the Mesoscale Discovery analyses and conducted the Mesoscale Discovery assays.

MKF performed the GFAP/Iba1 immunofluorescence and microscope GFAP/Iba1 imaging.

AL realized the schematic illustrating our proposed mechanism.

CO drafted the initial manuscript and prepared the figures.

CO, SDC, MKF and ACC significantly edited the final manuscript.

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List of Abbreviations

192-IgG-SAP	192-IgG-saporin
3'-UTR	3'-untranslated regions
5-choice task	5-Choice Serial Reaction Time Task
Aβ	Amyloid beta
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADRDA	Alzheimer's Disease and Related Disorders Association
ADAM17	Metalloproteinase domain 17
AICD	APP (amyloid precursor protein) intracellular domain
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPA-r	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANOVA	Analysis of variance
APH1	Anterior pharynx defective
APP	Amyloid precursor protein
APP/PS1 Tg	Amyloid precursor protein transgenic mouse (APP K670N/M671L; PSEN1 L166P mutations)
APPs	Amyloid precursor protein-derived peptides
AQP-4	Aquaporin-4

BACE-1	β-secretase 1
BBB	Blood-brain barrier
BChE	Butyrylcholinesterase
BDNF	Brain-derived neurotrophic factor
BFCNs	Basal forebrain cholinergic neurons
BLA	Basolateral amygdala
C99 or βCTF	Carboxy-terminal fragment
CA1	Cornu ammonis 1
CAA	Cerebral amyloid angiopathy
CaMKIs	Ca2+/calmodulin-dependent protein kinases
cAMP	Cyclic adenosine monophosphate
CBF	Cerebral blood flow
ChAT	Choline acetyltransferase
CHT1	High-affinity choline transporter
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRDs	Extracellular cysteine-rich domains
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
COL-IV	Collagen IV
CPT	Continuous Performance Test
Cpu	Caudate-putament
CVBM	Cerebrovascular basement membrane

DAG	Diacylglycerol
DBH	Dopamine b-hydroxylase
DLB	Dementia with Lewy bodies
DOPAC	Primary metabolite of dopamine
DR	Dorsal raphe
DS	Down syndrome
EC	Entorhinal cortex
EETs	Epoxyeicosatrienoic acids
E-LTP	Early phase of long-term potentiation (LTP)
EM	Electron microscopy
eNOS	endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA85	[18F]-F-A-85380
FDG	Fluorodeoxyglucose
FDG-PET	Fluorodeoxyglucose 18F-PET
FEOBV	18F-fluoroethoxybenzovesamicol
Fr1	Frontal cortex 1
Fr2	Frontal cortex 2
FTLD	Frontotemporal lobe dementia
GABA	γ-aminobutyric acid
GAD65	Glutamic acid decarboxylase 65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GP	Globus pallidus
GSK-3	Glycogen synthase kinase 3
HDB	Horizontal band of Broca
HRP	Horseradish peroxidase
HSAN IV	Human sensory and autonomic neuropathy type IV
HSAN V	Human sensory and autonomic neuropathy type V
Iba1	Ionized calcium-binding adaptor molecule 1
Ic	internal capsule
ICA	Internal carotid artery
ICV	Intracerebroventricular
IDE	Insulin degrading enzyme
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Ins(1,4,5)P3	Inositol-1,4,5-trisphosphate
IP3Rs type 3	Inositol triphosphate receptors
IPN	Interpeduncular nucleus
IR	Immunoreactive
ITI	Inter-trial interval
LC	Locus coeruleus
LDL	Low-density lipoprotein

LH	Limited hold
L-LTP	Late phase of long-term potentiation (LTP)
LOAD	Late-onset Alzheimer's disease
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein 1
ldt	laterodorsal tegmental nucleus
LTD	Long-term depression
LTP	Long-term potentiation
mAChRs	muscarinic acetylcholine receptors
MAPK	Mitogen-activated protein kinase
mBDNF	mature BDNF
M1 mAChR	Muscarinic acetylcholine receptor type 1
M2 mAChR	Muscarinic acetylcholine receptor type 2
M3 mAChR	Muscarinic acetylcholine receptor type 3
M4 mAChR	Muscarinic acetylcholine receptor type 4
M5 mAChR	Muscarinic acetylcholine receptor type 5
MBI	Mild behavioural impairment
MCA	Middle cerebral artery
MCI	Mild cognitive impairment
MCPO	Magnocellular preoptic field
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MIP	Maximum intensity projection

MMP	Matrix metalloprotease
MMP-1	Matrix metalloprotease-1
MMP-3	Matrix metalloprotease-3
MMP-9	Matrix metalloprotease-9
MMSE	Mini-Mental State Examination
mNGF	mature NGF
MRI	Magnetic resonance imaging
mRNA	messenger RNA
Ms	Medial septum
MSD	Mesoscale Discovery
MWM	Morris water maze
nAcc	nucleus accumbens
nAChRs	nicotinic receptors
nb	nucleus basalis
NBM	Nucleus basalis of Meynert
NCI	Non cognitive impairment
NDS	Normal donkey serum
NEP	Neprilysin
NeuN	Neuronal nuclei
NFTs	Neurofibrillary tangles
NFκB	Nuclear factor kappa-light-chain-enhancer of activated
NGF	Nerve growth factor
NGS	Normal goat serum

NIA-AA	National Institute on Aging Alzheimer's Association
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMDA	N-methyl-D-aspartate
NMDA-r	N-methyl-D-aspartate receptor
NMPB	[11C]N-methyl-4-piperidyl benzilate
NO	Nitric oxide
nNO	neuronal nitric oxide
NOL	Novel object location
NOR	Novel object recognition
NRP	Neuropilin
NSAIDs	Nonsteroidal anti-inflammatory drugs
NVU	Neurovascular unit
OF	Open field
P2RY12	Purinergic receptor P2Y12
p75ntr	p75 neurotrophin receptor
Par	Parietal cortex
PBS	Phosphate saline buffer
PET	Positron emission tomography
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1

PlGF	Placental growth factor
ppt	pedunculopontine tegmental nucleus
pre-MMP	precursor form of MMP
pre-proBDNF	precursor form of proBDNF
proBDNF	precursor BDNF
proNGF	precursor NGF
PS	Presenilin
PSD95	Postsynaptic density 95
PSEN1	Presenilin 1
PSEN2	Presenilin 2
p-Tau	Phosphorylated tau
PVMs	Perivascular macrophages
QNB	123I-iodo-quinuclidinylbenzilate
RAGE	Receptor for advanced glycation end products
RFP	Red fluorescent
ROI	Region of interest
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
Sal	Saline injected
SAP	192-IgG Saporin injected
sAPPα	Soluble amyloid precursor protein α
sAPPβ	Soluble amyloid precursor protein β
SART	Sustained Attention to Respond Task

SD	Stimulus duration
SI	Substantia innominata
Sig-1R	Sigma-1 receptor
SIVD	Subcortical ischemic vascular dementia
sLRP1	soluble form of LRP1
SMCs	Smooth muscle cells
SORL1	Sortilin-related receptor 1 gene
SN	Substantia nigra
SNPs	Single nucleotide polymorphisms
SP	Social preference
Tg	Transgenic
Tg-sal	Transgenic treated with saline
Tg-AF710B	Transgenic treated with AF710B
TH	Tyrosine hydroxylase
THA	Tacrine
Thiof-S	Thioflavin-S
TIMP1	Tissue inhibitor of metalloproteinases-1
TNFR	Tumor Necrosis Factor Receptor
tPA	tissue plasminogen activator
TREM2	Triggering receptor expressed on myeloid cells
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
TO	Time-out

V66M	Acid substitution (valine to methionine) at codon 66
VAChT	Vesicular acetylcholine transporter
VBB	Vertical band of Broca
VDB	Vertical diagonal band
VEGF-A	Vascular endothelial growth factor A
VEGFR-2	Vascular endothelial cell growth factor receptor 2
vGluT1	Vesicular glutamate transporter 1
VTA	Ventral tegmental area
VIP	Vasoactive intestinal peptide
WB	Western blot
Wt-sal	Wild-type treated with saline
Wt-AF710B	Wild-type treated with AF710B
αSMA	α-Smooth muscle actin
α-CTF or C83	C-terminal fragment known as the APP-CTF fragment
α7-nAChR	α-7 nicotinic acetylcholine receptor

CHAPTER 1 – Introduction

1.1 History of Alzheimer's disease

The German neuropathologist Alois Alzheimer described the first case of "presenile dementia" in 1907, with the publication of a report entitled "On an unusual illness of the cerebral cortex" ((Alzheimer, 1907); English translation (Stelzmann et al., 1995)). In this study, he described the case of a 51-year-old woman placed in an insane asylum in Frankfurt. Alzheimer reported that the woman's symptoms were extreme jealousy towards her husband, followed by progressive memory loss, disorientation, visual hallucinations, aggressiveness, and paranoia. After her death, four and half years after her admission to the asylum, Alzheimer performed a post-mortem neuropathological analysis of the brain. The brain was highly atrophic with vascular arteriosclerotic changes. In addition, histological analyses revealed "striking changes of the neurofibrils" characterized by their "unique thickness and capacity for impregnation" using Bielschowsky's silver stain. One-third of neurons in the cerebral cortex displayed these neurofibrillary pathological changes accompanied by the disappearance of the upper neuronal layer. He further proposed that fibrillar changes were the result of the deposition of a "metabolic substance" inside neurons and the occurrence of "minute miliary foci" (currently defined as amyloid plaques) in the cerebral cortex (Alzheimer, 1907).

The work that supported Alzheimer's conclusion was driven by reports of other contemporary brilliant neuroscientists such as Kraepelin, Nissl, Lewy, Cerletti, Perusini and Bonfiglio, which contributed to this discovery (Boller and Forbes, 1998). As a matter of fact, the group led by Arnold Pick had described "lobar atrophy" 15 years before Alzheimer's paper (Pick, 1892). In the same year of Pick's publication, the presence of brain "foci" was noticed by Paul Blocq and Georges Marinesco in the brain of an old individual with epilepsy (Blocq and Marinescu, 1892). Furthermore, Emil Redlich described the presence of "miliary sclerosis" in two cases of senile dementia and referred to them as plaques (Redlich, 1898). Blocq, Marinesco and Redlich did not further investigate the relevance of these aggregates as they believed that the plaques represented a modified glial cell (Blocq and Marinescu, 1892, Redlich, 1898).

In the same year that Alzheimer communicated his observations, Oskar Fischer described the presence of plaques in 12 of 16 cases of senile dementia, and for the first time, he described the

neuritic plaque (FISCHER, 1907). He proposed that the presence of plaques and neuritic plaques was associated with a clinical condition named “presbyophrenia”, a dementia subtype characterized by disorientation, short-memory impairment and euphoria (reviewed in (Goedert, 2009)). Following Perusini's work (Perusini, 1911) which confirmed that senile plaque represented a specific finding in senile dementia cases, the name “Fisher's plaques” began to appear in the literature (reviewed in (Boller and Forbes, 1998)).
While the work of Nissl and Perusini was important (Macchi et al., 1997), Alois Alzheimer was the driving force of the group, and it was considered legitimate to name this dementia Alzheimer’s Disease (AD) (Boller and Forbes, 1998).

1.2 The burden of Alzheimer's disease

Globally, 47 million people are estimated to be affected by dementia, and it is projected to rise to 135 million by 2050 (Prince et al., 2013). In Canada, dementia afflicts around half a million people, and this number will double by 2031 (Wong et al., 2016, Nichols et al., 2019). This increased number of people affected with dementia is mostly driven by the demographic shift toward an older population (Manuel et al., 2016). As such, the true cost of AD must include not only the death toll but also the burden placed on families and institutional caretakers. Furthermore, this rising number of individuals suffering from AD will double the cost of the Canadian healthcare system, from 9.2 to 18.2 billion dollars, by 2031 (Manuel et al., 2016).

The heterogeneity of disease manifestation and progression among patients has been identified as a critical issue (Ferretti et al., 2018). Not all individuals are affected by AD in the same proportion: twice as many females than males die of dementia each year, and the extent to which this effect is maintained after correcting for the longer lifespans of females is controversial (Ferretti et al., 2018). In addition to sex differences, racial disparities are understudied in Canada, and data from the United States shows that African and Hispanic Americans have a greater risk for AD, while Asian Americans may be protected; meanwhile, conflicting data exist for Indigenous North Americans (Matthews et al., 2019, Wong and Amano, 2019).

Overall, these numbers highlight the urgent need for new AD therapeutics, even symptomatic ones, and the essential nature of the investigations into AD pathobiology that will render them possible. Phenotypic and genetic factors can guide patient selection, stratification and clustering for AD

diagnosis (Ferretti et al., 2018). Future research studies aim to account for sex differences, ethnoracial factors and other disparities markedly understudied (Babulal et al., 2019).

1.3 Genetic and sporadic Alzheimer's disease

AD can be divided into different types based on the age of onset and genetic predisposition. Early onset (EOAD) or familial AD is rare and usually manifests by age 60 and presents a genetic etiology of up to 100% (Cacace et al., 2016, Hoogmartens et al., 2021).

Sporadic or late-onset AD (LOAD) accounts for over 95% of cases and begins after the age of 65 years. Key genetic and environmental risk factors seem to trigger sporadic cases, and in LOAD patients, the genetic etiology is responsible for up to 82% (Cacace et al., 2016).

Although EOAD and LOAD patients develop AD at different ages, they show similar clinical symptoms, and neuropathological features and both occur in familial and sporadic patients, with aging as the most important risk factor.

1.3.1 Early-Onset Alzheimer’s disease

Autosomal dominant EOAD families were essential for the identification of the three genes responsible for AD: amyloid precursor protein (APP) and presenilin 1 and 2 (PSEN1 and PSEN2), which are key players in the Aβ pathology further discussed (reviewed in (Hoogmartens et al., 2021, Selkoe and Hardy, 2016)).

The pathogenic missense mutations and whole gene duplications have been identified in APP. They are mostly located near the β- or γ-secretase cleavage sites or in the Aβ sequence of the APP protein (Dai et al., 2018), resulting in the overproduction of either total Aβ or shifting towards the production of the more toxic Aβ1-42 peptide (Hoogmartens et al., 2021). Relevant APP mutations are: the London mutation, which was the first to be discovered (V171I, increased Aβ42/40 ratio and Aβ42)(Goate et al., 1991), Indiana mutation (V717F, increased Aβ42/40 ratio)(Murrell et al., 1991), Florida mutation (I716V, increased Aβ42/40 ratio and Aβ42)(Eckman et al., 1997), and the Swedish mutation (a double mutation, KM670/671NL, increased total Aβ, increased Aβ42 and Aβ40)(Mullan et al., 1992). Other mutations are located within the Aβ sequence where they enhance self-aggregation of Aβ peptides as in the Dutch mutation (E693Q) (Levy et al., 1990, Van Broeckhoven et al., 1990, Wisniewski et al., 1991).

Another example of a population with EOAD is individuals with Down Syndrome (DS), who, given triplication of chromosome 21 and hence triplication of APP, will develop AD neuropathology and dementia by age 40-60 (reviewed in (Lott and Head, 2019, Fortea et al., 2021)). Individuals with DS display Aβ accumulation early in their lifespan, followed by the appearance of NFT by age 30-40. For this reason, DS individuals represent a significant population to study the long asymptomatic phase of AD (Fortea et al., 2021). Individuals with DS without full triplication of APP do not develop AD but display the DS phenotype (Doran et al., 2017, Prasher et al., 1998). On the other hand, individuals with mini-duplications of the APP gene do not have DS but develop AD in their 50's (Rovelet-Lecrux et al., 2006).

Interestingly, the protective A673T mutation in the second Aβ amino acid decreases APP cleavage by β-secretase (Jonsson et al., 2012). Carriers of this protective mutation have a lower risk of developing AD as well as age-related cognitive decline (Jonsson et al., 2012).

In addition to these three causal AD genes, other variants are present in familial AD. Genome-wide association studies (GWAS) led to the identification of common genetic variants with reduced penetrance associated with AD risk. One example of a mutation with reduced penetrance is a common variant in the sortilin-related receptor 1 gene (SORL1) (Reitz et al., 2011) associated with EOAD (Pottier et al., 2012). SORL1, present on the endosomes, sorts APP receptors and avoids redirecting APP toward the β-secretase cleavage pathway (Rogaeva et al., 2007).

1.3.2 Late-Onset Alzheimer's disease

The ε allele of the apolipoprotein E (APOE) gene is a genetic modifier identified as a major genetic risk factor for LOAD. The first study to uncover the APOE role came from the cardiovascular field. Plasma concentration of ApoE of healthy control subjects was reported to depend on the APOE isoform carried by the subject. Humans with two alleles ε2 (APOE2/2 genotype) express the highest levels of plasma ApoE and humans with two alleles ε4 (APOE4/4 genotype) express the lowest plasma ApoE levels (Utermann et al., 1980). At the same time, individuals with APOE2/2 also exhibit the lowest risk of developing AD and individuals with APOE4/4 have a higher risk of AD (Corder et al., 1994). These results were confirmed in both plasma and brain tissue of AD cases (Poirier, 2005, Bertrand et al., 1995).

In addition to ε3 and ε4 alleles, ε3 carriers, representing the most common APOE genotype, have neutral outcomes concerning AD risk (Arboleda-Velasquez et al., 2019). ApoE is the main component of lipoproteins in plasma (reviewed in (Hatters et al., 2006)). In carriers of ε3 and ε4 alleles, ApoE binds to low-density lipoprotein (LDL) receptors with high affinity, and in carrier ε2, this binding is 50- to 100- times weaker, inducing increased plasma cholesterol levels and premature cardiovascular disease (Hatters et al., 2006, Mahley, 1995). ε4 allele is present in more than half of sporadic AD cases. This isoform leads to Aβ aggregation and impaired clearance, leading to an increase in Aβ toxic effects (for a detailed review (Liu et al., 2013)). Although APOE ε4 allele is associated with LOAD (Farrer et al., 1997), in comparison to APP, PSEN1, and PSEN2 mutations, it is not considered necessary nor sufficient to cause the disease and was therefore categorized as a risk allele for AD (Cacace et al., 2016).

Other isolated mutations were associated with the inflammatory response (Guerreiro et al., 2013a, Karch and Goate, 2015). In particular, mutations have been found associated with CD33, a receptor expressed on myeloid and microglial cells, which activation leads to monocytic inhibition and decreases phagocytosis (Crocker et al., 1997). Increased CD33 levels have been detected in AD brains and positively correlate with cognitive decline (Karch et al., 2012). In addition, missense mutations in the triggering receptor expressed on myeloid cells 2 (TREM2) lead to impaired microglial phagocytosis and an increased risk of developing LOAD (Guerreiro et al., 2013b, Jonsson et al., 2013).

1.4 Clinical progression of Alzheimer's disease

1.4.1 Subjective Cognitive Impairment

Subjective cognitive impairment (SCI) is a cognitive decline reported by a patient's subjective report. In this report, the patient usually describes deficits in remembering names and recalling where things were placed. However, these patients perform well in psychometric and mental status tests such as the Mini-Mental State Examination (MMSE) (reviewed in (Reisberg et al., 2008)).

The term SCI has been suggested as the stage that preceded mild cognitive impairment (MCI) in the evolution of AD (Reisberg, 1986, Reisberg and Gauthier, 2008), and it was estimated to last approximately 15 years before the subsequent MCI stage in the evolution of AD (Reisberg et al., 2008).

Although some studies have cast doubt on the validity of SCI as a predictor of detectable cognitive impairments, cognitive decline, or dementia (Hollands et al., 2015, Thompson et al., 2015), the emerging classification of Mild Behavioural Impairment (MBI) proposes that a precursor state to MCI; delineated by the presence of anxiety, depression, and other mild neuropsychiatric symptoms (Gallagher et al., 2011, Ismail et al., 2017, Yokoi et al., 2019).

1.4.2 Mild Cognitive Impairment

MCI is the stage that precedes dementia in the most widely used classifications (Gauthier et al., 2006).

MCI individuals have a cognitive decline which does not interfere with daily activities (Petersen et al., 1999). However, individuals with MCI have a 3 to 7-fold increased risk of developing dementia, particularly of the Alzheimer's type (Gauthier et al., 2006, Petersen, 2004, Bennett et al., 2002). Indeed, MCI patients exhibit a faster rate of cognitive decline in episodic memory, semantic memory, and processing speed, while working memory is relatively spared (Bennett et al., 2002).

Several conditions can lead to the MCI condition, such as vascular, traumatic brain injury and medical causes of cognitive decline (Albert et al., 2011).

1.4.3 Dementia

After MCI, when individuals enter the moderate stage of dementia (the longest phase), they have difficulties communicating and performing daily tasks. Behavioral and personality changes, such as agitation, apathy, depression, hallucinations, and suspiciousness, are common at these stages (Albert et al., 2011, Mega et al., 1996). The late stages of AD are characterized by neuropsychiatric symptoms such as dysphoria, delusions and paranoia (Mega et al., 1996). As the disease progresses, motor impairments affecting speech, walking, and swallowing make the patient bed-bound (Association, 2019).

Generally, life expectancy after AD dementia diagnosis is 4 to 8 years. However, in some cases, certain individuals can live 20 years or more (Association, 2019).

The National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer’s Disease and Related Disorders Association (ADRDA) established the clinical

diagnosis of AD in 1984. It was later revised in 2011 by the National Institute on Aging Alzheimer's Association (NIA-AA), which introduced three terminologies to classify individuals with AD (reviewed in (McKhann et al., 2011, Jack Jr et al., 2011)).

The first classification is probable AD with amnestic dementia, in which individuals display learning and recall impairments. The second one is probable AD with non-amnestic dementia, characterized by visuospatial, language and executive deficits. The third one is probable or possible AD dementia with evidence of the AD pathophysiological process, used only for research purposes.

Besides the recent developments that allow early detection of possible or probable AD, a definite diagnosis depends on histological post-mortem confirmation.

1.5 Preclinical stages of Alzheimer's disease

The preclinical stage of AD, which precedes the clinical stage, gained much importance in research since it could be fundamental for the early detection and prevent AD progression. Unfortunately, the time between the detection of Aβ abnormalities and the onset of cognitive decline has not been established yet; but it has been suggested to have a duration of 15-30 years (Sperling et al., 2014, Wang et al., 2019, Villemagne et al., 2013).

Based on the hypothetical model discussed below and illustrated in **Figure 1-1** (Jack Jr et al., 2013) the NIA-AA has proposed some guidelines to describe the preclinical AD stage in research settings (Sperling et al., 2011).

The first detectable abnormality in AD preclinical stages is a drop in Aβ42 levels in the cerebrospinal fluid (CSF) (Tapiola et al., 2009, Skoog et al., 2003); and an increased Aβ binding in the brain as revealed by positron emission tomography (PET) (Jack Jr et al., 2008, Jack Jr et al., 2009). In fact, as amyloid accumulates in the brain, its amyloid levels decrease in the CSF. Following changes in Aβ, increased tau and p-tau levels in the CSF become detectable (Tapiola et al., 2009, Buerger et al., 2006); and correlate to neuronal loss and NFT formation (Jack Jr et al., 2013).

Subsequently, a reduction of cerebral glucose metabolism in the posterior cingulate, precuneus, and temporoparietal cortices has been measured in vivo by fluorodeoxyglucose 18F-PET (FDG-

PET) uptake (Sperling et al., 2011, Iturria-Medina et al., 2016). At the same time, magnetic resonance imaging (MRI) studies showed brain atrophy, affecting mostly the lateral temporal, posterior cingulate, medial parietal cortices and hippocampus (Sperling et al., 2011).

Today, it is not possible to predict whether an individual will develop AD with the current preclinical CSF AD biomarker signature. Interestingly, deregulation in pre and post-synaptic proteins (Scheff et al., 2016, Zolochevska et al., 2018), in the nerve growth factor (NGF) metabolic pathway (Pentz et al., 2020), in brain-derived neurotrophic factor (BDNF) (Boots et al., 2017), in oxidative stress (Scheff et al., 2016) and inflammation (Flores-Aguilar et al., 2021) have been observed in cognitively normal individuals with AD pathology.

For this reason, developing more sensitive assays and including other biomarkers could allow the identification of earlier pathological changes in the AD continuum.

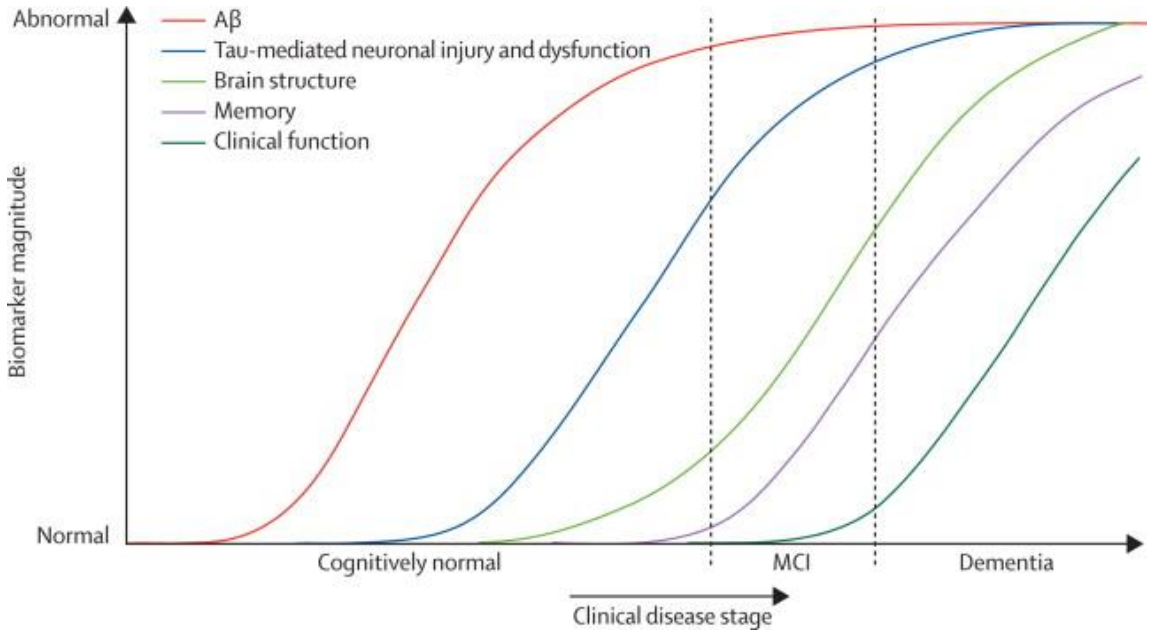

Figure 1-1. Hypothetical model of dynamic biomarkers of preclinical and clinical Alzheimer's disease.

First, Aβ measured in CSF, or PET amyloid imaging becomes abnormal. This is followed by CSF tau abnormalities. FDG PET and MRI are the last biomarkers to be altered shortly before the appearance of cognitive impairment. Abbreviations: Aβ, amyloid-β; CSF, cerebrospinal fluid; PET, positron emission tomography; FDG, fluorodeoxyglucose; MCI, mild cognitive impairment. Image reprinted from (Jack Jr et al., 2013), Journal The Lancet Neurology, with permission from Elsevier.

1.6 Neuropathological hallmarks of Alzheimer's disease

The major neuropathological hallmarks of AD brains are the deposition of amyloid plaques in the parenchyma and neurofibrillary tangles inside the neurons. In addition, the AD brain is characterized by the presence of brain atrophy, synaptic loss, inflammation, vascular pathology and neurotransmitter dysfunction.

1.6.1 Amyloid pathology

The plaques, previously described by Alzheimer (Alzheimer, 1907), were much later studied by Robert Terry and Michael Kidd with electron microscopy techniques, and they demonstrated that they are composed of an amyloid protein (Kidd, 1964, Terry et al., 1964). Almost 20 years later, Glenner and Wong isolated the amyloid protein from the cerebrovasculature of AD (Glenner and Wong, 1984a, Glenner and Wong, 1984b). This 4 kDa protein, nowadays, is known as the "amyloid-beta" (Aβ) peptide and is composed of 40-42 amino acids Aβ fragments. The Aβ42 fragments, more prone to form aggregates, are widespread in the cortex throughout the six layers (Serrano-Pozo et al., 2011), and they are also present in the hippocampus (Miller et al., 1993).

Two types of amyloid plaques exist. The first type (1) are diffuse amyloid plaques lacking distinct edges, Congo Red and Thioflavine-S negative and not commonly surrounded by astroglia, microglia or dystrophic neurites. These plaques are more abundant in preclinical stages of AD (Dickson and Vickers, 2001). Nevertheless, the presence of diffuse amyloid plaques does not guarantee the occurrence of AD.

The second type (2) is dense core or mature amyloid plaques, CongoRed and Thioflavin-S dyes positive and commonly surrounded by activated glia and dystrophic neurites are generally present (Serrano-Pozo et al., 2011).

Although the deposition of amyloid plaques in the AD brain follows an unpredictable progression, researchers have conducted histological studies aiming to stage AD progression by amyloid plaque deposition. Heiko Braak and Eva Braak were the first to propose a three stages classification criteria following amyloid deposition (Braak and Braak, 1991). In the first stage (stage A), they detected amyloid deposits in the frontal, temporal and occipital lobes. Subsequently, amyloid plaques appear in the isocortical association areas with a mild involvement of the hippocampus

(stage B). In the third stage (stage C), amyloid deposits spread to all cortical areas, to the molecular layer of the cerebellum and to subcortical nuclei.

A second, more detailed criteria that followed the amyloid deposition was proposed by Thal and collaborators (Thal et al., 2002). This five-stage classification started with amyloid plaque deposition in isocortical areas (stage 1), followed by deposits in allocortical areas such as the entorhinal cortex, hippocampus, amygdala and the insular and cingulate cortices (stage 2). Subsequently, the amyloid plaques appear in the striatum, basal forebrain cholinergic nuclei, thalamus, hypothalamus and white matter (stage 3) and after in some structures in the brainstem such as the substantia nigra, reticular formation of the medulla oblongata, superior and inferior colliculi and the red nucleus (stage 4). In the end, amyloid plaques spread to other areas in the brainstem, such as the raphe nuclei, locus coeruleus and the molecular layer of the cerebellum (stage 5).

1.6.1.1 APP processing and Aβ generation

In the late 1980s, Robakis and colleagues found that the β-amyloid identified by Glenner and Masters was cleaved post-translationally from a larger precursor: the amyloid precursor protein (APP) (Robakis et al., 1987a, Robakis et al., 1987b). This finding was supported by the studies of Tanzi and Kang (Tanzi et al., 1987, Kang et al., 1987). In the same year, Kang and Goldgaber discovered that the APP protein was a product of a gene mapped to chromosome 21 (Kang et al., 1987, Goldgaber et al., 1987). The APP gene structure was later identified by Yoshikai and colleagues (Yoshikai et al., 1990).

APP codifies for a transmembrane type I glycoprotein, and it is evolutionary conserved across different species indicating a common but presently unresolved physiological role (reviewed in (Nhan et al., 2015)). APP undergoes alternative splicing, and three isoforms (APP751, APP770, and APP695) are produced, with APP695 expressed mainly in neurons. Two main pathways are responsible for APP processing: the amyloidogenic pathway, in which toxic and more likely to aggregate Aβ peptides are generated, and the non-amyloidogenic pathway, where not toxic Aβ peptides are produced (**Figure 1-2**) (Nhan et al., 2015).

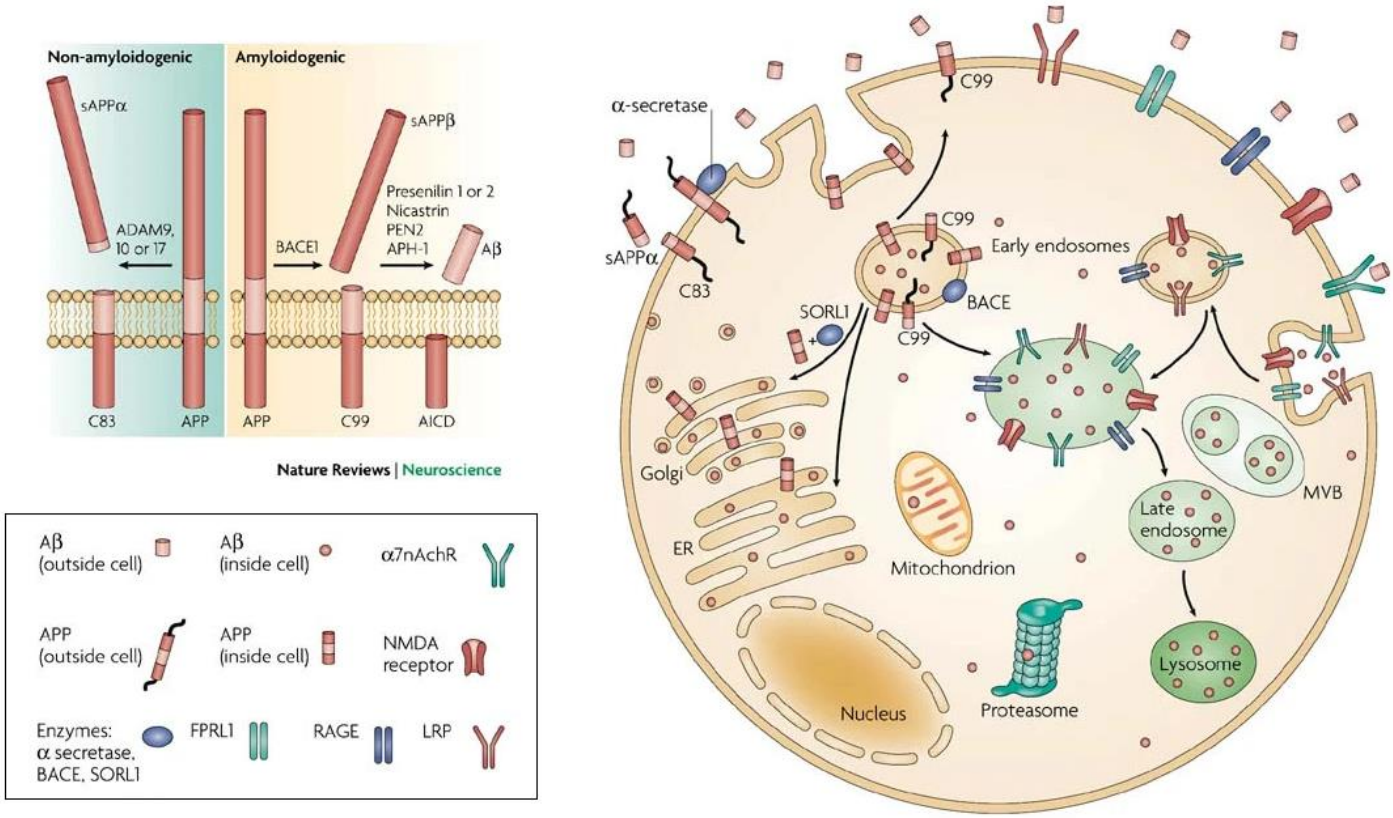

Figure 1-2. Schematic representation of amyloid precursor protein (APP) processing.

APP proteolysis through the amyloidogenic and non-amyloidogenic pathways is shown on the left. Sites of cellular Aβ production are shown on the right. Abbreviations: Aβ, Amyloid beta; α7-nAChR, α-7 nicotinic acetylcholine receptor; APP, Amyloid precursor protein; NMDA, N-methyl-D-aspartate; BACE-1, β-secretase 1; SORL1, Sortilin-related receptor 1 gene; RAGE, Receptor for advanced glycation end products; LRP, Lipoprotein receptor-related protein 1; sAPPα, Soluble amyloid precursor protein α; sAPPβ, Soluble amyloid precursor protein β. Images reprinted and adapted from (LaFerla et al., 2007), Journal Nature Reviews Neuroscience, with permission from Springer Nature.

The non-amyloidogenic pathway involves APP sequential cleavage by α and γ-secretases. A Member of the α-secretases is the ADAM (disintegrin and metalloprotease) family, such as ADAM9, ADAM10 and ADAM17 (also known as tumour necrosis factor α-converting enzyme, TACE) (Lammich et al., 1999). α-secretase cleaves APP in the middle of the Aβ region, releasing the soluble ectodomain named soluble-APPα (sAPPα) and a membrane-bound intracellular C-terminal fragment known as the APP-CTF fragment (α-CTF or C83) and therefore preventing the formation of toxic amyloid-beta peptides and favouring synaptogenesis (Bell et al., 2008, Kojro and Fahrenholz, 2005). Subsequently, γ-secretase, a macromolecular protein complex consisting of presenilin (PS), presenilin enhancer (PEN2), nicastrin, and anterior pharynx defective (APH1), cleaves the carboxyl-terminal fragment generating the p3 peptide and the APP intracellular domain (AICD or C59) (Nhan et al., 2015).

On the other hand, the amyloidogenic pathway involves sequential cleavage by β and γ-secretases. β-secretase is the only enzyme involved in APP processing (Vassar et al., 1999) and is represented by BACE-1 (β-site APP cleaving enzyme also known as Asp-2 and memapsin-2). First, BACE-1 cleaves between residues 671 and 672, generating the N-terminus of Aβ and releasing a part of the APP ectodomain (sAPPβ). The generated carboxy-terminal fragment (C99 or βCTF) is then cleaved by γ-secretase; this last step generates the Aβ peptide and the AICD fragment (Nhan et al., 2015). Secreted forms of Aβ tend to aggregate and form oligomers and then the fibrils, called amyloid plaques.

1.6.1.2 Intraneuronal Aβ

Evidence from AD-vulnerable brain regions, along with transgenic animal models of the amyloid-like pathology, indicates that the accumulation of intraneuronal Aβ precedes its extracellular deposition and plaques formation (Gouras et al., 2010, Gouras et al., 2000, LaFerla et al., 2007).

In neurons, the production of Aß typically occurs on membranes of subcellular compartments (LaFerla et al., 2007), where APP, BACE-1 and γ-secretase are expressed. In particular, Aß40 primarily localizes to the trans-Golgi network, while Aß42 localizes to the endoplasmic reticulum (Hartmann et al., 1997). Various Aß peptides are also observed in endosomes (Koo and Squazzo, 1994), in multivesicular bodies (Takahashi et al., 2002) and within exosomes (Rajendran et al., 2006).

An enormous plurality of roles has been suggested for the physiological role of the various Aß peptides. For example, APP peptides have been shown to regulate synaptic plasticity (Priller et al., 2006, Bell et al., 2008); and interestingly, conservation analysis of APP proteins revealed that the cell-adhesion motifs of APP are highly conserved, indicating that this might be central to their functions (EJ, 2000). However, whether this function represents a central one remains to be determined.

1.6.1.3 Aβ Clearance

In the brain, Aβ clearance is mediated by different systems, cell types and enzymes (reviewed in (Zuroff et al., 2017)). Some examples are intracellular degradation within lysosomes, the ubiquitin-proteasome system and autophagy (Zuroff et al., 2017). The insulin-degrading enzyme (IDE) (Mukherjee et al., 2000) and neprilysin (NEP) (Kanemitsu et al., 2003) can degrade soluble Aβ40, and Aβ42 in the extracellular space or they can be phagocytized and catabolized by monocytic cells (Koronyo-Hamaoui et al., 2009, Simard et al., 2006).

A mechanism of Aβ clearance in the brain is mediated by the BBB. This mechanism's clearance from the brain to blood is mediated by the low-density lipoprotein receptor-related protein-1 (LRP1). In the opposite direction (from the blood to the brain) operates the receptor for advanced glycation end products (RAGE) (reviewed in (Deane et al., 2009)). Interestingly in AD, at the BBB, RAGE levels are increased, and the soluble form of LRP1 (sLRP1) binding of peripheral Aβ is reduced, therefore preventing Aβ clearance from the brain (Deane et al., 2009).

In addition to clearance across the BBB, clearance by the glymphatic system might be an alternate mechanism to clear Aβ from the brain to the periphery (Iliff et al., 2012). Experimental evidence from mice suggests that CSF enters the brain from the subarachnoid space along the periarterial space surrounding penetrating arteries (Iliff et al., 2013). Aβ contained within the CSF is removed from the brain along paravenous spaces and is returned to the blood. This depends on glial water influx mediated through the astrocyte aquaporin 4 (AQP4) water channel (Iliff et al., 2012, Jessen et al., 2015).

Failure in any of the above mechanisms and the rate of Aβ clearance (Zlokovic et al., 2000) may contribute to amyloid deposition and development and/or progression of AD.

1.6.1.4 Cerebral amyloid angiopathy

The majority of AD brains (80%) display mild cerebral amyloid angiopathy (CAA) at autopsy (Serrano-Pozo et al., 2011), which represents the deposition of Aß on and within the walls of cerebral blood vessels (Joachim et al., 1988, Jellinger, 2002). CAA is typically produced from deposited Aß40 peptides rather than Aß42 (Herzig et al., 2004), and parietal and occipital cortices are mainly affected (Serrano-Pozo et al., 2011).

Post-mortem brain analyses revealed that amyloid deposition begins in the basement membrane and subsequently is observed in the tunica media, inducing a morphological change of the vessels to a double-barrel (Vonsattel et al., 1991). Vessels afflicted with CAA are also more prone to microhemorrhages (Vinters, 1987), and show a reduced ability to respond to the demands of local tissues (Christie et al., 2001).

1.6.1.5 The amyloid hypothesis

The hypothesis that the A4 peptide (or Aβ) could be the trigger cause of AD was suggested by Glenner and Wong (Glenner and Wong, 1984a, Glenner and Wong, 1984b). However, it was not until the discovery that certain mutations favoured the development of AD in APP that the so-called “amyloid hypothesis” was proposed by John Hardy (Hardy and Higgins, 1992) and revisited 10 years later by Hardy and Selkoe (Hardy and Selkoe, 2002). It was also hypothesized that this abnormal accumulation arises from an improper balance between the production and clearance of Aβ (Hardy and Selkoe, 2002, Kang et al., 2000).

The amyloid hypothesis poses Aβ pathological accumulation as the etiological factor that drives a cascade of events, such as synaptic loss, neurofibrillary tangles formation, and inflammation, eventually leading to AD development. The rationale behind the amyloid hypothesis is based on distinct scenarios where Aβ overproduction causes genetic or sporadic AD along with the chronological neuropathological hallmarks presentation (Aβ deposition precedes neurofibrillary tangles formation). In addition, experimental injection of Aβ oligomers in non-human primates can promote tau hyperphosphorylation (Wakeman et al., 2022, Forny-Germano et al., 2014, Geula et al., 1996).

Almost 30 years have passed since the formulation of the amyloid hypothesis, and it is not universally accepted. The main critique is that neurofibrillary tangles pathology correlates better with cognitive impairment than the number of amyloid plaques.

The amyloid hypothesis was revisited since a large body of literature showed that the most toxic amyloid species are Aβ oligomers rather than plaques (Hardy and Selkoe, 2002). Many studies supported this concept and found a strong correlation between soluble cortical Aβ levels and cognitive impairment (McLean et al., 1999). Multiple hypotheses have been proposed to explain how Aβ oligomers promote cognitive deficits. One hypothesis proposed that Aβ oligomers can cause disruption of long-term potentiation (LTP) formation, promoting cognitive deficiencies in AD-like transgenic models (Selkoe, 2002).

1.6.2 Neurofibrillary tangles

Alzheimer was the first to identify neurofibrillary tangles (NFTs) in AD brains (Alzheimer, 1907). NFTs are formed by paired helical filaments (Kidd, 1963, Terry et al., 1964) of abnormally misfolded and hyperphosphorylated tau protein (Goedert et al., 1988, Grundke-Iqbal et al., 1986).

Tau is a microtubule-binding protein normally located within neurons (Weingarten et al., 1975). Tau is important for microtubule assembly and stabilization and plays a physiological role in intracellular transport, neurite development and cell polarity (Mandelkow and Mandelkow, 1998). There are six tau isoforms in the adult human Central Nervous System (CNS), including three isoforms that have four microtubule-binding repeats (4R tau) and three isoforms that lack the second repeat (3R tau) (Goedert et al., 1989b). These isoforms are derived from alternative splicing of exons 2, 3, and 10 of the MAPT gene (Wang and Mandelkow, 2016).

In AD, both 3R and 4R tau species are implicated in AD (Goedert et al., 1992), and the hyperphosphorylation of tau could be the consequence of increased kinase activity and deficiencies in phosphatase activation (Wang and Mandelkow, 2016). In AD, in addition to phosphorylation, tau undergoes other modifications (such as N-truncation, ubiquitination, glycation or oxidation) that can decrease the affinity of tau for microtubules, increasing the propensity of tau to become insoluble and aggregate (Garcia-Sierra et al., 2008, Abraha et al., 2000, Bramblett et al., 1993). For this reason, tau can impair neural functions through loss of normal function as well as pathological gain-of-function, forming toxic tau aggregates. At advanced AD stages, following the death of NFT-bearing neurons appear tau "ghost" tangles (Mena et al., 1991).

NFT formation follows a predictable spatiotemporal pattern in AD, which Braak characterized into six stages (Braak and Braak, 1991). In stage 1, NFTs appear in the perirhinal region; in stage II, they are present in the entorhinal cortex, the cornu ammonis 1 (CA1) region of the hippocampus and the anterodorsal nucleus of the thalamus. Stage III is characterized by the accumulation of NFTs in limbic regions such as the hippocampal subiculum. During stage IV, NFTs appear in the amygdala, thalamus, claustrum and, to a small extent, in isocortical association areas. In stage VI, NFTs spreads to all isocortical areas involving motor, visual, and sensory areas in the final stage VI.

The extent of NFT deposition correlates with the degree of cognitive impairment in AD, unlike amyloid plaque deposition (Arriagada et al., 1992, Giannakopoulos et al., 2003). For this reason, NFT formation was suggested to reflect better the ongoing neurodegeneration in the AD brain.

1.6.3 Brain atrophy and synaptic loss

AD brain atrophy is well-established; in particular hippocampal atrophy is among the core biomarkers used in research studies to study the progression of AD pathology (Pini et al., 2016, Jack Jr, 2011). Hippocampal volume was shown to be reduced by 10-15 % in mild cognitive impairment (MCI) (Shi et al., 2009) and by 15-30% in mild AD (van der Flier et al., 2005). On the other hand, hippocampal atrophy lack specificity for AD as it can be present in other dementias, such as vascular dementia, Parkinson's dementia and frontotemporal lobar degeneration (Pini et al., 2016).

Cortical atrophy seems to closely recapitulate the neuropathological staging of NTF pathology (Braak and Braak, 1991). Indeed, the medial temporal lobe, including the entorhinal cortex, is affected earlier, and it extends to the cortex following a temporal–parietal–frontal trajectory; meanwhile, the motor areas are generally spared until the late stages of the disease (Apostolova et al., 2007, Dickerson et al., 2009, Frisoni et al., 2009, Lerch et al., 2005). Moreover, this topographical progression correlates with disease severity and the appearance of clinical symptoms (Pini et al., 2016).

Atrophy also affects subcortical areas and, in particular, early atrophy of basal forebrain cholinergic nuclei could represent an early biomarker for AD diagnosis, highlighting the potential disease-modifying properties of cholinergic treatments, further discussed.

The gross and obvious atrophy of the AD brain is accompanied by synaptic loss. Synapses loss was revealed by electron microscopy studies in biopsies from the frontal, temporal entorhinal cortices and hippocampus of AD brains (Scheff et al., 1990, Davies and Maloney, 1976, Scheff and Price, 1993, Scheff et al., 1993, Scheff et al., 1996). Such synaptic loss correlated strongly with cognitive impairment (Terry et al., 1991).

Immunohistochemical techniques allowed the quantification of pre and post-synaptic markers. The presynaptic protein synaptophysin was reported to be decreased in the hippocampus and cortices of mild AD brains since stages of MCI (Masliah et al., 1991, Masliah et al., 2001, Scheff et al., 2007).

The occurrence of synaptic loss has been attributed to amyloid and tau toxicity. Transgenic models overexpressing APP display a decrease in synaptic proteins even before the accumulation of amyloid plaques (for a review (Bell et al., 2006)). Similarly, pathological tau has been shown to promote synapse loss before the formation of tau aggregates in a transgenic tau rodent model (Yoshiyama et al., 2007). Moreover, synaptic pruning by microglial cells has been acknowledged as a possible mechanism contributing to synapse loss in transgenic models of amyloid-like pathology (Hong et al., 2016).

1.6.4 Inflammation

The relative immune privilege of the brain is given by three anatomical protective barriers surrounding the adult CNS (Abbott et al., 2010, Engelhardt et al., 2017). The arachnoid barrier (1),

located at the brain surface between the dura mater and the CSF drained subarachnoid space (SAS), is impermeable to fluids and separates the vessels and lymphatics in the dura from the SAS. The blood-CSF barrier (2) separates the CSF from fenestrated microvessels. It limits the passage of molecules between the cells; however, the fenestrated endothelium allows for a rapid flow of water to aid the production of CSF. Lastly, the Blood Brain Barrier (BBB) (3) is located at the level of cerebral blood capillaries. Such endothelial cells are connected by tight junctions and form a barrier that regulates the transfer of molecules from the blood to the brain parenchyma. The BBB will be further discussed with the concept of the neurovascular unit.

In AD, the presence of inflammation was first proposed by Oskar Fischer, who observed that the deposition of a "foreign substance" in the cerebral cortex induced a local inflammatory response leading to amyloid plaque formation and nerve degeneration (FISCHER, 1907, Fischer, 1912). Subsequently, the presence of an inflammatory component in AD brains was strongly suggested by the presence of complement proteins (C1q, C3 and C4) (Eikelenboom and Stam, 1982) and activated microglia, expressing IL-1β and surrounding amyloid plaques (McGeer et al., 1987, McGeer et al., 1988, Griffin et al., 1989, Rogers et al., 1999).

Today, we know that deposition of amyloid plaques and NFT is accompanied by a chronic neuroinflammatory process characterized by the activation of astrocytes, microglia and immune complexes (Akiyama et al., 2000, Heneka et al., 2015, Wyss-Coray and Rogers, 2012). A few examples of the well-known inflammatory reaction in AD brains are the upregulation of inflammatory cytokine pathways, activation of the complement cascade, protein nitration, and oxidative stress (reviewed in (Akiyama et al., 2000)). Furthermore, neurons express several receptors for chemokines and cytokines, suggesting that they possess the necessary machinery to orchestrate an immune response (Bajetto et al., 2002).

The presence of chronic inflammation in AD is indicative of a failed resolving inflammation response (Wang et al., 2015). A loop between Aβ production and inflammatory responses could lead to altered microglial activation and possible dysfunction of its phagocytic properties. It has been proposed that the extravasation of peripheral immune cells, such as macrophages, might play a role in effectively phagocytizing pathological protein aggregates and, therefore, halting the inflammatory response (Simard et al., 2006, Naert and Rivest, 2011, Thériault et al., 2015).

Epidemiological studies revealed that cognitively healthy individuals who underwent a long treatment with non-steroidal anti-inflammatory drugs (NSAIDs) had a reduced risk of developing AD (Etminan et al., 2003, McGeer and McGeer, 2013, Szekely et al., 2004). Unfortunately, the majority of anti-inflammatory clinical trials administered at clinical AD stages failed to demonstrate significant changes in cognitive outcomes and in some cases, induced a worsening of the disease (Rogers et al., 1993, Scharf et al., 1999, Aisen, 2000, Group, 2007, Group, 2008). This apparent contradiction can be explained by the existence of two phases of CNS inflammation in the continuum of AD pathology. The first phase is an early proinflammatory and disease-aggravating process. The second phase is a late chronic stage with fully activated microglia and invasion of peripheral monocytes with some phagocytic activity (Cuello, 2017, Rogers, 2018). Furthermore, it is unlikely that any anti-inflammatory treatment could elicit a beneficial outcome when severe brain damage is already well established.

1.6.5 Neurotransmitter dysfunction

Some evidence suggests that NFT pathology might start in the locus coeruleus (LC) (Braak and Del Tredici, 2011). The LC is a small nucleus located deep in the pons lining the fourth ventricle in the human (Baker et al., 1989, German et al., 1988). LC represents the only source of noradrenaline to the hippocampus and cortex (Schwarz and Luo, 2015). Given the extensive innervation of the LC, it regulates several physiological mechanisms such as arousal, response to stress, attention, memory and learning (Berridge and Waterhouse, 2003, Sara, 2009). In addition, noradrenaline has been shown to act as an anti-inflammatory molecule in the CNS (reviewed in (Feinstein et al., 2016)).

In AD, a loss of LC neurons (Forno, 1978, Bondareff et al., 1982) along with a drop in noradrenalin levels (Iversen et al., 1983) is present since preclinical AD stages (Kelly et al., 2017). Moreover, while LC neuronal loss occurs during MCI, volumetric shrinkage is already detectable before the appearance of cognitive impairments, starting at Braak score 0 (Theofilas et al., 2017).

In AD, an extensive loss of pyramidal neurons is also present as the glutamatergic system is severely compromised (Butterfield and Pocernich, 2003). Glutamate, the main excitatory neurotransmitter in the CNS, is highly involved in learning and memory (Greenamyre and Porter, 1994, Morris, 2013). In AD brains, a reduction in glutamate concentration (Ellison et al., 1986,

Rupsingh et al., 2011, Sasaki et al., 1986), N-methyl-D-aspartate receptor (NMDA-r) levels (Greenamyre et al., 1987), and a loss of cortical and hippocampal glutamatergic uptake sites, interpreted as nerve terminal loss (Procter et al., 1988, Cowburn et al., 1988) were observed.

A study by Bell and colleagues proposed that glutamatergic deregulation begins with a paradoxical upregulation of glutamatergic synaptic boutons reported in the cortex of MCI individuals, followed by a depletion of such boutons after clinical AD presentation (Bell et al., 2007). Further studies suggest that glutamate clearance is affected in AD and results in excitotoxicity (Francis, 2003). Memantine, a non-competitive NMDA-r antagonist, is thought to alleviate the toxicity of glutamate release.

In addition to the glutamatergic, GABAergic neurons are affected with a large loss (70%) of GABAergic terminals in cortical and hippocampal AD brains (Hardy et al., 1987). However, other studies found no changes in GABA and GABA receptor levels in the cingulate or cerebellar cortex but a reduction in the superior, frontal and temporal cortex (Simpson et al., 1988, Chu et al., 1987, Rossor et al., 1982).

In addition to the mentioned systems, the cholinergic system, involved in higher brain functions such as learning, memory, and attention (Drachman, 1977, Everitt and Robbins, 1997), was proposed to be one of the earliest to fail in AD as demonstrated by simultaneously by Bowen and by Davies and Maloney in 1976 (Davies and Maloney, 1976, Bowen et al., 1976) and reviewed by (Giacobini et al., 2022)). Interestingly, in the initial stages of the pathology and upregulation of cholinergic presynapses is present. This upregulation is preceded by an upregulation of glutamatergic and GABAergic terminals followed by their sharp decline (Bell and Cuello, 2006, Bell et al., 2003, Bell et al., 2006) (as shown in **Figure 1-3**. This phenomenon of synaptic upregulation has been interpreted as a compensatory mechanism balancing increasingly toxic amyloid β protein levels (Bell et al., 2006) as it has been reported in APP transgenic animal models before plaque formation (Wong et al., 1999, Mucke et al., 1994, Hernandez et al., 2001).

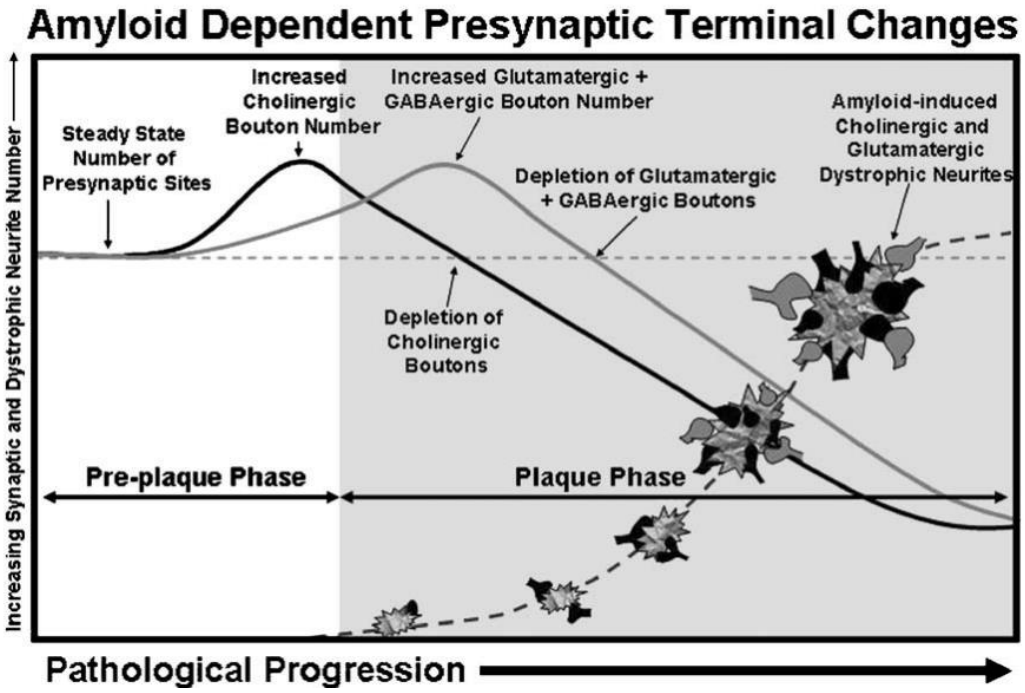

Figure 1-3. Schematic representation of the neurotransmitter-specific involvement of presynaptic boutons across progressive stages of the AD-like amyloid pathology.

Image reprinted from (Bell and Cuello, 2006), European Journal of Pharmacology, with permission from Elsevier.

Less studied is the contribution of monoaminergic systems to the development of AD. However, deficits in serotonergic, dopaminergic, histaminergic, and melatonergic system metabolites have been reported to occur in AD (reviewed in (Šimić et al., 2017, Trillo et al., 2013)).

It has been very debated whether cholinergic or noradrenergic degeneration appears first. A subgroup of individuals with AD, without major cholinergic degeneration, already displayed LC neuronal loss (Wilcock et al., 1988). However, this view has been challenged by a series of investigations reporting that the LC and nucleus basalis of Meynert, a main basal forebrain cholinergic nucleus, display the same extent of neuronal loss (Arendt et al., 2015, Lyness et al., 2003).

1.7 Is the basal forebrain relevant in Alzhimer's disease pathology?

We previously described the presence of several neuropathological hallmarks of AD. The neurotransmitter dysregulation, particularly acetylcholine (ACh), appears early in the disease progression.

Interestingly, four of six FDA-approved drugs to treat symptomatic AD are acetylcholinesterase inhibitors (AChEI). These drugs improve the telencephalic cholinergic tone by blocking extracellular acetylcholine degradation (Giacobini, 1987).

The rationale behind using these drugs in AD is the gradual atrophy and later loss of the major source of cholinergic innervation to the forebrain (Bowen et al., 1976, Davies and Maloney, 1976, Whitehouse et al., 1981). These neurons, named basal forebrain cholinergic neurons (Mesulam et al., 1983b, Mesulam et al., 1983a, Bigl et al., 1982, Struble et al., 1986) have a crucial role in learning, memory and attention mechanisms (Drachman and Leavitt, 1974, Bartus, 1979, Everitt and Robbins, 1997, Ballinger et al., 2016, Zaborszky et al., 1999).

Even though AChEIs still represent a breakthrough in the clinical treatment of AD (Giacobini, 2003), as they are valuable drugs in ameliorating symptoms of patients with mild to moderate dementia, they cannot significantly delay AD onset.

The cholinergic system will be the main focus of this thesis and several studies will report how this system is gaining popularity again as one of the earliest players in AD pathology. Its insight may provide new therapeutic avenues.

1.8 Acetylcholine in the Central Nervous System

ACh was indirectly discovered in the periphery by Otto Loewi in 1926, who used various perfusates to stimulate frog hearts. The released substance in the perfusates was named "vagustoff" (Loewi and Navratil, 1926) and identified as ACh by Sir Henry Dale (Dale, 1914). It was later demonstrated in the 1930s that ACh was released from parasympathetic, preganglionic and motor neuron fibers (Feldberg and Gaddum, 1934, Feldberg, 1945, Dale et al., 1936) and its presence in the brain was first reported in the same years (Chang and Gaddum, 1933). However, it took additional work to understand the presence of ACh in the CNS fully, and many years following Loewi's discovery, the cholinergic innervation of the cerebral cortex was poorly known.

Presently, it is well known that ACh is a neurotransmitter in the brain that induces facilitatory effects on the principal excitatory cells of the neocortex, the pyramidal neurons (Picciotto et al., 2012). The function of ACh is to bring neurons closer to the firing threshold, which ensures efficient synaptic transmission and quick responses to signals during wake and sleep (Jones, 2004).

In the subsequent sections, I will describe how ACh is synthesized in neurons, outlining the CNS cholinergic markers, the historical context of their discovery, and its role in the central cholinergic system in health and AD.

1.8.1 Cholinergic markers

1.8.1.1 Choline acetyltransferase (ChAT)

ACh is synthesized in the neuronal soma from its precursors, choline and glucose, taken up by neuronal terminals after crossing the Blood-Brain Barrier (BBB) (Freeman and Jenden, 1976). Following uptake, glucose is converted to Acetyl CoA, and then to ACh when choline acetyltransferase (ChAT) (Nachmansohn and Machado, 1943) transfers an acetyl door group from the Acetyl CoA to choline in an O-acetylation reaction (Potter et al., 1968, White and Wu, 1973). ACh from the cell soma is subsequently transported to the neuronal processes (reviewed in (Dahlström, 1983)). Eighty percent of ChAT activity is localized in the cytoplasm, while the remaining 20% is associated with membranes (Benishin and Carroll, 1983). Although the functional significance of its localization is still unclear, the membrane-bound ChAT seems critical to supply ACh synthesis when choline uptake is decreased (Cooke and Rylett, 1997). ChAT is

considered a reliable, widely used marker of cholinergic neurons since the production of an anti-ChAT monoclonal antibody (Eckenstein and Thoenen, 1982). A schematic representation of ACh synthesis in **Figure 1-4.**

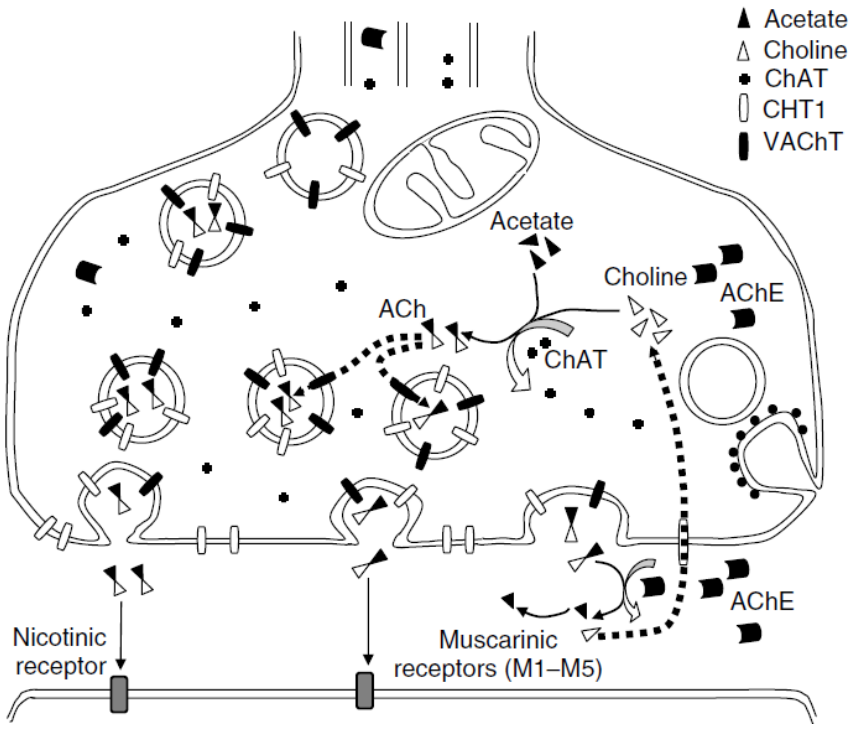

Figure 1-4. Schematic representation of a typical CNS cholinergic synapsis.

Abbreviations: ACh, acetylcholine; ACh, acetylcholinesterase; ChAT, choline acetyltransferase; CHT1, choline transporter 1; VAChT, vesicular acetylcholine transporter; AChE, acetylcholinesterase; M1–M5, muscarinic receptors. Image reprinted from (Cuello, 2009), Encyclopedia of Neuroscience, with permission from Elsevier.

1.8.1.2 Vesicular acetylcholine transporter (VAChT)

In neurons, ACh exists as both "free" and "bound" forms (Hebb and Whittaker, 1958), corresponding to cytoplasmic and vesicular pools. Basal release of ACh, occurring at rest, derives from cytoplasmic stores. On the other hand, the stimulated release of ACh concentrated in the pre-synaptic vesicles requires extracellular calcium (reviewed in (Parsons et al., 1993)). The transmembrane protein, expressed in synaptic vesicles and responsible for storing ACh displaying an active proton-driven uptake (Parsons and Koenigsberger, 1980, Michaelson and Angel, 1981), has been named vesicular acetylcholine transporter (VAChT). The cDNA encoding VAChT was cloned in the early 1990s in mice, rats, and humans (Bejanin et al., 1994, Erickson et al., 1994, Roghani et al., 1994). Interestingly, the sequence encoding VAChT is located downstream of the gene encoding ChAT (Bejanin et al., 1994, Erickson et al., 1994), and generally, the expression of ChAT and VAChT is coordinated.

1.8.1.3 Acetylcholinesterase (AChE)

Once released, ACh binds with post-synaptic receptors on cholinoceptive cells (such as glutamatergic neurons and GABAergic interneurons). To terminate cholinergic neurotransmission, acetylcholinesterase (AChE) enzymatically causes the rapid hydrolysis of ACh into acetate and choline (reviewed in (Brimijoin, 1983)). Histochemical techniques to measure AChE represented the first opportunity to identify cholinergic neurons and significantly improve the mapping of cholinergic pathways (Koelle and Friedenwald, 1949, KARNOVSKY and Roots, 1964). However, because of AChE presence either in cholinergic and cholinoceptive neurons (Mesulam and Geula, 1988), this enzyme is not considered a reliable cholinergic marker as compared with ChAT or VAChT.

1.8.1.4 High-affinity choline transporter (CHT1)

The amount of ACh synthesized *de novo* in presynaptic terminals is largely controlled by the capacity of a transporter to import choline since inhibition of this transporter depletes brain ACh levels (Happe and Murrin, 1993). Therefore, CHT1 represents a rate-limiting step in ACh synthesis and is responsible for the choline transport into the presynaptic vesicles (Okuda and Haga, 2003). cDNA clones encoding for this high-affinity choline transporter (CHT1) were isolated from the nematode *Caenorhabditis elegans*, rats and humans (Okuda et al., 2000, Okuda and Haga, 2000, Apparsundaram et al., 2000). In rats and primates, CHT1 cellular distribution showed strong

immunoreactivity in the cholinergic neuron terminals and cell bodies, resembling the VAChT distribution pattern (Okuda and Haga, 2003).

1.8.1.5 Butyrylcholinesterase (BChE)

Although homozygous knock-out mice for ChAT, CHT1, or VAChT die at birth (Brandon et al., 2003, de Castro et al., 2009, Ferguson et al., 2004), AChE knock-out −/− mice (strain 129Sv) lived for an average of 4 months, with a characteristic phenotype hypothesized to depend on ACh buildup (Duysen et al., 2002). Indeed, these mice showed weak muscles, no mounting behaviour, pinpoint pupils, deafness, seizures and lack of housekeeping behaviour, suggesting an absence of higher cognitive functions (Duysen et al., 2002). The capability of the mice to live despite the complete absence of AChE suggested the existence of an enzyme with a partially redundant function, thus compensating for the lack of AChE activity. This enzyme is known as Butyrylcholinesterase (BChE).

BChE represents a very small percentage of the total cholinesterase activity in different tissues, and humans lacking BChE activity are healthy (Primo-Parmo et al., 1996, Darvesh et al., 2003). Even though BChE did not undergo compensatory increases in the AChE knock-out −/− mice (Li et al., 2000); inhibition of BChE in these mice was lethal (Xie et al., 2000), suggesting that BChE partly substitutes AChE by hydrolyzing acetylcholine (Mesulam et al., 2002).

1.8.2 The cholinergic system: The Basal Forebrain Cholinergic nuclei

There are two large groups of cholinergic neurons in the brain. The largest group is located within the laterodorsal and pedunculopontine tegmental nuclei**.** The laterodorsal tegmental nucleus targets medially placed limbic regions of the diencephalon and brainstem (reviewed in (Woolf, 1991)). The pedunculopontine cholinergic neurons project to almost every subcortical brain region such as to the thalamus, tectum, substantia nigra, basal forebrain, basal ganglia, reticular formation, raphe nuclei, locus coeruleus, cranial nerve nuclei, pons, and deep cerebellar nuclei (Woolf, 1991). The second group of cholinergic neurons in the brain represents the major source of cholinergic innervation to the forebrain and it will be the main focus of this thesis. The two groups of cholinergic neurons are illustrated in **Figure 1-5**.

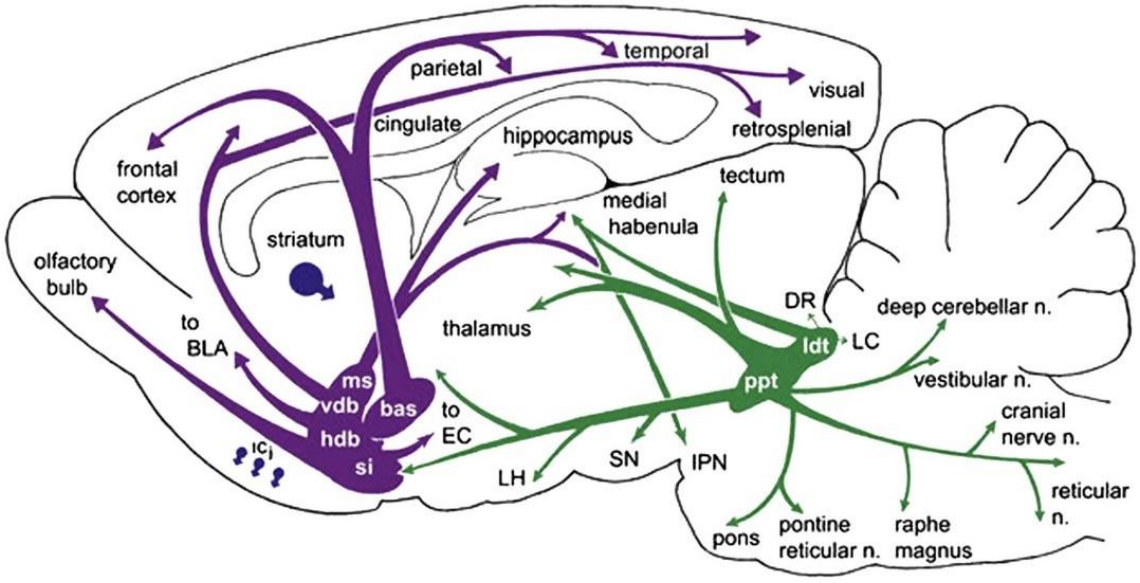

Figure 1-5. Parasagittal section of a rat brain with a schematic representation of the cholinergic system.

Abbreviations: BLA, Basolateral amygdala; ms, medial septum; vdb, ventral diagonal band; hdb, horizontal diagonal band; si, substantia innominata; bas, nucleus basalis; EC, entorhinal cortex; LH, lateral hypothalamus; SN, substantia nigra; IPN, interpeduncular nucleus; ppt, pedunculopontine tegmental nucleus; ldt, laterodorsal tegmental nucleus; DR, dorsal raphe. Image from (Perez-Lloret and Barrantes, 2016) adapted from (Woolf, 1991)).

The basal forebrain cholinergic nuclei were initially identified by Meynert (1872), and Kolliker and were named after observing the aggregation of large neurons at the basal nucleus (1896). Studies conducted by Mesulam and colleagues in primate brains (Mesulam et al., 1983a) and rat brains (Mesulam et al., 1983b) suggested a subdivision of the basal forebrain nuclear complex into four areas. This classification is currently still in use and comprises: the medial septal nucleus (MS, Ch1) and the vertical band of Broca (VBB, Ch2), which innervate the hippocampus; the horizontal band of Broca (HDB, Ch3), which projects to the olfactory bulb as well as to the medial and mostly limbic cortex; and the nucleus basalis (nb), substantia innominata (SI) and magnocellular preoptic field (MCPO) (cumulatively referred to as Ch4), whose axons project respectively to the cerebral cortex, amygdala/limbic cortex and limbic cortex. In primates, the delineation of the SI is relatively straightforward, and the nb, named nucleus basalis of Meynert (NbM) after its discoverer, is localized under the anterior commissure in the SI.

The human nucleus basalis, which contains 200,000 neurons in each hemisphere (Arendt et al., 1985), can be subdivided into neuronal clusters or sectors called anteromedial (am), anterolateral (al), anterointermediate (ai), intermediodorsal (id), intermedioventral (iv), and posterior (p) (Mesulam and Geula, 1988). The topography of these nuclei is not as clear for rats (Lehmann et al., 1980), and investigators have often used this term to include neurons of SI. Studies of SI-specific lesions (Heckers et al., 1994) and more recent studies with a Cre-Mice-mCherry (Agostinelli et al., 2019), with Cre-Recombinase injected in the SI, showed cholinergic projections to the basolateral amygdala (BLA). For this thesis, we adopted for rodents the same division proposed by Mesulam. The term nb was used to refer to the large group of cholinergic neurons in the innermost region of the internal globus pallidus (as in **Figure 1-6**).

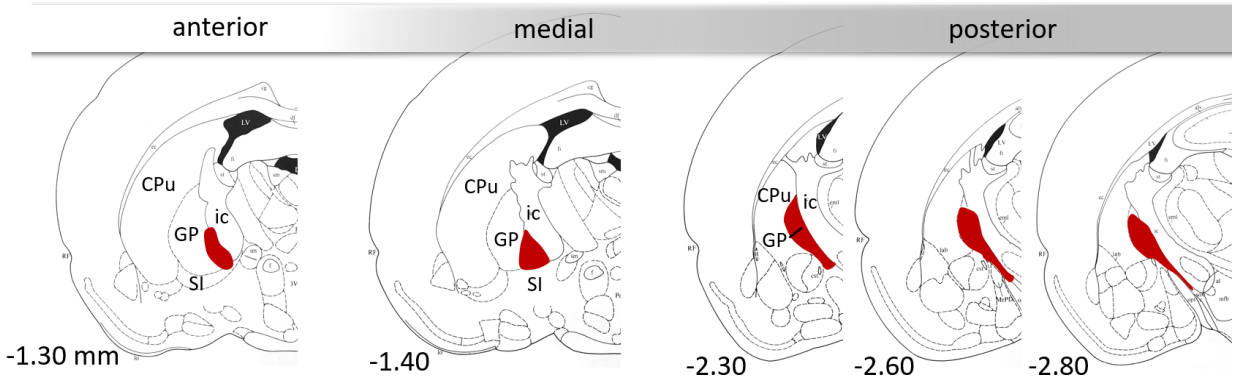

Figure 1-6. Coronal sections of the nucleus basalis in the rat brain.

The nucleus basalis is represented in red. The anteroposterior coordinates are reported from the Bregma (from -1.30 mm to -2.80 mm) according to the Paxinos and Watson Atlas (Paxinos and Watson, 2006). Abbreviations: Cpu, caudate-putament; GP, globus pallidus; ic, internal capsule; SI, substantia innominata. Figure adapted from (Paxinos and Watson, 2006).

1.8.2.1 Anatomy of basal forebrain cholinergic neurons (BFCNs) in rats and primates

Studies conducted using retrogradely labelled tracers (e.g. Horseradish peroxidase), AChE histochemistry, and ChAT immunohistochemistry in rhesus monkeys were able to show the topographical arrangments of the BFCNs projections and the proportion of cholinergic neurons for each area (Mesulam et al., 1983a), which resembled the human anatomy (Saper and Chelimsky, 1984). In rhesus monkeys, hDBB represents the region with the lower representation of cholinergic neurons (only 1%). The proportion of cholinergic neurons rises in MS (10%), reaching 70% in the vDBB and 90% in the nb (Mesulam et al., 1983a).

Another study conducted in the same year identified the proportion of cholinergic neurons in rats, which is lowest in the hDBB (20-25%); in MS is 40-50%, rising to 50-75% in the vDBB and reaching 90% in the nb as in monkeys (Mesulam et al., 1983b).

The non-cholinergic component of the basal forebrain nuclei of the rodent brain is mostly represented by g-aminobutyric acid (GABA)-ergic, glutamatergic, peptidergic, and tyrosine hydroxylase (TH)-positive neurons (reviewed in (Mesulam, 2013)). Some non-cholinergic neurons are interneurons; others project to the cerebral cortex (Freund and Meskenaite, 1992, Gritti et al., 1993). In addition, a neuronal population of "cholino-GABAergic" neurons that showed a co-release of the two neurotransmitters ACh and GABA (Saunders et al., 2015), have been identified, but their role has not been fully investigated.

1.8.2.2 Basalocortical pathway and nucleus basalis afferents

The first studies that investigated the basalocortical pathway, consisting of the nb innervation to the cortex, were conducted using horseradish peroxidase (HRP) injected into the cortex (Lehmann et al., 1980, Divac, 1975, McKINNEY et al., 1983). As previously mentioned, approximately 90-95% of neurons projecting from the nb of rats and monkeys are cholinergic (Rye et al., 1984, Mesulam et al., 1986). These projections are highly topographically organized. Particularly, cholinergic neurons in the anterior portion of the nb project to the frontal cortex, while the ones in the central and posterior area project to parietal and occipital cortices in monkeys (Mesulam et al., 1986). The topographic organization was confirmed in rats following injections of the anterograde tracer *Phaseolus vulgaris leucoagglutinin* (Luiten et al., 1987). Conversely, GABAergic neurons represent the majority of the non-cholinergic component, and in the rat, some of these project to the cerebral cortex, innervating cortical inhibitory interneurons (Gritti et al., 1993, Freund and

Meskenaite, 1992). The importance of the non-cholinergic neurons in the nb has been highlighted by showing that selective immunotoxic lesions of cholinergic neurons in the nb yield behavioural and physiological impairments less severe than those obtained by excitotoxic lesions, which destroy all cell types (Berger-Sweeney et al., 1994, Wenk et al., 1994).

The human nb contains numerous varicose axons that are TH-positive, serotonergic, and dopamine b-hydroxylase (DBH) positive (noradrenergic) (Smiley and Mesulam, 1999). In rats, these axons likely represent afferents from the TH-neurons of the ventral tegmental area (VTA)/substantia nigra and the noradrenergic neurons of the locus coeruleus (25%) (Martinez-Murillo et al., 1988) as well as the serotonergic neurons of the midbrain raphe (10 %) (Jones and Cuello, 1989). In addition, a very small proportion (less than 1%) of the pedunculopontine and lateral tegmental neurons have been shown to project to the nb (Jones and Cuello, 1989).

The nb also receives inputs from the hypothalamus and amygdala (Mufson and Mesulam, 1984, Price and Amaral, 1981). Through this circuitry, the nb cell group can influence cholinergic transmission in all parts of the cerebral cortex in a manner that is selectively sensitive to neural activity within components of the limbic system.

1.8.2.3 Septohippocampal pathway and MS/VDB afferent

The hippocampus consists of four interconnected subdivisions comprising the dentate gyrus, CA1, CA2 and CA3 regions, while the retrohippocampal region consists of the subicular and entorhinal subdivisions (Swanson, 1978).

The septohippocampal pathway, consisting of projections from the MS to the hippocampus, was extensively studied using retrograde labelling with HRP injected into the hippocampus and alternative tracers that combined ChAT and AChE immunohistochemistry (Alonso and Köhler, 1984, Rye et al., 1984, Woolf et al., 1984). The septohippocampal pathway consists of four routes (Swanson, 1978). One of the three routes ascending dorsally is the fimbria-fornix, representing 60% of hippocampus innervation and projecting to the dorsal hippocampal region. The second dorsal route is the fimbria, which innervates the hippocampus diffusely, and a third is the supracallosal, constituting 30% of innervation targeting the more caudal region of the hippocampus (Swanson, 1978). Finally, a fourth ventral route sends projections from the ventral

part of the vDBB to the hippocampus temporal lobe and constitutes 10% of hippocampal innervation (Gage et al., 1984).

The cholinergic projection from the MS/VDB predominantly innervates the hilar region of the dentate gyrus, while the other population of noncholinergic GABAergic neurons project to the hippocampus (Źborszky et al., 1986). Along with the septohippocampal pathway, the dentate gyrus receives input, mostly glutamatergic, from the entorhinal cortex through the perforant pathway (Swanson, 1978).

Cholinergic neurons of MS/VDB receive afferents from cholinergic neurons of VTA and catecholaminergic input from LC (Swanson, 1978). In addition, the pathway from HDB, medial habenula and paraventricular hypothalamic nuclei and to the hippocampus to the MS has been noted (Swanson, 1978, Źborszky et al., 1986).

1.8.2.4 Cholinergic projection from HDB to the olfactory bulb and afferents

Cholinergic neurons in the rostral portion of HDB project to the olfactory bulb (Źborszky et al., 1986, Woolf et al., 1984, Gaykema et al., 1990) while neurons in the medial region project mostly to the cortex (cingulate, retrosplenial, entorhinal, perirhinal cortices) and interpeduncular nucleus (Woolf et al., 1984). The remaining neurons of the HDB project to the basolateral amygdala (Woolf et al., 1984, Woolf and Butcher, 1982).

In turn, the afferents received by the HDB come from the olfactory nucleus, basolateral amygdala, different cortical regions (orbitofrontal, cingulate, entorhinal, insular and piriform cortices), VTA, dorsal and median raphe, pedunculopontine and laterodorsal tegmental nuclei (reviewed in (Woolf, 1991)).

1.8.3 Acetylcholine receptors

ACh signals through two classes of receptors: metabotropic muscarinic receptors (mAChRs) and ionotropic nicotinic receptors (nAChRs), which will be discussed below.

1.8.3.1 Muscarinic receptors

The action of ACh is in part mediated by a class of G-protein-coupled receptors termed muscarinic ACh receptors (mAChRs) (reviewed in (Caulfield, 1993, Caulfield and Birdsall, 1998, Wess, 1993)). This receptor family consists of 5 different subtypes referred to as M1-M5 mAChRs. ACh

binding to M1, M3 and M5 induces G-protein activation, which stimulates the breakdown of phosphatidylinositol (PI), generating second messengers (diacylglycerol and inositol-1,4,5-triphosphate). On the other hand, ACh binding to M2 and M4 leads to a G-protein activation that inhibits the accumulation of intracellular cAMP by reducing the activity of adenylyl cyclase. Moreover, mAChRs are located both pre- and postsynaptically throughout the brain, producing diverse consequences for brain activity (Wess et al., 2003).

In the body periphery, mAChRs mediate functions such as slowing the heartbeat, contracting smooth muscles and stimulating glandular secretion (Caulfield, 1993, Caulfield and Birdsall, 1998, Wess, 1993). In addition, members of the mAChR family are widely expressed throughout the brain and spinal cord (Levey, 1993) (for detailed distribution, see **Table 1-1**). Knock-out mouse models of each mAChR offered insight into each receptor function. Interestingly, all knock-out models were viable and fertile, although they each displayed deficits specific to each receptor knock-out (Wess, 2004).

Tissue	Receptor subtype	Protein levels	
		Rat (%)	Human (%)
Cortex	**M1**	**34-40**	**35-60**
	M2	19-37	25-30
	M3	10	5-10
	M4	15	20
hippocampus	**M1**	**47**	**60**
	M2	17	25
	M3	10	5
	M4	15	20
Striatum	**M1**	**29**	37
	M2	12-29	16
	M3	6	0
	M4	**29**	**50**
Thalamus	M1	6-14	-
	M2	**42-49**	
	M3	6	
	M4	15	
Brainstem and Cerebellum	M1	2-5	-
	M2	**70-84**	
	M3	5	
	M4	1	

Table 1-1. Relative proportions of M1-M4 muscarinic receptors (mAChRs) in the different brain areas.

Protein levels of the muscarinic receptor subtypes measured in rat (Levey, 1993) and human brains (Flynn et al., 1995). The subtype with the larger expression in each brain area is in bold. "–" indicates not reported.

The M1 subtype is widely expressed in rats and humans in the pyramidal neurons at all cortical layers, in the hippocampus and the striatum (Caulfield, 1993, Caulfield and Birdsall, 1998, Flynn et al., 1995). The possibility that ACh may modulate excitatory neurotransmission in cortical neurons via M1 receptors (Mrzljak et al., 1993) was raised since M1-immunoreactivity was found to be associated with symmetrical synapses (in which the postsynaptic density is similar in width with the presynaptic membrane), characteristic of cortical cholinergic pathways, and asymmetrical synapses (in which the postsynaptic density is thicker than the presynaptic membrane), typical of excitatory amino acid pathways (Peters and Folger, 2013). In agreement with the M1-subtype distribution, M1 -/- mice showed impairments in learning and memory (Anagnostaras et al., 2003) as well as signs of hyperactivity (Miyakawa et al., 2001). Interestingly, pharmacological manipulation with selective M1 agonists has been proposed as a therapeutic approach to improve cognitive outcomes in AD (Fisher et al., 2003) and will be further discussed.

M2 and M3 receptor subtypes are moderately expressed through the brain, with lower expression in the cortex, hippocampus and striatum when compared to the M1-type receptors. In particular, the M2 subtype has the highest expression in the thalamus, cerebellum and brainstem, basal forebrain and septum in rats and monkeys (Caulfield, 1993, Smiley et al., 1999). The M2 subtype is also associated with non-cholinergic terminals suggesting that it may act as a presynaptic receptor through which ACh may modulate the release of other transmitters (Mrzljak et al., 1993). M2 -/- knock-out mice showed memory deficits, reduction in LTP (Seeger et al., 2004), alteration of ACh release possibly mediated by presynaptic M2 autoreceptors (Tzavara et al., 2003) and altered analgesic response (Gomeza et al., 1999a) compared to the controls.

The less expressed M3 subtype is mostly implicated in appetite regulation. Indeed, M3-/- mice displayed a decrease in body weight and fat mass (Yamada et al., 2001b). This effect has been attributed to the abundant expression of this receptor in the hypothalamus, playing a central role in food intake regulation and energy homeostasis (Yamada et al., 2001b).

The M4 subtype is most expressed in the striatum in rats and humans (Flynn et al., 1995, Levey, 1993). M4 -/- mice showed locomotor hyperactivity, but less pronounced than M1-/- mice (Gomeza et al., 1999b). As M4 mAChRs are co-expressed with D1 dopamine receptors in striatal

projection neurons, it was proposed that the activation of M4 receptors inhibits the D1 dopamine receptors responsible for promoting locomotor activity (Gomeza et al., 1999b).

The M5 receptor subtype is primarily expressed in dopaminergic neurons of the midbrain ventral tegmental area (VTA) (Vilaró et al., 1990), which project to the nucleus accumbens (nAcc) and prefrontal cortex, playing a role in mediating response to rewarding stimuli (Carlezon Jr et al., 1996). Moreover, these receptors are present in the vascular endothelial cells (Furchgott and Zawadzki, 1980). Interestingly, M5 -/- mice showed reduced sensitivity to the rewarding effect of morphine (Basile et al., 2002) and an alteration in the diameter of cerebral arteries and arterioles (Yamada et al., 2001a).

1.8.3.2 Nicotinic receptors

Neuronal nicotinic receptors (nAChRs) are ionotropic channels composed of five out of the twelve different existing subunits: α2 to α10 and β2, β3, β4. When the channels are constituted from five identical subunits, these receptors are called homomeric, and when they have at least one α and one β subtype, they are defined as heteromeric (reviewed in (Picciotto et al., 2012, Colangelo et al., 2019)).

These receptors are not clustered at postsynaptic membranes opposed to sites of ACh release, but they are dispersed along intracellular compartments and on the surface of neurons, including presynaptic terminals (McGehee et al., 1995), cell bodies, and axons (Hill et al., 1993, Colangelo et al., 2019). In addition, stimulation of nAChRs can increase the release of glutamate, GABA, dopamine, ACh, norepinephrine, and serotonin (McGehee et al., 1995, Wonnacott, 1997).

Five α subunits (3–7) and three β subunits (2–4) are expressed in the human brain. The most abundant receptor subtypes in the neocortex are the receptor α7, specifically associated with regulating glutamate neurotransmission (Jones and Wonnacott, 2004), and the α4β2 channel (reviewed in (Colangelo et al., 2019)).

Although mouse knock-out models for α4, β2 or α7 are viable and do not show a marked phenotype (Picciotto et al., 2001, Paylor et al., 1998, Franceschini et al., 2002), aged β2 knock-out mice showed loss of neurons and an increase of glial cells in the hippocampal region (Zoli et al., 1999). In addition, α7 knock-out mice showed cognitive deficits only in a task that required sustained

attention (Young et al., 2004). α7 nAChRs are involved in inflammation in the peripheral nervous system, as signalling from the Vagus nerve inhibits cytokine production by releasing ACh, which binds the α7 receptors in macrophages (Pavlov and Tracey, 2005). Since α7 nAChRs are expressed in neurons, astrocytes and microglia, it was proposed that ACh could play a role also in the CNS inflammation (Conejero-Goldberg et al., 2008).

Interestingly, nicotine improves memory performance and learning in rodents, vigilance, and rapid information processing in humans (Rezvani and Levin, 2001, Buccafusco et al., 2005). In addition, nicotine restores cognitive deficits caused by a lesion in the cholinergic system (Picciotto and Zoli, 2002).

1.8.4 The physiological function of Basal Forebrain Cholinergic neurons

Along with lesions of BFCNs, microdialysis was applied to understand which cognitive processes are associated with the activation of cholinergic neurons (reviewed in (Pepeu and Giovannini, 2004)). In this technique, dialysis tubing is implanted in the brain area of interest in rats performing different behavioural tasks. Perfusates are collected at regular intervals and ACh levels are tested. Microdialysis represented a powerful tool for studying the physiological role of ACh in certain cognitive tasks and the specific brain regions involved (Pepeu and Giovannini, 2004). As such, cholinergic input was implicated in a wide variety of cognitive processes.

Cortical ACh function has been linked to the control of circuits underlying attention (Lehmann et al., 2003, Dalley et al., 2004, Robbins et al., 1989, Baxter and Chiba, 1999, McGaughy et al., 2002, McGaughy and Sarter, 1998, Harati et al., 2008, Ljubojevic et al., 2018) and cue detection (Parikh et al., 2007). ACh may increase the "signal-to-noise" ratio in cortical networks, making neurons more sensitive to external stimuli (Hasselmo and Sarter, 2011).

In the hippocampus, ACh can induce spatial-memory-critical theta rhythms (Mitchell et al., 1982) and is involved in neuronal plasticity (Sugisaki et al., 2011, Yun et al., 2000).

Several studies have shown that ACh is associated with stress responses (Picciotto et al., 2012) as stress increases ACh release in a brain region-specific manner (Mark et al., 1996). For instance, hippocampal and cortical ACh levels can increase following restraint stress in rats, while ACh levels in the amygdala are unchanged, although an increase in cholinergic tone can also reduce BLA activity through activation of mAChRs (Power and Sah, 2008).

In addition, BFCNs constitute an important component of the neuromodulatory system controlling brain states.

Indeed, cholinergic projections to the thalamus and neocortex are implicated in the generation of the fast β and γ oscillations, characterizing brain states of waking and REM sleep, and in the blockage of low-frequency oscillations (spindles), defining the slow-wave sleep (reviewed in (Steriade and Descarries, 2006, Jones, 1993)).

1.9 Cholinergic deficits in Alzheimer's disease

The first studies associating the hippocampus with memory came from Scoville, Penfield and Milner, who observed that human patients with bilateral lesions in the hippocampus presented memory deficits (Scoville and Milner, 1957, Penfield and Milner, 1958). A few years later, pharmacological approaches attributed the amnestic effects to ACh. Indeed, rats injected with scopolamine, an antagonist of mAChRs, which acted in the central and peripheral nervous system, performed significantly poorly in a running maze test compared to controls (Pazzagli and Pepeu, 1965).

The central cholinergic system was suggested to be impaired in aging, starting with the pioneering work of Drachman. Drachman and his collaborator Leavitt demonstrated that volunteer subjects receiving scopolamine showed memory impairment similar to impairment present in aged individuals, while peripheral cholinergic antagonists and central agonists did not (Drachman and Leavitt, 1974). Furthermore, he showed that the impairments induced by scopolamine were reversed by physostigmine, a cholinesterase inhibitor (Drachman, 1977). In a later study, he also showed that when scopolamine was given to cognitively normal young subjects, it resulted in impairments during a dichotic listening task in a similar way to untreated aged subjects (Drachman et al., 1980). Studies conducted in the same years on rats (Heise et al., 1976) and monkeys (Bartus, 1978, Bartus and Johnson, 1976) confirmed the amnesic effect of scopolamine.

Bowen and collaborators made progress investigating cholinergic neurons by showing that ChAT activity was reduced in dementia and correlated with brain morphological changes (White et al., 1977), memory impairment and plaque load (Perry et al., 1978b). Simultaneously, a reduction of ChAT activity levels in the cortex and hippocampus of AD brains was observed by Perry (Perry

et al., 1977b, Perry et al., 1977a) and by Davies and Maloney, along with a reduction in AChE activity levels (Davies and Maloney, 1976, PERRY et al., 1978a).

Subsequently, it was shown that levels of ACh (Richter et al., 1980) and the CHT1 (Bowen and Davison, 1978) were also lower in AD patients. However, mAChR density was not generally affected, indicating that cholinergic pathology may be largely presynaptic (White et al., 1977, Perry et al., 1977a, Perry et al., 1978b).

A loss of cortical cholinergic innervation in AD has been extensively reported in the literature (Mesulam and Geula, 1994, Geula and Mesulam, 1996, Geula and Mesulam, 1989), and it tends to be the most accentuated (76–85%) within the temporal lobe, including areas 20, 21, 22, and 28 of Brodmann (Geula and Mesulam, 1996). An intermediate loss (45%–75%) has been shown to be present in the frontal and parietal association areas, in the insula and temporal pole; whereas the anterior cingulate gyrus, primary motor, primary somatosensory, and primary visual cortex displayed less accentuated (4–28%) loss of cholinergic fibers. In the hippocampus, fiber density was reduced in all sectors, and in the amygdala, ChAT-IR varicosities showed a marked depletion in the cortical, accessory basal, and lateral nuclei (Emre et al., 1993).

The most obvious explanation for the ChAT activity reduction was the loss of cholinergic neurons (Terry and Davies, 1980), but other possibilities were suggested. In this debate, Whitehouse and collaborators demonstrated with a Nissl-staining a reduction of the “magnocellular” neurons in the nucleus basalis of Meynert of AD patients compared to aged controls; however, they could not differentiate between cholinergic and non-cholinergic neurons nor could they differentiate neuronal atrophy from neuronal loss (Whitehouse et al., 1981, Whitehouse et al., 1982). A similar study on the MS and DBB in an autopsy case of AD confirmed a similar reduction of these magnocellular neurons (Nakano, 1982). Later investigations using a monoclonal antibody against ChAT and staining for AChE confirmed that cholinergic cells were affected, though they did not die. Rather, they had significant shrinkage of their cell bodies (Pearson et al., 1983), similar to the shrinkage of the nb cell bodies resulting from extended stroke lesions in the rat (Sofroniew et al., 1983). These observations led Cuello and Sofroniew to propose that the pathological involvement of the nb in AD results from retrograde atrophy following a cortical lesion (Cuello and Sofroniew, 1984).

1.9.1 The Cholinergic Hypothesis

The above foundational studies on the cholinergic system led Bartus to propose "The Cholinergic Hypothesis of Geriatric Memory Dysfunction", which supported the role of cholinergic dysfunction in memory decline during aging (Bartus et al., 1982). This influential theory, nowadays cited as "The Cholinergic Hypothesis", proposed to explain the cognitive decline symptoms of aging rather than being an aetiological theory of AD. One year later, Coyle formulated the "Cholinergic Hypothesis of AD", highlighting the significant involvement of the cholinergic system in AD pathology (Coyle et al., 1983). Of note, as age is the greatest risk factor for AD (Raji et al., 2009), cholinergic involvement in aging served as a foundational understanding for applying this hypothesis to the context of AD.

1.9.2 Cholinergic drugs in Alzheimer's disease

Following the formulation of the cholinergic hypothesis, many studies focused on compensating for the reduced synaptic availability of ACh. This led to the development of inhibitors of AChE (AChEI), which prolong the presence of Ach released in the synaptic cleft.

Initial studies in the 1970s suggested that physostigmine could improve memory in normal subjects (Davis et al., 1978, Drachman, 1977). However subsequent clinical trials in AD patients showed that the cognitive improvement was short-lasting, the results were not consistent across all the studies, and adverse effects (gastrointestinal, including nausea, vomiting, diarrhea, as well as dizziness and headache) remained common reasons for withdrawal from the clinical trials (reviewed in (Coelho and Birks, 2001)).

Physostigmine was followed by Tacrine (tetrahydroaminoacridine), a drug developed earlier for agitation and postoperative delirium. A 1986 clinical study with Tacrine, treating moderate to severe AD patients for a year, showed symptomatic improvement and alleviation of peripheral side effects (Summers et al., 1986). Tacrine was approved in the U.S. by the FDA in 1993, though liver toxicity issues led to its discontinuation in 2013. However, the results with Tacrine enabled the development of subsequent generations of AChEI (Giacobini et al., 1991).

Three AChEIs, donepezil, rivastigmine and galantamine, were approved by the FDA and have been widely used for years for the symptomatic management of AD. In 1996, donepezil was shown to be effective for AD cognitive symptoms (with a reduced decline on the ADAS and MMSE tests)

without any sign of hepatotoxicity (Rogers and Friedhoff, 1996). Rivastigmine, which irreversibly inhibits AChE as well as butyrylcholinesterase (Sugimoto et al., 1995), was shown to be clinically applicable in 1999 with an improvement of cognitive symptoms and reduced peripheral cholinergic side effects (Gottwald and Rozanski, 1999).

The latest to be introduced (Raskind et al., 2000), galantamine, is both AChEI (Bores et al., 1996) and an allosteric modulator of nicotinic receptors (Schrattenholz et al., 1996, Albuquerque et al., 1997). Because it binds to nicotinic receptors, it has been proposed to have additional mechanisms that may activate non-cholinergic pathways impaired in AD (Francis et al., 1999).

Even though AChEIs are valuable drugs in alleviating symptoms of patients with mild to moderate dementia (Giacobini, 2003), they cannot significantly delay AD onset.

1.9.3 New clinical evidence supporting the Cholinergic Hypothesis

New methodologies have allowed the study of cholinergic deficits during the early and prodromal stages of AD (reviewed in (Hampel et al., 2018)) reinforcing the cholinergic hypothesis and the early contribution of the basal forebrain in AD pathology. These new strategies included structural MRI, the clinical use of cholinergic PET ligands, and follow-up on prolonged use of cholinergic and anticholinergic drugs.

1.9.3.1 Evidence from MRI clinical studies

Studies with high-resolution structural MRI revealed that the basal forebrain cholinergic system declines in volume in early adult life, with atrophy that affects the nucleus basalis of Meynert in MCI and mild AD and the whole basal forebrain with the onset of cognitive symptoms in AD (Grothe et al., 2012, Teipel et al., 2011). In MCI, basal forebrain volume reduction predicts conversion to AD (Brüggen et al., 2015) and has a similar or superior diagnostic accuracy to the hippocampal volume (Grothe et al., 2012, Kilimann et al., 2014, Teipel et al., 2014).

Because cholinergic hypofunction affects amyloid pathology and tau hyperphosphorylation (Fisher, 2012), the relationship between the basal forebrain and these neuropathological markers has been investigated. In pre-symptomatic AD subjects, basal forebrain atrophy correlates with amyloid deposition (Grothe et al., 2014) better than hippocampal atrophy (Teipel et al., 2014). It is detectable at least 4.5 years prior to the onset of clinical symptoms (Hall et al., 2008). In addition,

basal forebrain atrophy has been shown to predict and precede the degeneration of the entorhinal cortex (Schmitz et al., 2016).

Basal forebrain atrophy also predicts cortical degeneration topographically, following basal forebrain projections (Schmitz et al., 2018). It is also accompanied by CSF biomarkers of pTau, amyloid-beta (Schmitz et al., 2018, Fernández-Cabello et al., 2020) and of neuroinflammatory markers (Schmitz et al., 2020).

1.9.3.2 Evidence from Cholinergic PET ligands

Cholinergic positron emission tomography (PET) ligands permit the observation of cholinergic degeneration *in vivo*. [11C]-MP4A PET, a marker for AChE activity, revealed reduced activity in AD brains (Iyo et al., 1997) starting during the MCI stage (Rinne et al., 2003) and predicted conversion to AD (Marcone et al., 2012). In addition, [11C]-MP4A PET revealed a reduction of the ascending cholinergic system from the nucleus basalis of Meynert in AD, which correlated with the scores on the Mini-Mental State Exam (MMSE) (Shinotoh et al., 2000).

VAChT PET tracer 18F-fluoroethoxybenzovesamicol (FEOBV) is largely used in current clinical studies to follow the cholinergic degeneration *in vivo* (Petrou et al., 2014, Aghourian et al., 2017, Albin et al., 2018), and it is considered a possible biomarker of early AD that could be used in diagnosis. Indeed, FEOBV prevailed over an amyloid-beta (18F-NAV4694) and a glucose metabolism tracer (18FFluorodeoxyglucose), to distinguish AD patients from controls and showed significant correlations with cognitive scores (Aghourian et al., 2017). In agreement with this study, our laboratory reported a reduction of VAChT levels in post-mortem brain samples in non-cognitively impaired individuals, which correlated with cognitive scores (Pentz et al., 2020). Other VAChT tracers have shown a reduction in AD but are unsuited for widespread use in humans due to poor selectivity over receptors, slow brain kinetics or fast metabolism (Giboureau et al., 2010).

Muscarinic PET tracers have been studied in both monkeys and humans. [^{11}C]N-methyl-4-piperidyl benzilate (NMPB), a nonselective muscarinic receptor ligand, showed no regional changes in muscarinic receptor concentrations in AD (Zubieta et al., 2001). Attempts have been made to develop more selective muscarinic agonists. More recently, another tracer specific for M1/M4 receptors, 123I-iodo-quinuclidinylbenzilate (QNB), showed subunit- and-region-specific changes in AD (Colloby et al., 2015).

Tracers for nicotinic ACh receptors have yielded conflicting results. Indeed, [18F]-F-A-85380 (FA85), showed a reduction of FA85-binding sites globally in aging and in the hippocampus of AD patients but no changes in the cortex despite the significant loss of volume (Lagarde et al., 2017). On the contrary, 11C-nicotine binding was reduced in AD in the temporal cortex, frontal cortex, and hippocampus, and this decrease correlated with the MMSE score (Nordberg et al., 1995). However, 2-[18F]fluoro-A-85380 (2-FA), used to measure the α4β2 nicotinic subtype, revealed no change in early AD and no correlation with cognitive scores in one study (Ellis et al., 2008), while in another study, a reduction of this receptor was measured in AD and MCI that converted to AD in the later course (Kendziorra et al., 2011). Another tracer specific for the α4β2 nicotinic subtype, (-)-[^{18}F]Flubatine PET, showed a reduction of this receptor in AD patients, which correlated with tests of executive function and attention (Meyer et al., 2018). Finally, tracers specific to α7 nicotinic receptors showed reduced binding in AD and correlated with memory and frontal function scores (Nakaizumi et al., 2018).

While methods still need to be refined to create more selective compounds capable of targeting receptor subtypes, cholinergic changes can be tracked in AD, with important implications for AD pathogenesis.

1.9.3.3 Evidence from the use of cholinergic and anticholinergic drugs

Treatment with AChEIs can reduce hippocampal atrophy (Dubois et al., 2015), cerebral cortex thinning (Cavedo et al., 2016), and basal forebrain atrophy (Cavedo et al., 2017), though this did not show any significant difference in neuropsychological performance.

Conversely, elderly individuals taking common anticholinergic medications such as tricyclic antidepressants, first-generation antihistamines, and bladder antimuscarinics showed an increased risk of MCI and AD (Gray et al., 2015). In addition, anticholinergic drugs are associated with increased brain atrophy and cognitive decline in otherwise healthy-aged adults (Risacher et al., 2016).

Interestingly, microdoses of the antimuscarinic agent scopolamine (0.20 mg) could represent an accurate diagnostic test for prodromal AD (Snyder et al., 2014). This cognitive stress test was preliminarily tested in adults with elevated brain amyloid loads, and it led to cognitive impairment detection at 3 hours post-dose, followed by a full cognitive recovery within 5 hours (Snyder et al., 2014).

The evidence above suggests that cholinergic degeneration in AD might constitute an early disease-aggravating response and that a more sophisticated cholinergic therapy could modify the course of AD.

1.9.4 Acetylcholine modulates Amyloid Precursor Protein processing

A relationship between ACh and amyloid pathology has been largely investigated and could explain the correlation between decrease in basal forebrain volume and the increase in cortical amyloid deposition (Grothe et al., 2014).

In a study by Nitsch and colleagues (Nitsch et al., 1992), the connection between muscarinic receptors and APP production was demonstrated. This study used human embryonic kidney cell lines transfected with the human M1 and M3 mAChR genes and stimulation of these receptors with carbachol increased the production of APP a few minutes post-stimulation (Nitsch et al., 1992).

In another study, M1 stimulation (carbachol and AF102B) in PC12 cells activated two transduction pathways: the protein kinase C (PKC)-dependent and mitogen-activated protein kinase (MAPK)-dependent pathways. These pathways operate in parallel and converge with transduction pathways of NGF and bFGF (MAPK-dependent pathways), stimulating α-secretase and enhancement of αAPP secretion (Haring et al., 1998).

Other studies were conducted in cell cultures transfected with M1 mAChR and treated with nonselective muscarinic, M1-selective orthosteric agonists, and M1-selective allosteric agonists (Jones et al., 2008, Müller et al., 1997, Shirey et al., 2009) supported αAPPs secretion.

In line with this evidence, interfering with mAChR signalling resulted in a decrease in αAPPs production. For example, injection of 192 IgG-saporin into the medial septum or an M1 selective agonist Pirenzepine, caused uncoupling of the M1 muscarinic receptor with its G-protein complex, decreasing the responsiveness of M1 to stimulation (Potter et al., 1999).

Chronic treatment with the M1 agonist AF267B, an M1-selective muscarinic agonist, and physostigmine, following 192 IgG-saporin lesions in the nucleus basalis decreased the Aβ deposition in the cerebral cortex of rabbits (Beach et al., 2003) and 3xTg-AD mice (Caccamo et al., 2006).

To confirm the effect was mediated by the M1-receptor, in APP(Swe/Ind) transgenic mice knock-out for M1 showed an increased amyloidogenic APP processing, with more production of the

neurotoxic βAPPs and increased amyloid pathology (Davis et al., 2010). Expression of M1 on the M1 knock-out was able to rescue this phenotype (Davis et al., 2010). The pathways activated by a muscarinic agonist are shown in **Figure 1-7**.

Muscarinic agonists have also been shown to impact hyperphorylated tau *in vitro* (Sadot et al., 1996a) and *in vivo* (Caccamo et al., 2006) with a mechanism mediated by the activation of PKC and inhibition of glycogen synthase kinase 3 (GSK-3) (Caccamo et al., 2006, Forlenza et al., 2000) which will be further highlighted in the Discussion.

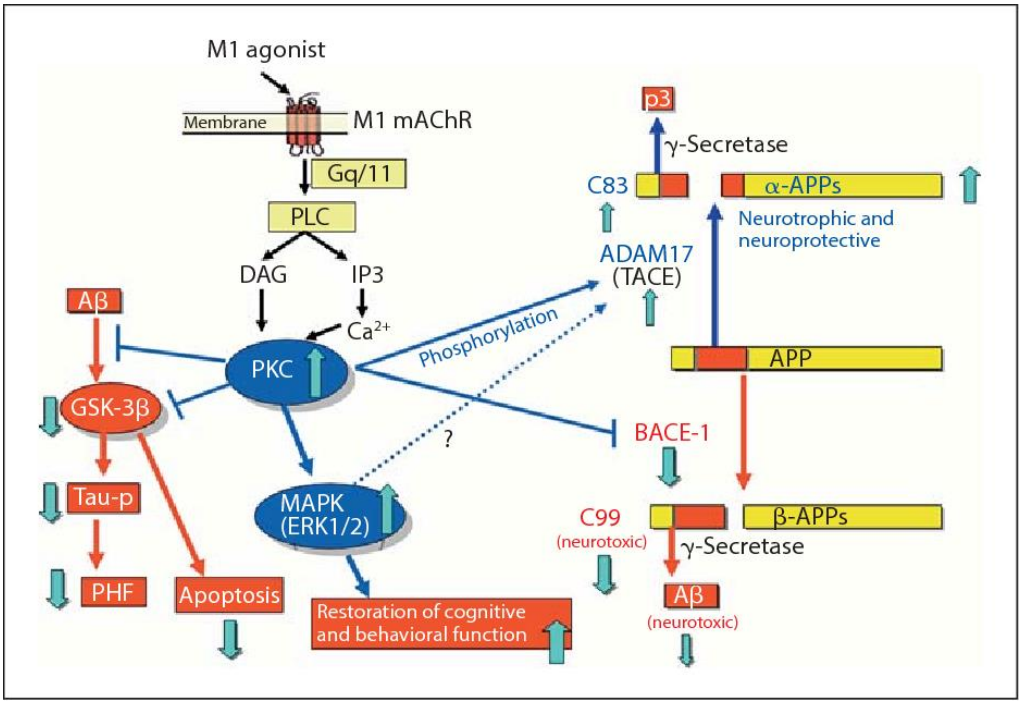

Figure 1-7. M1-Agonist binding modulates pathways of APP processing and tau phosphorylation.

Stimulation of M1 could activate pathways of protein kinase C (PKC), inducing the (a) activation of α-secretase (ADAM17) and blockage of β-secretase (BACE1), favouring the non-amyloidogenic pathway and the αAPP secretion. In addition, (b) it would block glycogen synthase kinase-3β (GSK-3β), responsible for tau hyperphosphorylation, and activate (c) the mitogen-activated protein kinase (MAPK)-dependant responsible for the restoration of cognitive and behavioural function. Image reprinted from (Fisher, 2008), Neurodegenerative Diseases, with permission from Karger.

1.10 Nerve Growth Factor (NGF)

The discovery of the Nerve Growth Factor (NGF) is the result of the studies of Rita Levi-Montalcini. Levi-Montalcini received her medical degree in 1936 at the University of Turin and then became a postgraduate student in the laboratory of Giuseppe Levi, an anatomist tutor of two other future Nobel Prize winners (Salvatore Luria and Renato Dulbecco). In this laboratory, Levi-Montalcini investigated the relationship between the developing CNS and its peripheral targets. The results led her to suggest that the failure of neurons to grow in the absence of peripheral target tissue was because of a degenerative process (Levi-Montalcini, 1997, Levi-Montalcini and Levi, 1944). In the meantime, the neuroembryologist Victor Hamburger at the Washington University in St Louis arrived at a different conclusion. Removing the developing limbs of chick embryos, he concluded that the differentiation of nerve cells depends largely on their destination (Hamburger, 1934). To resolve their scientific disagreements, Levi-Montalcini joined Victor Hamburger's group. Working together, they confirmed the original hypothesis proposed years earlier by Levi-Montalcini (Hamburger and Levi-Montalcini, 1949).

In her subsequent studies, Levi-Montalcini showed that size differences of ganglia in different spinal cord segments could depend on the regulation of neuronal degeneration. Indeed, she proposed that the sensory ganglia of the neck are small because they send out more neurites than the periphery can support, whereas brachial ganglia are larger because they innervate the additional mass of the limb. Based on these findings, Levi-Montalcini hypothesized that the lack of a specific endogenous biological mediator could cause cell death (apoptosis) (Levi-Montalcini, 1950)

In 1949, one of the postgraduate students of Victor Hamburger showed that a transplanted tumour grew well in chick embryos and nearby peripheral nerves invaded the tumour mass. Levi-Montalcini followed up on these studies and hypothesized that the transplanted tissues released a diffusible agent that stimulated the growth and differentiation of the developing neurons inducing the hyperinnervation of internal organs (Levi-Montalcini, 1952). This endogenous biological mediator was named Nerve Growth Factor (NGF) (Levi-Montalcini, 1987).

In the early 1950s, Levi-Montalcini collaborated with biochemist Stanley Cohen to characterize the biochemical properties of NGF. They performed experiments using snake venom, which would destroy any nucleic acid to determine whether NGF was a nucleic acid or a protein. Unexpectedly, they observed that snake venom produced more neural outgrowth than they saw with neurons

incubated with tumour extract. Since NGF was present in the snake venom, Cohen realized that it might be worthwhile to look at the mammalian analog of the snake venom gland. When they looked at the mouse salivary gland, they discovered it was a rich source of NGF (Levi-Montalcini and Cohen, 1956).

In 1960, Levi-Montalcini and Cohen successfully isolated and purified the NGF from the mouse salivary gland and demonstrated that it was a protein (Levi-Montalcini, 1964, Cohen, 1960). Together, they won the Nobel Prize in 1986, and their discovery was described as a fascinating example of how a skilled observer can create a concept out of apparent chaos' (Aloe, 2004).

1.10.1 Trophic dependency of Basal Forebrain Cholinergic neurons

In the adult CNS, NGF expression diminishes to basal levels and is no longer essential for the survival of neurons, including BFCNs. Indeed, cortical excitotoxins applied to the hippocampus, a BFCN target and the brain area providing NGF, induce BFCN atrophy but not neuronal death (Sofroniew et al., 1990).

The continuous supply of NGF to BFCNs is critical in maintaining their cholinergic phenotype and function (Gibbs and Pfaff, 1994, Holtzman et al., 1992, Pioro and Cuello, 1990, Sobreviela et al., 1994). Following CNS injuries, such as stroke or fimbria transaction, the application of NGF was able to recover ChAT activity and cholinergic synapses, preserving the morphological integrity of basal forebrain cholinergic cell bodies and preventing behavioural deficits (Williams et al., 1986, Kromer, 1987, Venero et al., 1994, Figueiredo et al., 1996, Garofalo et al., 1992, Kolb et al., 1997, Cuello, 1996). The same effect was obtained using a selective partial agonist of NGF receptors (Bruno et al., 2004). Endogenous NGF is also critical for the proper maintenance of the cholinergic synapses, as blocking the NGF function with NGF immunization or TrkA receptor blockade reduced the number of the pre-existing cortical cholinergic synaptic terminals (Debeir et al., 1999). Further research confirmed the previous points, demonstrating that mice with one mutant NGF allele alongside a single working copy have decreased BFCNs numbers and cholinergic terminals in target tissues (Chen et al., 1997).

This strong experimental evidence reinforces the idea that NGF is crucial in regulating the cholinergic phenotype and is responsible for maintaining and remodelling cortical synaptic contacts.

1.10.2 Molecular biology of NGF

In humans, the NGF gene spans 45 Kbp and four introns on chromosome 1 (Francke et al., 1983). The transcription from two different promoters and alternative splicing leads to two major and two minor pre-proNGF molecules. The major forms of pre-proNGF, 34 and 27 kDa, are cleaved respectively to 32 kDa and 25 kDa (by removing a signal sequence in the Golgi apparatus), representing the proNGF isoforms (Edwards et al., 1988).

Of these two isoforms, the 25 kDa predominates, constituting 70-80% in most tissues, and the 32 kDa isoform makes up for the remaining 20-30% (Selby et al., 1987). In addition, proNGF is subjected to post-translational modification at three potential sites for N-linked glycosylation (Seidah et al., 1996b), which seems to play a role in proNGF secretion and stability (Suter et al., 1991). ProNGF is known to form dimers (Paoletti et al., 2006), and complexes with several proteins, including mature NGF (mNGF) (Kliemannel et al., 2004), transport molecules such as the soluble protein α_2-macroglobulin (Barcelona and Saragovi, 2015) and receptor complexes (Nykjaer et al., 2004). For these reasons, proNGF-immunoreactive species exist in most tissues as unprocessed forms of 27 kDa and 32 kDa, as well as 41 (Seidah et al., 1996b, Bruno and Cuello, 2006) and 53 (Pedraza et al., 2005, Lakshmanan et al., 1989) kDa forms commonly reported in human and rat brains. Higher molecular weight forms were also reported, but their significance is unclear.

1.10.3 Nerve Growth Factor Metabolism

For decades following the discovery of NGF in the human and rat brain, it was assumed from cell culture experiments that proNGF was only cleaved intracellularly by furin and released as mNGF (Seidah et al., 1996b, Blöchl and Thoenen, 1995). This principle was turned over by Bruno and Cuello using an *ex vivo* superfusion approach based on Otto Loewi's experiment, in which they showed that proNGF (a 41 kDa), and not mature NGF, was secreted from rat cortex cells in response to cholinergic stimulation with carbachol, glutamate and KCl (Bruno and Cuello, 2006). This secretion of proNGF was dependent on intracellular calcium mobilization as adding a calcium chelator cell permanent (BAPTA-AM) abrogated proNGF release in response to carbachol (Bruno and Cuello, 2006). In addition, alongside proNGF, all the proteins and zymogens responsible for its conversion to mNGF and subsequent degradation (Bruno and Cuello, 2006) were also secreted, as shown in **Figure 1-8**. In summary, mNGF derives from the extracellular maturation of proNGF

by the action of plasmin, which is derived from the inactive zymogen, plasminogen, by the action of tissue plasminogen activator (tPA). This maturation step is regulated by the endogenous tPA inhibitor, neuroserpin. mNGF is a transient molecule in the synaptic cleft, and receptors rapidly take it up; meanwhile, unbound mNGF is quickly degraded by the metalloprotease 9 (MMP-9) (Bruno and Cuello, 2006) and metalloprotease 3 (MMP-3) (Pentz et al., 2021b). This explains the predominance of proNGF over NGF in the brain (Fahnestock et al., 2001). MMP-9 is derived from the precursor form (pre-MMP) by the action of plasmin and regulated by the non-specific metalloproteinase inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP1). MMP-3 can be released in response to cholinergic stimulation (Reina et al., 2011, Pentz et al., 2021b) and its consensus cleavage sequences overlap significantly with MMP-9 sequences, which are observed in the mNGF molecule (Eckhard et al., 2016). MMP-3 could play an indirect role by activating MMP-9 (Ogata et al., 1992). In addition, *in vitro* processing of proNGF to mNGF is also mediated by Furin and MMP-7 (Seidah et al., 1996b, Lee et al., 2001), though whether this occurs *in vivo* is not clear (Bruno and Cuello, 2006).

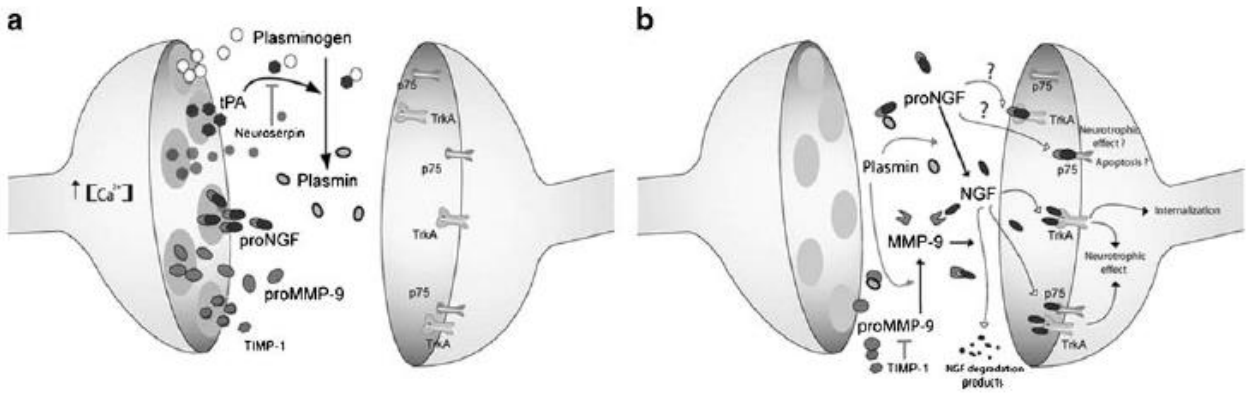

Figure 1-8. Schematic rapresentation of the NGF metabolic pathway.

Abbreviations: NGF, Nerve growth factor; proNGF, precursor NGF; tPA, tissue plasminogen activator; TIMP1, Tissue inhibitor of metalloproteinases-1; MMP-9, Matrix metalloprotease-9; TrkA, Tropomyosin receptor kinase A; p75ntr, p75 neurotrophin receptor. Image from (Bruno and Cuello, 2006), PNAS.

1.10.4 NGF and proNGF signalling

mNGF binds with higher affinity (Kd = 10^{-11} M (Hempstead et al., 1991, Cordon-Cardo et al., 1991)) the Tropomyosin receptor kinase A (TrkA), and induces the dimerization (Jing et al., 1992) and autophosphorylation (Cunningham et al., 1997) of adjacent TrkA monomers within their cytoplasmic domains. This causes the activation of four major signal transduction pathways: PI3K–AKT, ERK, MAPK, and PLCγ, implicated in growth, survival and synaptic plasticity (Kaplan and Miller, 1997). TrkA signalling is required for the health of BFCNs and for the maintenance of their cholinergic character (expression of TrkA, VAChT and ChAT) (Venero et al., 1994, Gibbs and Pfaff, 1994, BERSE et al., 1999). Conversely, mNGF has a much lower affinity for the p75 neurotrophin receptor (p75ntr; Kd = 10^{-9} M) (Chao et al., 1986, Johnson et al., 1986).

In contrast, proNGF has a high affinity for p75ntr and a low affinity for TrkA. p75ntr is part of the Tumor Necrosis Factor Receptor (TNFR) family characterized by extracellular cysteine-rich domains (CRDs) and intracellular death domains where proNGF binds to the third CRD (Kd = 1.6 x 10^{-10}) (Yan and Chao, 1991). In contrast to other members of the TNFR family, the intracellular "death" domain does not recruit caspase-9 in response to proNGF binding, rather its effects depend on the presence of coreceptors. Indeed, when proNGF binds with p75ntr complexed with the sortilin, they form a ternary complex shown to induce apoptosis *in vitro* (Lee et al., 2001; Nykjaer et al., 2004). This effect depends on the p53, ceramide, and c-Jun N-terminal kinase pathways (Roux and Barker, 2002).

Impairment of proNGF cleavage with α2-antiplasmin or the presence of the furin cleavage-resistant form of proNGF directly impacts the cholinergic phenotype and cognition (Allard et al., 2012, Allard et al., 2018). These results suggest that the effect on the cholinergic phenotype could be due to a lack of mNGF signalling.

In addition, some groups have reported proNGF to have (pseudo)trophic effects (Fahnestock et al., 2004), when it binds to the p75ntr in the absence of sortilin, subsequently activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Rho (Hamanoue et al., 1999, Bibel et al., 1999). The trophic versus apoptotic effects of proNGF could depend on the relative abundance of TrkA and sortilin (Ioannou and Fahnestock, 2017), and even if proNGF could exhibit trophic effects on BFCNs, it would be five-fold less trophic compared to mNGF (Fahnestock et al., 2004). For this reason, the relative levels of proNGF and mNGF reflecting the status of the

NGF metabolic pathway could play a critical role in determining the phenotypic maintenance of BFCNs.

1.10.5 NGF in Alzheimer's disease

Given that the cholinergic neurons of nb are highly dependent on NGF supply, it was initially hypothesized that in AD, there is an NGF trophic failure (Appel, 1981). Initial studies demonstrated that NGF mRNA was unchanged in AD (Goedert et al., 1986, Goedert et al., 1989a, Fahnestock et al., 1996) and that levels of overall NGF were elevated (Scott et al., 1995, Crutcher et al., 1993) but since the notion of the existence of a precursor and mature NGF isoforms was unknown at the time, detection methods rendered a combined read-out of the two.

It was later demonstrated that proNGF was predominant over mNGF in the brain (Fahnestock et al., 2001), and proNGF was upregulated in AD starting at stages of MCI (Fahnestock et al., 2001, Bruno et al., 2009a, Peng et al., 2004, Cuello et al., 2019). AD and MCI brains showed a reduction in mNGF protein levels (Pentz et al., 2020) along with its receptor TrkA (Pentz et al., 2020, Boissière et al., 1997, Mufson et al., 1996, Mufson et al., 2000).

In AD, there is diminished conversion of pro-NGF to mNGF mediated by increased neuroserpin activity and enhanced mNGF degradation depending on MMP9 activity (Bruno et al., 2009a, Bruno et al., 2009b). Indeed, in AD, a reduction in tPA, plasminogen and plasmin protein levels and increased neuroserpin levels have been revealed (Fabbro and Seeds, 2009, Bruno et al., 2009a, Pentz et al., 2020). In addition, a strong upregulation of MMP-9 activity and increased levels of MMP-3 and TIMP-1 in CSF of AD patients was found (Hanzel et al., 2014a). In particular, MMP-3 is significantly increased in AD brains and plasma compared to control subjects, and in close proximity to amyloid plaques (Yoshiyama et al., 2000, Horstmann et al., 2010).

The following sections will discuss how this NGF metabolic dysregulation in AD could depend on the two main pathological hallmarks in AD: Aβ and tau.

1.10.6 NGF dysmetabolism and amyloid pathology

An important role of amyloid beta (Aβ) in regulating NGF metabolism was supported by studies demonstrating that NGF dysmetabolism can be induced in wildtype rats by infusing Aβ oligomers (Bruno et al., 2009a). Similarly, in a rat model of AD-like amyloidosis, NGF dysmetabolism started

concomitantly with the onset of cognitive deficits recapitulating the human AD pathology (Iulita et al., 2017).

Individuals with Down syndrome (DS), a condition characterized by lifelong amyloidosis (Head and Lott, 2004), showed dysregulation of NGF metabolism in the frontal cortex, which correlated with Aβ pathology (Iulita et al., 2014b). The dysregulation of NGF metabolism was also found in the periphery with proNGF upregulation in plasma (Iulita et al., 2016), and proNGF upregulation in CSF of asymptomatic people with DS compared to non-trisomic controls (Pentz et al., 2021a). The increase in cortical proNGF in AD is accompanied by alterations in plasminogen, tPA and neuroserpin (Iulita et al., 2014b), and a similar pattern of NGF-metabolic alterations was revealed in the hippocampus of aged trisomic mice from the Ts65Dn line (Iulita et al., 2014b).

In addition, NGF dysmetabolism was observed during MCI stages, as wall as in individuals who were non-cognitively impaired but had high amyloid load (Pentz et al., 2020, Bruno et al., 2009b) and proNGF unpregulation correlated with Aβ pathology (Pentz et al., 2020).

A connection between Aβ pathology, NGF dysregulation and atrophy of BFCNs has been proposed in the *feed-forward pathological cycle* hypothesis (Florencia Iulita and Claudio Cuello, 2016). Amyloidosis could induce NGF dysmetabolism, depriving BFCNs of trophic support (Bruno et al., 2009a). Consequently, atrophic BFCNs would produce and release less ACh onto cortical neurons (Schliebs and Arendt, 2011). Since cholinergic signalling through M1 receptors reduces the amyloidogenic processing of APP (Nitsch et al., 1992), by depleting M1 signalling, this feedback loop could accelerate amyloidosis and, therefore, the course of AD. In addition to the signalling to M1-mAChRs, cholinergic signalling regulates functional hyperperfusion (Claassen and Jansen, 2006), which will be further discussed, and though nicotinic receptors could have an anti-inflammatory role (Nizri et al., 2006).

1.10.7 NGF dysmetabolism and tau pathology

BFCNs develop tau pathology relatively early in disease progression, and the impairment of retrograde transport has been proposed as a mechanism responsible for BFCNs vulnerability. Indeed, pre-tangle pathology appears to coincide with the downregulation of trkA and other cholinergic genes (Tiernan et al., 2018a) as well as the alteration of BFCN morphology prior to cell death (Tiernan et al., 2018b).

It is unclear whether diminished trophic support or impairment of retrograde transport would happen first in the context of tau pathology. In support of the hypothesis that trophic withdrawal causes aged BFCNs to be more at risk for tau pathology, it has been shown that NGF deprivation can increase tau expression (Sadot et al., 1996b) and phosphorylation (Nuydens et al., 1997) in cholinergic cells while proNGF/p75 signalling can induce tau hyperphosphorylation (Shen et al., 2018). Interestingly, the reduction of NGF maturation induces an impairment of retrograde transport and loss of cholinergic markers (Allard et al., 2018).

The second hypothesis is that some mechanism causing tau pathology would reduce the capacity to receive trophic support. Indeed, TrkA-NGF complexes are internalized by clathrin-dependent mechanisms to form a signalling endosome (Howe et al., 2001) and transported along microtubules by dynein (Heerssen et al., 2004), in several types of vesicles (Bhattacharyya et al., 2002). Tau is a microtubule-stabilizing protein, and tauopathy is associated with the disruption of axonal transport (Ishihara et al., 1999). As such, tau-mediated disruptions of axonal transport could underlie disruptions of trophic support, and such deficits have been associated with cholinergic degeneration in a mouse model of Down Syndrome (Cooper et al., 2001). However, these deficits have not been investigated early in the disease progression and could be consequential to a lack of trophic support.

1.11 Brain-derived neurotrophic factor (BDNF)

BDNF was originally isolated from the pig brain by Yves Barde and colleagues in 1982 (Barde et al., 1982). It was able to support the survival of cultured sensory neurons and, combined with NGF, their effect was additive (Barde et al., 1982). Many follow-up studies in the early 1990s demonstrated that BDNF was involved in memory-related modifications of synaptic transmission and long-term potentiation (LTP), in the morphological maintenance of pyramidal neurons and other neurons. BDNF was also found to enhance learning, memory and neurogenesis. A model of its function was proposed in **Figure 1-9.** However, most initial studies were conducted on BDNF without distinguishing between the two isoforms and only later the BDNF precursor and mature forms were discovered.

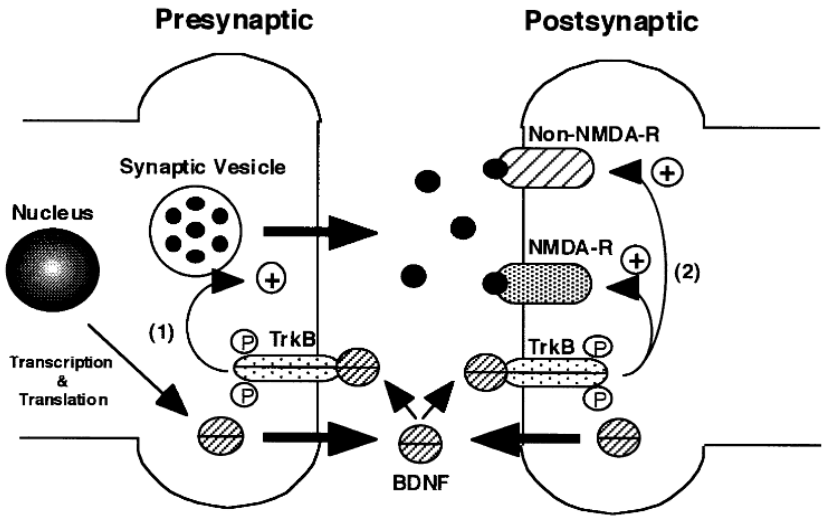

Figure 1-9. A model of BDNF functions in learning and memory.

Neuronal activity increases BDNF gene expression and releases BDNF from presynaptic and postsynaptic sites in an activity-dependent manner. BDNF binds to TrkB receptors located on presynaptic axons facilitating the neurotransmitter release via synapsin phosphorylation (1). BDNF also binds to postsynaptic TrkB, which leads to the activation of NMDA and non-NMDA receptors (2). BDNF increases the phosphorylation of the NMDA receptor subunits and the expression of AMPA receptor subunits (3). In addition, the modulation of growth and complexity of dendrites (4) could contribute to BDNF-dependent learning and memory processes. Abbreviations: BDNF, Brain-derived neurotrophic factor; TrkB, Tropomyosin receptor kinase B; NMDA, N-methyl-D-aspartate; AMPA-r, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Image from (Yamada et al., 2002), Life Sciences, with permission from Elsevier.

1.11.1 BDNF, LTP and synaptic plasticity

BDNF is involved in both the early and late phases of LTP (respectively E-LTP and L-LTP) (reviewed in (Lu and Gottschalk, 2000)). In the early phase of LTP, intracellular calcium concentrations increase rapidly, which induces the activation of protein kinases (Bliss and Collingridge, 1993). BDNF application facilitates E-LTP while the reduction of BDNF levels attenuates it (Figurov et al., 1996, Korte et al., 1995). BDNF enhances synaptic transmission by facilitating synaptic vesicle docking, acting on synaptophysin and synaptobrevin at the pre-synaptic level (Pozzo-Miller et al., 1999) and eliciting rapid postsynaptic through activation of protein kinases. Indeed, postsynaptic transmission is blocked by a tyrosine kinase receptor (TrkB) inhibitor (Levine et al., 1995). In a second L-LTP phase, the cAMP and cAMP response element-binding protein (CREB) signalling pathways are recruited to synthesize proteins involved in structural changes of hippocampal synapses (Kandel, 2001). L-LTP can never be induced in BDNF mutant mice (Korte et al., 1998, Tartaglia et al., 2001), and it requires conversion from the precursor to the mature BDNF form (Pang et al., 2004).

1.11.2 BDNF and phenotypic neurons maintenance

Neuronal morphology, given by the complexity, shape and size of the dendritic arbour, influences the functional properties of neuronal circuits. BDNF is well-known to affect the dendritic morphology and spine density of neurons (reviewed in (Horch, 2004)). BDNF acts preferentially on active neurons, as demonstrated in ferret cortical brain slices, in which inhibition of spontaneous electrical activity and synaptic transmission prevented dendritic arborizations increase (McAllister et al., 1996).

Autocrine BDNF produced by cortical pyramidal neurons expressing green fluorescent protein (GFP) elicited dramatic sprouting of basal dendrites and decreased dendritic spines (Horch et al., 1999). On the other hand, the effect of paracrine BDNF was studied with ‘donor neurons’ of ferret cortex brain slices expressing red fluorescent (RFP) BDNF, co-cultured with ‘recipient neurons’ expressing GFP. BDNF from ‘donor neurons’ increased dendritic branching of ‘recipient neurons’ in a distance-dependent manner (Horch and Katz, 2002).

Furthermore, in cultured rat hippocampal neurons, acute and gradual increases in BDNF promoted neurite elongation and spine head enlargement. In contrast, a sustained increase in BDNF facilitated neurite branching and spine-neck elongation (Ji et al., 2010).

1.11.3 BDNF, learning and Memory

As expected from the important role of BDNF in synaptic plasticity (Kang and Schuman, 1995), inhibition of BDNF signalling in rodents by gene knock-out impaired spatial learning and memory assessed by the Morris water maze task in young and aged wild-type mice (Linnarsson et al., 1997). An impairment in fear conditioning was demonstrated in inducible BDNF knock-out mice generated by crossing a floxed BDNF mouse with a Cre recombinase mouse combining the doxycycline-inducible system (Monteggia et al., 2004). These mice were bred in the presence of doxycycline, which was removed when the mice were 3 months of age, inducing a deletion of BDNF in the hippocampus (Monteggia et al., 2004). Furthermore, a continuous intracerebroventricular infusion of antisense BDNF oligonucleotide resulted in an impairment of spatial learning assessed by radial arm maze, and rats that had previously acquired spatial memory, treated with the oligonucleotide, showed impairment in both reference and working memory (Mizuno et al., 2000). In addition, BDNF receptor knock-out mice exhibited a severe impairment in spatial learning tasks (Minichiello et al., 1999).

1.11.4 BDNF and non-glutamatergic neurons

BDNF is critical not only for pyramidal neurons but also for neuronal growth and differentiation of BFCNs (Alderson et al., 1990, Morse et al., 1993). In addition, it regulates GABAergic synaptic transmission (Porcher et al., 2018, Elmariah et al., 2004, Sakata et al., 2009) by the expression of presynaptic GABA synthetic enzyme GAD65 (Sanchez-Huertas and Rico, 2011). Moreover, it seems to be a critical factor for neurogenesis (Rossi et al., 2006, Chan et al., 2008).

1.11.5 Molecular biology of BDNF

In adult brains, BDNF levels are dynamically regulated mostly by neuronal activity-dependent mechanisms (Poo, 2001). At the transcriptional level, Bdnf has eight distinct promoters that initiate the transcription of multiple different mRNA transcripts, but each encodes for an identical BDNF protein (Timmusk et al., 1993, Greenberg et al., 2009). In the cortex, most of the neuronal activity inducing Bdnf expression depends on promoter IV transcription (Timmusk et al., 1994). It is not clear why multiple RNA transcripts exist, but it may be essential to obtain mRNAs with different stability or subcellular localization of either mRNA or protein (Greenberg et al., 2009). Additionally, Bdnf transcripts are polyadenylated at either of two alternative sites, leading to a

population of mRNAs with short 3'-untranslated regions (3'-UTR) and others with a long 3'-UTR (Timmusk et al., 1993). As demonstrated with disruption of the long 3'-UTR in a mouse strain (Bdnf flox/flox), the short 3'-UTR Bdnf mRNA was restricted to the soma, whereas the long 3'-UTR Bdnf mRNA was localized in the dendrites for local translation (An et al., 2008).

BDNF mRNA is transcribed into a precursor (pre-proBDNF) in the endoplasmic reticulum. Following cleavage of the signal peptide, proBDNF (32-kDa) is transported to the Golgi and packed into vesicles (Mowla et al., 2001). Most proBDNF is not constitutively released (Mowla et al., 1999), but is directed towards the regulated pathway, in which it is secreted in response to neuronal activity (Mowla et al., 1999, Poo, 2001). In the extracellular space, proBDNF is converted into mBDNF (14-kDa) by plasmin through the tPA-dependent activation of plasminogen (Lee et al., 2001, Seidah et al., 1996a). Other studies have shown that proBDNF is proteolytically cleaved intracellularly by enzymes such as furin and secreted as the mBDNF, but to which extent intracellular and extracellular processing happen is unclear (reviewed in (Lessmann et al., 2003)).

1.11.6 BDNF and proBDNF signalling

As discussed for mNGF, mBDNF binds two receptors with different affinities: with higher affinity to the tropomyosin-related kinase receptor type B (TrkB), and lower affinity to the p75NTR (Lessmann et al., 2003). ProBDNF, like proNGF, binds with higher affinity the p75NTR receptor, sortilin and with lower affinity TrkB (Teng et al., 2005, Fayard et al., 2005). The interaction of proBDNF with sortilin is important in intracellular trafficking since the lack of binding redirects BDNF from the regulated secretory pathway to the constitutive secretory pathway (Chen et al., 2005). The molecular pathways which were activated by the binding of neurotrophins to p75NTR and sortilin were previously discussed for NGF, while those activated by the binding of mBDNF to TrkB are illustrated in **Figure 1-10.**

In light of the discovery of two different BDNF forms, some studies aimed to clarify how each isoform impacts LTP and long-term depression (LTD). In electrophysiological studies, mBDNF, but not the uncleavable proBDNF in tPA and plasminogen knock-out mice, rescued L-LTP (Pang et al., 2004). As well, when the neurons were subjected to high-frequency stimulation (a condition that induces LTP), a substantial amount of mBDNF was detected (Nagappan et al., 2009). On the

other hand, in conditions that induce LTD, it was secreted mostly proBDNF (Nagappan et al., 2009). These results demonstrate how high-frequency neuronal activity could regulate opposing functions of BDNF isoforms. Furthermore, to determine if proBDNF alters synaptic activity *in vivo*, it was infused in wild-type and p75NTR mutant mice. ProBDNF significantly enhanced LTD in wild-type, but did not show any effect on p75NTR knock-out animals (Woo et al., 2005). Interestingly, complete deletion of the Bdnf gene, did not impact LTD (Matsumoto et al., 2008), suggesting that complete deletion may annul these opposing functions.

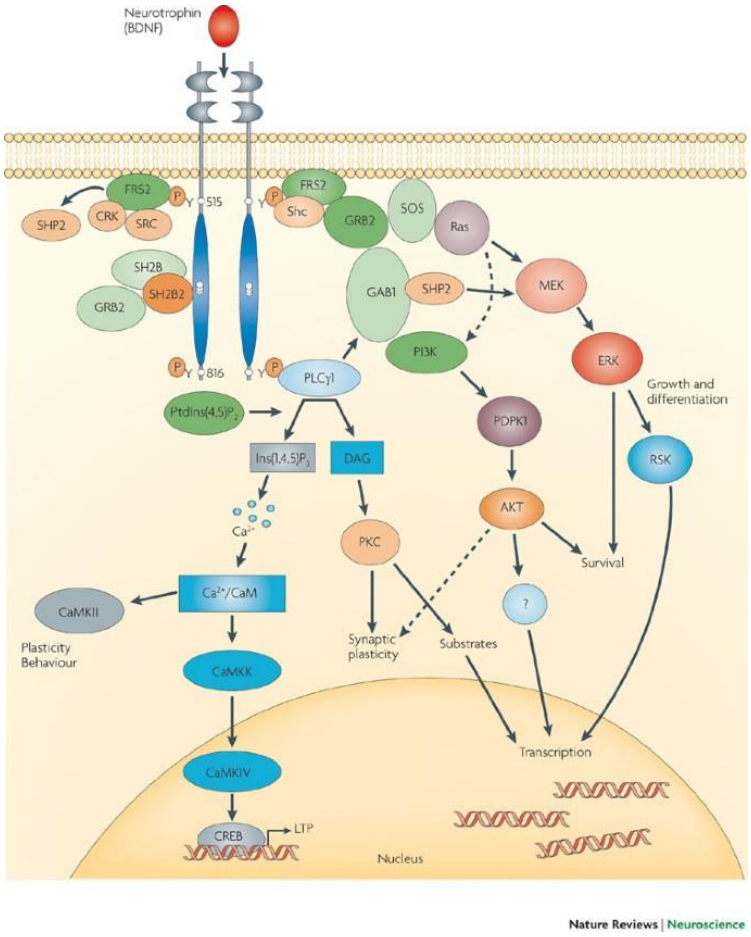

Figure 1-10. Major TrkB-signalling-activated pathways.

There are three main signalling pathways activated by binding of mBDNF to TrkB receptor: (1) Ras–mitogen-activated protein kinase (MAPK) signalling cascade, which promotes neuronal differentiation and growth through MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK). A second pathway (2) is the activation of the phosphatidylinositol 3-kinase (PI3K) cascade, which promotes the growth of neurons and other cells through protein kinase B (AKT). The third pathway is the activation of phospholipase Cγ1 (PLCγ1) which results in the generation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) and Ins(1,4,5)P3 promotes the release of Ca2+ from internal stores and subsequent activation of Ca2+/calmodulin-dependent protein kinases (CaMKIs), cyclic AMP-responsive element-binding protein (CREB) and synaptic plasticity. Image from (Minichiello, 2009), Nature Reviews Neuroscience with permission from Springer Nature.

1.11.7 BDNF in Alzheimer's disease

Along with mood disorders such as depression, BDNF alterations are associated with neurological disorders, including AD. Patients with severe AD have decreased BDNF mRNA and protein levels in the hippocampus, frontal cortex, and parietal cortex (Holsinger et al., 2000, Phillips et al., 1991, Peng et al., 2005, Michalski and Fahnestock, 2003b, Hock et al., 2000, Garzon et al., 2002) and reduction in both pro- and mBDNF levels are present during earlier stages of MCI (Peng et al., 2005, Michalski and Fahnestock, 2003a). AD post-mortem studies suggest that mBDNF is present in lower amounts in the cortex than proBDNF and decreases more sharply than proBDNF in the prodromal stages of AD (Peng et al., 2005). AD animal models confirmed that BDNF expression alteration occurs early, before plaque deposition (Iulita et al., 2017, Francis et al., 2012). Furthermore, BDNF protein levels correlate strongly with cognitive abilities (Peng et al., 2005, Fahnestock et al., 2002), and it has been proposed to represent a candidate biomarker for early AD detection (Beeri and Sonnen, 2016).

On the other hand, cortical levels of TrkB are reported to be unchanged in AD (Savaskan et al., 2000, Michalski et al., 2015, Boissière et al., 1997). It is unclear why TrkB levels are not affected, but one explanation could be the lack of exclusivity of TrkB in binding BDNF, as it can also bind Neurotrophin 4/5 (NT-4/5) (Bothwell, 1995), which is not affected in AD. A case contrary to the TrkA receptor density, which is regulated by NGF (Venero et al., 1994, Gibbs and Pfaff, 1994, Figueiredo et al., 1995).

1.12 Vascular-cholinergic hypothesis

Regulation of cerebral blood flow is fundamental to the functional activity of the brain. When the activity of a certain brain region rises, it also increases the blood flow to that region, allowing substrate delivery and the removal of metabolic products in a mechanism called hyperemia (Iadecola, 2004). Pharmacological studies using cholinergic drugs brought to light an additional role for the cholinergic neurons: the regulation of cerebral perfusion. Indeed, anticholinergic drugs such as Scopolamine given to young people reduced cerebral perfusion by 20% in the frontal cortex (Honer et al., 1988). This vascular-cholinergic hypothesis proposed that the cholinergic deficit not only affects the innervation of cortical neurons but also the innervation of cerebral blood vessels and consequently regulates cerebral vasodilatation and cerebral blood flow. It was even

proposed 30 years ago that sporadic AD is a vascular disorder caused by impaired cerebral perfusion (De la Torre and Mussivan, 1993, de la Torre, 2004).

1.12.1 Evidence of vascular innervation by cholinergic neurons

1.12.1.1 Use of ChEIs in AD

Computed tomography using radionuclides has been an important tool in providing information on regional changes in cerebral blood flow in AD patients (Claassen and Jansen, 2006). ChEIs given to individuals with AD resulted in an increase in the posterior parietal-temporal and superior frontal perfusion (Geaney et al., 1990, Ebmeier et al., 1992). Interestingly, it was later confirmed that a regional increase in perfusion was not a consequence of increased regional metabolism produced by cortical neuron stimulation. Indeed, physostigmine increased the cerebral blood flow in the cerebral cortex and the subcortical structures in young and aged individuals. On the contrary, cerebral glucose consumption decreased significantly in most regions (Blin et al., 1997). In addition, AD patients receiving ChEIs had improved cerebral blood flow that preceded beneficial effects on glucose metabolism by months (Nordberg et al., 1998, Nordberg, 1999).

1.12.1.2 Biochemical and immunohistochemical studies

After the initial pharmacological studies, immunohistochemical and electron microscopy (EM) studies followed. The most important evidence that supported the vascular-cholinergic hypothesis was the presence of cholinergic terminals in the cortical cerebral blood vessels shown in rats by Hamel and colleagues (Vaucher and Hamel, 1995). Indeed, injection of the anterograde tracer *Phaseolus vulgaris leucoagglutinin* in the substantia innominata showed that neuronal terminals in all cortical subdivisions surrounded microvessels (Vaucher and Hamel, 1995). Further immunohistochemical studies showed a loss of cholinergic terminals in the arterioles in the temporal lobe of AD brains compared to the age-matched controls (Tong and Hamel, 1999). In addition, along with the initial studies of the effect of ACh on vessel vasodilatation (Furchgott and Zawadzki, 1980), mAChRs were identified as components of the BBB. mAChRs were observed in perivascular astrocytes (subtypes M 1-5), smooth muscle cells (subtypes M 1,2,4,5), and endothelial cells (subtypes M 1,2,3,5) (Luiten et al., 1996, Garcia-Villalon et al., 1991, Elhusseiny et al., 1999, Furchgott and Zawadzki, 1980) in the brains of animals and humans.

1.12.2 The Neurovascular unit (NVU)

The NVU is a structure composed of several cell types, including endothelial cells, pericytes, vascular smooth muscle cells (SMCs), astrocytes, microglia, and neurons, which connect the brain parenchyma to the cerebral vasculature. The interactions among these NVU cell types are important for a variety of physiological processes such as angiogenesis, vessel maintenance and permeability, metabolic support, and regulation of blood flow (reviewed in (McConnell et al., 2017, Schaeffer and Iadecola, 2021)). The composition of the NVU varies across capillaries, arteries and veins.

Capillaries are small vessels with a diameter under 10 µm containing a single layer of endothelial cells surrounding the vessel lumen, wrapped by a thin layer of cerebrovascular basement membrane (CVBM). This structure is irregularly surrounded by pericytes whose proposed contractile properties are still controversial (Fernández-Klett et al., 2010) and externally carpeted by a layer of astrocytic endfeet (McConnell et al., 2017) (**Figure 1-11**). Arteries are found on the brain surface and have extrinsic innervation (Hamel, 2006). They are wrapped by a layer of endothelial cells surrounded by multiple layers of SMCs, or a single layer in the case of arterioles, with contractile properties, and finally with a CVMB (Schaeffer and Iadecola, 2021). In addition, arteries are externally covered by astrocytic endfeet (Filosa et al., 2016) (**Figure 1-12**).Veins and venules are made up of endothelial cells wrapped by a thin layer of CVMB and carpeted by astrocytic endfeet. They occasionally have pericytes but lack SMCs (reviewed in (Schaeffer and Iadecola, 2021)).

In addition to that, capillaries and arteries mostly receive intrinsic innervation (Hamel, 2006). The cell bodies of these nerve fibres are mostly located in the BF (cholinergic fibers), the LC (noradrenergic fibers) and the raphe nucleus (serotoninergic system) (Hamel, 2006). At the NVU, nerve fiber terminals have been shown to be <1.0 µm from the vessel walls (Tong and Hamel, 1999). They can regulate the vascular tone by either synapsing directly on SMA, endothelial cells, pericytes or via astrocytic endfeet (Mulligan and MacVicar, 2004, Toribatake et al., 1997, Hamilton et al., 2010).

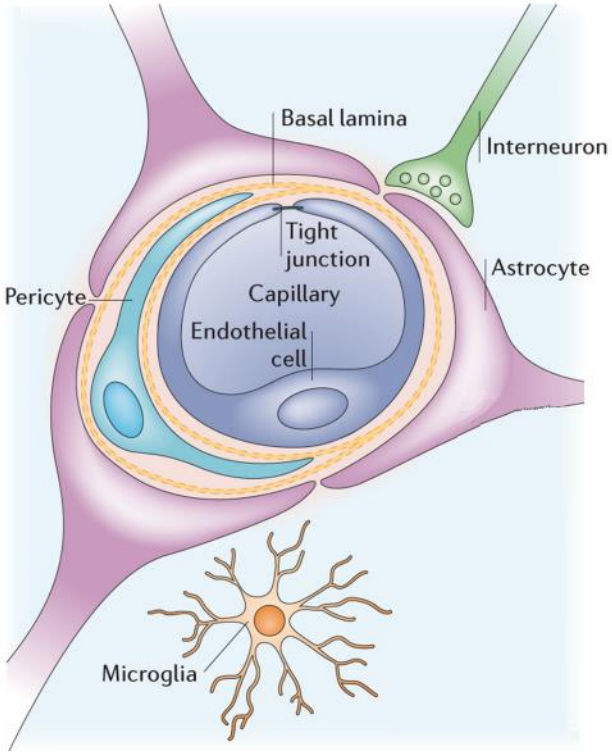

Figure 1-11. Representation of a capillary cross-section within a single NVU.

The NVU shows the following features: 1) Brain endothelial cells lining cerebral vessels. 2) Tight junctions between endothelial cells "sealing" the vessels. 3) A continuous basal lamina/basement membrane encases endothelial cells with pericytes between endothelial cells and astroglial endfeet. 4) Astrocytic endfeet wrapping the lamina. 5) Resident microglia with long cellular processes to survey their microenvironment. 6) Local interneurons innervating cerebral vasculature and can induce vessels to change their tone. Image modified from (McConnell et al., 2017).

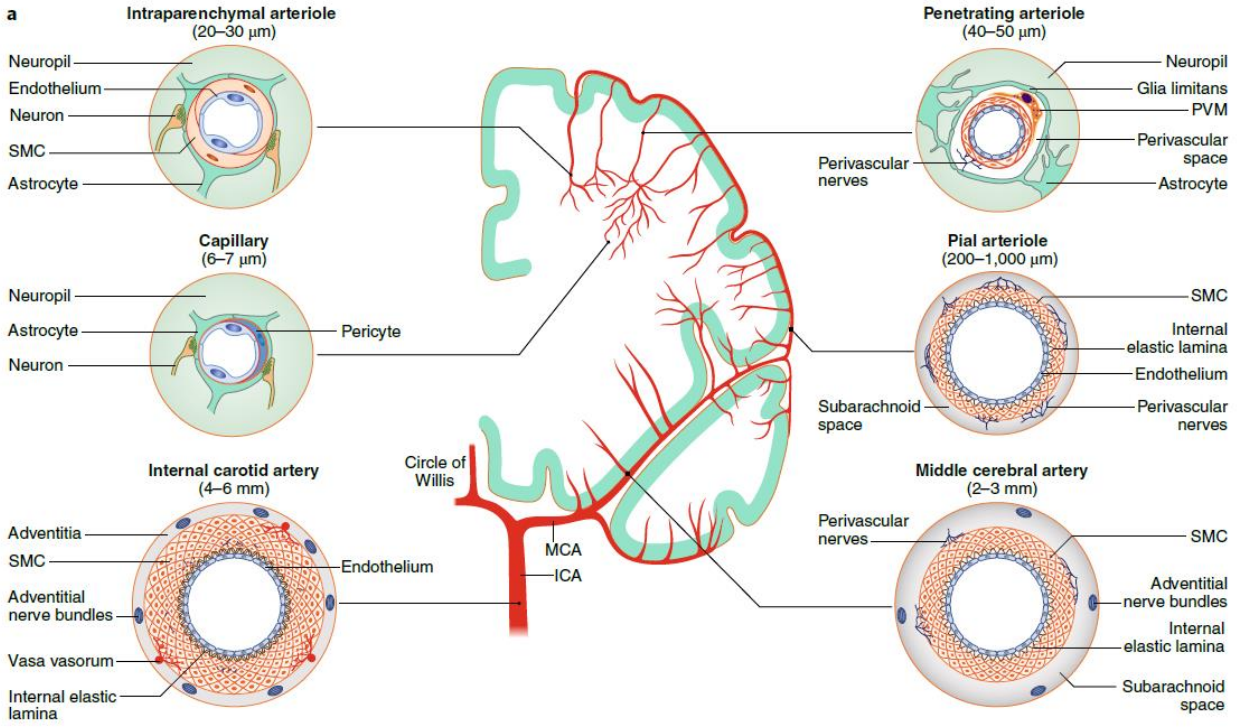

Figure 1-12. Heterogeneity of cerebral arteries.

Representation of different types of arteries present in the human brain. As the vessel becomes smaller (arterioles), the vascular basement membrane fuses with the glial basement membrane and perivascular nerves are replaced by nerve terminals from subcortical pathways (intrinsic innervation). In capillaries, SMCs are replaced by pericytes. Vascular diameters refer to the human cerebral circulation. Abbreviations: MCA, middle cerebral artery; PVMs, perivascular macrophages; SMCs, smooth muscle cells; ICA, internal carotid artery. Image from (Schaeffer and Iadecola, 2021), Nature Reviews Neuroscience, with permission from Springer Nature.

1.12.3 Coordinated vascular response

Far from the traditional view proposing that cerebral blood flow depends solely on the release of vasoactive agents from neurons, it is becoming clear that this cannot be attributed to a single cell type. The cerebral blood flow would result from multiple processes involving different cell types of the NVU (Iadecola, 2004). Indeed, synaptic activity generates vasoactive mediators in neurons and astrocytes, which act on local blood vessels to produce vasodilation of arterioles and possibly capillaries at the activated site. Furthermore, vasodilation in the activated area is propagated upstream by intramural vascular signalling to balance the pressure in the cerebrovascular tree. Variations in intravascular pressure induced by the changes in flow and resistance are compensated by adjustments of smooth muscle cells (Iadecola, 2004). This model is shown in **Figure 1-13.**

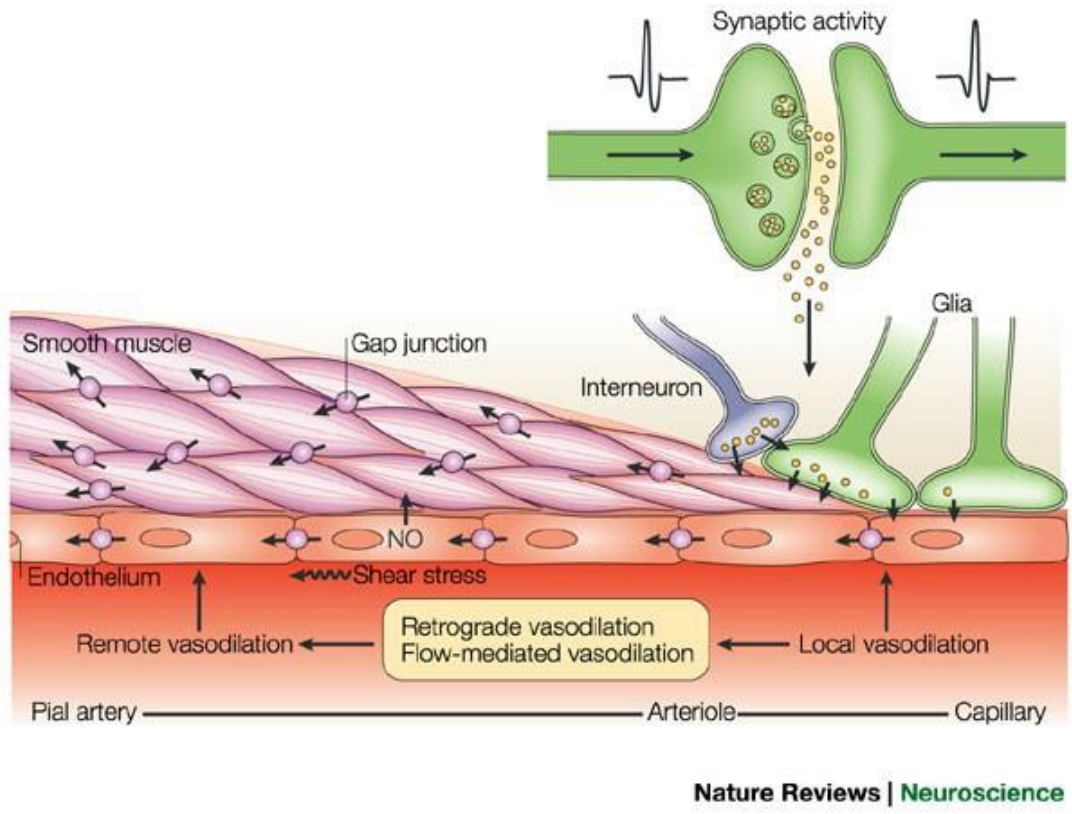

Figure 1-13. Cellular mechanisms for the propagation of vasodilation in the vessels from the activated site.

Image from (Iadecola, 2004), Nature Reviews Neuroscience, with permission from Springer Nature.

1.12.4 BFCNs in the cerebral blood flow regulation

It is well-studied that glutamate induces an increase in Ca2+ in neurons and glia, activating the synthesis of powerful vasodilators, including nitric oxide (NO), metabolites of cyclooxygenase-2 (COX-2) such as prostaglandins and cytochrome P450 epoxygenase products, epoxyeicosatrienoic acids (EETs) (Iadecola, 2004)). In astrocyte endfeet, the increase of Ca2+ is produced by the activation of metabotropic glutamate receptors (mGluRs), and this response propagates to the neighbouring astrocytes through gap junctions (Iadecola, 2004).

ACh is known to induce vasodilation (Furchgott and Zawadzki, 1980). Experiments of BFCNs immunolesion with 192 IgG-Saporin in rats showed a global decrease (24-40%) in cerebral blood flow in the parietal and temporal regions (Waite et al., 1999), while other studies utilizing muscarinic antagonists (Elhusseiny and Hamel, 2000) and muscarinic M5 knock-out mice (Yamada et al., 2001a) showed similar results.

In particular, BFCNs can directly regulate vasodilatation through the release of the potent vasodilator NO, from endothelial cells (eNO), but not from neurons (nNO) (Zhang et al., 1995). In addition, BFCNs stimulation has an indirect effect by inducing selective activation of Somatostatin and vasoactive intestinal peptide (VIP) neurons (Kocharyan et al., 2008, Cauli et al., 2004) as shown in **Figure 1-14**. A pharmacological study showed that blockade of mAChRs and either NMDA or GABA receptors had no additive attenuating effect, indicating that glutamate and GABA effects are downstream to ACh from BFCNs (Lecrux et al., 2012).

In addition, multiple mAChRs also exist on perivascular astrocytes and it cannot be excluded that these cells contribute to the cerebral blood flow response as demonstrated for glutamate (Hamel, 2006). Indeed, ACh is responsible for releasing EETs, a product of the P450 epoxygenase pathway, selective for astrocytes (Lecrux et al., 2012), and two specific gliotoxins were able to decrease the ACh-induced cerebral blood flow (Lecrux et al., 2012).

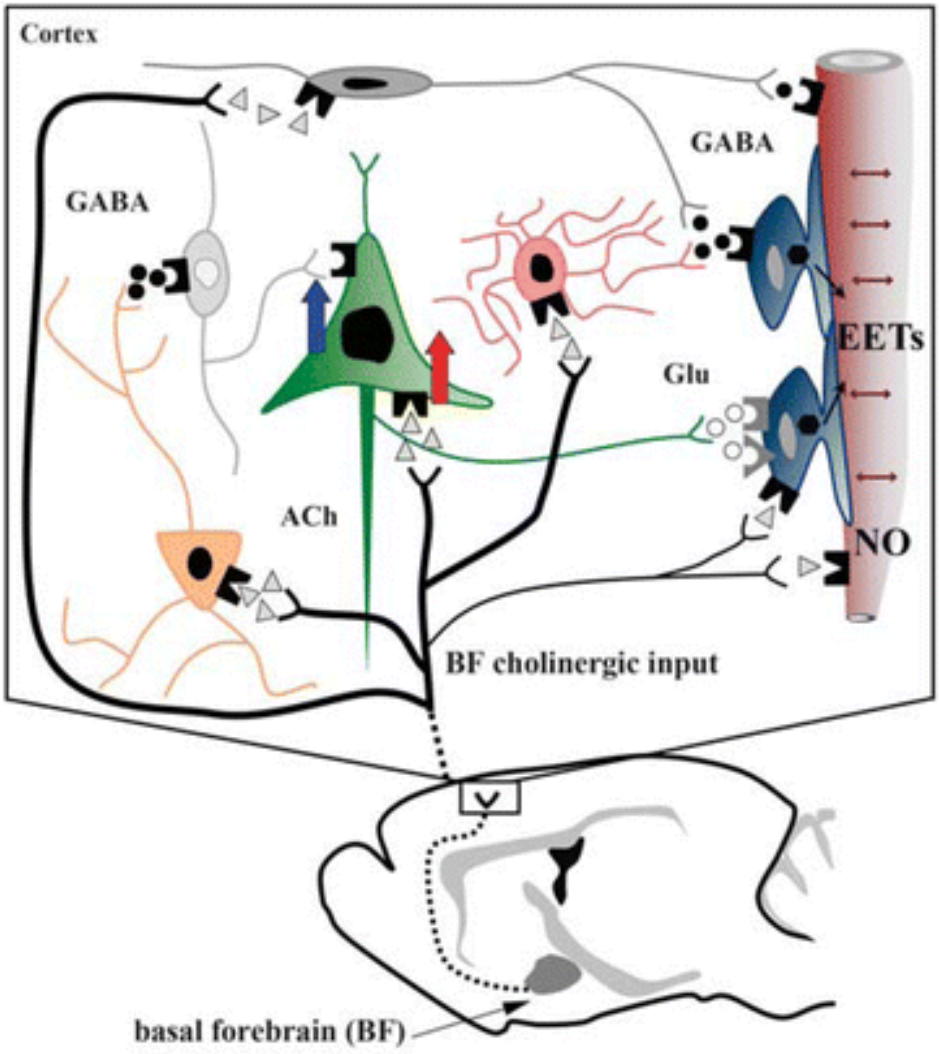

Figure 1-14. Schematic representation of how glutamate, GABA and astrocytes may interact in response to basal forebrain stimulation.

BF send input to pyramidal neurons (in green), somatostain (yellow), neuropeptide Y (pink), and vasoactive intestinal polypeptide (grey) neurons. Additionally BF can make synapses with astrocytes and vessels. Abbreviations: BF, Basal forebrain; ACh, Acetylcholine; GABA, γ-aminobutyric acid; Glu, glutammate; EETs, Epoxyeicosatrienoic acids; NO, Nitric oxide. Image from (Lecrux et al., 2012).

1.12.5 BFCN regulation of vascular endothelial growth factor (VEGF)

First identified in rodent tumour cell lines, the vascular endothelial growth factor (VEGF) has been shown to increase vascular permeability (Senger et al., 1983) and, for this reason, is also referred to as vascular permeability factor. In mammals, the VEGF family comprises VEGF-A (named VEGF, which has received the most attention), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) (reviewed in (Koch and Claesson-Welsh, 2012, Lange et al., 2016) (**Figure 1-15**). Years after its discovery, VEGF was identified in the rat brain (Monacci et al., 1993) and was shown to be expressed by the vascular endothelium, astrocytes and neurons (reviewed in (Ruiz de Almodovar et al., 2009)).

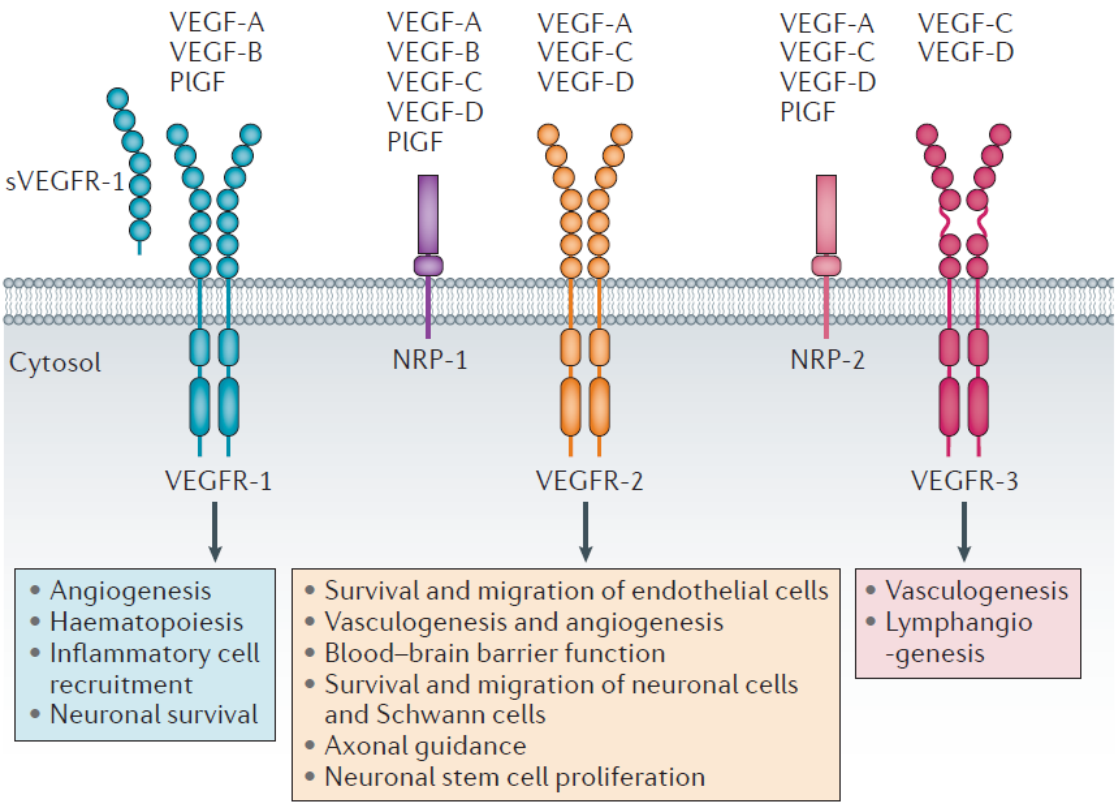

Figure 1-15. The VEGF family of growth factors.

VEGF family members bind to cell surface receptors VEGFR-1, VEGFR-2 and VEGFR-3. Several members also bind to non-tyrosine kinase receptors of the NRP family, which function as co-receptors for the VEGFRs. Abbreviations: VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial cell growth factor receptor; NRP, Neuropilin. Image from (Lange et al., 2016), Nature Reviews Neurology, with permission from Springer Nature.

VEGF relevance in the context of the cholinergic hypothesis depends on its regulation by cholinergic input. Studies using organotypic hippocampal slice cultures showed that tacrine (THA) treatment rescued hippocampal neurons from excitotoxicity-induced long-lasting hippocampal cell damage possibly mediated by VEGF since the effect was significantly reduced by a selective inhibitor of vascular endothelial cell growth factor receptor 2 (VEGFR-2) (Inada et al., 2014). In addition, studies in primary neurons and primary astrocytic cultures from mice showed that different mechanisms regulate VEGF release. THA treatment increased VEGF mRNA expression in neurons in a manner that was reversed by mecamylamine, a nicotinic ACh receptor antagonist, whereas in primary astrocytes, carbachol, but not THA increased VEGF mRNA expression and secretion in a manner that was inhibited by scopolamine, a mAChR inhibitor (Kimura et al., 2018). The increase of VEGF in medial septal cholinergic neurons was confirmed *in vivo* by daily administration of THA (2.5 mg/kg, i.p.) for seven days (Kimura et al., 2018). Moreover, it was shown that NGF controls neuronal expression of VEGF (Calza et al., 2001) and there exists a common signal transduction pathway between VEGF and NGF (reviewed by (Nico et al., 2008)).

VEGF seems to play several important functions: it is a (1) vascular factor that affects vascular tone by controlling the release of vasorelaxant nitric oxide by endothelial cells (eNOS) (Storkebaum and Carmeliet, 2004). The isoforms cooperate in an angiogenetic process (2) establishing chemoattractive gradients which promote blood vessel sprouts from pre-existing blood vessels (Ruhrberg et al., 2002). VEGF also plays a role (3) in regulating BBB permeability, increasing the leakiness of the microvascular wall (Mayhan, 1999). Moreover, it is a (4) neurotrophic factor which plays a role in hippocampal synaptic plasticity and memory consolidation in mice (Cao et al., 2004, Licht et al., 2011, De Rossi et al., 2016). Notably, VEGFR2 is expressed in hippocampal neurons *in vivo* (De Rossi et al., 2016, Luck et al., 2019).

VEGF family members exert their effects via downstream signalling pathways, such as the MEK–MAPK pathway (proliferation and migration), the PI3K–Akt pathway (survival), and the Src–eNOS pathway (permeability) (reviewed in (Koch and Claesson-Welsh, 2012)).

1.12.6 Neurovascular unit dysfunction in Alzheimer's disease

A full understanding of the underlying mechanisms for cerebral blood flow reduction in AD is unclear. Indeed, hypoperfusion could be consequential to several mechanisms, such as the constriction of brain arterioles (Niwa et al., 2001), loss of vascular density (Farkas and Luiten, 2001) and changes in the neurovascular coupling (Hamel, 2006).

In addition, Aβ and tau play a key role in NVU dysfunction (Schaeffer and Iadecola, 2021). In particular, Aβ induces a pro-inflammatory response by perivascular glia cells and the production of free radicals (Park et al., 2017). Furthermore, Aβ showed to induce vasoconstriction of cerebral arteries (Niwa et al., 2001) and capillaries in a mouse model of AD and in AD human brains (Nortley et al., 2019). In addition, APP/PS1 mice showed transient occlusion of vessels by neutrophils adhesion to capillaries and blockage of blood flow (Cruz Hernández et al., 2019).

On the other hand, hyperphosphorylated tau affects the neurovascular coupling involving a tau-induced dissociation of neuronal nitric oxide synthase (nNOS) from postsynaptic density 95 (PSD95) and reducing the production of NO during glutamatergic synaptic activity (Park et al., 2020).

Thesis objective and rationale

Basal forebrain cholinergic neurons (BFCNs), essential for learning, memory and attention, represent the main source of cholinergic innervation to the cortex and hippocampus. These neurons degenerate early in Alzheimer's disease (AD), contributing to early cognitive deficits. Interestingly, Acetylcholinesterase Inhibitors (AChEIs), which potentiate any remaining cholinergic signalling, can provide temporary relief without modifying disease progression.

Some studies have shown that AChEIs reduce the rate of atrophy of the hippocampus, cortex, and basal forebrain, while patients taking anticholinergic medications have worse disease outcomes in AD. This suggests that BFCN degeneration may play a significant role in aggravating AD pathology. Therefore, the main goal of this thesis is to study the role of BFCNs in the function of neurotrophins, vasculature and experimental cholinergic therapeutics to prevent AD. Toward this objective, the following evidence has been taken into consideration:

1. Rats represent a good model to study the interaction between BFCNs innervation and neurotrophins release, given the similarity of their basal forebrain cholinergic system to the humans and their superior cognitive abilities compared to mice. One side of the relationship between neurotrophins and BFCNs has been very well-studied. It was demonstrated that BFCNs depend on levels of mature nerve growth factor (mNGF) and, in less extent, on the mature brain-derived neurotrophic factor (mBDNF), produced by cortical and hippocampal neurons and retrogradely transported to the cell bodies.

2. Synapses of BFCNs are localized in the proximity of cortical cerebral blood vessels and form synapses with different cell types, such as vascular smooth muscle cells, endothelial cells, and astrocytic end-feet. Synapses of BFCNs represent a component of the neurovascular unit regulating vascular tone and cerebral blood flow.

3. The acetylcholine muscarinic receptor M1 is present mostly in the CNS, is not downregulated in AD brains and can modulate the amyloidogenic pathway. For these reasons, agonists that selectively target this receptor would avoid adverse effects, given by

stimulation of other muscarinic receptors outside the CNS. Additionally, some M1 receptor agonists have been shown to delay AD-like progression in preclinical trials.

Based on this evidence, we have generated the following hypotheses:

I. Neurotrophins not only influence BFCNs, but the input of BFCNs influences the release of neurotrophins in a reciprocal interaction relationship.

II. Synapses of BFCNs are part of the neurovascular unit and influence vessel diameters, vascular endothelial growth factor A, astrocytes and microglial cells.

III. A new cholinergic treatment, a partial agonist of the M1 muscarinic receptor, with the additional property of modulating the sigma-1 receptor, prevents AD-like hallmarks in a transgenic rodent model of amyloid-like pathology.

Chapters 2,3, and 4 of this thesis will cover the testing and outcome of the above hypotheses.

CHAPTER 2

Long-term nucleus basalis cholinergic depletion induces attentional deficits and impacts cortical neurons and BDNF levels without affecting the NGF synthesis

Chiara Orciani, Helene Hall, Rowan Pentz, Morgan K Foret*, Sonia Do Carmo*, A. Claudio Cuello

*these authors contributed equally to this work

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Abstract

Basal forebrain cholinergic neurons (BFCNs) represent the main source of cholinergic innervation to the cortex and hippocampus and degenerate early in Alzheimer's disease (AD) progression. Phenotypic maintenance of BFCNs depends on levels of mature nerve growth factor (mNGF) and mature brain-derived neurotrophic factor (mBDNF), produced by target neurons and retrogradely transported to the cell body. Whether a reciprocal interaction where BFCN inputs impact neurotrophin availability and affect cortical neuronal markers is unknown. To address our hypothesis, we immunolesioned the nucleus basalis (nb), a basal forebrain cholinergic nuclei projecting mainly to the cortex, by bilateral stereotaxic injection of 192-IgG-Saporin (the cytotoxin Saporin binds p75ntr receptors expressed exclusively by BFCNs) in 2.5-month-old Wistar rats.

At six months post-lesion, Saporin-injected rats (SAP) showed an impairment in a modified version of the 5-Choice Serial Reaction Time Task (5-choice task). Post-mortem analyses of the brain revealed a reduction of Choline Acetyltransferase-immunoreactive neurons compared to wild-type controls. A diminished number of cortical vesicular acetylcholine transporter-immunoreactive boutons was accompanied by a reduction in BDNF mRNA, mBDNF protein levels, markers of glutamatergic (vGluT1) and GABAergic (GAD65) neurons in the SAP-group compared to the controls. NGF mRNA, NGF precursor and mNGF protein levels were not affected. Additionally, cholinergic markers correlated with the attentional deficit and BDNF levels. Our findings demonstrate that while cholinergic nb loss impairs cognition and reduces cortical neuron markers, it produces differential effects on neurotrophin availability, affecting BDNF but not NGF levels.

2.1 Introduction

Basal forebrain cholinergic neurons (BFCNs) provide the major source of cholinergic innervation to the forebrain (Mesulam et al., 1983b, Mesulam et al., 1983a, Bigl et al., 1982, Struble et al., 1986). It is well-established, in both humans and experimental animal models, that BFCNs play a crucial role in learning, memory and attention mechanisms (Drachman and Leavitt, 1974, Bartus, 1979, Everitt and Robbins, 1997, Ballinger et al., 2016, Zaborszky et al., 1999). The gradual degeneration of BFCNs in ageing, and their severe atrophy and loss in Alzheimer's disease (AD) pathology (Bowen et al., 1976, Davies and Maloney, 1976, Whitehouse et al., 1981), contribute significantly to cognitive impairment in this neurodegenerative condition. The involvement of BFCNs in AD cognitive impairment is further illustrated by the fact that four out of six FDA-approved drugs are acetylcholinesterase inhibitors (AChEI). These drugs improve the telencephalic cholinergic tone by blocking extracellular acetylcholine degradation (Giacobini, 1987), thus offering transient cognitive relief even at advanced stages of AD (Giacobini et al., 2022). Accordingly, the cholinergic hypothesis suggests that dysfunction of BFCNs contributes considerably to the cognitive decline observed in the geriatric population (Bartus et al., 1982, Coyle et al., 1983, Cuello and Sofroniew, 1984, Francis et al., 1999); a hypothesis extended to AD and supported by the beneficial effects of AChEI.

New interest in the basal forebrain cholinergic system has been provoked by the evidence of basal forebrain atrophy at prodromal stages of AD (Teipel et al., 2011), which precedes and predicts atrophy of the entorhinal and cerebral cortices (Schmitz et al., 2016) as well as the cortical spread of the AD pathology (Fernández-Cabello et al., 2020). In addition, long-term use of anticholinergic drugs increases the risk of dementia and AD (Gray et al., 2015), and results in brain atrophy and cognitive decline (Risacher et al., 2016), further supporting the key role of BFCNs on brain health. In line with the above, AChEIs reduce the rate of hippocampal (Dubois et al., 2015), and basal forebrain atrophy (Cavedo et al., 2017) while eliciting an increased cortical thickness (Cavedo et al., 2016, Hampel et al., 2017, Hampel et al., 2018). The symptomatic benefits of AChEIs used in AD patients could be explained by the trophic effects exerted by acetylcholine (ACh) on cortical neurons. Indeed, stimulation of the M1 and M3 muscarinic ACh receptors (mAChRs) located in both pyramidal glutamatergic projections and local γ-aminobutyric acid (GABA)-ergic neurons modulates cellular excitability and network synchronization in the neocortex and hippocampus.

Further, this stimulation promotes the production of amyloid precursor protein-derived peptides (APPs) (Nitsch et al., 1992, Haring et al., 1998) through the alpha-secretase pathway (or non-amyloidogenic pathway) (Davis et al., 2010), therefore preventing the formation of toxic amyloid-beta peptides and favouring synaptogenesis (Bell et al., 2008, Kojro and Fahrenholz, 2005). Conversely, the loss of mAChRs promotes pathogenic amyloid processing (Davis et al., 2010, Medeiros et al., 2011).

The phenotypic maintenance of BFCNs is tightly dependent on the trophic support of endogenous nerve growth factor (NGF) (Sofroniew et al., 1990, Hefti, 1986, Cuello et al., 1992, Cuello, 1993) released by pyramidal neurons (Bruno and Cuello, 2006). Along with NGF, brain-derived neurotrophic factor (BDNF), is widely expressed in BFCN target areas (Phillips et al., 1990), supports BFCNs (Alderson et al., 1990, Morse et al., 1993, Boissière et al., 1997) and is essential for the phenotypic maintenance of pyramidal neurons (McAllister et al., 1996, Horch and Katz, 2002). In addition, BDNF sustains both early and late phase of long-term potentiation (LTP) (Figurov et al., 1996, Korte et al., 1995, Korte et al., 1998, Tartaglia et al., 2001) and is markedly involved in learning and memory (Kang and Schuman, 1995, Korte et al., 1998, Broad et al., 2002, Hariri et al., 2003). Both NGF and BDNF are secreted in an activity-dependent manner (Mowla et al., 1999, Bruno and Cuello, 2006) from cortical and hippocampal neurons as precursors, respectively proNGF and proBDNF and converted in the extracellular space into the bioactive mature NGF (mNGF) (Bruno and Cuello, 2006, Bruno et al., 2009a) and mature BDNF (mBDNF) (Lee et al., 2001, Seidah et al., 1996a, Pang et al., 2004).

These neurotrophins have been shown to be affected in AD. In particular, AD and mild cognitive impairment (MCI) brains showed a reduction in mNGF protein levels (Pentz et al., 2020) along with its receptor tropomyosin receptor kinase A (TrkA) (Pentz et al., 2020, Boissière et al., 1997, Mufson et al., 1996, Mufson et al., 2000) and increased proNGF levels (Fahnestock et al., 2001, Bruno et al., 2009a, Peng et al., 2004, Cuello et al., 2019). In addition, decreased BDNF mRNA levels (Holsinger et al., 2000, Phillips et al., 1991), proBDNF and mBDNF protein levels were reported in MCI and AD brains (Peng et al., 2005, Michalski and Fahnestock, 2003a, Hock et al., 2000). Neurotrophin dysregulation was also recapitulated by transgenic animal models during early stages of intraneuronal amyloid pathology (Iulita et al., 2017, Cuello et al., 2012, Francis et al., 2012, Peng et al., 2009).

As neurotrophins are released in response to neuronal activity, we hypothesized that the selective demise of cholinergic neurons of the nucleus basalis (nb), should have a significant effect on the cortical expression of either the precursor and mature NGF and BDNF forms.

Towards this goal, we selectively immuno-depleted BFCNs of the nb in wt rats with a stereotaxic injection of 192-IgG-saporin (192-IgG-SAP) consisting of an anti-p75ntr receptor monoclonal antibody conjugated to the cytotoxin saporin (Wiley 1991). After 6 months since the immunolesion, we assessed the effect of cholinergic neuron depletion on behaviour by performing a modified 5-Choice Serial Reaction Time Task (5-Choice task), which is extensively employed to measure sustained attention in rodents (Higgins and Silenieks, 2017, Robbins, 2002). Finally, we assessed levels of mRNA, pro and mature NGF and BDNF along with neuronal markers of the glutamatergic (vGluT1), GABAergic (GAD65) and catecholaminergic (TH) systems.

This study revealed that the long-term loss of BFCNs has significant consequences on the cortical expression and protein levels of BDNF as well as on the neurotransmitter systems beyond the cholinergic synaptic network.

2.2 Methods

2.2.1 Experimental design

One sex-balanced group of twelve 2.5 month-old Wistar rats (Charles River Laboratories; RRID:RGD_2312511) was stereotactically injected in the nb with 192-IgG-saporin (SAP), while nine were injected with PBS vehicle as a control (Sal). Six months post-lesion, rats were block randomized and housed in pairs then food-deprived to 85% of their weight in preparation for the 5-choice task (**Figure 2-1A)**. Food deprivation was maintained during the testing period. After completing behavioural testing, the animals were sacrificed, and their brains were preserved for histological and biochemical analyses. One rat was excluded from the histological analyses as the post-fixation was not optimal (n=20), while the biochemical analyses were performed in all rats (n=21). We used an analogous sample size of previous studies in which the neurotrophins were altered ((Allard et al., 2012) n1=6, n2=6; (Iulita et al., 2017) n1=10, n2=7). In (Allard et al., 2012), proNGF was increased following treatment with α2-antiplasmin (effect size, d=2.3) and in (Iulita et al., 2017), McGill-APP-transgenic rats showed decreased BDNF mRNA (effect size, d=1.7) compared to wild-type control. We have chosen the lower effect size between the two studies (d=

1.7) and we computed a post-hoc calculation obtaining a power (β-1) = 0.955 in a two tailed t-test (with n1=9, n2=12, α=0.05). The McGill University Animal Care Committee approved all experimental procedures (Ethical Approval Number: MCGL-3860).

2.2.2 Stereotaxic surgery

Surgical procedures were conducted under isoflurane anesthesia (isofluorane vaporizer was initially set at 4% and decreased to 2.5% for maintenance), and animals were mounted on a stereotaxic frame. The injections were performed with a 5-μL microsyringe (cat. no. 7633-01, Hamilton, USA) fitted with a 32-gauge needle (cat. no. 7762-05, Hamilton, USA) through a burr-hole drilled in the skull. Stereotaxic coordinates were measured from Bregma, according to the Paxinos and Watson Atlas (Paxinos and Watson, 2006) and were anteroposterior (AP) -1.8; mediolateral (ML) ±3.2; dorsoventral (DV) -7.5. 192-IgG-Saporin (2.6 mg/ml, Advanced Targeting Systems, cat. no. IT-01) was injected at a concentration of 0.5 μg/μl (1.0 μl/hemisphere), while PBS was injected as a control. Following stereotaxic surgery rats were injected with carprofen (5 mg/ml, given 0.1 ml/100 g) for three days to minimize animal suffering.

2.2.3 5-Choice Serial Reaction Time Task

Training and testing were conducted in rat five-hole operant boxes (cat. no. 80600A-CP, Lafayette Instruments, Lafayette, USA), using the FiveChoice program (version 5.3, Cambridge, UK) and the Whisker control system (Cardinal and Aitken, 2010) under diurnal conditions (12 h light). In the training phase, the rats learned to receive a reward in the food magazine after a correct response (**Supplemental Methods**). The testing phase occurred over multiple stages, and whenever each rat reached a certain number of correct responses with only a few failures to respond (omissions) in a daily session, it was moved to the next stage (see the experimental design in **Figure 2-1A**, details in **Supplemental Table 2-1** and **Supplemental Methods**). The first trial was initiated when the rat pushed the food magazine panel to collect the pellet. Each session ended after 100 correct trials or a maximum of 200 total trials and not more than 30 min of testing time. The number of sessions (days) required to complete each stage was calculated for each animal in both groups. Some challenges introduced were: a variable delay of the stimulus initiation (inter-trial interval), a fixed period after the stimulus offset for a response (limited hole hold period) and a punishment (time-out) after an incorrect response, an omission, or a premature response. Once the food pellet

was delivered, or the time-out was over, the next trial was initiated when the rat entered the food magazine. The global score was calculated for each animal as the sum for each stage ((average correct responses/average omissions+1)/total trials completed)). The +1 was added to avoid a denominator equal to 0. The experimenter was unaware of the animal's group during behavioral testing.

2.2.4 Brain tissue collection

Upon completion of the behavioural test, rats were deeply anesthetized with a mix of chloral hydrate and sodium pentobarbital (6.5 mg and 3 mg, respectively, per 100 g of body weight) delivered by intraperitoneal injection and perfused transcardially with chilled saline solution for 2 min. The brain was removed, and the cortex, hippocampus and cerebellum were dissected from one hemisphere. This tissue was flash-frozen and kept at −80 °C for qRT-PCR and Western blot. The other hemisphere was post-fixed in 4% PFA at 4°C for 24 hours and transferred to 30% sucrose in 0.1 M phosphate buffer for cryopreservation before being processed for immunohistochemical analysis. The fixed hemisphere was sectioned on a microtome (Leica SM 2000R; Germany) into 40-μm thick sections and stored at -20 °C in a cryoprotectant solution of ethylene glycol and sucrose in PBS (pH 7.4). All the following immunohistochemical analyses were performed on free-floating sections following cryoprotectant solution removal by washing in PBS.

2.2.5 Gene expression analysis

Messenger RNA (mRNA) expression was analyzed by reverse transcription–coupled real-time quantitative polymerase chain reaction (qRT-PCR). RNA was extracted from cortical tissue (15 mg) using the RNeasy Mini Kit (cat. no. 74104, Qiagen, USA). For reverse transcription, 500 ng total RNA was used to synthesize cDNA using iScript Reverse Transcription Supermix for RT-qPCR (cat. no. 1708840, Bio-Rad, USA). Quantitative RT-PCR was performed in a total volume of 10 μL using the SsoAdvanced Universal SYBR Green Supermix (cat. no. 1725270, Bio-Rad, USA) and using the CFX Connect Real-Time PCR Detection System (cat. no. 1855201, Bio-Rad, USA) and CFX Manager (cat. no. 1845000, Bio-Rad, USA). Fold changes in gene expression compared to PBS vehicle-treated rats were calculated, normalizing the values for each gene of

interest to housekeeping genes (GAPDH, Actin), using the 2 (Delta Delta C[T]) method (Livak and Schmittgen, 2001). PCR primer information is reported in the **Supplemental Methods.**

2.2.6 Western blotting (WB)

For all proteins besides mNGF (subjected to a chloroform/methanol protein extraction following (Locke et al., 2020); detailed in **Supplemental Methods**), 20 mg of frontoparietal cortical samples were manually homogenized in lysis buffer (cat. no. 9803, Cell Signaling, USA) containing a complete protease inhibitor cocktail (cat. no. 11836153001, Roche, USA). Manual homogenization was followed by two 5 s pulses of sonication. The homogenates were centrifuged at 13,000 rpm, for 45 min at 4 °C, the supernatants were collected, and the protein concentration was determined by Bradford *assay* (DC™ Protein Assay kit, cat. no. 5000111, BioRad, USA). The homogenates (50 μg) were mixed with Sample Buffer (Tris-HCl 250 mM, pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue) and boiled for 5 minutes. The samples were loaded on SDS-polyacrylamide gels and were transferred (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, cat. no. 1703940, BioRad, USA) to PVDF membranes (cat. no. 1620177, BioRad, USA) for 1 h at 350 mA. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated with the primary antibody in TBS-T overnight at 4 °C. Peroxidase-conjugated secondary antibody, dissolved in TBS-T, was applied for 1 h at room temperature. WB was developed with an enhanced chemiluminescence substrate (Western Lightning® Plus-ECL, cat. no. NEL111001EA, PerkinElmer Inc., USA) and imaged with Amersham Imager 600 (GE Healthcare, USA) and ChemiDoc™ Touch system (cat. no. 12003154, Bio-Rad, Canada). Densitometry was quantified with TotalLab CLIQS Software (TotalLab, UK) and ImageLab (cat. no. 17006130, Bio-Rad, Canada), and the values were normalized to the loading control GAPDH. An additional sample was loaded in each gel as an internal loading control. The values were expressed as fold change relative to the control group.

2.2.7 Immunohistochemistry (IHC)

Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 30 min at 80 ºC, and sections were then cooled for 20 minutes at room temperature (RT). Endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 and 10% methanol in PBS for 30 minutes. For ChAT

immunostaining, sections were blocked in 5% anti-horse serum for 1 hour and then incubated 48 hours at 4 ºC with goat anti-ChAT antibody in 1% BSA-PBS-T. For VAChT immunostaining, sections were blocked in 5% anti-goat serum (NGS, RRID: AB_2336990) for 1 hour and incubated 48 hours at 4 ºC, with rabbit anti-VAChT antibody in 5% NGS-PBS-T. Sections were incubated for 1 hour at room temperature with secondary biotinylated antibodies (in 1% BSA-PBS-T for ChAT labelling and 5% NGS-PBS-T for VAChT labelling). Providers and dilutions of antibodies are available in **Supplemental Methods**. Following washes, sections were then incubated with a VECTASTAIN Elite ABC HRP kit (cat. no. PK-6100, Vector Laboratories, Burlingame, CA, USA), and the staining was visualized using DAB (6 mg/mL) and 1% H_2O_2 as a chromogen. The sections were mounted on gelatin-coated slides and dehydrated in ascending ethanol concentrations, cleared in xylene and coverslipped using Entellan mounting medium (cat. no. 107961, EMD; Millipore) and #1 coverslips.

2.2.8 Immunofluorescence (IF)

Sections were incubated at 80 °C in 10 mM citrate buffer (pH 6.0) for 30 minutes then cooled for 20 minutes at RT. They were then washed with PBS and permeabilized using 50% ethanol for 20 minutes, followed by washes with PBS-T (containing 0.2% Triton-X-100), and blocked for 1 hour at RT in 10% NGS. The sections were then incubated with primary antibodies for 48 hours at 4 °C: NeuN, proNGF and vGluT1. After primary antibody incubation, sections were washed in PBS-T and incubated with Alexa Fluor secondary antibody for 2 hours RT. Provider and dilutions of antibodies are available in **Supplemental Methods**. The antigen retrieval was omitted for GAD65, TH, and TrkB immunostaining: sections were blocked in 10% Donkey Serum (NDS, RRID: AB_2337258) and donkey Alexa Fluor secondary antibodies. Following washes, sections were incubated for 5 minutes with 0.3% Sudan black in 70% ethanol to reduce autofluorescence. Sections were then washed three times for 5 minutes in PBS-T, then three times for 5 minutes in PBS before mounting onto gelatin-coated slides and coverslipped with Aqua-Poly/Mount (cat. no. 18606-100, Polysciences) and #1.5 coverslips.

2.2.9 Brightfield imaging and analysis

The ChAT and VAChT stained sections were imaged by bright-field microscopy using a 10x and 40x oil objective, respectively. Sections were imaged using Z-stacks with a Zeiss AxioImager M2

Imaging microscope equipped with an AxioCam 506 colour digital camera (Zeiss, Canada) and using Zeiss ZenPro software v.2.3 (Zeiss, Canada). For the ChAT quantification, thirteen sections per animal were chosen through the anterioposterior extent of the nb (cholinergic neurons in the internal globus pallidus innermost region; bregma -1.80 / -2.80 mm) and five sections for the Medial Septum/Ventral Diagonal Band (MS/VDB, bregma +1.00 / +0.48 mm). The neurons were manually counted (Cell Counter, Fiji software, Image J). For the VAChT+ terminals, three images were collected for each region (Frontal cortex 2, Frontal cortex 1 and Parietal cortex; **Figure 2-3B**), in three technical replicates, for a total of twenty-seven images per animal. Each image was taken in lamina IV-V, with a field of 315 × 252.44 μm (bregma +2.70 / +3.20 mm). The steps to count the cholinergic varicosities (Fiji software) are illustrated in the flowchart (**Supplemental Figure 2-2**, following (Iulita et al., 2017)). The average size was expressed by the VAChT-IR area/count of cholinergic varicosities, and the circularity was calculated as 4pi(Area)/(Perimeter^2) with a value from 0 to 1, where 1 indicates a perfect circle.

2.2.10 Confocal imaging and analyses

Images were acquired using a 63x oil HC LP APO objective in the parietal cortex (layer V) with a confocal microscope, Leica TCS SP8 (Leica, Canada) using the LAS X Software (Leica, Canada). Eight images per animal were collected in Z-stacks with an optical section of 0.896 μm and an interval of 1 μm (184.71 x 184.71 μm; resolution 1024x1024 for TrkA, TrkB, vGluT1, TH, GAD65, and 512x512 for proNGF). Images were imported to Fiji software (ImageJ) and slices were merged into one stack (stack/Z projection/Maximum Intensity Projection) for each channel. Briefly, to quantify the immunolabelling of TrkA and TH, we converted each micrograph to a binary image and measured the immunoreactive-area. For vGluT1, GAD65 and TrkB the intensity value was divided by the immunoreactive area which was generated by converting the image to a binary mask. For proNGF, the immunoreactivity was quantified by measuring the intensity that colocalized with NeuN. A detailed imaging processing is showed in **Supplemental Figure 2-2 and Supplemental Figure 2-3**. Images were processed and analyzed after blinding to treatment.

2.2.11 Statistical analysis

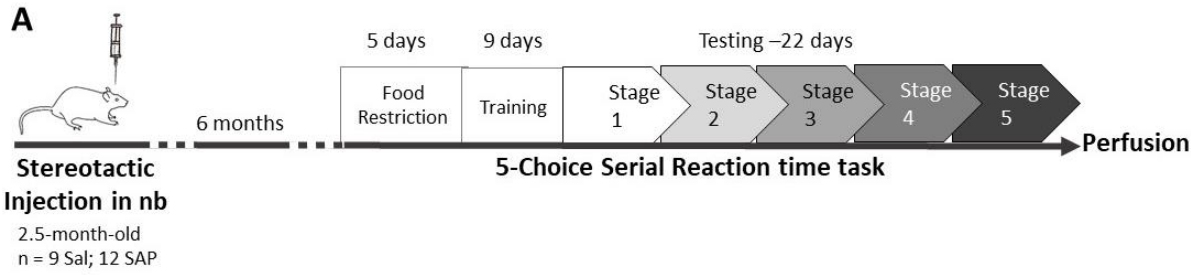
The software GraphPad Prism v8 (La Jolla, CA, USA) was used for statistical analysis. Data were displayed in columns showing individual values, means and SEM. Data normality was verified

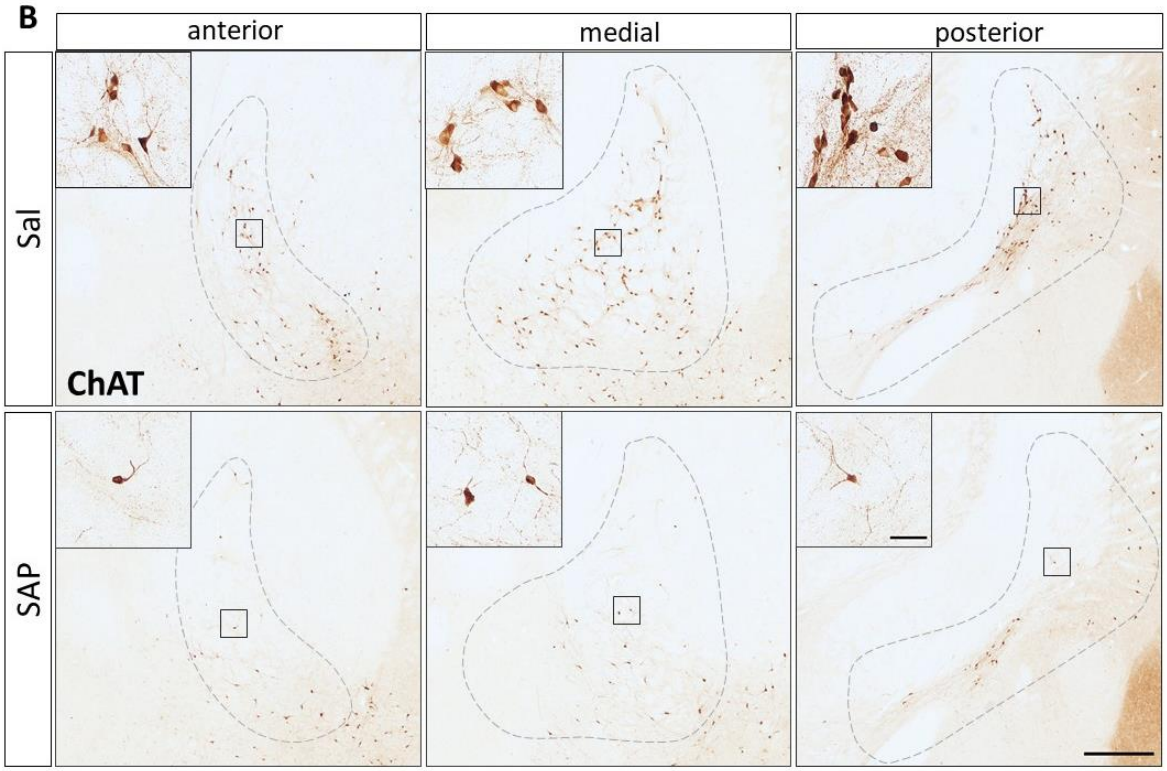
with the Shapiro-Wilk normality test. If data obey assumptions of parametric statistics, a two-tailed unpaired t-test was used for 2-group comparisons, and the Welch's correction was applied for unequal variances. Whenever the values were all the same within the group (case of sessions number equal to 1), we performed the one-sample t-test. For more than two group comparisons, we performed a *2-way* analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons. We excluded outliers with the Grubbs's test. Pearson correlation analysis was used to investigate associations between variables. If data did not obey the assumption of parametric statistics, the analysis was performed with nonparametric tests (Mann-Whitney test and Spearman correlation coefficient). We did not apply a correction for in the correlation matrix, instead all the P values were reported. For each experiment, the statistical analysis is specified in the figure legends and tables. Significance was set at $p < 0.05$.

2.3 Results

2.3.1 Lesion of the nb with 192-IgG SAP induced a loss of ChAT-immunoreactive neurons

The immunotoxin-induced selective depletion of cholinergic neurons of the nb was confirmed by immunohistochemical analysis. The stereotaxic injection of 192-IgG SAP directly in the nb resulted in a significant loss of ChAT-immunoreactive (IR) neurons across all nb regions 6 months post-injection, as illustrated in (**Figure 2-1B**). Quantification of ChAT-IR neurons showed that the loss of cholinergic neurons was in the order of 71% for the antero-medial region and 79% for the posterior region of the nb (**Figure 2-1C).** Importantly, the procedure did not significantly affect the density of ChAT-IR neurons in MS/VDB projecting to the hippocampus, indicating that the cholinergic depletion was restricted to the nb (**Supplemental Figure 2-1**).





C

Number ChAT+	Sal (Mean ± SEM)	SAP (Mean ± SEM)	P value	Reduction
Anterior-medial	701.8 ± 38.51	203.5 ± 22.49	P < 0.0001	71 %
Posterior	627.4 ± 36.19	129.5 ± 6.974	P < 0.0001	79 %
Total	**1329 ± 69.72**	**334.4 ± 24.17**	**P < 0.0001**	**75 %**

Figure 2-1. Decreased number of cholinergic neurons in the nb following immunolesion with 192-IgG-Saporin.

(A) Schematic representation of the experimental design: 192-IgG-SAP was stereotactically injected in 2.5 month-old rats (n=9 controls, 4 males and 5 females; n=12 Saporin-injected, 5 males and 7 females). Following 6 months of ageing post-injection, rats were submitted to behavioural

testing before being sacrified at 10 months. (B) Photomicrographs display ChAT-IR in the anterior, medial and posterior nb from Sal-treated and SAP-treated rats. Low magnification scale bar is 500 µm; high magnification scale bar is 50 µm. (C) Table summarizing quantification of ChAT-IR neurons in the anteriormedial and posterior nb regions. Data analysis: Sal rats, n=9; SAP rats, n=11. Anterior-medial region, unpaired t-test, t=11.67, df=18. Posterior region, unpaired t-test with Welch's correction, t=13.51, df=8.596; total quantification, Mann Whitney test , U = 0. The P values are reported in the table. Abbreviations: Sal, saline injected; SAP, 192-IgG Saporin injected; nb, Nucleus Basalis; ChAT, choline acetyltransferase.

2.3.2 Cognitive impairment following long-term lesion of the nb

Given the well-established participation of BFCNs in cognitive function, we then investigated the impact of significant cholinergic neuron loss induced by 192-IgG-SAP on sustained attention (Sarter et al., 2001) using the widely used 5-choice task (Robbins, 2002, Bari et al., 2008). In each daily session of the 5-choice task, animals performed a maximum of 200 trials in which they responded with a nose-poke to a brief visual stimulus presented randomly in one of the five apertures. In our test design, the animals were individually moved to a more difficult stage when they reached a defined number of correct responses and few missed trials in a daily session (the criteria were set as described in **Supplemental Table 2-1**). If the rats did not reach the criterion to pass, they would perform the session again on the following day. Therefore, a larger number of sessions in the same stage indicates an attentional impairment.

In the first two testing stages, with a light stimulus duration (SD) of 60 and 25 sec, respectively, both experimental groups required a similar number of sessions to reach the criterion and move to the next stage (**Figure 2-2A**). Subsequently, in stage 3, in conditions of increased attentional demand, when SD was reduced to 10 sec, along with variable intervals of light and reducing the response time limit to 5 sec, the SAP-immunolesioned group showed an attentional impairment requiring a significantly higher number of sessions to complete the test compared to the Sal-treated group (**Figure 2-2A**). In the following stage 4, in which the SD was reduced to 5 sec, the two experimental groups performed similarly (**Figure 2-2A**). However, the introduction of an extra challenge (a time-out of 5 sec following an incorrect response) in the last testing stage, required a significantly higher number of sessions to the SAP-immunolesioned group compared to the Sal-treated group to reach the criterion (**Figure 2-2A**) indicating again a deficit in tasks with high attentional demand.

To evaluate the overall attentional performance of the two groups, the correct responses and omissions for each stage were combined in a global score with a higher score indicating better performance. As expected, we found that the global score was significantly higher in the Sal-treated group compared to the SAP-immunolesioned group (**Figure 2-2C;** representative image of the performance in **Figure 2-2B**).

A

N. sessions			
Testing stages	Sal (Mean ± SEM)	SAP (Mean ± SEM)	P value
1	4.778 ± 0.8941	5.636 ± 1.047	0.5512
2	1.00 ± 0.0	1.200 ± 0.1333	0.1679
3	1.00 ± 0.0	1.727 ± 0.3042	0.0379 *
4	2.125 ± 0.4795	2.636 ± 0.8008	0.9258
5	1.00 ± 0.0	1.800 ± 0.2906	0.0224 *

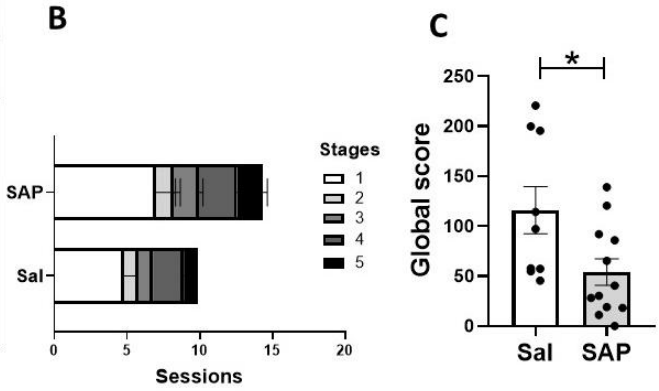


Figure 2-2. Long-term nb cholinergic neuron depletion induced cognitive deficits in the 5-choice task.

(A) Table showing the daily session number required by the rats in each stage to reach the criterion. The P values, mean and SEM are reported in the table. Data analysis: Sal rats, n=9; SAP rats, n=12. Stage 1, unpaired t-test, t=0.6073, df=18; stage 2, one-sample t-test, t = 1.5000, df = 9. Stage 3, One sample t-test, t = 2.3905, df = 10. Stage 4, Mann Whitney test, U = 42.50. Stage 5, One sample t-test, t = 2.7530, df = 9. (B) Representative image of all 5-choice task stages. (C) The global score is a combination of the overall 5-choice task cognitive performance (see **Methods**); Mann Whitney test, P = 0.0339 *, U = 24.

2.3.3 Long-term lesion of the nb induced loss and morphological changes of VAChT-IR terminals in frontal and parietal cortices.

Given the significant loss of ChAT-IR neurons of the nb in the SAP-immunolesioned group and the resulting attentional deficits, we then assessed the number and morphology of cortical cholinergic VAChT-IR presynaptic boutons in the frontal and parietal cortices (**Figure 2-3B**). As expected, the number of cholinergic varicosities was reduced in the SAP-immunolesioned group compared to the Sal-treated group in frontal cortex 2 (Fr2), frontal cortex 1 (Fr1) with the highest loss in the parietal cortex (Par) (**Figure 2-3C, A**). On the other hand, the average size of the VAChT-IR varicosities increased in the SAP-immunolesioned compared to the Sal-treated group in all three cortical areas (**Figure 2-3D, A**). In addition, the cholinergic pre-synaptic boutons in the Par cortex appeared significantly more round in SAP-immunolesioned as compared to the Sal-treated group (**Figure 2-3E, A**).

Overall, the Par cortex was the most affected cortical area and displayed greater loss of VAChT-IR synaptic boutons as well as morphological changes in the remaining VAChT-IR synaptic boutons.

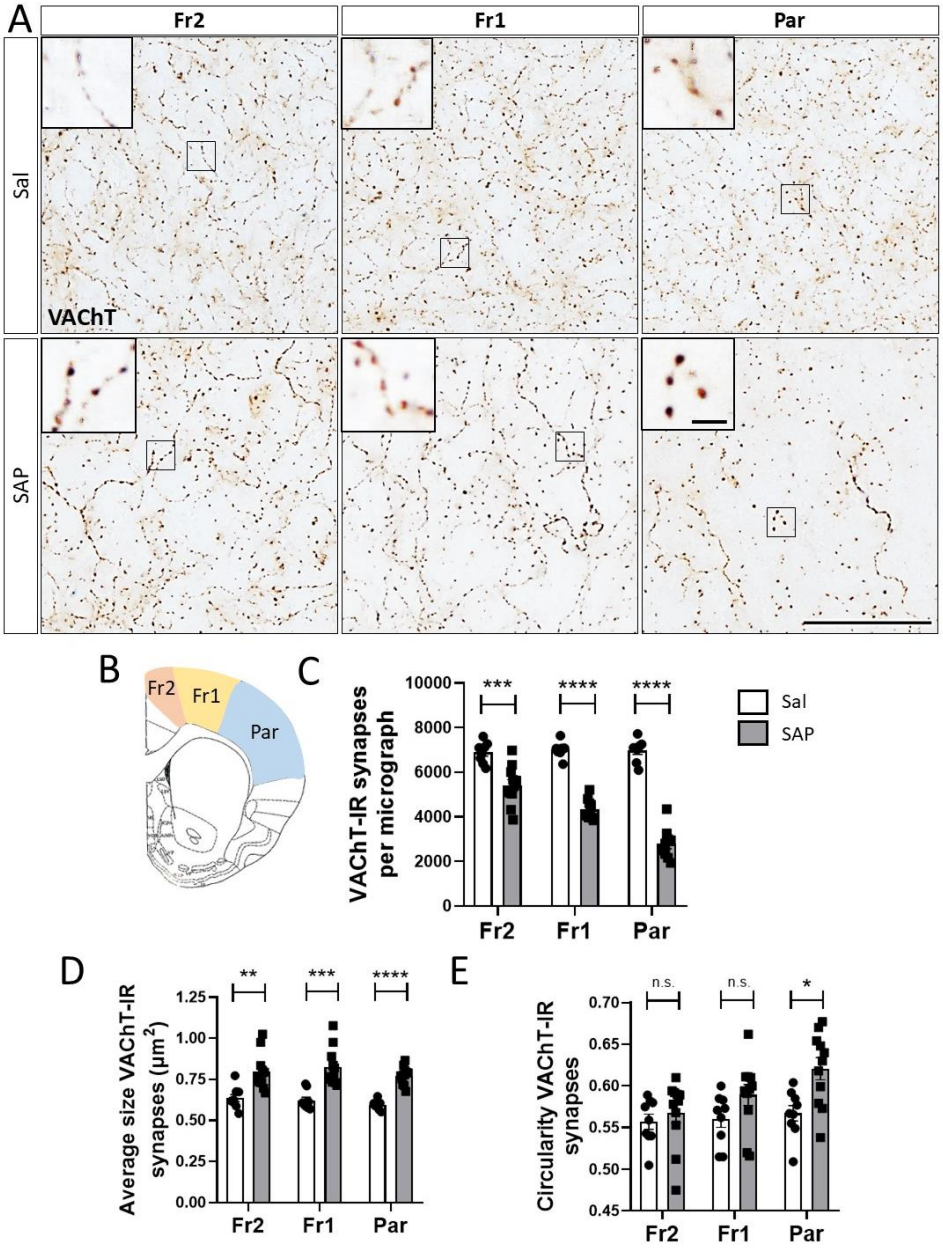


Figure 2-3. Cortical VAChT-IR terminals were reduced and showed morphological changes following nb demise.

(A) Micrographs illustrating VAChT-IR terminals at different cortical regions (scalebar 50 µm); and the high magnifications showing morphological differences of cholinergic pre-synaptic boutons among experimental groups (scalebar 5 µm). (B) Schematic representation of the investigated frontal (Fr2, Fr1) and parietal (Par) cortical regions. (C) The three cortical areas analysed in the SAP-immunolesioned group showed a reduction of VAChT-IR varicosities

compared to the Sal-treated group. 2-way-ANOVA; main effect cortical region ($P<0.0001$, $F(1.734, 27.75) = 45.01$); main effect immunolesion ($P<0.0001$, $F(1, 17) = 118.7$); interaction ($P<0.0001$, $F(2, 32) = 51.99$). Bonferroni's multiple comparisons; Fr2 $P = 0.0006$, $t = 4.779$, $DF = 15.96$; Fr1 $P<0.0001$, $t = 14.30$, $DF = 16.00$; Par $P<0.0001$, $t = 15.00$, $DF = 15.95$. (D) A significant increase of cholinergic terminal size was reported in all three cortical regions. 2-way-ANOVA; main effect immunolesion ($P<0.0001$, $F(1, 18) = 35.96$, $DF = 1$); main effect cortical region ($P= 0.111$, $F(1.250, 22.49) = 2.659$, $DF = 1$); interaction (0.5278, $F(2, 36) = 0.6505$, $DF = 2$). Bonferroni's multiple comparisons: Fr2 $P=0.003$, $t = 3.981$, $DF = 16.45$; Fr1 $P= 0.0004$, $t = 5.122$, $DF = 14.96$; Par $P <0.0001$, $t = 9.303$, $DF = 15.08$. (E) Graph showing the roundness of the varicosities was affected only in the Par cortex of the SAP-immunolesioned group compared to the control group. 2-way-ANOVA; main effect cortical region ($P<0.0001$, $F(1.942, 34.96) = 26.08$, $DF = 2$); interaction ($P<0.0001$, $F(2, 36) = 11.98$, $DF = 2$); main effect immunolesion ($P= 0.064$, $F(1, 18) = 3.888$, $DF = 1$). Bonferroni's multiple comparisons: Fr2 $P>0.999$, $t = 0.6942$, $DF = 17.50$; Fr1 $P= 0.278$, $t = 1.777$, $DF = 17.83$; Par $P= 0.012$, $t = 3.303$, $DF = 17.12$. For all the analyses Sal rats, n=9; SAP rats, n=11. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected, VAChT, Vesicular acetylcholine transporter; Fr2, Frontal Cortex 2; Fr1, Frontal Cortex 1; Par, Parietal Cortex.

2.3.4 Long-term immunolesion of the nb did not impact the NGF metabolic pathway

We next examined the impact of a partial lesion of the nb on the cortical NGF metabolic pathway, and on the NGF receptor TrkA, as NGF is essential for BFCNs phenotypic maintenance.

As expected, the loss of cortical VAChT-IR in the SAP-immunolesioned group was accompanied by a loss of Par cortex TrkA receptors in the axonal terminals projecting from BFCNs (Holtzman et al., 1995) (**Figure 2-4A, F**). Since TrkA receptor density is regulated by NGF (Venero et al., 1994, Gibbs and Pfaff, 1994, Figueiredo et al., 1995) , we measured expression and protein levels of precursor and mNGF. No differences in cortical NGF transcript expression were found between SAP-immunolesioned and Sal-treated groups (**Figure 2-4B**). The protein levels of mNGF were measured by WB with an extraction protocol (**see Supplemental Methods**) adapted to the low amount present in the brain (Bruno and Cuello, 2006) and no significant difference between SAP-immunolesioned and Sal-treated group was observed (**Figure 2-4C**).

Furthermore, we investigated levels of proNGF, and two proNGF-IR bands were detected **(Figure 2-4J**). We investigated whether the levels of the 32 kDa isoform, representing the post-translationally modified form (Reinshagen et al., 2000), and the 27 kDa band, corresponding to the unprocessed proNGF were different in the SAP-immunolesioned compared to the controls and whether one isoform was more affected than the other. Neither the proNGF 27 and 32 kDa isoforms, showed differences in their levels in the SAP-immunolesioned compared to the Sal-treated group (**Figure 2-4D**). We confirmed our result measuring the overall levels of proNGF by IF in the Par cortex (**Figure 2-4E, F**). We also assessed whether Neuroserpin, a key enzyme of the NGF metabolic pathway, was impacted. Neuroserpin regulates the conversion of proNGF to mNGF (Bruno and Cuello, 2006). The native isoform at 55 kDa, the cleaved isoform at 45 kDa and the active isoform (Yepes and Lawrence, 2004) were measured by WB (**Figure 2-4J**). There were no differences in neuroserpin transcripts nor in the two protein isoforms between SAP-immunolesioned and Sal-treated groups (**Figure 2-4 G-I)**.

Overall, we confirmed a loss of TrkA due to the immunolesion but in contrast to our expectations, there were no changes in proNGF and mNGF levels.

2.3.5 Long-term immunolesion of the nb impacted cortical BDNF RNA and protein levels.

Since BFCNs show widespread expression of BDNF in their target cortical and hippocampal areas (Phillips et al., 1990) and regulate BDNF levels in the hippocampus (Gil-Bea et al., 2011, Kokaia et al., 1996), we investigated cortical BDNF gene expression and protein levels following nb immunolesion. BDNF gene expression was significantly decreased in the SAP-immunolesioned compared to the Sal-treated group (**Figure 2-5A**). Then, we assessed protein levels of the two BDNF precursor isoforms and mature BDNF, detectable by WB in the human (Michalski and Fahnestock, 2003b) and rat brains (Iulita et al., 2017). The molecular bands at 40 and 32 kDa, representing the proBDNF, were unchanged between SAP-immunolesioned and control group (**Figure 2-5B, C**). However, the 14 kDa band, corresponding to mBDNF, was significantly reduced in the SAP-immunolesioned compared to the Sal-treated group (**Figure 2-5D**). Lastly, the levels of the BDNF receptor TrkB in the Par cortex were comparable in the two groups (**Figure 2-5E, F**).

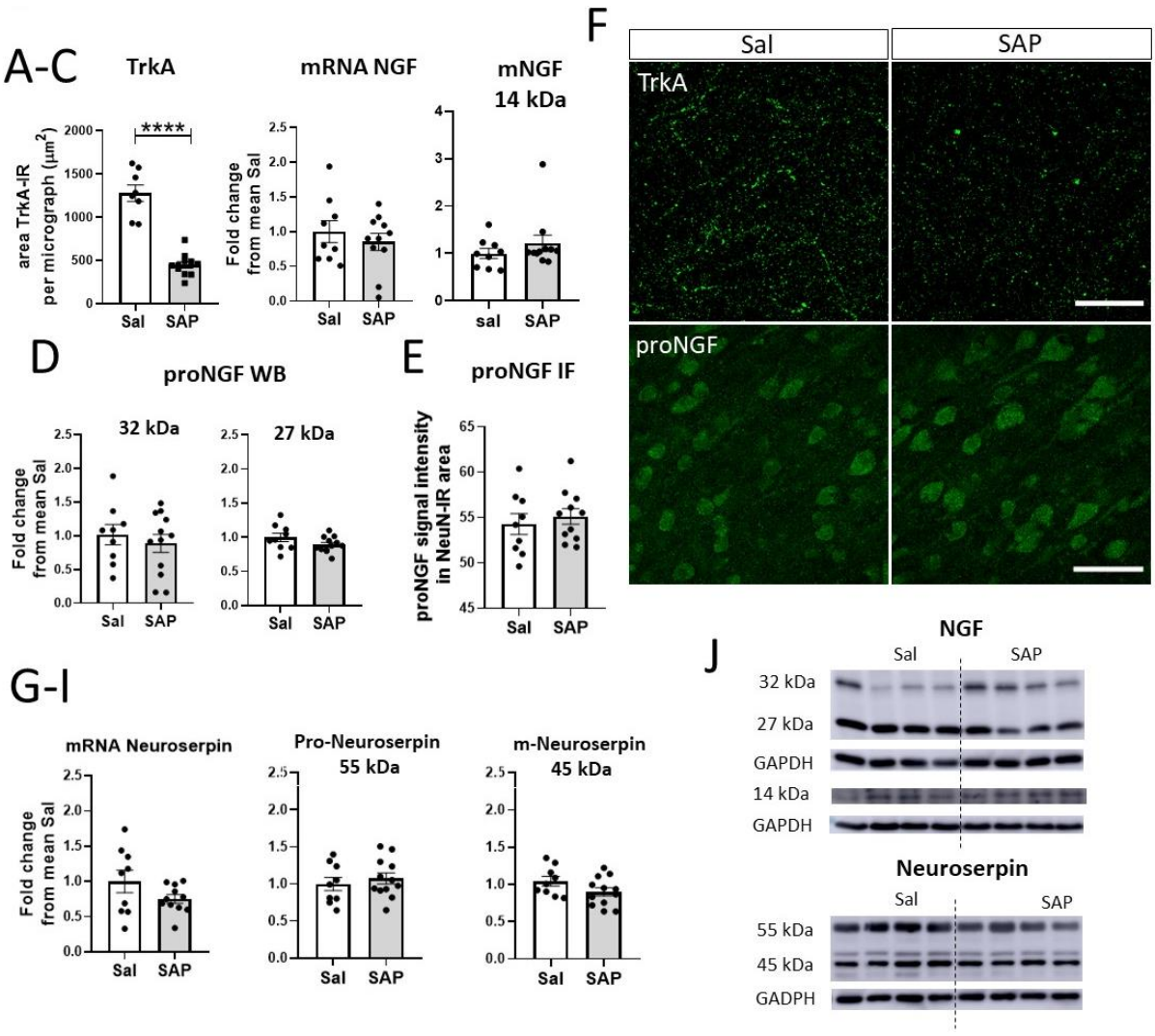


Figure 2-4. Nb immunolesion did not impact cortical gene expression and protein levels of NGF.

(A) Cortical levels of TrkA showed a reduction in the SAP-immunolesioned compared to the Sal-treated group (P <0.0001, t=8.112, df=9.430). (B-E) mRNA cortical levels of NGF were not altered in the SAP-immunolesioned group compared to the controls (P = 0.4620, t=0.7517, df=18) nor protein levels assessed by western blot of mNGF (P = 0.3702, U = 37), proNGF 32 kDa (P = 0.5292, t=0.6409, df=19), proNGF 27 kDa (P = 0.1212, t=1.622, df=19), and proNGF measured by IF (P = 0.5543, t=0.6027, df=18. (F) Representative images of IF for proNGF (scale bar 50 µm) and TrkA (scale bar 12 µm). (G-J) No changes in mRNA levels of Neuroserpin (P = 0.1752 , t=1.456, df=10.29) and protein levels of Neuroserpin 55 kDa and 45 kDa were found (respectively P =0.5348, t=0.6322, df=19; P =0.1127, t=1.663, df=19). Data analysis: For WB/PCR analyses

Sal rats, n=9; SAP rats, n=12, for IF analyses Sal rats, n=9; SAP rats, n=11. An unpaired t-test was performed for all comparisons, except for protein levels of mNGF in which the Mann Whitney test was performed. An unpaired t-test with Welch's correction was applied to compare TrkA and Neuroserpin mRNA levels. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected; mRNA, messenger RNA; WB, western blot; mNGF, mature Nerve Growth Factor; proNGF, precursor NGF; TrkA, Tropomyosin receptor kinase A.

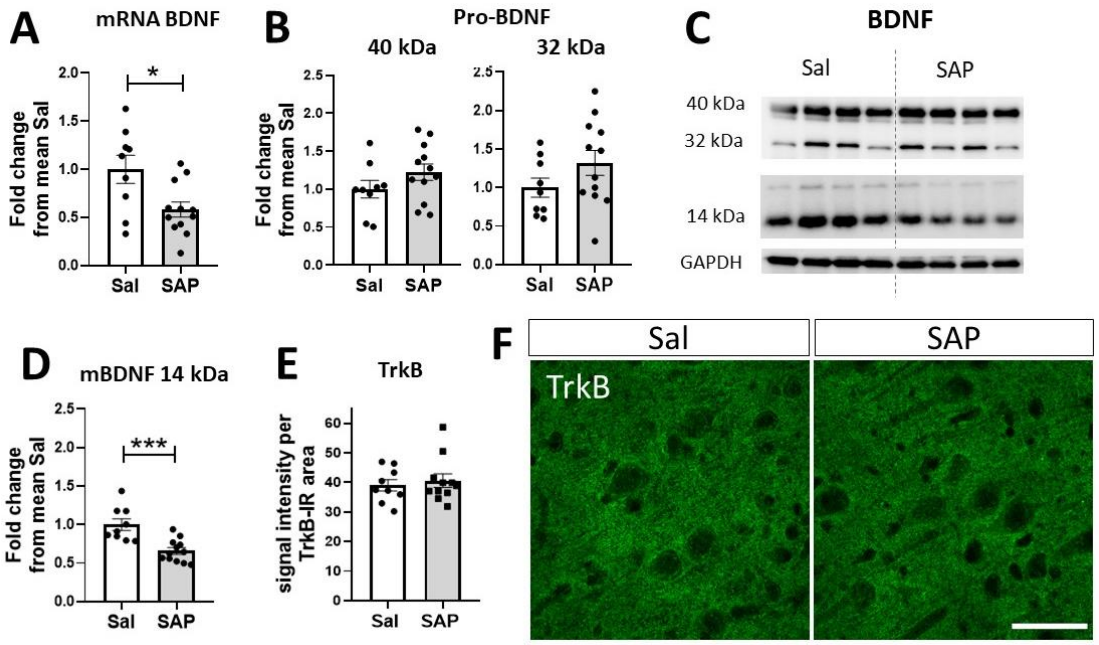


Figure 2-5. Expression of BDNF and mBDNF protein levels were downregulated following nb depletion.

(A) Cortical mRNA levels of BDNF were reduced in the SAP-immunolesioned group compared to the controls (P = 0.014, t=2.707, df=19). (B) Levels of proBDNF 40 kDa and 32 kDa were not significantly different between the two groups (respectively, P = 0.178 , t=1.398, df=19 and P =0.153 , t=1.488, df=19). (C) Representative WB images of proBDNF and mBDNF. (D) mBDNF protein levels were significantly reduced in the SAP-immunolesioned compared to the controls (P = 0.0004, t=4.284, df=19) (E) Cortical levels of TrkB assessed by IF did not show any difference between SAP-immunolesioned and Sal-treated (P = 0.940 , U = 48). (F) Representative images of IF for TrkB in the Par (scale bar 50 µm). Data analysis: For WB/PCR analyses Sal rats, n=9; SAP rats, n=12, for IF TrkB, Sal rats, n=9; SAP rats, n=11. An unpaired t-test was performed for all comparisons, except for TrkB protein levels which was assessed by Mann Whitney test. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected; WB, western blot; BDNF; Brain-derived neurotrophic factor; mBDNF, mature Brain-derived neurotrophic factor; proBDNF, precursor Brain-derived neurotrophic factor; TrkB, Tropomyosin receptor kinase B.

2.3.6 Long-term immunolesion of the nb impacted expression of glutamatergic and GABAergic neuronal markers.

Similar to cholinergic neurons, glutamatergic and GABAergic neurons are dysregulated in the AD-like amyloid pathology showing an initial upregulation in presynaptic bouton density followed by a sharp decline (Wong et al., 1999). However, in an AD-like amyloidosis model, glutamatergic and GABAergic synaptic compromise appears later, after the cholinergic synaptic loss as the pathology progresses (Bell et al., 2003, Bell et al., 2006).

Aged rats exhibiting cognitive impairment revealed reduced cortical cholinergic synaptic bouton density and an altered ratio of excitatory/inhibitory inputs (Casu et al., 2002, Wong et al., 1998, Wong et al., 2006). To investigate whether 6-months post-cholinergic depletion decreases glutamatergic synapses, we measured levels of vesicular glutamate transporter 1 (vGluT1) in the rat cerebral Par cortex (Minelli et al., 2003) as this transporter is required for exocytic glutamate release at presynaptic terminals (Fremeau Jr et al., 2004). We also measured in the Par cortex levels of glutamate decarboxylase 65 (GAD65) which reveals GABAergic terminals (Esclapez et al., 1994). Additionally, as a primary metabolite of dopamine (DOPAC) was found to be upregulated 7 days after a cholinergic depletion (Waite et al., 1994), in agreement with the catecholaminergic–cholinergic balance hypothesis (reviewed in (van Enkhuizen et al., 2015)), we investigated the levels of tyrosine hydroxylase (TH), a marker of catecholaminergic terminals and neurons. We found a significant reduction of vGluT1 (**Figure 2-6A, B**) and GAD65 (**Figure 2-6A, C**) in the SAP-immunolesioned group compared to the Sal-treated group and a trend toward upregulation of cortical TH terminals in the SAP-immunolesioned group (**Figure 2-6A, D**).

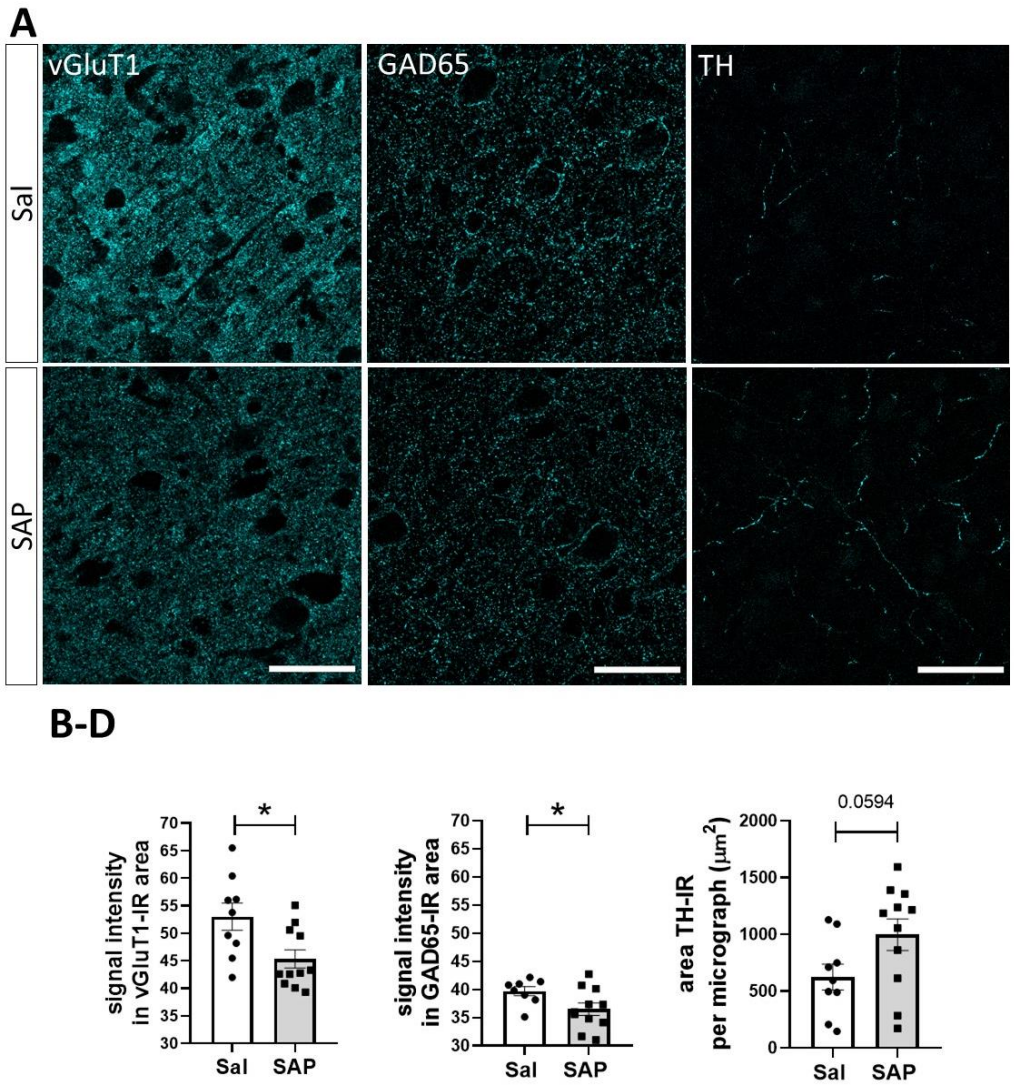


Figure 2-6. Nb cholinergic immunolesion impacted cortical glutamatergic and GABAergic markers.

(A) Representative micrographs of vGluT1, GAD65 and TH in the parietal cortex layer V (Scale bar 50 µm for vGluT1 and GAD65, 25 µm for TH). (B,C) Protein levels of vGluT1 (P=0.0158; t=2.665, df=18) and GAD65 (P=0.0441; t=2.174, df=17) were reduced in the SAP-immunolesioned as compared to the controls. (D) TH showed a trend towards increase (P=0.0624; t=1.986, df=18) following nb cholinergic immunolesion. Data analysis: Sal rats, n=9; SAP rats, n=11. Unpaired t-tests were performed for all comparisons. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected; Par, Parietal cortex; vGluT1, Vesicular glutamate transporter 1;

GAD65, glutamic acid decarboxylase 65; TH, Tyrosine Hydroxylase*;* MIP, Maximum Intensity Projection; nb, Nucleus basalis.

2.3.7 Cholinergic markers correlate with global score and BDNF levels

We investigated whether the reduction of the global score and BDNF levels correlated with cholinergic depletion of the nb. Interestingly, the numbers of ChAT-IR neurons and VAChT-IR terminals in the most affected areas (Par and Fr1) showed significant positive correlations with the global score, and mBDNF, (**Figure 2-7, Supplemental Table 2-2**); as well with BDNF transcript expression (**Supplemental Table 2-2**). In addition, vGluT1, showed a trend towards a positive correlation with ChAT-IR neurons (P = 0.075) and with mBDNF (P = 0.082) (**Supplemental Table 2-2**).

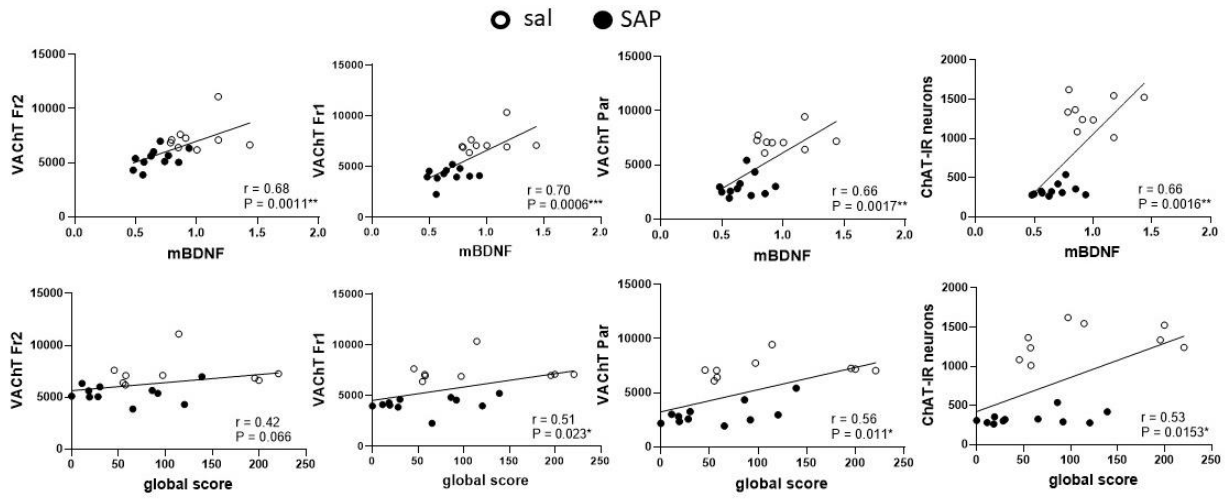


Figure 2-7. Correlation between VAChT-IR synapses and ChAT-IR neurons with global score and mBDNF.

VAChT-IR in the frontal and parietal cortical areas as well as ChAT-IR neurons of the nb revealed significant positive correlations with global score and mBDNF. All the correlations are reported in the **Supplemental Table 2-2.** For mBDNF and global score Sal rats, n=9; SAP rats, n=12, for VAChT and ChAT, Sal rats, n=9; SAP rats, n=11. Abbreviations: VAChT, Vesicular acetylcholine transporter; Fr2, Frontal Cortex 2; Fr1, Frontal Cortex 1; Par, Parietal Cortex; nb, Nucleus Basalis; ChAT, choline acetyltransferase; mBDNF, mature Brain-derived neurotrophic factor.

2.4 Discussion

The basal forebrain cholinergic system plays a major role in higher CNS functions and its atrophy in AD contributes to cognitive impairment (reviewed in (Giacobini, 2003, Giacobini et al., 2022)).

Historically, experimental basal forebrain cholinergic denervation has contributed to understanding its physiological functions and participation in neurodegenerative conditions. Initial basal forebrain cholinergic studies were carried out with mechanical and chemical lesions (reviewed in (Smith, 1988, Pepeu and Pepeu, 1994) and later replaced by a more selective immune-toxicity approach by the intracerebral injection of 192-IgG-SAP. This approach targets BFCNs selectively without affecting the non-cholinergic component or the rodent cholinergic interneurons (reviewed in (Petrosini et al., 2014)). Several studies have addressed the effects of 192-IgG-SAP injections on brain neurotrophins (Gu et al., 1998, Yu et al., 1996, Gil-Bea et al., 2011, Kokaia et al., 1996). However, in these reports the cholinergic denervation was achieved by an intracerebroventricular (ICV) administration of 192-IgG-SAP, affecting the hippocampus, olfactory bulb and cerebellum, with many off-target effects. In addition, they showed a transient change in neurotrophin levels a few days after 192-IgG-SAP injection without discriminating over precursor and mature neurotrophins forms and rendering a combined read-out of the two (Gu et al., 1998, Yu et al., 1996, Gil-Bea et al., 2011, Kokaia et al., 1996).

To clarify the impact of nb BFCNs on neurotrophin regulation and the characteristic cholinergic participation in attentional behaviour, we performed the selective immunolesion of the nb with 192-IgG-SAP. We focused on assessing attentional behavior as this component is highly dependent on cholinergic tone (Chudasama et al., 2004, Risbrough et al., 2002). Our studies demonstrated that a 6-month depletion of nb cholinergic neurons, rendered significant attentional deficits which were accompanied by a reduction of mature BDNF (mBDNF) and a loss of cortical neurotransmitter-specific markers, while NGF metabolism appeared to be preserved. Importantly, in these studies, the extent of cholinergic marker depletion correlated with the attentional deficits, the diminished BDNF levels and a reduction in glutamatergic markers.

2.4.1 Cognitive deficits and correlation with cholinergic terminals

Involvement of the nb in sustained attention, defined as continuous readiness to respond to unpredictable events (Wilkins et al., 1987, Sarter et al., 2001), has been previously established

(Lehmann et al., 2003, Dalley et al., 2004, Robbins et al., 1989, Baxter and Chiba, 1999, McGaughy et al., 2002, McGaughy and Sarter, 1998, Harati et al., 2008, Ljubojevic et al., 2018). The present study builds on these previous findings by providing evidence that depletion of nb cholinergic neurons provokes a long-lasting impairment on sustained attention, as detected with the 5-choice task even after 6 months post-lesion. Given the experimental conditions, in which there was no decrease in the stimulus duration within the sessions, it does not exclude that a learning component could have affected the 5-choice task performance of the SAP-immunolesioned rats.

A more extensive lesion of the nb (>90%) could have had a larger effect on sustained attention; however, an extended immunolesion would have, inevitably, depleted the cholinergic neurons of the neighbouring MS/VDB nuclei. Although cholinergic MS/VDB neurons are not essential for attention (Lehmann et al., 2003), their depletion may affect spatial behavior in the 5-choice test since they project to the hippocampus (Gaykema et al., 1990, Woolf et al., 1984). Such neurons are also involved in learning and memory (Frielingsdorf et al., 2006, Baxter and Gallagher, 1996, Shen et al., 1996). For these reasons, we have purposely chosen to perform an immunolesion restricted to the nb. The 5-choice task correlates well with attentional tasks performed in humans; in particular, as shown by the continuous performance test (CPT) and sustained attention to respond task (SART) (Conners et al., 2003, Robertson et al., 1997) which accurately reveal sustained attention decline during early stages of AD (Huntley et al., 2017, Saunders and Summers, 2011). Furthermore, we found that the global attentional impairment not only correlates with depletion of ChAT-IR neuronal cell bodies, as previously demonstrated by applying the 5-choice task paradigm (McGaughy et al., 2002), but also with the diminished density of cortical cholinergic presynaptic elements in the cerebral cortex, as revealed by VAChT-IR varicosities. Recent studies have been monitoring the density of cholinergic presynaptic elements *in vivo* by applying the VAChT PET tracer 18F-fluoroethoxybenzovesamicol (FEOBV) in clinical studies (Petrou et al., 2014, Aghourian et al., 2017, Albin et al., 2018). FEOBV prevailed over an amyloid-beta (^{18}F-NAV4694) and a glucose metabolism tracer (^{18}F-Fluorodeoxyglucose), to distinguish AD patients from controls showing significant correlations with cognitive scores (Aghourian et al., 2017). This indicates that VAChT could serve as a possible biomarker of early AD diagnosis.

2.4.2 Lack of changes in the NGF metabolic pathway

Several studies showed a transient total NGF upregulation which returns to physiological levels following a fimbria-fornix transaction, hippocampal lesion and selective partial nb lesion (Korsching et al., 1986, Gu et al., 1998, Yu et al., 1996, Hellweg et al., 1997). However, no changes in NGF expression nor the protein level of the precursor and bioactive NGF were found after a 6-month cholinergic immune-toxin lesion of the nb.

As expected, the partial immunolesion of the nb involving 75% of cholinergic neurons led to a reduction of VAChT-IR terminals of 27, 44 and 58% in Fr2, Fr1 and Par cortex respectively. This result was consistent with the loss of cholinergic fibers reported for the corresponding areas in human AD brains (Geula and Mesulam, 1996, Geula and Mesulam, 1989, Geula et al., 2021).

There is a possibility that a long-term reduction of ACh release alone does not significantly impact the NGF metabolic pathway, and likely requires additional disruptive mechanisms (such as accumulation of intracellular Aβ). An aspect which would be in agreement with our previous study in aged cognitively impaired rats (Bruno and Cuello, 2012). The application of Aβ oligomers *per se* have shown to be sufficient to induce a NGF metabolic pathway dysregulation (Bruno et al., 2009a).

Interestingly, in MCI brains, that already present dysregulation in the NGF metabolic pathway (reviewed in (Florencia Iulita and Claudio Cuello, 2016)) also have an upregulation of cholinergic markers (DeKosky et al., 2002, Ikonomovic et al., 2003). Furthermore, transient upregulation of cholinergic fibers has also been shown at early stages of the AD-like amyloid pathology in transgenic models (reviewed in (Bell and Cuello, 2006)). In addition, an assessment of cortical synaptic density in MCI and AD brains has shown a significant decrease in synaptic number compensated by an enlargement of synaptic size (Scheff et al., 1990, Scheff et al., 2007). This phenomenon has been interpreted as a compensatory mechanism balancing increasingly toxic amyloid β protein levels (Bell et al., 2006) as it has been reported in APP transgenic animal models before plaque formation (Wong et al., 1999, Mucke et al., 1994, Hernandez et al., 2001).

2.4.3 Impact on BDNF and cortical neuronal markers

In this study, we reported that long-term loss of the nb induces a decrease of BDNF mRNA without changes in NGF mRNA, which has been widely documented in AD brains (Phillips et al., 1991,

Holsinger et al., 2000, Goedert et al., 1986, Fahnestock et al., 1996). Such a difference between BDNF and NGF mRNA expression has also be reported in a rat model of the pre-AD-like amyloid pathology (Iulita et al., 2017). Interestingly, AD post-mortem analyses suggested that mBDNF is present in lower amounts in the cortex than proBDNF and decreases more sharply than proBDNF in the prodromal stages of AD (Peng et al., 2005). Furthermore, consistent with human studies (Peng et al., 2005, Buchman et al., 2016, Fahnestock et al., 2002), we observed that reduction in BDNF correlated with cognitive deficits, BDNF mRNA and mBDNF protein levels.

Reduced expression and protein levels of mBDNF in SAP-immunolesioned rats, may be responsible for depletion of cortical neurotransmitter markers other than the cholinergic system, as proposed in **Figure 2-8**.

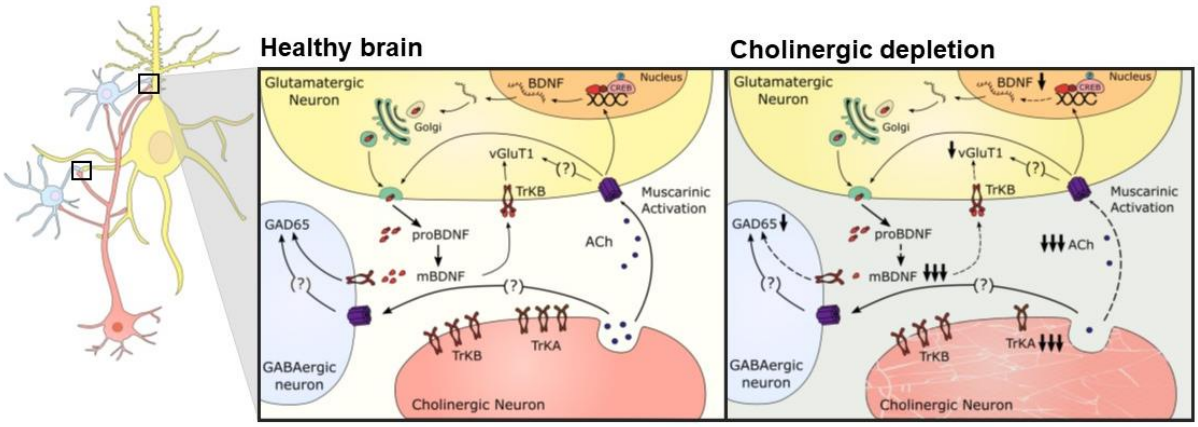


Figure 2-8. Schematic of the BDNF mechanism in wild-type and with nb cholinergic depletion.

Under normal conditions of ACh release (left panel), BDNF is normally transcribed, translated and released in the extracellular space as proBDNF, where it is converted to mBDNF. mBDNF binds the high-affinity TrkB receptor, triggering signalling cascades responsible for neuron plasticity and LTP. A long-term lack of ACh (right panel), could lead to a reduction of BDNF transcription and conversion to mBDNF. Although TrkB levels are not compromised, markers of glutamatergic (vGluT1) and GABAergic (GAD65) may be downregulated by the lack of trophic support. Legend arrows: discontinued arrows show the pathways participating. Arrows with question mark indicate other possible pathways activated by ACh. Abbreviations: ACh, Acetylcholine; BDNF; Brain-derived neurotrophic factor; proBDNF, Brain-derived neurotrophic factor; mBDNF, mature Brain-derived neurotrophic factor; TrkA, Tropomyosin receptor kinase A, TrkB, Tropomyosin receptor kinase B; vGluT1, Vesicular glutamate transporter 1; GAD65, glutamic acid decarboxylase 65.

Post-synaptic muscarinic receptors are localized in glutamatergic and GABAergic neurons, and on dendritic shafts (M1 in particular) and spines (Hersch and Levey, 1995, Sarter et al., 2009). In contrast, the M2 receptor appears to be located in cholinergic pre-synaptic sites likely suppressing neurotransmitter release (Hounsgaard, 1978, de Sevilla et al., 2002, Mrzljak et al., 1993, Salgado et al., 2007). ACh has the potential to increase the excitability of glutamatergic neurons (McCormick and Prince, 1985, Desai and Walcott, 2006, Metherate et al., 1988) and it is very well-known that neuronal activity increases BDNF expression (Ernfors et al., 1991, Isackson et al., 1991, Castrén et al., 1998, Zafra et al., 1990). The cooperation of glutamatergic and cholinergic input may be responsible for BDNF regulation (Navakkode and Korte, 2012) in cortical and hippocampal neurons. mBDNF, binding to autocrine and paracrine TrkB receptors (Horch et al., 1999, Horch and Katz, 2002), exerts effects on the glutamatergic neurons (reviewed in (Leßmann, 1998)) as well as on interneurons (Ohba et al., 2005, Sakata et al., 2009).

On the other hand, we did not find changes in TrkB receptor levels. Interestingly, studies in BDNF knock-out mice (He et al., 2004) and post-mortem analyses on AD brains (Boissière et al., 1997, Savaskan et al., 2000, Michalski et al., 2015) reported diminished expression of BDNF compared to controls, in absence of alteration in TrkB. This could be explained by the known ligand promiscuity of TrkB receptors that, besides BDNF, could be activated by NT-4/5 (Bothwell, 1995); in addition, TrkB could be activated by BDNF-independent transactivating mechanisms (Rantamäki et al., 2011, Di Lieto et al., 2012).

The reduction of the glutamatergic (vGluT1) and GABAergic (GAD65) markers we observed might be a secondary consequence to the diminished levels of BDNF as suggested by other studies (Melo et al., 2013, Sanchez-Huertas and Rico, 2011).

2.5 Conclusions

Our study supports the notion of a neuroprotective role of cholinergic neurotransmission in the adult and fully differentiated cerebral cortex. We showed that long-lasting cholinergic deprivation negatively affects cognitive performance, and decreases levels of BDNF, GABAergic and glutamatergic markers. Of significance, the long-term cholinergic component does not appear to affect NGF biosynthesis as it is the case of AD pathology, thus highlighting the important role of NGF regulation in AD.

2.6 Conflict of interest

The authors declare that they have no conflict of interests.

2.7 Acknowledgments

We would like to thank Dr. Veronika Zlatkina and Dr. Michael Petrides for permission to use the five-hole operant boxes; Refilwe Mpai in explaining the use of the FiveChoice program. We also thank Dr. Alfredo Ribeiro Da Silva for access to the Zeiss AxioImager M2 Imaging microscope. We acknowledge the McGill Microscope Facility Advanced BioImaging *Facility* (ABIF) in particular, Dr. Joel Ryan for the technical support and data analysis. We thank Agustina Lavagna for the schematic illustrating our proposed mechanism. CO was the holder of the Associazione Levi-Montalcini fellowship. HH was a recipient of a Fonds de Recherche du Quebec-Sante postdoctoral fellowship. RP was the recipient of a Student Fellowship from the McGill Integrated Program in Neuroscience and CIHR Doctoral Award. MKF was the recipient of a CIHR Doctoral Award. SDC is the holder of the Charles E. Frosst/Merck Research Associate position. ACC is the holder of the McGill University Charles E. Frosst/Merck Chair in Pharmacology and a member of the Canadian Consortium of Neurodegeneration in Aging.

2.8 Supplemental methods

5-CSRTT training phase

In the first phase, each rat was located for 20 minutes in a single box where ten reward pellets were placed in the food tray and two pellets in each of the five apertures. During this session, all five stimulus lights, the house light and the tray light remained illuminated. The training phase was repeated until the rats ate all the pellets provided. In a second phase, rats received two food magazine training sessions of 20 minutes each, in which one food pellet/min was delivered. The house-light was turned on during this phase. The training phase lasted seven days.

Extraction protocol mNGF

Fifty µl of homogenized tissue was added 200 µl of methanol, 50 µl of chloroform and 150 µl of water. The solution was centrifuged at 13,000 g for 5 minutes at room temperature. The upper aqueous phase was discarded. Subsequently, 200 µl of methanol was added, the solution was vortexed and centrifuged at 13,000 g for 5 minutes. The pellet (100 µg of protein) was collected and resuspended in denaturing buffer (6 M urea, 1 mM EDTA, 50 mM TEAB pH 8.5). The concentrated protein was used for western blots as described in the methods section.

Primers for qPCR

gene	primer	Sequence 5'→ 3'	T Annealing
NGF	Fw	CAA CAG GAC TCA CAG GAG CA	56.0
	Rev	GTC CGT GGC TGT GGT CTT AT	
BDNF	Fw	GCG GCA GAT AAA AAG ACT GC	61.8
	Rev	GCA GCC TTC CTT CGT GTA AC	
Neuroserpin	Fw	ATG AGG CTG GTG GCA TCT AC	54.5
	Rev	GAT CAG CTG TGG TTT GAG CA	
GAPDH	Fw	TGA TGG GTG TGA ACC ACG AG	56.0
	Rev	TCA TGA GCC CTT CCA CGA TG	
Beta-Actin	Fw	AGC CAT GTA CGT AGC CAT CC	56.0
	Rev	CTC TCA GCT GTG GTG GTG AA	

Antibodies (Ab)

Primary Ab	Supplier	Conc.	Secondary Ab	RRID*	Supplier	Conc.
WB						
Anti-proNGF	ANT-005, Alomone	1:2000	Goat anti-rabbit- HRP	RRID:AB_2040021	Jackson Immunoresearch	1:10,000
Anti-NGF	Ab9795, Abcam	1:1500	Goat anti-rabbit- HRP	RRID:AB_296626	Jackson Immunoresearch	1:10,000
Anti-Neuroserpin	Ab33077, Abcam	1:4000	Goat anti-rabbit- HRP	RRID:AB_956293	Jackson Immunoresearch	1:10,000
Anti-BDNF	Ab108319, Abcam	1:2000	Goat anti-rabbit- HRP	RRID:AB_10862052	Jackson Immunoresearch	1:10,000
Anti-GAPDH	MAB374, Millipore	1:10,000	Goat anti-mouse-HRP	RRID:AB_2107445	Jackson Immunoresearch	1:10,000
IHC						
Anti-ChAT	AB144P, Millipore	1:1500	Horse anti-goat	RRID:AB_2079751	Vector Laboratories	1:200
Anti-VAChT	139 103, SySy	1:8000	Goat anti-rabbit	RRID:AB_887864	Vector Laboratories	1:200
IF						
Anti-TrkA	AF1056-SP, R&D	1:70	Donkey anti-goat Alexa Fluor 647	RRID:AB_2283049	Thermo Fisher Scientific	1:800
Anti-TrkB	AF1494-SP, R&D	1:100	Donkey anti-goat Alexa Fluor 647	RRID:AB_2155264	Thermo Fisher Scientific	1:800
Anti-NeuN	ABN90P, Millipore	1:2000	Goat anti-guinea pig Alexa Fluor 647	RRID:AB_2341095	Thermo Fisher Scientific	1:800
Anti-proNGF	Ab9040, Millipore	1:300	Goat anti-rabbit Alexa Fluor 568	RRID:AB_262175	Thermo Fisher Scientific	1:800
Anti-vGluT1	MediMabs	1:200	Goat anti-mouse Alexa Fluor 488	RRID:AB_1808396	Thermo Fisher Scientific	1:800

Anti-GAD65	GAD6, DSHB**	1:100	Donkey anti-mouse Alexa Fluor 488	RRID:AB_2314499	Thermo Fisher Scientific	1:800
Anti-TH	AB152, Millipore	1:2000	Donkey anti-rabbit Alexa Fluor 568	RRID:AB_390204	Thermo Fisher Scientific	1:800

* Research Resource Identifiers

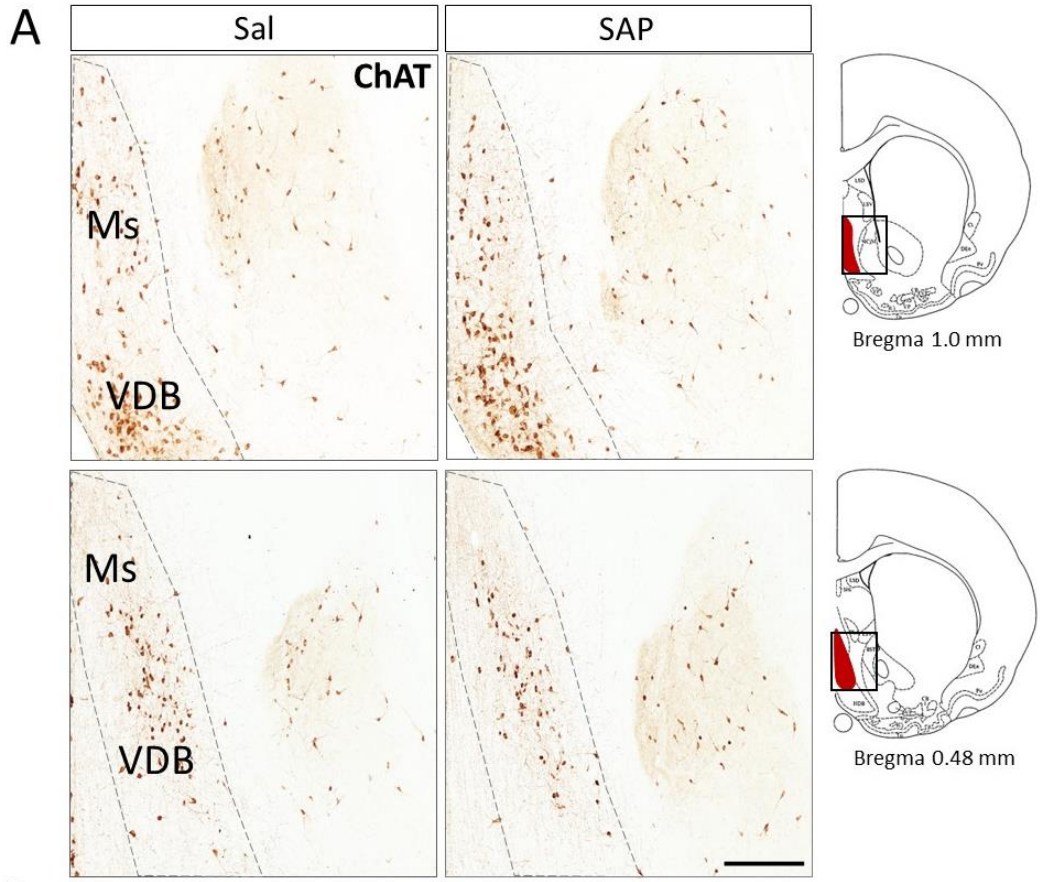
** Developmental Studies Hybridoma Bank

2.9 Supplemental figures and tables

Testing stages	Daily Session				Criterion Trials/Daily Session
	SD	ITI	LH	TO	
1	60 s	Fix	no	no	≥ 60 % correct, ≤ 30 % omissions
2	25 s	Fix	no	no	≥ 60 % correct, ≤ 30 % omissions
3	10 s	Variable	5 s	no	≥ 70 % correct, < 25 % omissions
4	5 s	Variable	5 s	no	≥ 70 % correct, < 25 % omissions
5	5 s	Variable	5 s	yes	≥ 70 % correct, < 25 % omissions

Supplemental table 2-1. Testing stages of 5-choice task.

The conditions for the different stages and the criteria to reach in each daily session are reported. Each animal repeated the same session of 200 trials maximum until it reached the criterion to move to the next stage. Abbreviations: SD, stimulus duration; ITI, inter-trial interval; LH, limited hold; TO, incorrect time-out. Fix ITI was set at 1s; Variable ITI was set at 0.625, 2.812, 5.0, 7.188, 9.375s respectively.

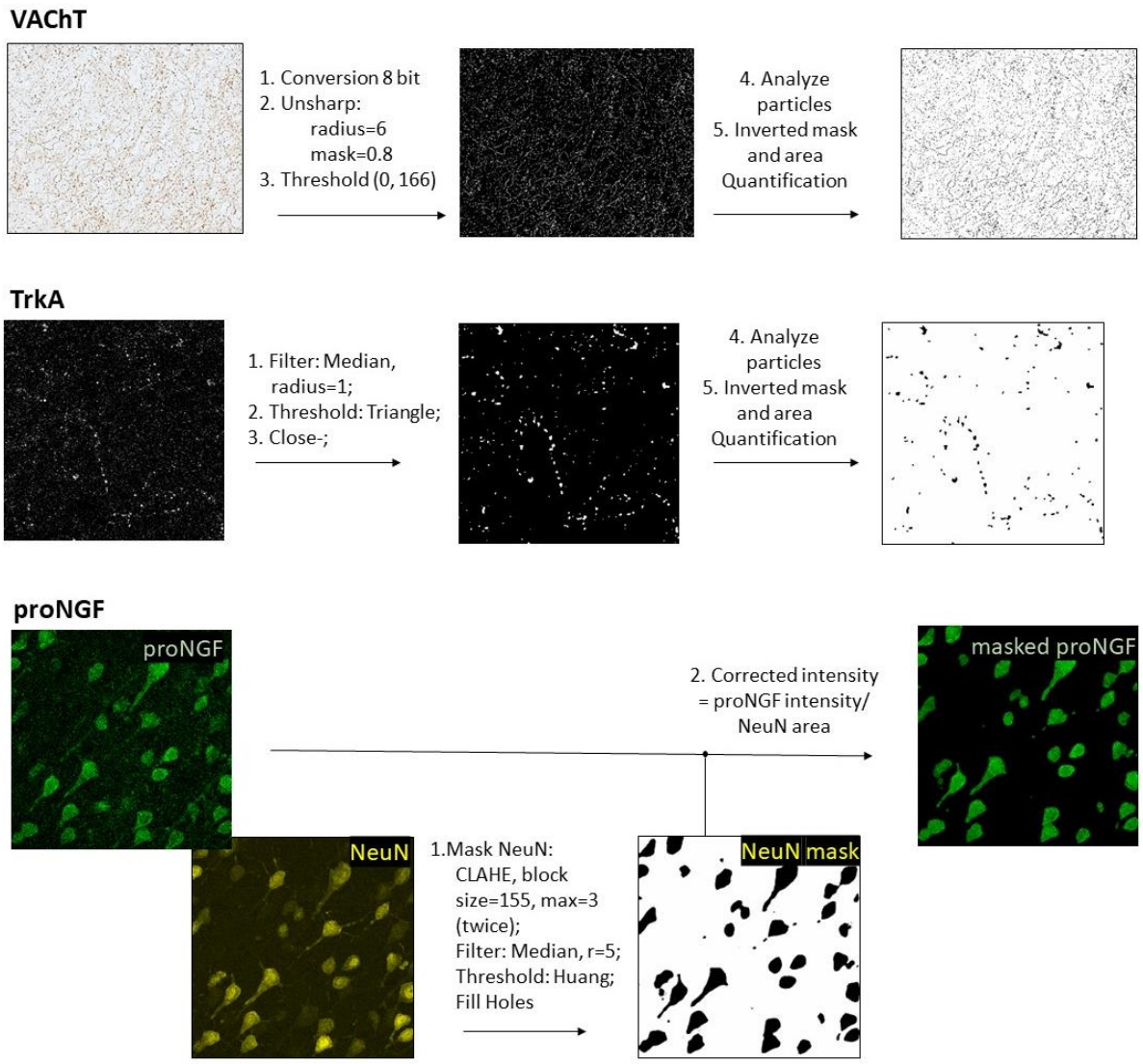


B

Number ChAT+	Sal (Mean ± SEM)	SAP (Mean ± SEM)	P value
Ms/VDB	483.0 ± 23.62	541.2 ± 159.3	0.6970

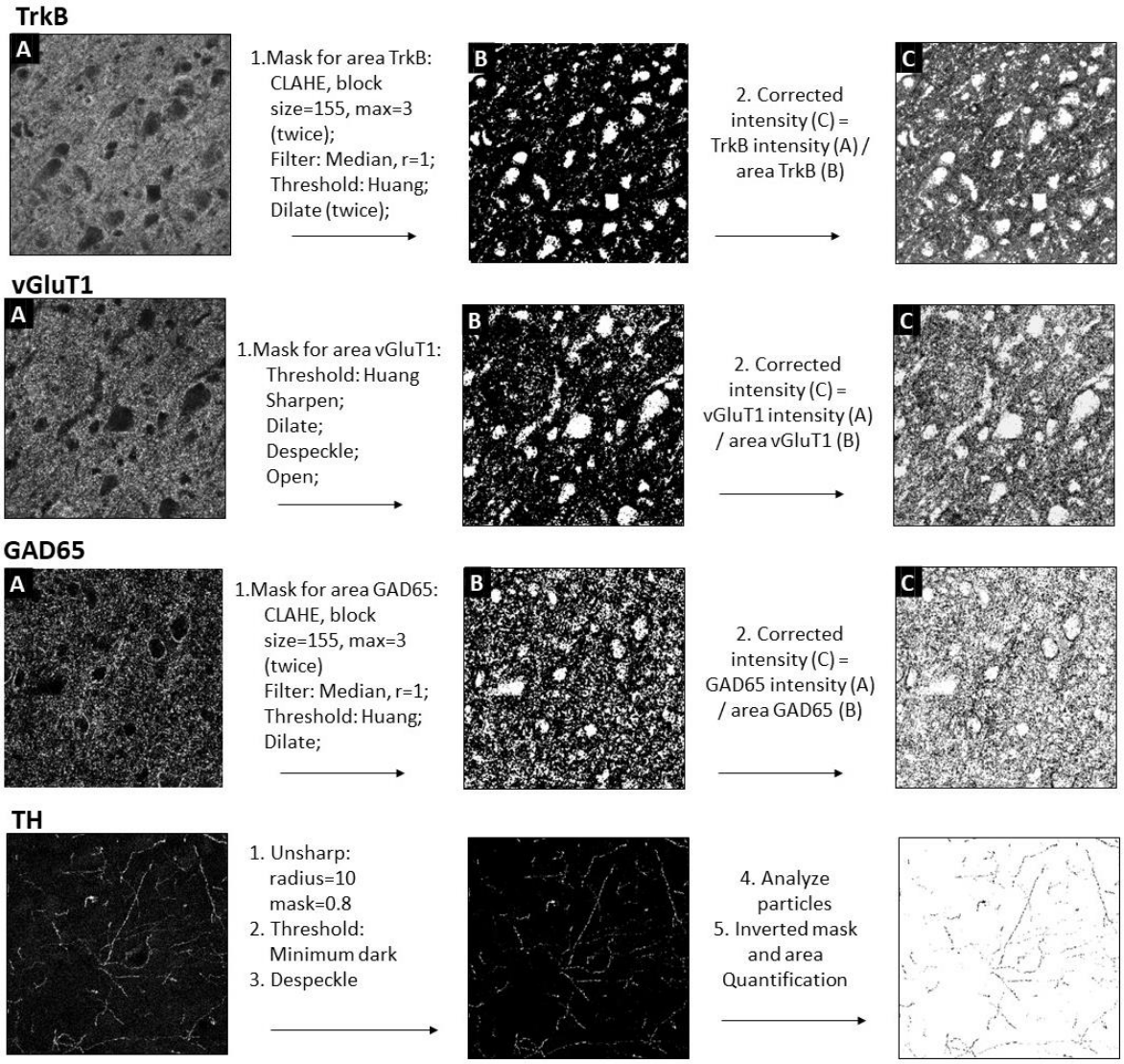
Supplemental figure 2-1. Cholinergic cells of the Ms/VDB were not affected following immunolesion of the nb.

(A) Micrographs of ChAT-IR neurons in Ms and VDB (left) and localization according to the Paxinos and Watson Atlas (right). Scale bar 250 µm. (B) No differences were found in the ChAT-IR cell count between the groups. Data analysis: Sal rats, n=6; SAP rats, n=6. Mann Whitney test, P = 0.6970, U = 12.50. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected; Ms, medial septum, VDB, vertical diagonal band.



Supplemental figure 2-2. Flowchart illustrating the major steps employed in the quantification of VAChT, TrkA and proNGF.

Far right panels (C) indicate area images where binary black pixels were quantified. Abbreviations: VAChT, vesicular acetylcholine transporter; TrkA, Tropomyosin receptor kinase A; proNGF, precursor nerve growth factor.



Supplemental figure 2-3. Flowchart illustrating the major steps employed in the quantification of TrkB, vGluT1, GAD65, TH.

Far right panels (C) indicate area images where binary black pixels were quantified. Abbreviations: TrkB, Tropomyosin receptor kinase B; vGluT1, Vesicular glutamate transporter 1; GAD65, glutamic acid decarboxylase 65; TH, *Tyrosine Hydroxylase.*

	ChAT neurons	VAChT Par	VAChT Fr1	VAChT Fr2	global score	mRNA BDNF	mBDNF	vGluT1	GAD65
ChAT neurons	p = 0 r = 1								
VAChT Par	**p = ≤ 0.0001** r = 0.83	p = 0 r = 1							
VAChT Fr1	**p = ≤ 0.0001** r = 0.88	**p = ≤ 0.0001** r = 0.96	p = 0 r = 1						
VAChT Fr2	**p = 0.00033** r = 0.72	**p = ≤ 0.0001** r = 0.88	**p = ≤ 0.0001** r = 0.88	p = 0 r = 1					
global score	**p = 0.015** r = 0.53	**p = 0.011** r = 0.56	**p = 0.023** r = 0.51	p = 0.068 r = 0.42	p = 0 r = 1				
mRNA BDNF	**p = 0.019** r = 0.52	**p = 0.022** r = 0.51	**p = 0.014** r = 0.54	p = 0.215 r = 0.29	p = 0.144 r = 0.33	p = 0 r = 1			
mBDNF	**p = 0.0016** r = 0.66	**p = 0.0017** r = 0.66	**p = 0.00061** r = 0.70	**p = 0.0011** r = 0.68	p = 0.511 r = 0.15	p = 0.061 r = 0.42	p = 0 r = 1		
vGluT1	p = 0.075 r = 0.41	p = 0.135 r = 0.35	-	-	p = 0.191 r = 0.31	p = 0.208 r = 0.29	p = 0.082 r = 0.40	p = 0 r = 1	
GAD65	p = 0.339 r = 0.23	p = 0.663 r = 0.10	-	-	p = 0.450 r = -0.18	p = 0.582 r = -0.13	p = 0.779 r = -0.07	p = 0.810 r = -0.06	p = 0 r = 1

Supplemental table 2-2. Correlation between cholinergic markers, global score and BDNF.

Unadjusted correlations between all variables showing a significant reduction following cholinergic loss in the nb and demonstrate the intercorrelation between cholinergic markers (in blue) global score, BDNF mRNA and mBDNF (in green). Relationships where $p < 0.05$ are bolded. (–) indicate that vGluT1 and GAD65 were analysed in the parietal but not in the frontal cortices. Data analysis: Sal rats, n=9; SAP rats, n=12 for all analyses besides IHC/IF in which SAP rats, n=11. Pearson correlation analysis was applied to investigate associations between all the variables except for ChAT neurons, VAChT Par, VAChT Fr2 and global score, for which Spearman correlations were calculated. Abbreviations: ChAT, choline acetyltransferase; VAChT, Vesicular acetylcholine transporter; Fr2, Frontal Cortex 2; Fr1, Frontal Cortex 1; Par, Parietal Cortex; BDNF; Brain-derived neurotrophic factor; mBDNF, mature Brain-derived neurotrophic factor; vGluT1, Vesicular glutamate transporter 1; GAD65, glutamic acid decarboxylase 65.

Connecting text: Chapter 2 to 3

In Chapter 2, we investigated whether a long-term specific immunolesion of the nucleus basalis (nb) affects neurotrophins, cortical neuronal markers and cognition in Wistar rats. Our studies demonstrated an attentional deficit accompanied by a reduction of cortical markers of glutamatergic (vGluT1), and GABAergic (GAD65) neurons. Furthermore, we found that the cholinergic nb loss produced differential effects on neurotrophin availability with a reduction in BDNF expression and mature BDNF levels without affecting NGF. Additionally, cholinergic markers correlated with the attentional deficit and BDNF. Finally, we proposed that BDNF dysregulation, consequent to the nb loss, could be responsible for the loss of neuronal markers and cognitive impairment.

Subsequently, since basal forebrain cholinergic neurons are known to regulate vascular tone and cerebral blood flow regulating multiple processes and making synapses with multiple cell types, we explored the effect of cholinergic nb loss on the neurovascular unit. We hypothesized that cholinergic synapses would influence vessel diameters, vascular endothelial growth factor A, and the density of astrocytes and microglia cells in the cerebral cortex. The results of this investigation will be presented in the next chapter of this Thesis.

CHAPTER 3

Long-term nucleus basalis cholinergic depletion affects cortical vessels, Vascular Endothelial Growth Factor-A and glial cells in Wistar rats

Chiara Orciani, Sonia Do Carmo, Morgan K Foret, A. Claudio Cuello

Manuscript in preparation for submission

Abstract

Cholinergic innervation of cerebral blood vessels is important for the regulation of vascular tone and cerebral blood flow. Basal forebrain cholinergic neurons (BFCNs) provide the major source of cholinergic innervation to the basal forebrain and degenerate early in Alzheimer's disease (AD) progression. BFCNs synapses are localized in proximity of cortical cerebral blood vessels and represent the intrinsic innervation of the neurovascular unit (NVU), as they form synapses on different cell types of the NVU such as vascular smooth muscle cells, endothelial cells, and astrocytic end-feet. However, whether the multiple cell types of the NVU are affected by BFCN input remains unknown. To address this question, we immunolesioned the nucleus basalis (nb), a basal forebrain cholinergic nucleus projecting mainly to the cortex, by bilateral stereotaxic injection of the cholinergic immunotoxin 192-IgG-Saporin in 2.5-month-old Wistar rats. Postmortem brain analyses, at 7 months post-lesion, revealed that a reduction of cortical vesicular acetylcholine transporter-immunoreactive boutons was accompanied by a reduction in the average diameter of capillaries and pre-capillary arterioles in the cortex and a decrease of vascular endothelial growth factor A expression and protein levels. Furthermore, the immunolesion significantly increased the density of astrocytes and microglia in the cortex. Astrocytic end-feet showed higher colocalization with arterioles following cholinergic immunolesion. In addition, microglia cells in the parietal cortex correlated with cholinergic loss and showed morphological changes indicative of an intermediate state of activation. Our findings demonstrate that cholinergic nb loss impairs vessels and different cell types belonging to the NVU.

3.1 Introduction

Basal forebrain cholinergic neurons (BFCNs) provide the major source of cholinergic innervation to the basal forebrain (Mesulam et al., 1983b, Mesulam et al., 1983a, Bigl et al., 1982, Struble et al., 1986) and play a crucial role in learning, memory and attention mechanisms (Drachman and Leavitt, 1974, Bartus, 1979, Everitt and Robbins, 1997, Ballinger et al., 2016, Zaborszky et al., 1999). BFCN synapses are involved in cerebral blood flow (CBF) regulation (De la Torre and Mussivan, 1993, de la Torre, 2004), which is fundamental to the functional activity of the brain (Iadecola, 2004). Indeed, initial studies demonstrated that acetylcholine (ACh), released by cholinergic neurons, affects vessel vasodilatation (Furchgott and Zawadzki, 1980). It was later shown that BFCNs could regulate the vascular tone by forming synapses directly on vascular smooth muscles cells (SMCs), endothelial cells, pericytes or via astrocytic end-feet (Mulligan and MacVicar, 2004, Toribatake et al., 1997, Hamilton et al., 2010). All these cell types have ACh muscarinic receptors (mAChRs) in human and animal brains (Luiten et al., 1996, Garcia-Villalon et al., 1991, Elhusseiny et al., 1999, Furchgott and Zawadzki, 1980).

BFCN synapses are present in proximity of cortical cerebral blood vessels (Vaucher and Hamel, 1995, Tong and Hamel, 1999) and, along with neurons located in the locus coeruleus and the raphe nucleus, represent the intrinsic innervation of the neurovascular unit (NVU) (Hamel, 2006). The NVU is a structure connecting the brain parenchyma to the cerebral vasculature, composed of several cell types, such as endothelial cells, pericytes, SMCs, astrocytes, microglia, and neurons. The NVU is the main keeper of brain homeostasis. The NVU regulates the CBF to ensure that it meets the brain's energetic needs; it is the site of the blood-brain barrier (BBB), which controls the bidirectional trafficking of molecules (Iadecola, 2017). In addition, it is involved in the trafficking of immune cells and is the main conduit for removing potentially toxic by-products of brain metabolism, including β-amyloid and tau (Iadecola, 2017).

The gradual degeneration of BFCNs in aging, and their severe atrophy and loss in Alzheimer's disease (AD) pathology (Bowen et al., 1976, Davies and Maloney, 1976, Whitehouse et al., 1981), contribute significantly to cognitive impairment. In line with this, the cholinergic hypothesis suggests that dysfunction of BFCNs contributes considerably to the cognitive decline observed in the geriatric population (Bartus et al., 1982, Coyle et al., 1983, Cuello and Sofroniew, 1984,

Francis et al., 1999). In addition, the *vascular-cholinergic hypothesis* (reviewed in (De la Torre and Mussivan, 1993, de la Torre, 2004)) suggests that the loss of BFCNs, which are responsible for cerebral vasodilatation and CBF, could impair cerebral perfusion (Tong and Hamel, 1999), contributing to the etiology and the progression of AD.

This double involvement —on cognition and brain perfusion— of BFCNs in AD is further illustrated by the fact that four out of six FDA-approved drugs are acetylcholinesterase inhibitors (AChEIs); drugs that improve the telencephalic cholinergic tone by blocking extracellular ACh degradation (Giacobini, 1987). Indeed, AChEIs not only offer transient cognitive relief even at advanced stages of AD (Giacobini et al., 2022), but they also increase perfusion in the posterior parietal-temporal and superior frontal cortex in individuals with AD (Geaney et al., 1990, Ebmeier et al., 1992). This effect on perfusion preceded the beneficial effects on glucose metabolism by months (Nordberg et al., 1998, Nordberg, 1999, Blin et al., 1997). In contrast, anticholinergic drugs such as Scopolamine, when given to young people, result in brain atrophy and cognitive decline, reducing cerebral perfusion by 20% in the frontal cortex (Honer et al., 1988).

The basal forebrain cholinergic system generated renewed interest since basal forebrain atrophy has been reported at prodromal stages of AD (Teipel et al., 2011). BFCNs atrophy precedes and predicts atrophy of the entorhinal and cerebral cortices (Schmitz et al., 2016) as well as the cortical spread of the AD pathology (Fernández-Cabello et al., 2020). At the same time, vascular pathology has been suggested to be the earliest indicator of AD development (Iturria-Medina et al., 2016, Jack Jr et al., 2010). These new studies in the context of AD were accompanied by an improved understanding of the NVU physiological functionality. Indeed, it is becoming clear that CBF does not depend solely on the release of vasoactive agents from neurons, but it results from multiple processes involving the different cell types of the NVU (Iadecola, 2004).

We recently demonstrated that a 7-month selective loss of cholinergic neurons of the nb reduces cortical synaptic markers, affects BDNF availability and impairs cognition (Orciani et al., 2022). Given that BFCN synapses provide innervation to the NVU (Hamel, 2006), we hypothesized that the selective long-term demise of nb cholinergic neurons would also alter the organization of the NVU. In particular, we postulated that selective long-term loss of cholinergic nb neurons would

affect not only the structure of the vessels, but also other cell types that participate in the NVU, such as astrocytes and microglia.

Towards this goal, we selectively immuno-depleted BFCNs of the nb in wt rats with a stereotaxic injection of 192-IgG-saporin (192-IgG-SAP) consisting of an anti-p75ntr receptor monoclonal antibody conjugated to the cytotoxin saporin (Wiley 1991). Seven months following the immunolesion, we assessed the effect of cholinergic neuron depletion on the vessels, in particular on capillaries and arterioles. Furthermore, we investigated the effect of the immunolesion on a vascular factor implicated in regulating the vascular tone, the overall astrocyte count, and the astrocytic end-feet. Lastly, we assessed overall changes in the number of microglial cells and their morphology.

This study revealed that the long-term loss of BFCNs of the nb has significant consequences on the average diameter of capillaries and pre-capillary arterioles in the cortex and induced a reduction of vascular endothelial growth factor A (VEGF-A) expression and protein levels. Furthermore, we found that the immunolesion significantly affected astrocytes and microglia, which were increasingly present in areas that were subjected to larger cholinergic terminal loss, such as the parietal cortex. Astrocytic end-feet showed higher colocalization with arterioles following cholinergic immunolesion. In addition, microglia cells in the parietal cortex correlated with cholinergic loss and showed morphological changes indicative of an intermediate active state.

3.2 Methods

3.2.1 Experimental design

Two and a half-month-old Wistar rats (Charles River Laboratories) were randomized into 2 sex-balanced groups to receive stereotaxic injection in the nb of either 192-IgG-saporin (SAP) (n=12, 6 M and 6 F) or PBS vehicle as a control (Sal) (n=9, 5 M and 4 F). Seven months post-immunolesion, the animals were sacrificed, and their brains were preserved for histological and biochemical analyses. One rat was excluded from the histological analyses as the post-fixation was not optimal (n = 20), while the biochemical analyses were performed in all rats (n = 21). The McGill University Animal Care Committee approved all experimental procedures (Ethical Approval Number: MCGL-3860).

3.2.2 Stereotaxic surgery

Surgical procedures were conducted under isoflurane anesthesia (isofluorane vaporizer was initially set at 4% and decreased to 2.5% for maintenance), and animals were mounted on a stereotaxic frame. The injections were performed with a 5-µl microsyringe fitted with a 32-gauge needle through a burr-hole drilled in the skull. Stereotaxic coordinates were measured from Bregma, according to the Paxinos and Watson Atlas (Paxinos and Watson, 2006) and were anteroposterior (AP) -1.8; mediolateral (ML) ±3.2; dorsoventral (DV) -7.5. 192-IgG-Saporin (2.6 mg/ml, Advanced Targeting Systems) was injected at a concentration of 0.5 µg/µl (1.0 µl/ hemisphere), while PBS was injected as a control. Following stereotaxic surgery, rats were injected with carprofen (5 mg/ml, given 0.1 ml/100 g) for 3 days to minimize animal suffering.

3.2.3 Brain tissue collection

Seven months post-lesion, rats were deeply anesthetized with a mix of chloral hydrate and sodium pentobarbital (6.5 mg and 3 mg, respectively, per 100 g of body weight) delivered by intraperitoneal injection and perfused transcardially with saline solution for 2 min. The brain was removed, and the cortex, hippocampus and cerebellum were dissected from one hemisphere. The tissue was flash-frozen and kept at −80 °C for qRT-PCR and Western blot. The other hemisphere was post-fixed in 4% PFA at 4°C for 24 hours and transferred in 30% sucrose in 0.1 M phosphate

buffer before being processed for immunohistochemical analysis. The fixed hemisphere was sectioned on a microtome (Leica SM 2000R; Germany) into 40-μm thick sections and stored at -20 °C in a cryoprotectant solution of ethylene glycol and sucrose in PBS (pH 7.4).

3.2.4 Gene expression analysis

Messenger RNA (mRNA) was extracted from cortical tissue (15 mg) using the RNeasy Mini Kit (Qiagen, USA). For reverse transcription, 500 ng of total RNA was used to synthesize cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR was performed in a total volume of 10 μL using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and using the CFX Connect Real-Time PCR Detection System and CFX Manager (Bio-Rad). Fold changes in gene expression compared to vehicle-treated rats were calculated, normalizing the values for each gene of interest to housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actin, using the 2 (Delta C[T]) method (Livak and Schmittgen, 2001). Primer sequences: VEGF-A Fw, CTG GAC CCT GGC TTT ACT GC; VEGF-A Rev, ACT TCA CCA CTT CAT GGG CTT; GAPDH Fw, TGA TGG GTG TGA ACC ACG AG, GAPDH Rev, TCA TGA GCC CTT CCA CGA TG; Beta-Actin Fw, AGC CAT GTA CGT AGC CAT CC; Beta-Actin Rev, CTC TCA GCT GTG GTG GTG AA.

3.2.5 Western blotting (WB)

Twenty mg of frontoparietal cortical samples were manually homogenized in lysis buffer (Cell Signaling, USA) containing a complete protease inhibitor cocktail (Roche, USA). Manual homogenization was followed by two 5 second pulses of sonication. The homogenates were centrifuged at 13,000 rpm, for 45 min at 4 °C, the supernatants were collected, and the protein concentration was determined by Lowry *assay* (DC™ Protein Assay kit, BioRad, USA). The homogenates (50 ug) were mixed with Sample Buffer (Tris-HCl 250 mM, pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue) and boiled for 5 minutes. The samples were loaded on SDS-polyacrylamide gels and were transferred to PVDF membranes (BioRad, USA) for 1 h at 350 mA. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated with the following primary antibodies: anti-mouse VEGF-A (1:2000, ab1316, Abcam), anti-rabbit AQP IV (1:2000, 249-323, Alomone) in TBS-T overnight at 4 °C. Peroxidase-

conjugated secondary antibodies (1:10,000, Goat anti-rabbit and anti-mouse HRP, Jackson Immunoresearch), dissolved in TBS-T, were applied for 1 h at room temperature. WB were developed with an enhanced chemiluminescence substrate (Western Lightning® Plus-ECL, PerkinElmer Inc., USA) and imaged with Amersham Imager 600 (GE Healthcare, USA) and ChemiDoc™ Touch system (Bio-Rad, Canada). Densitometry was quantified with TotalLab CLIQS Software (TotalLab, UK) and ImageLab (Bio-Rad, Canada). The values were normalized to the loading control GAPDH (1:10000, MAB374, Millipore). An additional sample was loaded in each gel as an internal loading control. The values were expressed as fold change relative to the control group.

3.2.6 Immunofluorescence (IF)

Free floating, 40 µm coronal brain sections were washed using PBS and underwent heat-mediated antigen retrieval. For VAChT, Iba1 and GFAP antibodies, sections were incubated at 80 ºC in 10 mM citrate buffer (pH 6.0) for 30 minutes then cooled for 20 minutes at room temperature (RT). For collagen IV (COL-IV) and AQP IV we performed the antigen retrieval in pepsin (1 mg/ml) in HCl 0.01N for 6 minutes at 37 °C after a previous wash in water for 5 minutes at 37 °C. The sections were then washed with PBS and permeabilized using 50% ethanol for 20 minutes, followed by washes with PBS-T (containing 0.2% Triton-X-100), and blocked for 1 hour at RT in 10% NGS. The sections were then incubated with primary antibodies for 48 hours at 4 ºC: anti-GFAP (1:2000, SPM507, Novus Biological), anti-VAChT (1:3000, 139 103, Synaptic Systems), anti-Iba1 (1:500, Synaptic Systems), COL-IV (1:200, M3F7, Developmental Studies Hybridoma Bank), AQP IV (1:1000, 249-323, Alomone), alpha-SMA (1:600, NB300-978, Novus Biological). After primary antibody incubation, sections were washed in PBS-T, and incubated with Alexa Fluor 488 (goat-anti-mouse), Alexa Fluor 568 (goat-anti-rabbit), and Alexa Fluor 647 (goat-anti-guinea pig), or Alexa Fluor 488 (donkey-anti-mouse), Alexa Fluor 568 (donkey-anti-rabbit), and Alexa Fluor 647 (donkey-anti-goat), (all at 1:800, Thermo Fisher Scientific) for 2 hours RT. Following washes, sections were incubated for 5 minutes with 0.3% Sudan black in 70% ethanol to reduce autofluorescence. Sections were then washed three times for 5 minutes in PBS-T, then three times for 5 minutes in PBS before mounting onto coated slides and coverslipped (#1.5) with Aqua-Poly/Mount (Polysciences).

3.2.7 Confocal imaging and analyses

For the COL-IV, AQP-4 and αSMA, images were acquired using a confocal microscope Leica TCS SP8 (Leica, Canada) equipped with a 20x HC PL APO objective and using the LAS X Software (Leica, Canada). Twelve (AQP-4, αSMA) and eight images (COL-IV) per tissue section (two sections/technical replicates per animal) were collected in the parietal cortex, in Z-stacks with a frame of 600 x 600 µm, resolution of 512x512 pixels, an optical section of 0.896 µm, an interval of 1.3 µm and an averaging of two by line. The 40x oil HC LP APO objective was used to acquire with a frame of 291.19 x 291.19 µm capillary images (COL-IV), two images per animal in layer 5 of the parietal cortex, with the same confocal microscope, same interval, resolution, and optical section. Signal was detected with emission wavelengths of 500-550 nm (488 laser), 571-620 nm (561 laser), and 650-700 nm (640 laser).

For Iba1-GFAP-VAChT staining, images were acquired using a LSM800 Confocal Microscope AxioObserver (Zeiss, Germany) equipped with a 20x Plan Apochromat objective lens (NA = 0.80) and using ZEN Imaging software (ZEN Black). Six images in the frontal and six images in the parietal cortex per tissue section (two sections/technical replicates per animal) were collected using Z-stacks at an interval of 1 µm. Diode lasers of 488, 561, and 640 nm were used sequentially from longest to shortest wavelength, all with a pinhole size equivalent to 1 airy unit (AU) for each respective wavelength. Sixteen-bit images of 319.45 x 319.45 µm were acquired with an averaging of two by line. Signal was detected with emission wavelengths of 500-550 nm (488 laser), 571-620 nm (561 laser), and 650-700 nm (640 laser).

Images were imported to Fiji software (ImageJ), and slices were merged into one stack (stack/Z projection/Maximum Intensity Projection) for each channel and subsequent structural analysis. To quantify the immunolabelling of VAChT, COL-IV, αSMA, GFAP and Iba1, images were converted to binary. The number VAChT-immunoreactive (IR) synapses were counted with a home-built macro as in **Supplemental figure 3-1.** αSMA-IR vessel diameter was assessed as in **Supplemental figure 3-2**, but instead of using a defined region of interest (ROI), all the vessels were measured **Supplemental figure 3-1 (bottom panels)**. After the diameter was quantified, the arterioles were classified. Arterioles larger than 10 µm (cut-off determined based on capillary size) were defined as medium size (up to 20 µm) and ones beyond 20 µm were defined as larger size, as in (Michaloudi et al., 2006). The diameters of the capillaries COL-IV-IR were assessed

following the procedure explained in **Supplemental figure 3-2.** The IR area for COL-IV, αSMA, GFAP and the number of Iba1-IR cells were quantified as in **Supplemental figures 3-1, and 3-3**. For the microglia morphology, we measured the Iba1-IR average soma size, the overall processes, and the average processes length after skeletonization, as described in **Supplemental figure 3-3**. AQP-4 colocalization was quantified by measuring the intensity of colocalization with COL-IV and αSMA (ImageJ). Images were processed and analyzed after blinding to treatment.

3.2.8 Statistical analysis

The software GraphPad Prism v8 (La Jolla, CA, USA) was used for statistical analysis. Data were displayed in columns showing individual values, mean and SEM. Data normality was verified with the Shapiro–Wilk normality test. A 2-way-ANOVA or a mixed effect analysis followed by Bonferroni's multiple comparisons test was applied for quantification repeated in the frontal and parietal cortex. A two-tailed unpaired t-test was used for 2-group comparisons. The Welch's correction was applied for unequal variances. Associations between variables were investigated with Pearson correlation. If data normality was violated, the analysis was performed with nonparametric tests. For each experiment, the statistical analysis is specified in the figure legends and tables. Significance was set at $p < 0.05$.

3.3 Results

3.3.1 Long-term lesion of the nb induced loss of VAChT-IR terminals in frontal and parietal cortices.

Motivated by the significant loss of ChAT-IR neurons of the nb in long-term SAP-immunolesioned rats, recently reported by us (Orciani et al., 2022), and given that the cholinergic system has been extensively studied in the vasculature context in frontal and parietal cortices (Uchida et al., 2000, Sato et al., 2002), we assessed the number of cortical cholinergic VAChT-IR pre-synaptic terminals in these cortical areas (bregma +0.45 / +1.00 mm) (**Figure 3-1 A,B**). A significant reduction in the number of cholinergic pre-synapses was found in both frontal and parietal cortices in the SAP-immunolesioned group compared to the Sal-group (**Figure 3-1 C**). This result is in agreement with our previous findings (Orciani et al., 2022), as the reduction was more pronounced in the parietal cortex (loss of 52% VAChT-IR terminals) compared to the frontal cortex (loss of 37% VAChT-IR terminals) (**Figure 3-1 C**).

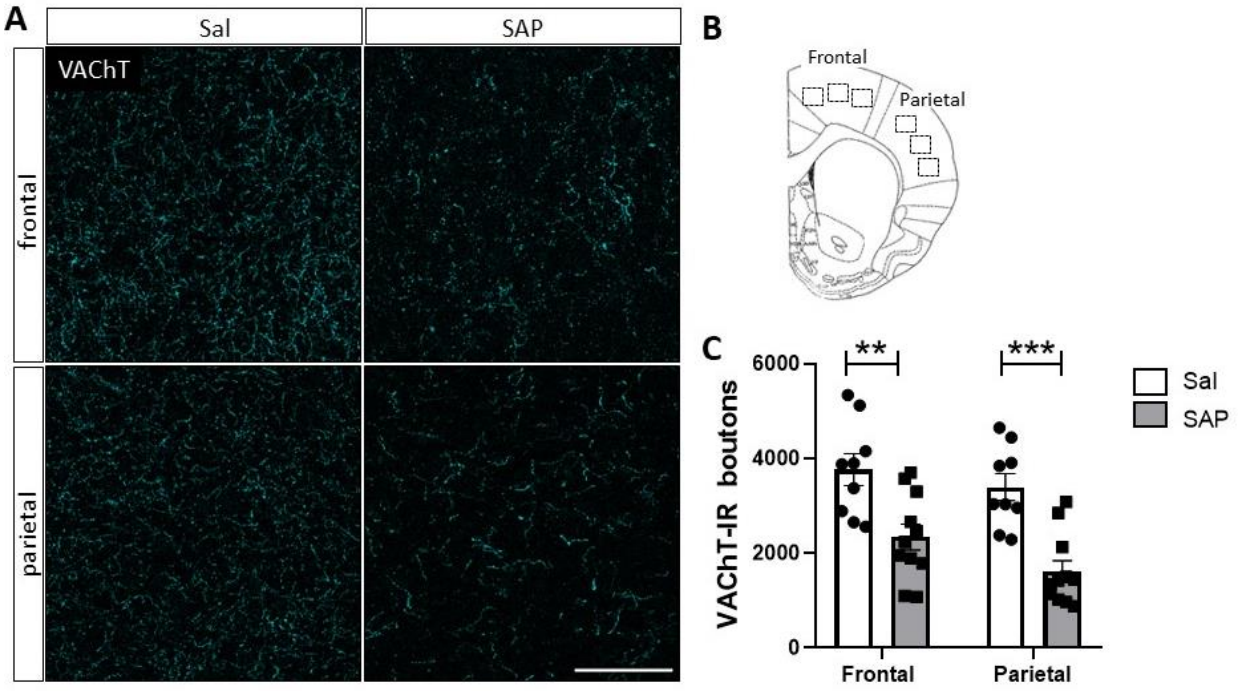


Figure 3-1. VAChT-IR terminals were reduced in frontal and parietal cortices following nb demise.

(A) Representative images of IF for VAChT-IR terminals in the frontal and parietal cortices (scale bars 100 μm). (B) Schematic representation of the investigated cortical regions. (C) The two cortical areas analyzed in the SAP-immunolesioned group showed a reduction of VAChT-IR varicosities compared to the Sal-treated group. Bonferroni's multiple comparisons test, frontal (p= 0.0019, t = 3.600, DF = 36.00), parietal (p= 0.0001, t = 4.496, DF = 36.00). Two-way ANOVA; main effect cortical region (p<0.0001, F (1, 18) = 28.68); main effect treatment (p=0.0005, F (1, 18) = 17.56); interaction (p=0.0998, F (1, 18) = 3.011). Data analysis: Sal rats, n = 9; SAP rats, n = 11. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected, VAChT, Vesicular acetylcholine transporter; ANOVA, analysis of variance.

3.3.2 Loss of VAChT-IR terminals affects pre-capillary arterioles and capillaries in the parietal cortex.

To assess the effect of cholinergic denervation on the vessels, we measured the area occupied by collagen IV-IR (COL-IV-IR) vessels in the parietal cortex, representing the area with the larger VAChT-IR depletion. COL-IV is a component of the thin cerebrovascular basement membrane (CVBM) layer (Timpl, 1996), wrapping the endothelial cells surrounding the vessel lumen. No difference between the groups was found in the area occupied by COL-IV-IR vessels (**Figure 3-2 A,B**).

Since cholinergic terminals form synapses mostly with arteries and capillaries (Nizari et al., 2019, Hamel, 2006), arterioles were subsequently investigated. Arterioles are characterized by the presence of an SMCs layer containing α-smooth muscle actin (αSMA) between endothelial cells and CVBM (reviewed in (Schaeffer and Iadecola, 2021)). There was no significant difference in the area covered by αSMA-IR vessels between SAP-immunolesioned group and controls (**Figure 3-2 A,C**) as well in the diameter of the medium-sized and the larger-sized arterioles (**Figure 3-2 A,D**). On the other hand, the diameter of the smaller arterioles preceding the capillaries, defined as pre-capillaries arterioles (Schaeffer and Iadecola, 2021), was significantly increased in the SAP-immunolesioned rats showing an average diameter of 8.40 μm (± 0.16 SEM) compared to the 7.78 μm (± 0.17 SEM) of the control group (**Figure 3-2 A,D**).

Given that the effect of SAP-immunolesion was limited to smaller αSMA-IR arterioles, we then investigated the diameter of the smaller COL-IV-IR vessels. The average diameter of the capillaries in the SAP-immunolesioned rats was 5.14 μm (±0.07 SEM), significantly lower than the 5.44 μm (±0.06 SEM) of the control group (**Figure 3-2 A,E**).

Overall, a long-term lack of pre-synaptic VAChT-IR terminals may affect the vascular tone of the smaller microvessels in the parietal cortex through its effect on the vessel diameter, a subject that warrants further investigation.

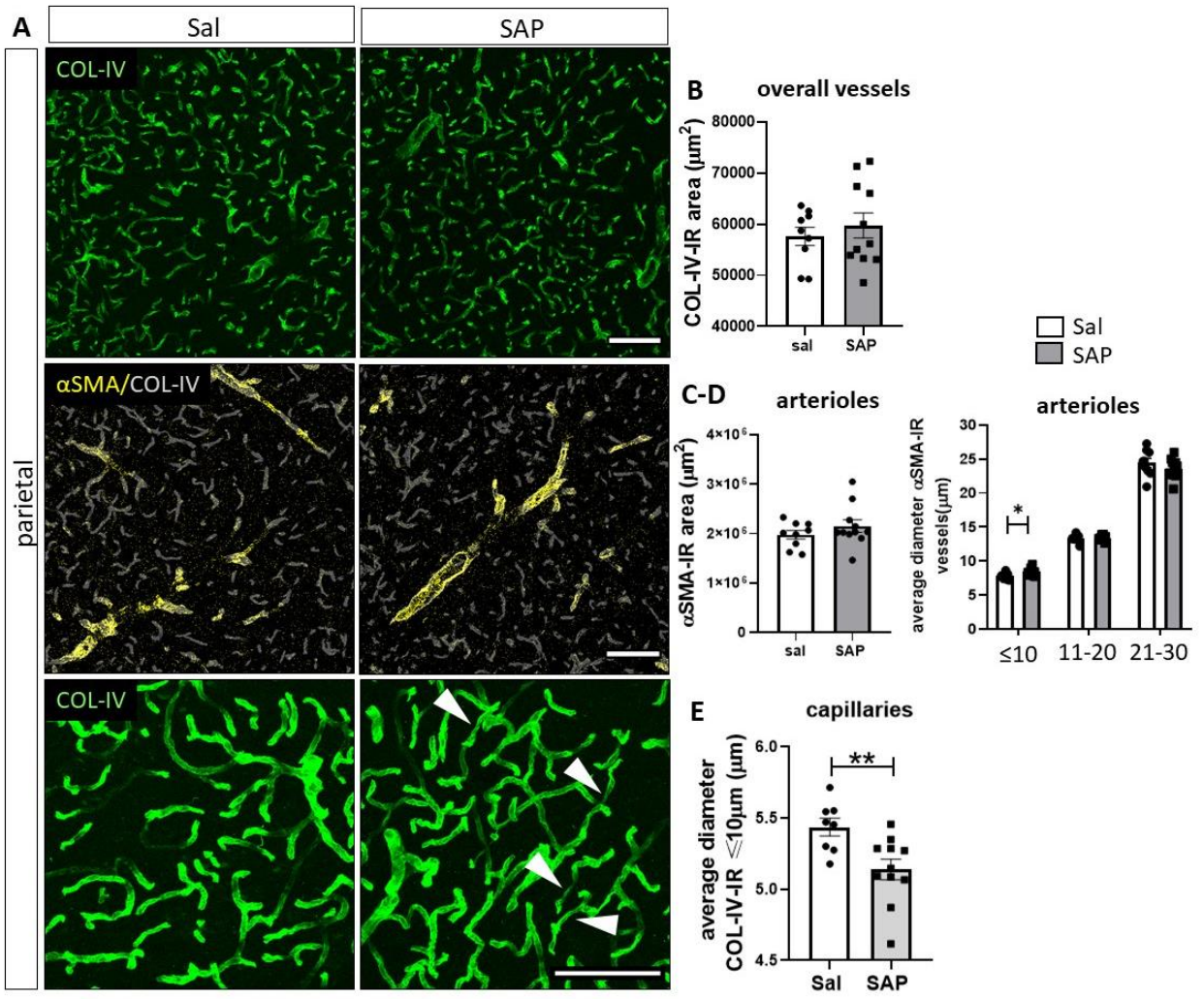


Figure 3-2. Loss of VAChT-IR terminals affects arterioles and capillaries in the parietal cortex.

(A) Representative IF images for COL-IV-IR and αSMA-IR vessels in the parietal cortex (scale bar 100 μm). (B) The area covered by COL-IV-IR vessels did not show any difference between SAP-immunolesioned and Sal-treated ($p = 0.5092$, $t=0.6735$, $df=18$). (C-D) Although the area covered by αSMA-IR vessels was not significantly different between the two groups ($p= 0.2947$, $t=1.079$, $df=18$), the average diameter of the pre-capillaries arterioles was significantly increased in the SAP-immunolesioned compared to the controls (D) ($p = 0.0463$, $t = 2.709$, $DF = 16.10$). Middle and larger-sized arterioles did not show any significant difference between the two groups (respectively, $p>0.9999$, $t = 0.1121$, $DF = 15.60$; $p= 0.8945$, $t = 1.079$, $DF = 14.60$). (E) The average capillary diameter was significantly reduced in the SAP-immunolesioned compared to the

controls ($p = 0.0082$, $t=2.992$, $df=17$). Data analysis: Sal rats, n = 9; SAP rats, n = 11. An unpaired t-test was performed for all comparisons, and for the diameter of arterioles, a mixed effect analysis followed by Bonferroni's multiple comparisons test was performed. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected; COL-IV, collagen IV; αSMA, α-smooth muscle actin.

3.3.3 Loss of VAChT-IR terminals affects vascular endothelial growth factor A.

Given the changes in the diameter of pre-capillary arterioles and capillaries in the SAP-immunolesioned group compared to the control group, we measured VEGF-A. VEGF-A is regulated by cholinergic input (Inada et al., 2014, Kimura et al., 2018) and has multiple effects on the vasculature. It is an angiogenetic factor inducing vessel sprouting (Ruhrberg et al., 2002) and can influence vessel diameter by acting on endothelial cell proliferation (Nakatsu et al., 2003). It has also been shown to regulate vascular tone through endothelial nitric oxide synthase (eNOS) (Storkebaum and Carmeliet, 2004) and is a mediator of Blood Brain Barrier (BBB) permeability (Mayhan, 1999). As expected, we found that mRNA expression and protein levels of VEGF-A were reduced in the SAP-immunolesioned group compared to the Sal-group (**Figure 3-3 A-C**).

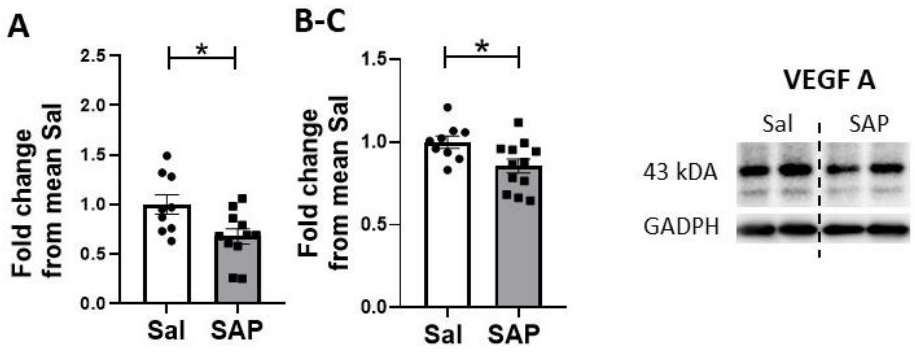


Figure 3-3. mRNA and protein levels of VEGF-A were down-regulated following nb depletion.

(A,B) Cortical mRNA levels of VEGF-A and protein levels were reduced in the SAP-immunolesioned group compared to the controls (respectively, p =0.0178, t=2.609, df=18; p =0.0236, t=2.461, df=19). (C) Representative WB images of VEGF-A and control gene GAPDH. Data analysis: Sal rats, n = 9; SAP rats, n = 11. An unpaired t-test was performed for all comparisons. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected, VEGF-A, vascular endothelial growth factor A; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; WB, western blot.

3.3.4 Loss of cholinergic innervation increased the number of astrocytes in the frontal and parietal cortices

Vessels are externally carpeted by a layer of astrocytic end-feet (McConnell et al., 2017, Filosa et al., 2016) and it has been shown that astrocytes participate in the structure and function of the NVU and BBB (Abbott et al., 2006, Lecuyer et al., 2016, Willis et al., 2004, Liu et al., 2018). Astrocytes play an active role in the modulation of the CBF following neuronal stimulation (reviewed in (Koehler et al., 2009)) in addition to their inflammatory response function (Sofroniew, 2015).

Therefore, we examined whether long-term loss of VAChT synaptic terminals alters the number of astrocytes in cortical areas. SAP-immunolesioned rats showed an increased GFAP-IR area in parietal and frontal cortices compared to the control group (**Figure 3-4 A,B**). However, there were no significant correlations between VAChT-IR synapses and the area covered by astrocytes (**Figure 3-4 C,D**). We next assessed the protein levels and coverage of astrocytic end-feet on the vessels by probing Aquaporin-4 (AQP-4), representing the most abundant water channel of CNS highly expressed by astrocytic end-feet (Nielsen et al., 1997). The overall protein levels of AQP-4 measured by WB in the cortex were not altered between the two experimental groups (**Figure 3-4 F-H**). As well, there were no differences in the colocalization of AQP-4 with the basement membrane of vessels (COL-IV, **Figure 3-4 J**). However, there was a significant increase in AQP-4 colocalization with the arterioles (αSMA, **Figure 3-4 K**).

These analyses showed that nb immunolesion increased in the cortex astrocytes density and the AQP4-IR astrocytic endfeet on the arterioles compared to the control group.

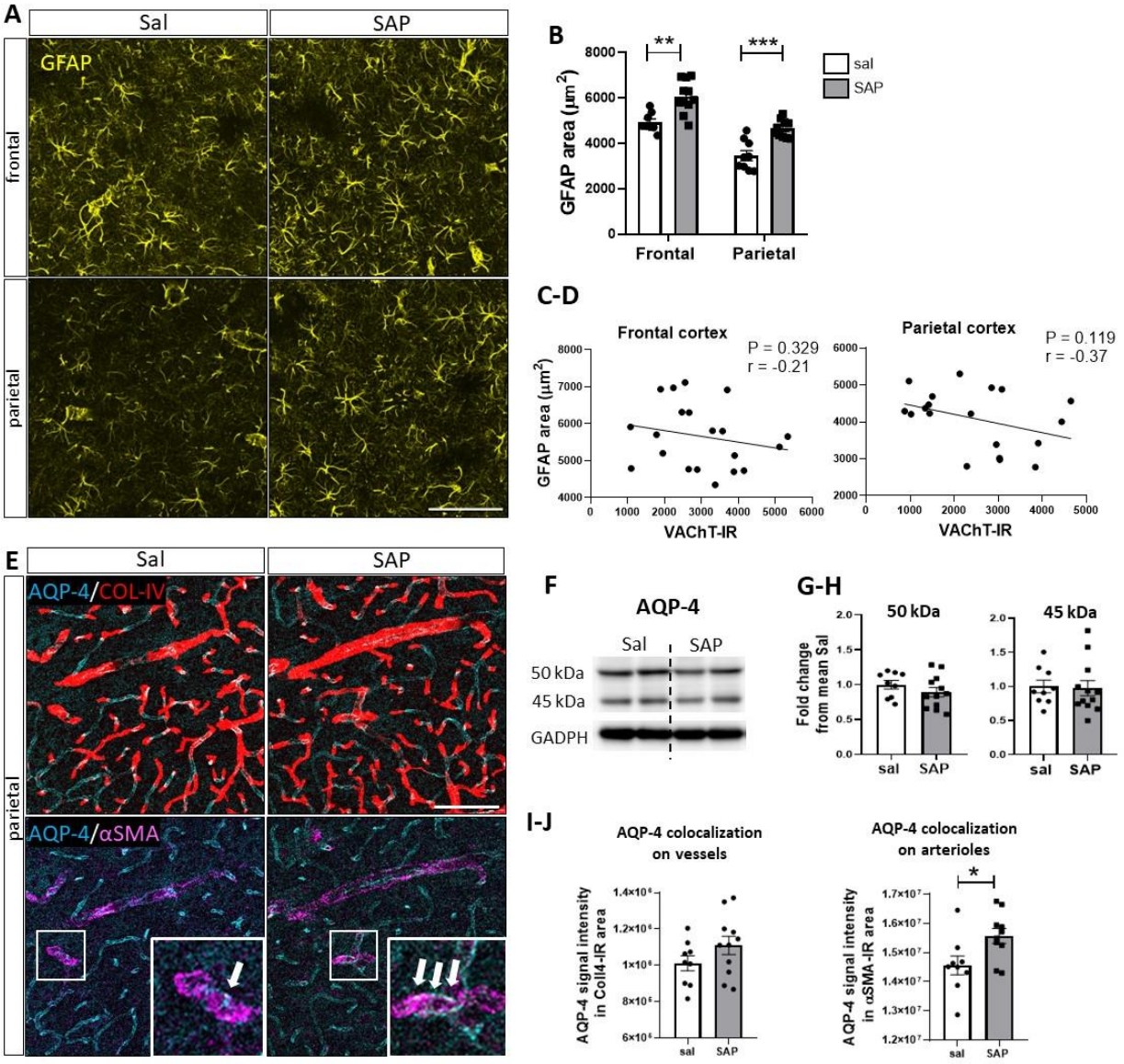


Figure 3-4. Nb cholinergic immunolesion promoted increase of cortical astrocytic cells and increased colocalization of astrocytic end-feet in arteries.

(A) Representative micrographs of GFAP in the frontal and parietal cortices, layer V (Scale bar 100 µm). (B) The two cortical areas analyzed in the SAP-immunolesioned group showed an increased area of GFAP-IR compared to the Sal-treated group (frontal, p = 0.0017, t = 4.234, DF = 14.00 and parietal, p= 0.0010, t = 4.509, DF = 14.00). Mixed effect analysis; main effect cortical region (p<0.0001, F (1, 20) = 59.23); main effect immunolesion (p<0.0001, F (1, 14) = 38.21); interaction (p= 0.8751, F (1, 14) = 0.02562). (C,D) VAChT-IR in the frontal and parietal cortical areas did not show a significant correlation with GFAP-IR area (frontal, p = 0.3729, R^2= 0.04433;

parietal, p = 0.1190, R^2= 0.1369). (F-H) the levels of AQP-4, assessed by WB, were not changed for either the two isoforms between the two groups (50 kDa, p = 0.2743, t=1.126, df=19 and 45 kDa, p= 0.8780, t=0.1556, df=19). (E) Representative colocalization images of AQP-4-IR with COL-IV-IR and with αSMA-IR in the parietal cortex (scale bar 100 μm, magnification 80 x 80 μm). (I-J) No colocalization was detected overall with the vessels (COL-IV, p= 0.1558, t=1.482, df=18), although increased levels of AQP-4 colocalization with arterioles was found (αSMA-IR, p = 0.0238, t=2.481, df=17). Data analysis: Sal rats, n = 9; SAP rats, n = 11. Pearson r, unpaired t-test and mixed effect analysis followed by Bonferroni's multiple comparisons was applied. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected, VAChT, Vesicular acetylcholine transporter; GFAP; Glial fibrillary acidic protein; AQP-4, aquaporin-4; COL-IV, collagen IV; αSMA, α-smooth muscle actin; WB, western blot.

3.3.5 Loss of cholinergic innervation correlated with the increased number of microglial cells in the parietal cortex.

Along with astrocytes, microglia also plays a double role in interacting with both neurons and blood vessels (Szalay et al., 2016), modulating CBF and neurovascular coupling (Császár et al., 2022) and are recruited to sites of BBB leakage within minutes (Lou et al., 2016, Jolivel et al., 2015). Therefore, we examined whether a long-term nb depletion affects microglia. We found a significant increase in microglia number in the parietal cortex of SAP-immunolesioned rats compared to the Sal-group **(Figure 3-5 A,B).** Furthermore, the increase in microglia in the parietal cortex correlated with the loss of VAChT-IR terminals **(Figure 3-5 D),** which was not the case for astrocytes **(Figure 3-4 D)**. The increased number of microglia was accompanied by changes in the microglial morphology in the parietal cortex as we found a significant increase in the average length of processes in the SAP-immunolesioned group compared to the control group (**Figure 3-5 E-F**). This change was not accompanied by differences in the average size of microglial soma (**Figure 3-5 G**). Consequentially, the ratio given by the cell body area divided by the average length of processes, representative of microglia activation, was significantly higher in the SAP-immunolesioned group compared to the control group (**Figure 3-5 H**).

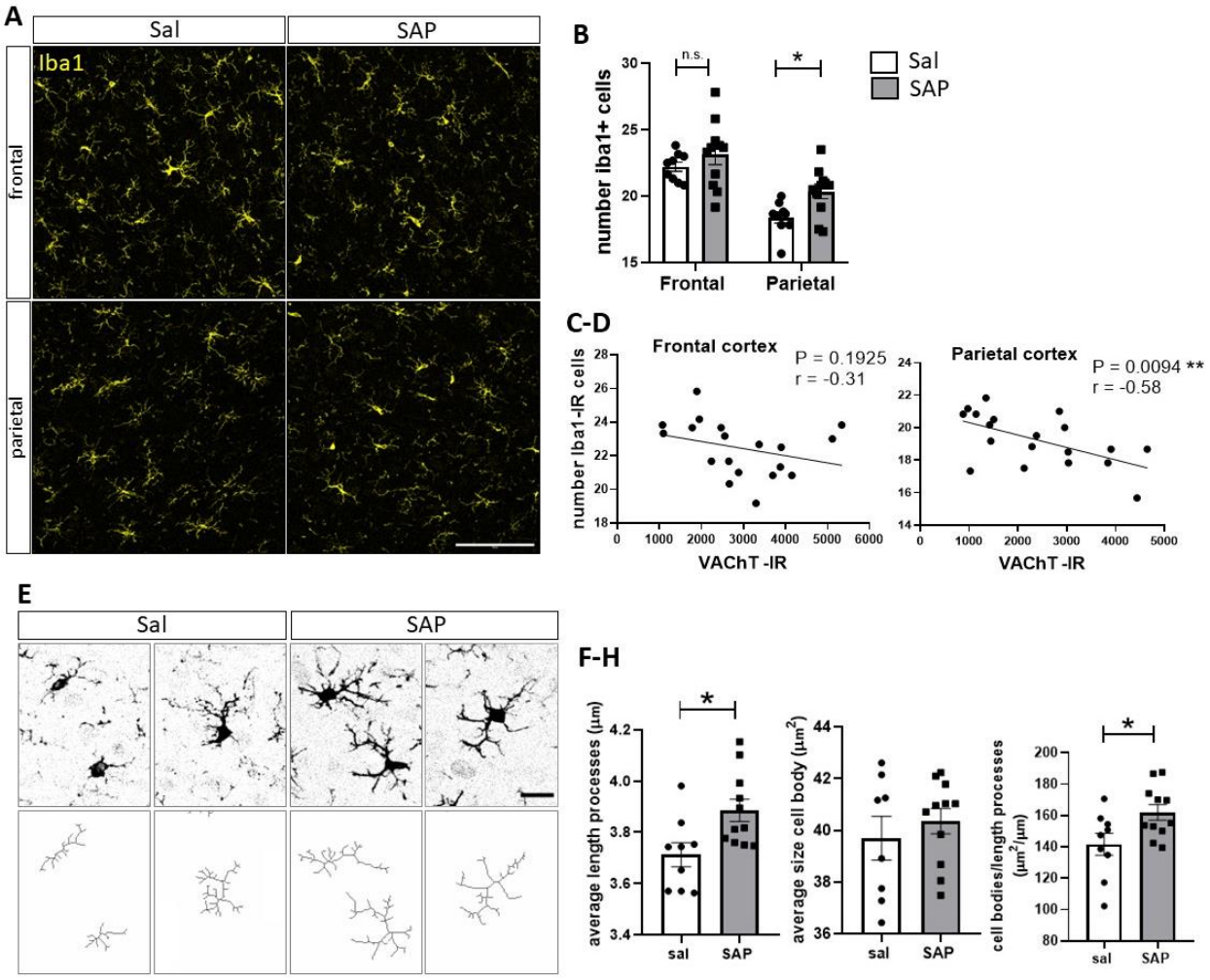


Figure 3-5. Loss of cholinergic synapses promoted microglia cells recruitment, correlated and showed morphological changes in the parietal cortex.

(A) Representative micrographs of Iba-1 in the frontal and parietal cortices, layer V (Scale bar 100 µm). (B) An increased number of Iba-1-IR cells was found in the parietal cortex (p= 0.0406, t = 2.428, DF = 36.00), but not in the frontal cortex (p = 0.5452, n.s., t = 1.114, DF = 36.00); in the SAP-immunolesioned group compared to the Sal-treated group. Mixed effect analysis; main effect cortical region (p <0.0001, F (1, 18) = 54.84); main effect immunolesion (p = 0.0476, F (1, 18) = 4.518); interaction (p=0.2499, F (1, 18) = 1.414). (C,D) VAChT-IR in the parietal cortex, but not in the frontal, showed a significant correlation with the number of Iba-1-IR cells (frontal, p = 0.1925, R^2=0.09776; Parietal: p = 0.0094, R^2= 0.3353). (F-H) Microglia in the SAP-immunolesioned group showed an increase in processes average length (p = 0.0147, t=2.697,

df=18) compared to the control group, and no differences were found between the groups when looking at the cell body size (p= 0.4827, t=0.7177, df=17) and the overall length of skeletonized microglia (p = 0.1878, t=1.369, df=18). (E) Representative images of microglia and their skeletonization (scalebar 20 μm). Data analysis: Sal rats, n = 9; SAP rats, n = 11. Pearson r, unpaired t-test and Mixed effect analysis followed by Bonferroni's multiple comparisons was used. Abbreviations: Sal, saline-injected; SAP, 192-IgG Saporin injected, VAChT, Vesicular acetylcholine transporter; Iba1, Ionized calcium-binding adaptor molecule 1.

3.4 Discussion

Brain activity and blood flow are strictly related: when the activity of a certain brain region rises, it also increases the blood flow to that region, allowing substrate delivery and the removal of metabolic products (reviewed in (Iadecola, 2004)). In the context of AD, hypoperfusion could be consequential to several mechanisms, such as the constriction of brain arterioles (Niwa et al., 2001); loss of vascular density (Farkas and Luiten, 2001) and changes in the neurovascular coupling (Hamel, 2006). In addition, Aβ and tau seem to play a key role in NVU dysfunction (Schaeffer and Iadecola, 2021).

Following the *vascular-cholinergic hypothesis* in AD (De la Torre and Mussivan, 1993, de la Torre, 2004), suggesting that the loss of BFCNs contributes to the etiology and AD progression, we postulated that the association between BFCNs dysfunction and cerebral hypofusion is mediated by a disruption of the cortical NVU structure. Indeed, previous studies on BFCNs using IgG-SAP immunolesion in rats, showed a global decrease (24-40%) in CBF in the parietal and temporal regions (Waite et al., 1999), this was also confirmed by studies utilizing muscarinic antagonists (Elhusseiny and Hamel, 2000) and mAChRs type 5 knock-out mice (Yamada et al., 2001a).

The interactions among NVU cell types are important for the CBF, and also for a variety of physiological processes such as angiogenesis, vessel maintenance and permeability, and metabolic support (reviewed in (McConnell et al., 2017, Schaeffer and Iadecola, 2021)). Studies performing intracerebroventricular short-term IgG-SAP immunolesion (1.5 months) did not find alteration of the NVU structure (Nizari et al., 2019), although they did observe a loss of CBF response and an accelerated pathology in a transgenic mouse model with cerebral amyloid angiopathy (Nizari et al., 2021).

Here, we were interested to see whether seven months after selective cholinergic nb depletion affected the cortical NVU structure. A change in the diameters of capillaries and smaller arterioles was found to be accompanied by a decrease of VEGF-A. Additionally, the nb immunolesion increased the number of astrocytes and microglia in the cortex. Astrocytic end-feet showed higher colocalization with arterioles; on the other hand, microglia cells in the parietal cortex correlated with cholinergic loss and showed morphological changes indicative of an intermediate state of activation.

3.4.1 Effect on vessels and VEGF-A

The tone generated by vascular SMCs in arteries depends on several factors, including artery length, local levels of metabolites, signalling molecules, communications with other cells, and neural inputs (Secomb, 2008). Although we did not find differences in the average diameter of the medium and larger size arterioles, we found an increase in diameter in the pre-capillary arterioles and a reduction of the diameter of the capillaries in the SAP-immunolesioned group compared to the controls. This opposite effect on the vascular tone in the smaller vessels could be explained by local autoregulatory adjustments to the intravascular pressure changes. This upstream and downstream coordination between continuous vascular segments is important to regulate the flow avoiding a flow steal from adjacent vascular territories (Schaeffer and Iadecola, 2021).
The overall effect on the vessels could be mediated by BFCNs through different mechanisms that are not mutually exclusive: (1) BFCNs can directly regulate vasodilatation through the release of the potent vasodilator NO, from endothelial cells (eNO) (Zhang et al., 1995) or (2) indirectly, inducing selective activation of vasoactive intestinal peptide (VIP) and somatostatin neurons (Kocharyan et al., 2008, Cauli et al., 2004), which have been shown to be lost following IgG-SAP immunolesion (Zhang et al., 1998).

An additional indirect mechanism of vascular tone regulation (3) is through the release of VEGF-A (Inada et al., 2014, Kimura et al., 2018) expressed by the vascular endothelium, astrocytes and neurons (reviewed in (Ruiz de Almodovar et al., 2009)). Indeed, VEGF mRNA expression has been shown to be increased *in vitro* following treatment with the AChEI tacrine given to primary neurons and the cholinergic agonist carbachol in primary astrocytes (Kimura et al., 2018) and *in vivo* in medial septal cholinergic neurons following daily administration of tacrine for 7 days (Kimura et al., 2018). Interestingly, the expression and protein levels of VEGF-A were reduced in cortical homogenates of SAP-immunolesioned rats compared to the Sal-group.

Although we have previously shown a loss of glutamatergic and GABAergic markers in SAP-immunolesioned rats (Orciani et al.), their effect on the vasculature seems to be downstream of ACh as demonstrated by a pharmacological study in which blockade of mAChRs and either NMDA or GABA receptors had no additive attenuating effect (Lecrux et al., 2012).

3.4.2 Effect on astrocytes

Multiple mAChRs exist on perivascular astrocytes, and the participation of these cells in the CBF can be mediated by ACh. ACh is responsible for releasing epoxyeicosatrienoic acids (EETs), a product of the P450 expoxygenase pathway, selective for astrocytes (Lecrux et al., 2012), and two specific gliotoxins were able to decrease the ACh-induced CBF (Lecrux et al., 2012).

Astrocytes provide their perivascular end-feet, representing a link between neurons and the vasculature, which is visualized with AQP-4. In the SAP-immunolesioned rats, overall levels of AQP-4 did not change, but an increased colocalization between AQP-4 and α-SMA, a marker of arteries, was found. This suggests that astrocytes may repolarize their projections on the vessels as a gain-of-function or loss-of-function effects. Indeed, AQP-4 is shown to be involved in the blood-brain permeability to water and a myriad of other crucial physiological conditions (Valenza et al., 2020). In addition, astrocytic end-feet have been shown to be involved in glucose transport (Rouach et al., 2008) and CBF modulation following the elevation of carbon dioxide levels in the blood (Howarth et al., 2017).

It cannot be excluded that the increase of astrocytes in the cortical areas results from the pro-inflammatory response mediated by lack of ACh. Indeed, the cholinergic system also regulates inflammation through the cholinergic anti-inflammatory pathway, primarily mediated by the alpha-7 nicotinic acetylcholine receptor (α7-nAChR) (Gamage et al., 2020, Piovesana et al., 2021, Reale and Costantini, 2021).

3.4.3 Effect on microglia

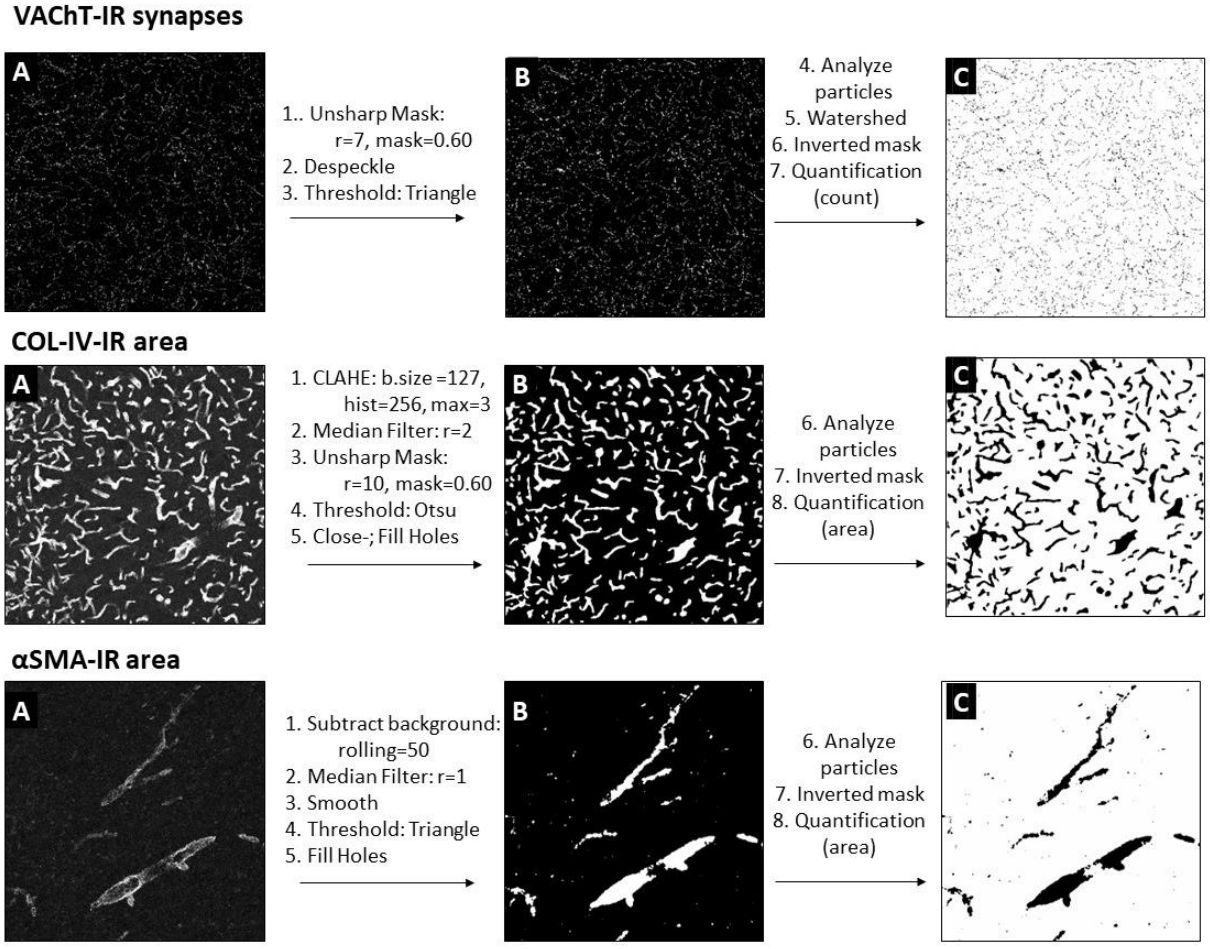
Intracerebroventricular administration of IgG-SAP resulted in microglia activation in both the septum and hippocampus 7 days (Roβner et al., 1995, Seeger et al., 1997) and 1.5 months after injection of the immunotoxin (Dobryakova et al., 2019). For the first time, we reported an increased amount of microglia, after 7 months post-nb immunodepletion. Microglia are a very well-known player in the inflammatory response, and ACh has been proposed to be a neurotransmitter with anti-inflammatory properties in the CNS, as demonstrated in the peripheral nervous system (Conejero-Goldberg et al., 2008, Pavlov and Tracey, 2005). Microglia cells can display different morphological states, and interestingly, we found a morphological change of

microglia corresponding to an intermediate state of activation (Davis et al., 2017, Flores-Aguilar et al., 2022), acquired before reaching an amoeboid shape, indicative of a full activation state. Unfortunately, the majority of studies assessing interactions between microglia and the vasculature are in the context of disease and in the healthy brain less is known (Mondo, 2020). Recently, a subgroup of juxtavascular microglia expressing purinergic receptor P2Y, G-protein coupled, 12 (P2RY12), has been shown to mediate chemotaxis whenever a BBB breakdown occurs (Lou et al., 2016). These microglia dynamically establish direct, purinergic contacts with endothelial cells, SMCs, pericytes, and astrocytes in both the mouse and the human brain modulating blood flow, neurovascular coupling, and hypoperfusion (Császár et al., 2022).

3.5 Acknowledgments

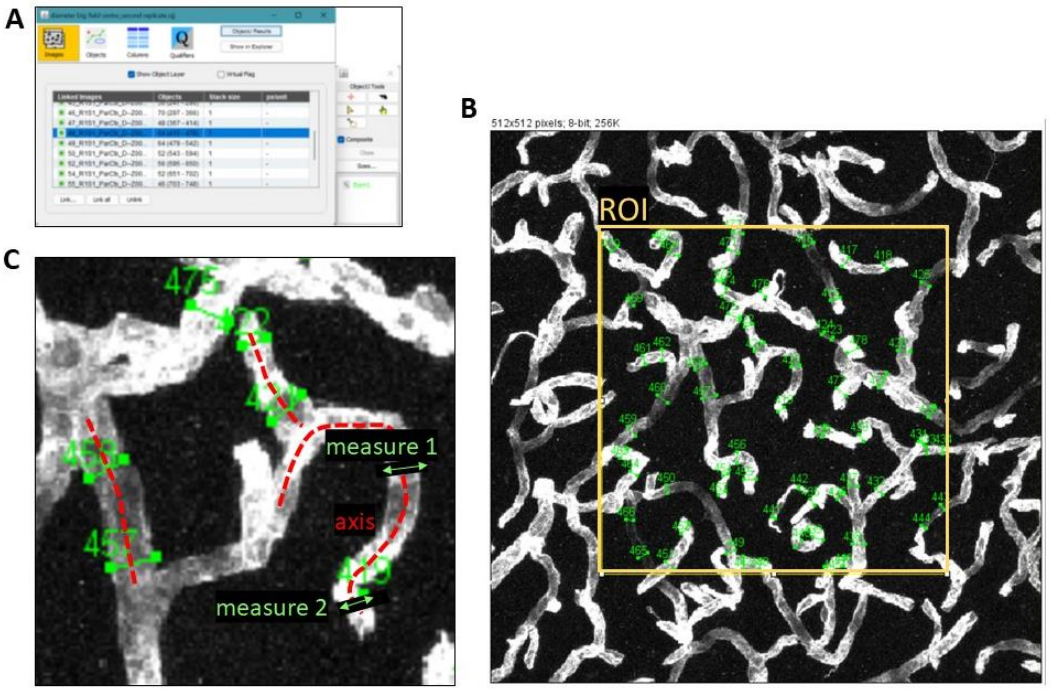
We acknowledge the McGill microscope facility Advanced BioImaging Facility (ABIF) Joel Ryan, for the data analysis and the technical support. CO was the holder of the Associazione Levi-Montalcini fellowship. MKF was the recipient of a CIHR Doctoral Award. SDC is the holder of the Charles E. Frosst/Merck Research Associate position. ACC is the holder of the McGill University Charles E. Frosst/Merck Chair in Pharmacology and a member of the Canadian Consortium of Neurodegeneration in Aging.

3.6 Supplemental figures



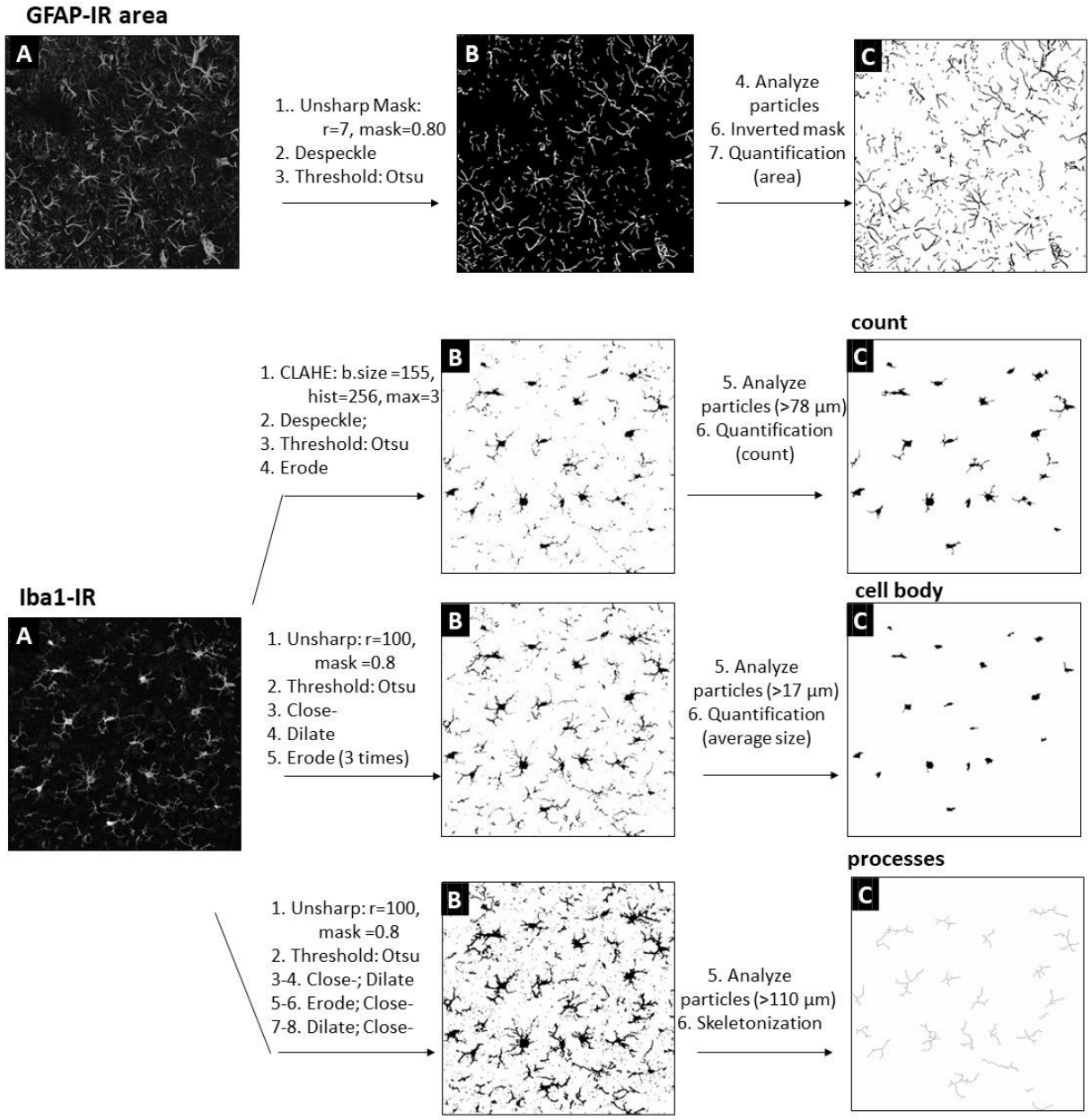
Supplemental figure 3-1. Flowchart illustrating the major steps employed in the quantification of VAChT-IR, COL-IV-IR and αSMA-IR.

Far right panels (C) indicate area images where binary black pixels were quantified. Abbreviations: VAChT, Vesicular acetylcholine transporter; COL-IV, collagen IV; αSMA, α-smooth muscle actin.



Supplemental figure 3-2. The diameter of capillaries assessed with Object J.

(A) interface of Object J (Fiji ImageJ). (B) The diameter of all vessels was measured in a defined region of interest (ROI, 183x183 µm, in *yellow*) with the same positioning in all images. The overall number of vessels measured for each image ranged between 32 and 70 (in *green*, the progressive number of measurements). (C) Magnification shows that the diameter was manually measured, averaging two measurements for each vessel segment perpendicular to their axes (in *red*). Subsequently, only vessels with a diameter of ≤ 10 µm were considered capillaries, in agreement with the existing literature on rodent brains (Stefanovic et al., 2008, Bennett et al., 2018). Abbreviations: ROI, region of interest.



Supplemental figure 3-3. Flowchart illustrating the major steps employed in the quantification of GFAP-and Iba1-IR cells.

Far right panels (C) indicate area images where binary black pixels were quantified. Abbreviations: GFAP; Glial fibrillary acidic protein; Iba1, Ionized calcium-binding adaptor molecule 1.

Connecting text: Chapter 3 to 4

In Chapter 3, we assessed the importance of basal forebrain cholinergic neurons in the context of the cortical vasculature studying the effect of a long-term specific cholinergic immunolesion of the nucleus basalis (nb) on the neurovascular unit.
Our findings demonstrated that cholinergic nb loss reduced the average diameter of cortical vessels and the expression of vascular endothelial growth factor A. In addition, we assessed that a long-term nb loss increased the density of astrocytes and microglia in the cortex. Astrocytic end-feet showed higher colocalization with arterioles following cholinergic immunolesion. In addition, microglia cells in the parietal cortex showed morphological changes indicative of an intermediate state of activation. These results indicate that cholinergic input affects cells also belonging to the neurovascular unit.

Furthermore, we explored whether a new cholinergic therapy has the potential to prevent an AD-like pathology in transgenic rats. The selective allosteric M1 muscarinic and sigma-1 receptor agonist AF710B takes advantage of that acetylcholine postsynaptic M1 muscarinic brain receptor levels to remain unchanged in AD. Selectivity for this receptor would avoid adverse effects due to stimulation of other muscarinic receptors outside the CNS. Additionally, its stimulation is capable of modulating amyloid pathology. The next chapter of this thesis will present whether this compound could prevent AD-like hallmarks in a transgenic rodent model of amyloid-like pathology.

CHAPTER 4

An M1 and sigma-1 receptor agonist prevents cognitive decline and Alzheimer's disease-like hallmarks in a transgenic rat model

Chiara Orciani, Sonia Do Carmo, Morgan K Foret, Helene Hall, Quentin Bonomo, Agustina Lavagna, Chunwei Huang, Augusto Claudio Cuello

Manuscript to be submitted

Abstract

The selective allosteric M1 muscarinic and sigma-1 receptor agonist, AF710B (aka ANAVEX3-71) takes advantage of the fact that the brain levels of acetylcholine postsynaptic M1 muscarinic receptors remain unchanged in AD. We have previously shown that AF710B treatment attenuated AD-like hallmarks in McGill-R-Thy1-APP transgenic rats when administered at advanced stages of the AD-like pathology. It remains unknown whether preventive strategies, delivered prior to the extensive disease pathology, may be more effective than treatment administered in later stages.

Therefore, we tested whether daily oral administration of AF710B (10 µg/kg) to seven-month-old, preplaque, McGill-R-Thy1-APP rats during seven months could prevent cognitive impairment as well as some of the AD-like pathological hallmarks.

Long-term AF710B treatment restored the cognitive abilities of McGill-R-Thy1-APP rats. These effects were accompanied by a reduction in the number of mature and diffuse amyloid plaques in the cortex and in the levels of Aβ42 and Aβ40 peptides. AF710B treatment also reduced microglia and astrocyte recruitment towards CA1 hippocampal Aβ-burdened neurons compared to vehicle-treated McGill-R-Thy1-APP rats and reduced microglia activation, promoting the release of anti-inflammatory cytokines and reducing pro-inflammatory cytokines. Lastly, AF710B treatment rescued the conversion of Brain-Derived Neurotrophic Factor precursor (proBDNF) to its mature form (mBDNF).

The long-lasting effect of AF710B in preventing cognitive decline of McGill-R-Thy1-APP rats and reducing the AD-like amyloid pathology, after a wash-out period (of 4 weeks) suggest preventive, disease-modifying properties of the compound.

4.1 Introduction

Basal forebrain cholinergic neurons (BFCNs) provide the major source of cholinergic innervation to the forebrain (Mesulam et al., 1983b, Mesulam et al., 1983a, Bigl et al., 1982, Struble et al., 1986) and play a crucial role in learning, memory and attention mechanisms (Drachman and Leavitt, 1974, Bartus, 1979, Everitt and Robbins, 1997, Ballinger et al., 2016, Zaborszky et al., 1999). The gradual degeneration of BFCNs in ageing, and their severe atrophy and loss in Alzheimer's disease (AD) pathology (Bowen et al., 1976, Davies and Maloney, 1976, Whitehouse et al., 1981), contribute significantly to cognitive impairment in this neurodegenerative condition. These observations led to the formulation of the cholinergic hypothesis (Bartus et al., 1982, Coyle et al., 1983, Cuello and Sofroniew, 1984, Francis et al., 1999). As a result of several years of research focused on developing cholinergic drugs as a treatment for AD (reviewed in (Giacobini et al., 2022)), four acetylcholinesterase inhibitors (AChEIs) were approved by the Food and Drug Administration. AChEIs, which increase cholinergic transmission by inhibiting acetylcholine (ACh) breakdown, gained recent attention since they have been shown to delay disease progression rather than being solely symptomatic drugs (Giacobini et al., 2022). On the other hand, AChEIs have limited efficacy, given the progressive atrophy and loss of cholinergic neurons in AD (White et al., 1977, Perry et al., 1977b, Perry et al., 1977a, Davies and Maloney, 1976, Richter et al., 1980).

The postsynaptic M1 muscarinic receptors (M1 mAChRs) have the highest potential to be a preclinical target amongst the CNS cholinergic receptors (Fisher, 2008) since they are mostly located in the brain (Levey, 1993), remain intact in AD (Araujo et al., 1988) and modulate amyloid precursor protein (APP) processing (Nitsch et al., 1992, Davis et al., 2010). Interestingly, M1 stimulation activates two transduction pathways: the protein kinase C (PKC)-dependent and mitogen-activated protein kinase (MAPK)-dependent pathways. These pathways operate in parallel to stimulate α-secretase, enhancing the secretion of neuroprotective αAPPs peptides (Haring et al., 1998, Jones et al., 2008, Müller et al., 1997, Shirey et al., 2009) and decreasing Aβ deposition (Beach et al., 2003), ultimately shifting APP processing towards the non-amyloidogenic pathway (Fisher et al., 2016).

AF710B, currently known as ANAVEX3-71, is a selective allosteric M1 muscarinic and sigma-1 receptor (Sig-1R) agonist (Fisher et al., 2016). Sig-1R is an intracellular chaperone at the

endoplasmic reticulum (ER) that modulates calcium signalling (Hayashi et al., 2011, Hayashi and Su, 2007). Sig-1Rs translocate from the ER when cells are subjected to prolonged stress and regulate a variety of functional proteins (Su et al., 2010, Morin-Surun et al., 1999). Overall, Sig-1Rs activation has been implicated in neuroprotective, anti-amnestic and anti-inflammatory functions (Marrazzo et al., 2005, Villard et al., 2011, Jia et al., 2018, Pal et al., 2012). AF710B was shown previously to attenuate cognitive decline and reduce amyloid pathology and neuroinflammation in 3xTg-AD mice (Fisher et al., 2016) and McGill-R-Thy1-APP (McGill-APP) transgenic (Tg) rats (Hall et al., 2018). However, it remains unknown whether preventive strategies, delivered prior to the extensive disease pathology, may be more effective than treatment administered in later stages.

For this reason, we tested whether a 7-month chronic treatment with low-dose AF710B (10 µg/kg) starting at early amyloid pathology stages could prevent cognitive impairment, plaque deposition, and neuroinflammation in McGill-APP Tg rats. Additionally, we investigated NGF and BDNF, two neurotrophins known to be affected in the brains of AD and MCI patients (Pentz et al., 2020, Fahnestock et al., 2001, Bruno et al., 2009a, Peng et al., 2004, Cuello et al., 2019, Peng et al., 2005, Michalski and Fahnestock, 2003a, Hock et al., 2000).

4.2 Methods

4.2.1 Animals

McGill-R-Thy1-APP homozygous Tg rats (n = 22), express the human APP751 gene carrying the Swedish double mutation (K670M and M671L) and the Indiana mutation (V717F) under the control of the murine Thy1.2 promoter (Leon et al., 2010). This Tg rat model shows intracellular pathology at 7 months old, with the first amyloid plaques gradually appearing in the cerebral cortex and hippocampus at 13 months of age (Leon et al., 2010, Wilson et al., 2016, Iulita et al., 2014a, Flores-Aguilar et al., 2022). Age-matched wild-type (wt) rats (n = 22) were used as controls. The genotype was assessed by quantitative polymerase chain reaction (qPCR) as previously described (Leon et al., 2010). The animals were housed two per cage and kept on a 12-hour light/ dark cycle with ad libitum access to water and standard rodent diet. The McGill University Animal Care Committee approved all experimental procedures.

4.2.2 Experimental design and drug treatment

Seven-month-old rats were divided into four sex-balanced groups according to their genotype and treatment (AF710B or phosphate saline buffer (PBS)) administered orally to the animals daily (wt-sal = 12, wt-AF710B = 10, Tg-sal = 10, Tg-AF710B = 12) (**Figure 4-1, Supplemental figure 4-1 A**). The dose of AF710B (10 µg/kg) and the experimental design were chosen based on a previous study (Hall et al., 2018). Treatment lasted 7 months, with bi-weekly drug adjustment based on their weight (**Supplemental figure 4-1 B**). When the treatment ended, animals were given a wash-out period of 4 weeks where no drug or vehicle was administered, prior to behavioral testing. After completing behavioural testing, the animals were perfused at 15.5 months old, and their brains were preserved for histological and biochemical analyses.

4.2.3 Open Field

The open field (OF) task was performed in an open field arena (80x80 cm, 45 cm high), where the rats were placed for 3 minutes. Tracking paths and time spent in the centre and perimeter of the box were recorded using a video tracking software (EthoVision XT10.1; Noldus, The Netherlands).

4.2.4 New Object Location and Novel Object Recognition

The novel object location (NOL) and novel object recognition (NOR) tasks were performed 24 h after the OF, as described previously (Iulita et al., 2014a, Habif et al., 2021). Briefly, rats were exposed to five objects of various shapes and colors (familiarization phase). Subsequently, one of the objects was moved to a new location (NOL) and in the following session one object was replaced by a new, unfamiliar object (NOR). During each of the 2-minutes phases, time spent exploring each object was recorded by the experimenter and we quantified the % time spent exploring the object in the new location and the novel object compared to the familiar objects. A 15-minute interval separated each trial.

4.2.5 Social Preference

The social preference (SP) protocol of Yang and collaborators (2011) (Yang et al., 2011) was adapted for rats. The OF arena was separated in 3 chambers (80 cm x 26.6 cm x 45 cm each). In

both external chambers a grid cage (26 x 12 x 15 cm) was placed. An opening of 10 cm was cut in the centre of each cardboard separation to allow the rats to freely explore the 3 chambers (**Figure 4-2 C**). The subject rat was introduced in the arena facing the wall of the middle chamber and was allowed to explore for 2 minutes (habituation phase). During the testing phase, an unfamiliar rat was placed in one grid cage, while a rat-shaped object was placed inside the other grid cage and the subject rat was allowed to explore the chambers for 4 minutes. A maximum interval of 15 minutes was allowed between habituation and testing phase. During each phase, time spent exploring each grid cage was recorded and we quantified the % time spent on the unfamiliar rat compared to the rat-shaped object. Tracking paths were recorded using a video tracking software (EthoVision XT10.1; Noldus, The Netherlands).

4.2.6 Morris water maze

To assess spatial learning and memory, rats were trained in the Morris water maze task (MWM) as described previously (Hall et al., 2018), 48 hours after the SP task. Briefly, rats were trained for five consecutive days (four trials per day) to locate a platform (10 cm in diameter) submerged 2 cm under the water surface. In each trial, rats were released from a different starting point (east/west/north/south) and given 120 seconds to find the platform and climb onto it. The sequence of release positions was changed each day. A 20-minute interval separated each trial. On day 6, the platform was removed from the tank, and the animals were given a final spatial probe trial in which rats were allowed to swim *ad libitum* for 60 seconds. Latency to locate the hidden platform, swim speed, total distance swim, and swim paths were recorded using a video tracking software (EthoVision XT10.1; Noldus, The Netherlands), and the number of platform zone crossings were counted during the probe test.

4.2.7 Brain tissue collection

Upon completion of the behavioural test, rats were deeply anesthetized with a mix of chloral hydrate and sodium pentobarbital (6.5 mg and 3 mg, respectively, per 100 g of body weight) delivered by intraperitoneal injection and perfused transcardially with cold saline solution for 2 min. The brain was removed, and the cortex, hippocampus and cerebellum were dissected from one hemisphere. The tissue was flash-frozen and kept at −80 °C for qRT-PCR and Western blotting. The other hemisphere was post-fixed in 4% PFA at 4°C for 24 hours and transferred to

30% sucrose in 0.1 M phosphate buffer. The fixed hemisphere was sectioned on a microtome (Leica SM 2000R; Germany) into 40-μm thick sections and stored at -20 °C in a cryoprotectant solution of ethylene glycol and sucrose in PBS (pH 7.4).

4.2.8 Sample preparation for Aβ levels and inflammatory mediators

Cortical tissue (40-50 mg) was homogenized by sonication in 8 volumes TBS buffer (150mM NaCl, 50mM Tris-HCl, 5mM EDTA, pH 7.6) containing protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail, Roche). Homogenates were ultracentrifuged at 100,000g for 1 hour. Supernatants (soluble fraction) were collected and stored at -80C. The pellets were resuspended in 5 volumes of guanidine buffer (5M guanidine HCl, 50mM Tris HCl, pH 8.0), sonicated and incubated at room temperature for 3 hours. The samples were then ultracentrifuged at 100,000g for 1 hour after which the supernatants (insoluble fraction) were collected and stored at -80C. Protein concentration of the soluble and insoluble fractions were assessed using the DC Protein Assay (BioRad) according to the manufacturers' instructions.

4.2.9 Analysis of TBS soluble and insoluble human Aβ38, Aβ40 and Aβ42 peptides

Levels of human Aβ38, Aβ40, and Aβ42 in cortical brain tissue and plasma of Tg rats were analyzed using the MesoScale Discovery V-plex Aβ Peptide Panel 1 (6E10) kit (MesoScale Discovery, Rockville, MD) according to the manufacturers' protocol (as in (Martino Adami et al., 2016)). Soluble cortical fractions were pre-diluted to 4 ug protein/ul and assayed at a 1:2 dilution in Diluent 35. Insoluble cortical fractions were pre-diluted to 10 ug protein/ul and assayed at a 1:50 to 1:1000 dilution. Plasma-EDTA samples were assayed at a 1:4 dilution. All samples were assayed in duplicate. The electrochemiluminescence signal was quantified using a MesoScale Discovery SECTOR Imager 6000. Analyte concentrations were calculated in reference to calibrators for each individual analyte, as determined from a standard curve generated by the MSD Discovery Workbench software v. 4.0 (Meso Scale Discovery). The dynamic range for Aβ38 was 8.12-12200 pg/ml, for Aβ40 4.89-15300 pg/ml and for Aβ42 0.328-1710 pg/ml. Values were normalized to total protein input and expressed as pg Aβ peptide/mg protein.

4.2.10 Analysis of inflammatory mediators

Soluble cortical fractions, prepared as described above, were pre-diluted to 4 ug protein/ul and assayed at a 1:2 dilution in Diluent 42, in duplicate. Levels of 9 cytokines (IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, TNF-α) were measured using the MesoScale Discovery Proinflammatory Panel 2 (rat) kit and the SECTOR Imager 6000 (MesoScale Discovery, Rockville, MD) (as in (Wilson et al., 2018)). Cytokine concentrations were calculated from a standard curve generated by the MSD Discovery Workbench software v. 4.0. Values were normalized to total protein input and expressed as pg cytokine/mg protein.

4.2.11 Western blotting

Frontoparietal cortical samples (20 mg) were manually homogenized in lysis buffer (Cell Signaling, USA) containing a complete protease inhibitor cocktail (Roche, USA). Manual homogenization was followed by two 5 s pulses of sonication. The homogenates were centrifuged at 20,780 g, for 45 min at 4 °C, the supernatants were collected, and the protein concentration was determined by the Lowry *assay* (DC™ Protein Assay kit, BioRad, USA). The homogenates (50 ug) were mixed with Sample Buffer (Tris-HCl 250 mM, pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue) and boiled for 5 minutes. The samples were loaded on SDS-polyacrylamide gels and were transferred to PVDF membranes (BioRad, USA) for 1 h at 350 mA. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated with the primary antibodies directed against BDNF (1:2000, Ab108319, Abcam), proNGF (1:2000, ANT-005, Alomone), and GAPDH (1:10,000, MAB374, Millipore) in TBS-T overnight at 4 °C. Peroxidase-conjugated secondary antibodies (1:10,000, Jackson Immunoresearch), dissolved in TBS-T, were applied for 1 h at room temperature. Immunoreactions were developed with an enhanced chemiluminescence substrate (Western Lightning® Plus-ECL, PerkinElmer Inc., USA) and imaged with Amersham Imager 600 (GE Healthcare, USA) and ChemiDoc™ Touch system *(*Bio-Rad, Canada*)*. Densitometry was quantified with TotalLab CLIQS Software (TotalLab, UK) and ImageLab *(*Bio-Rad, Canada*)* and the values were normalized to GAPDH as a loading control. An additional sample was loaded in each gel as an internal loading control. The values were expressed as fold change relative to the control group. Three and two technical replicates were performed respectively for BDNF and proNGF.

4.2.12 McSA1 immunostaining and Thioflavin-S staining

Sections were washed with PBS and permeabilized using 50% ethanol for 20 minutes, followed by washes with PBS-T (containing 0.2% Triton-X-100), and blocked for 1 hour at RT in 10% NGS. The sections were then incubated with anti-Aβ McSA1 (1:1500, MediMabs) and anti-NeuN (1:1500, ABN90P, Millipore) antibodies overnight at 4 °C. Sections were washed in PBS-T and incubated with Alexa Fluor-conjugated secondary antibodies for 2 hours RT. Sections were then washed three times for 5 minutes in PBS-T, then were rinsed two times for 5 minutes in deionized water and incubated in Thioflavin-S (Thio-S, 0.1% solution in 50% EtOH) for 5 minutes at room temperature. Subsequently sections were rinsed two times in 50% EtOH for 2 minutes and in PBS three times for 10 minutes. Sections were mounted on Gelatin Coated Slides and Coverslipped with Aqua Polymount.

4.2.13 Immunofluorescence (IF)

Free-floating sections were washed using PBS and underwent heat-mediated antigen retrieval. Sections were incubated at 80 ºC in 10 mM citrate buffer (pH 6.0) for 30 minutes, then cooled for 20 minutes at room temperature. They were then washed with PBS and permeabilized using 50% ethanol for 20 minutes, followed by washes with PBS-T (containing 0.2% Triton-X-100), and blocked for 1 hour at RT in 10% normal goat (NGS) or normal donkey (NDS) serum. The sections were then incubated with primary antibodies for 48 hours at 4 ºC: anti-GFAP (1:2000, SPM507, Novus Biologicals); anti-NeuN (1:1000, ABN90P, Merck), anti-Iba1 (1:1000, 019-19741, Wako), anti-VAChT (1:1500, 139 103, Synaptic Systems), anti-vGluT1 (1:200, MediMabs), anti-TrkA (1:120, AF1056-SP, R&D). After primary antibody incubation, sections were washed in PBS-T, and incubated with Alexa Fluor 488 (goat or donkey anti-mouse), Alexa Fluor 568 (goat or donkey anti-rabbit), and Alexa Fluor 647 (goat-anti-guinea pig or donkey-anti-goat) (all at 1:800, Thermo Fisher Scientific) for 2 hours room temperature. Following washes, sections were incubated for 5 minutes with 0.3 Sudan black in 70% ethanol to reduce autofluorescence. Sections were then washed three times for 5 minutes in PBS-T, then three times for 5 minutes in PBS before mounting onto subbed coated slides and coverslipped with Aqua-Poly/Mount (Polysciences).

4.2.14 Confocal imaging and analyses

For Thio-S-McSA1-NeuN staining, images were acquired using a 20x HC LP APO objective with a confocal microscope Leica TCS SP8 (Leica, Canada) using the LAS X Software (Leica, Canada). We collected seven images per animal for CA1, six images for the cortical layer V and one image for the subiculum within two subject replicates. The of 582.39 × 582.39 µm images were collected in Z-stacks with an optical section of 0.896 µm, an interval of 1.5 µm and a resolution of 512 × 512 pixels.

For VAChT-vGluT1-TrkA staining, images were acquired using a 63x oil HC LP APO objective objective with a confocal microscope Leica TCS SP8 (Leica, Canada). We collected six images per animal for CA1 within two subject replicates. The of 184.71 × 184.71 µm images were collected in Z-stacks with an optical section of 0.896 µm, an interval of 1.0 µm and a resolution of 1024 × 1024 pixels.

For Iba1-GFAP-NeuN staining, images were acquired using a 20x Plan Apochromat objective lens (NA = 0.80) with a LSM800 Confocal Microscope AxioObserver (Zeiss, Germany) using ZEN Imaging software (ZEN Black). Six images per animal were collected in CA1 (three images per section) using Z-stacks at an interval of 1 µm. Sixteen-bit images of 523.4 × 523.4 µm were acquired with a pixel dwell of 0.76 µs and an averaging of two by line.

For all imaging, the signal was detected with emission wavelengths of 500-550 nm (488 laser), 571-620 nm (561 laser), and 650-700 nm (640 laser). Images were subsequently imported to Fiji software (ImageJ), and slices were merged into one stack (stack/Z projection/Maximum Intensity Projection) for each channel. The immunolabelling of McSA1 plaques was quantified using the integrated density value in the McSA1-IR area that does not overlap with the NeuN-IR area using a home-built macro for ImageJ as in (**Supplemental figure 4-3 A**). The area of the plaques Thiof-S-positive is described in (**Supplemental figure 4-3 B**). The overall amount of Iba1-IR and GFAP-IR cells and the one in the proximity of NeuN is described in (**Supplemental figure 4-4)**; the Iba1-IR cell morphology in NeuN proximity is in (**Supplemental figure 4-4)**. For the Vesicular acetylcholine transporter (VAChT), the vesicular glutamate transporter 1 (vGluT1) and tropomyosin receptor kinase A (TrkA) the images were analyzed as shown in (**Supplemental figure 4-6 C)**. Images were processed and analyzed after blinding to treatment.

4.2.15 Statistical analysis

The software GraphPad Prism v8 (La Jolla, CA, USA) was used for statistical analysis. Data normality was verified with the D'Agostino-Pearson normality test. A two-tailed unpaired t-test was used for 2-group comparisons. The rats that did not explore at all (NOL, NOR) or had difficulty to swim (MWM) were removed from the analysis. For 4-group comparisons, 2-way-ANOVA followed by multiple comparisons analysis (Tukey) was applied and a mixed-effect analysis was performed, instead of ANOVA, when some values were missing. If data normality was violated, the analysis was performed with non-parametric tests or the data were transformed as reported in the legend of each figure. Significance was set at $p < 0.05$.

4.3 Results

4.3.1 AF710B prevents cognitive decline in McGill-APP rats

Following 7 months of drug treatment, and after a 1-month wash-out period, rats were tested in a battery of behavioural tests (**Figure 4-1**). The Open Field test showed no locomotor activity differences between the groups (**Supplemental figure 4-1 A-C**). In the NOL and NOR tests, rats typically spend more time exploring the object in the new location (NOL) and the novel object (NOR), based on a natural preference for novel location and object over familiar ones (Antunes and Biala, 2012). Although Tg rats were not impaired in the NOL task compared to the wt rats (**Supplemental figure 4-1 D, E**), when one of the familiar objects was substituted with a new object, Tg-sal spent significantly less time exploring the novel object compared to the wt-sal (**Figure 4-2 A,B**). In contrast, Tg-AF710B rats performed similarly to the Wt-sal animals (**Figure 4-2 A, B**).

In the SP task, the time rats spend interacting with an unfamiliar rat rather than exploring a rat-shaped object is measured (Yang et al., 2011) (**Figure 4-2 C**). Tg-sal rats showed a 16.6 % average less interaction time with an unfamiliar rat than the wt-sal (**Figure 4-2 D**). This deficit was rescued in Tg-AVAVEX, which spent 18.8% more time interacting with the unfamiliar rats compared to the Tg-sal (**Figure 4-2 D**). In addition, the overall time of exploration of either the unfamiliar rat and the object was significantly increased in the Tg-AF710B group compared to the Tg-sal (**Figure 4-2 E**). These findings are further illustrated by the cumulative heatmap paths obtained during the SP task (**Figure 4-2 F**).

In the MWM task, the rats' performance improved over the days of the acquisition phase, as demonstrated by a decrease in escape latency (**Figure 4-2 G, H**). Significant differences in learning were found across the groups in the last three days of acquisition and the escape latencies were averaged. As a result, in the last three days of acquisition, the Tg-sal required an average of 58 s to locate the hidden platform compared to the wt-sal, which required 36 s (**Figure 4-2 I**) and the Tg-AF710B performed significantly better than the Tg-sal, with an average latency of 40 s (**Figure 4-2 I**). In the probe test, we measured the number of platform zone crossings after removing the

hidden platform. Treatment with AF710B rescued the deficit observed in the Tg-sal (**Figure 4-2 J**).

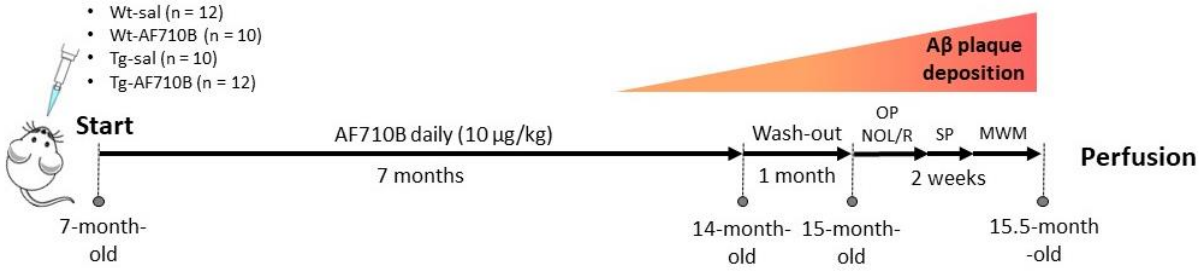


Figure 4-1. Schematic representation of the experimental design.

AF710B or saline was delivered to 7-month-old rats for 7 months. Following 1 month of wash-out, rats were submitted to behavioural testing before being euthanized at 15.5 months.

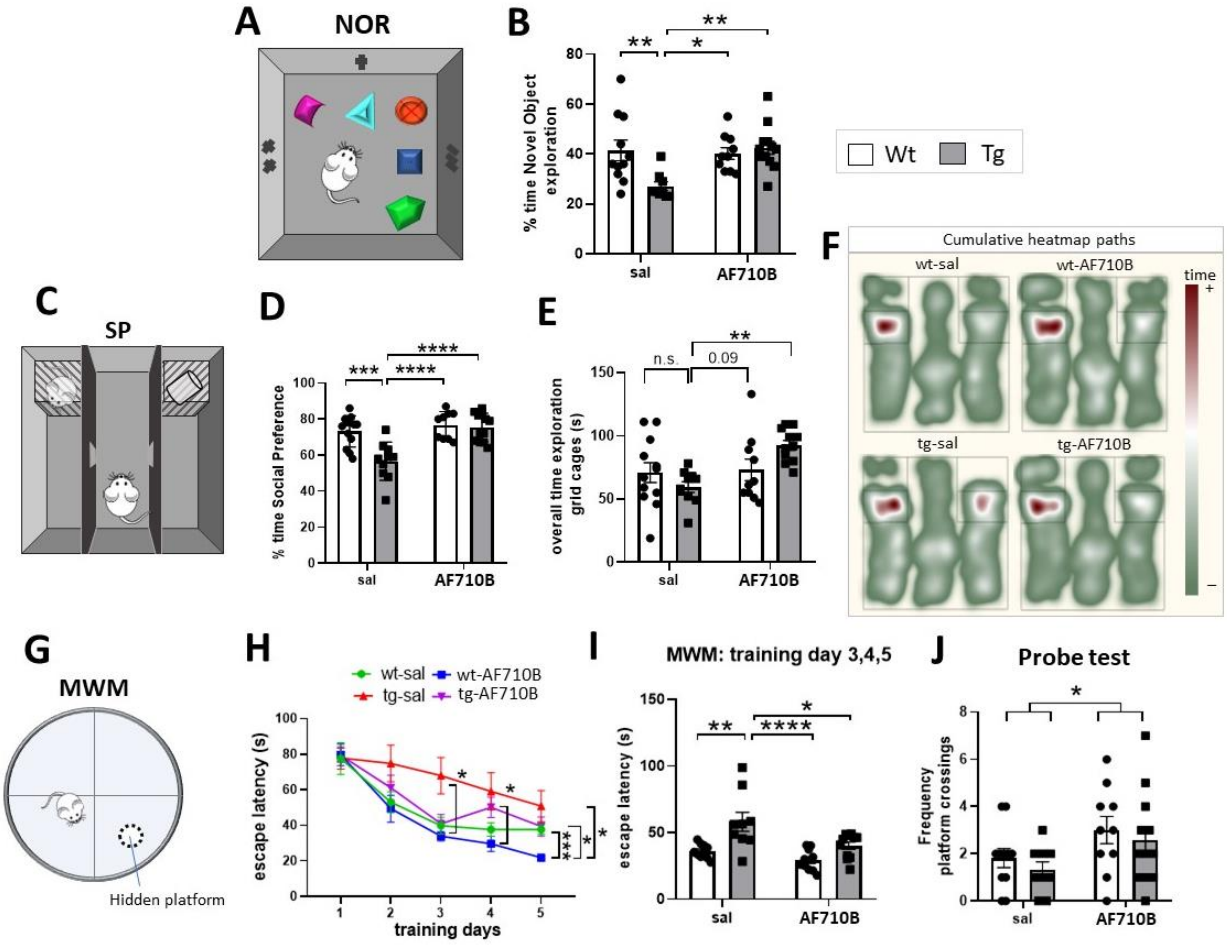


Figure 4-2. AF710B prevented cognitive impairment in McGill-APP rats.

(A, B) In the NOR, Tg-sal was significantly impaired compared to the wt-sal (P=0.0099, Df = 38.00). The impairment was rescued in the Tg-AF710B (P=0.004, Df = 38.00), and a significant effect was also present in the wt-AF710B compared to the Tg-sal (P=0.025, Df = 38.00). (2x2 ANOVA: treatment: P=0.022, F (1, 38) = 5.664; genotype: P=0.050, F (1, 38) = 4.112; interaction: P=0.008, F (1, 38) = 7.853)).

(C, D) Tg-sal group was impaired compared to the wt-sal in the SP test (P<0.001, Df = 39.00) and this deficit was rescued in the Tg-AF710B (P<0.0001, Df = 39.00). Also, Tg-sal performed worst compared to the wt-AF710B (P<0.0001, Df = 39.00). (2x2 ANOVA: treatment: P<0.001, F (1, 39) = 16.93; genotype: P=0.002, F (1, 39) = 10.76; interaction: P=0.007, F (1, 39) = 8.171). (E,F) The overall time exploring either the rats or the object was significantly higher in the Tg-AF710B compared with the Tg-sal (P=0.006, Df = 39.00), as also expressed by the representative cumulative heatmap paths (2x2 ANOVA: treatment: P=0.011, F (1, 39) = 7.206; genotype:

P=0.544, F (1, 39) = 0.3743; interaction: P=0.024, F (1, 39) = 5.530). (G, H) In the MWM, Tg-sal performed significantly worst on days 3 and 4 compared to wt-AF710B (respectively, P = 0.038, Df = 10.22; P = 0.045, Df = 19.26). In day 5, Tg-sal performed worst than Tg-AF710B (P = 0.036, Df = 13.15), wt-sal (P = 0.001, Df = 16.25) and wt-AF710B (P = 0.044, Df = 8.605). (I) In the average of the last 3 days of training, sal-Tg group was impaired compared to the sal-wt (P = 0.002, Df = 36.00), and Tg-AF710B showed a significant improvement compared to the Tg-sal (P = 0.012, Df = 36.00). In addition, Tg-sal performed significantly worst than wt-AF710B (P <0.0001, Df = 36.00). (2x2 ANOVA: treatment: P = 0.003, F (1, 36) = 10.20; genotype: P< 0.001, F (1, 36) = 17.54; interaction: P= 0.161, F (1, 36) = 2.046). (J) The probe test showed a significant difference in the treatment effect (2x2 ANOVA, P = 0.020, F (1, 38) = 5.917) (genotype: P= 0.373, F (1, 38) = 0.8132; interaction: P= 0.946, F (1, 38) = 0.004651). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test.

Altogether, long-term treatment with AF710B could prevent the cognitive decline of the Tg rats, as observed in the NOR, SP and MWM tasks. This effect was sustained after 1 month of drug discontinuation, highlighting the therapeutic disease-modifying effects of AF710B.

4.3.2 AF710B prevents extracellular Aβ deposition in McGill-APP rats

The McSA1 immunoreactivity in CA1 of hippocampus and cortical pyramidal layer V in McGill-APP rats starts intraneuronal and with the progression of amyloid pathology is accompanied by the widespread presence of amyloid plaques (Leon et al., 2010, Iulita et al., 2014a). The intensity of the extraneuronal McSA1-IR of Tg-treated with AF710B was significantly lower in CA1 (**Figure 4-3 A, C**) in the cortex (**Figure 4-3 A,D**) compared to the Tg controls. In this analysis, the proportion of rats in each group with extracellular Aβ pathology was assessed. The majority of Tg-sal (90%) showed at least one plaque in CA1 and 50% of Tg-sal showed extracellular pathology in the cortex (**Figure 4-3 B**). In contrast, in the Tg-AF710B group, only 33% of rats showed extraneuronal Aβ pathology in the CA1 and only 8% in the cortex (**Figure 4-3 B**).
Thio-S-stained mature amyloid plaques were found in the subiculum but not in the cortex and hippocampus in the Tg-Sal, and AF710B-treatment led to a reduction in the number of Thio-S-positive plaques (**Figure 4-3 A, E**). Furthermore, protein levels of the three Aβ isoforms (**Figure 4-3**) were measured in the soluble and insoluble fraction of cortical homogenates. Decreased levels of the more toxic, soluble and insoluble, Aβ42 were detected in the Tg-AF710B compared with the Tg-sal (**Figure 4-3 F,G**) as well as a significant reduction of the insoluble Aβ40 (**Figure 4-3 H**). Interestingly, the ratio Aβ42/Aβ40, was significantly higher in the Tg-sal compared to the Tg-AF710B (**Figure 4-3 J**). No changes were detected between the two groups in the levels of Aβ38 (**Supplemental Figure 4-3 D).**

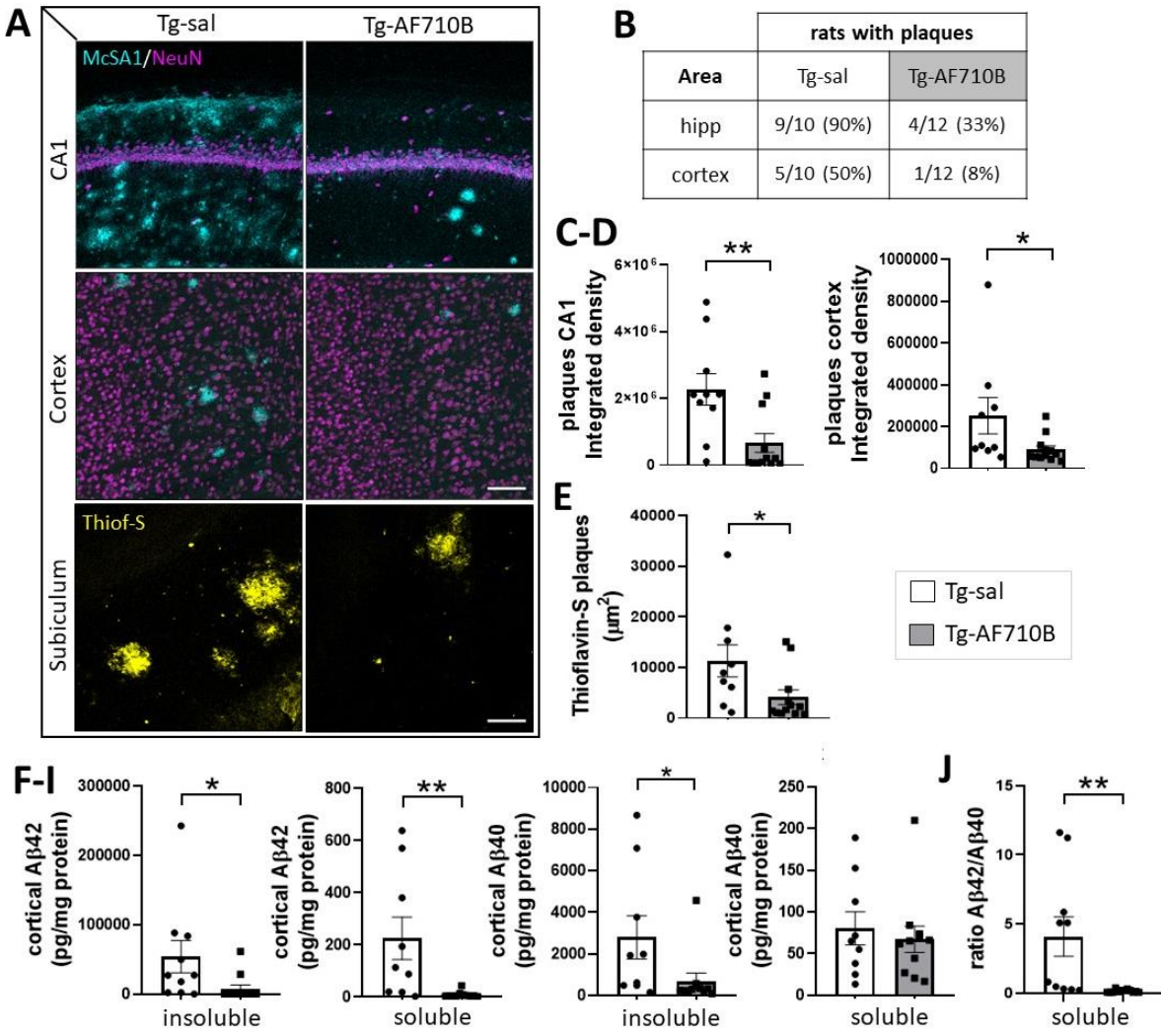


Figure 4-3. AF710B reduced amyloid pathology in McGill-APP rats.

(A) Representative images of IF for a McSA1 and NeuN in cortex and hippocampus (top) and Thiof-S staining of the subiculum (bottom) from Tg-sal and Tg-AF710B rats (scale bar 100 µm). (B) Summary table of the number of rats with plaques in the cortex and hippocampus. (C-E) Tg-AF710B showed a significant decrease in diffuse plaques signal intensity in the hippocampus (P = 0.003, U = 17), and cortex (P = 0.023, U = 22), as well as a significant decrease of Thiof-S positive plaques in the subiculum (P = 0.018, U = 21), compared to the Tg-sal. (F-I) Tg-AF710B showed a significant reduction in the insoluble and soluble fraction Aβ42 (respectively, P = 0.014, U = 23 and P = 0.003, U = 12) and a significant decrease of insoluble Aβ40 (P = 0.016, U = 18), without changes of the soluble fraction (P = 0.824, U = 46) compared to the Tg-sal. (J) The ratio

of the soluble Aβ42/40 was increased in the Tg-sal compared to the Tg-AF710B (P = 0.006, t=3.063, df=20). Statistical analysis: Mann-Whitney test and unpaired t-test for the ratio Aβ42/40.

4.3.3 AF710B reduces microglia and astrocyte recruitment towards neurons in the hippocampus

As Aβ-burdened neurons have been shown to induce an inflammatory reaction recruiting astrocytes and microglia (as previously demonstrated (Hanzel et al., 2014b, Ferretti et al., 2012)), the number of glial cells recruited towards CA1 neuronal layer was assessed (**Supplemental Figure 4-4)**. Tg-AF710B group showed a significant reduction in microglia **(Figure 4-4 A, C)** and astrocytes recruited towards the CA1 hippocampal region compared to the Tg-sal **(Figure 4-4 A,E)**. The density of microglia and astrocytes in the proximity of Aβ-burdened neurons was significantly reduced following AF710B treatment in a manner that was independent from the genotype. Interestingly, no differences across the four groups were found for the overall CA1 area covered by astrocytes and the number of microglia **(Figure 4-4 B, D**), confirming that the differences are due to glia recruitment by Aβ-burdened neurons and not an increased amount of glia.

4.3.4 AF710B rescues microglia morphological changes and the profile of inflammatory mediators

In AD animal models, the increased number of microglia recruited in the proximity of hippocampal neurons is accompanied by changes in their morphology (Ferretti et al., 2012), reflecting the functional state of the cell (Torres-Platas et al., 2014, Franco-Bocanegra et al., 2019, Martini et al., 2020). In the fully activated state, microglia are characterized by an ameboid shape, with an increased soma size and process retraction (Serrano-Pozo et al., 2013, Davies et al., 2017).

AF710B treatment decreased the microglia soma size and increased the length of the processes (**Figure 4-5 A, C-E, Supplemental figure 4-4 A**). Additionally, Tg-sal showed a significant increase in the ratio "overall soma area /overall processes length", compared to the wt-sal (**Figure 4-5 B**), corresponding to an activated state. Conversely, this ratio was significantly decreased in the Tg by AF710B treatment (**Figure 4-5 B**), indicating a less 'activated' state.

Additionally, the protein levels of the inflammatory mediators were examined. AF710B treated Tg rats showed a significant decrease in pro-inflammatory cytokines (particularly IL-1β and IL-6), and an increase in anti-inflammatory cytokines (such as IL-10 and IL-4) compared to the Tg-Sal group (**Figure 4-5 F-L**) with no changes for IL-13 and KC/GRO (**Supplemental figure 4-5**).

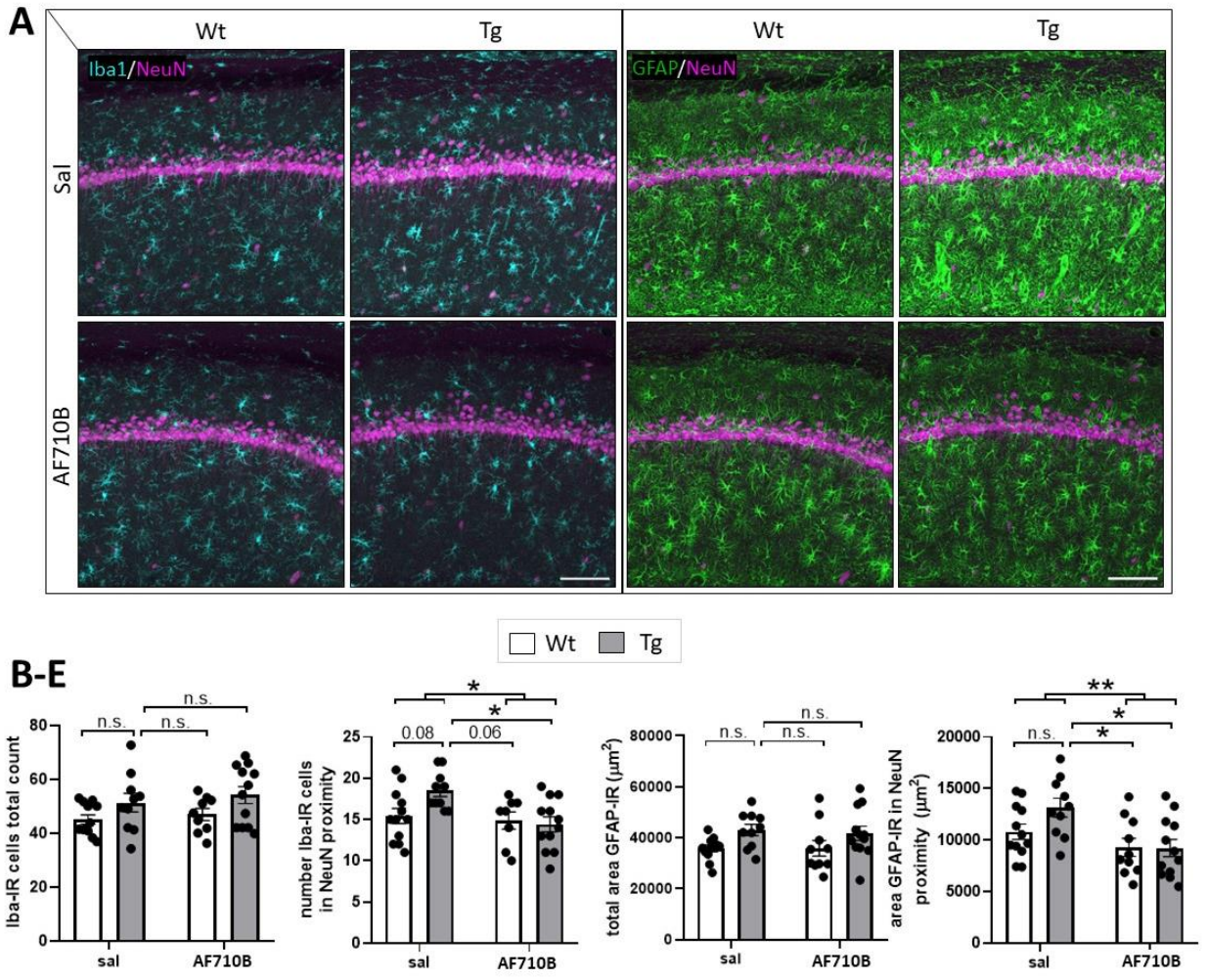


Figure 4-4. AF710B reduced microglia and astrocyte recruitment toward hippocampal neurons

(A) Representative images of CA1 IF for Iba1-IR cells and NeuN (left) and GFAP-IR cells and NeuN (right) from wt and Tg rats treated either with saline or AF710B (scale bar 100 µm). (B, D) There were no differences in the total amount of Iba1-IR cells and GFAP-IR area between the four groups (2x2 ANOVA for Iba1, treatment: P= 0.402, F (1, 39) = 0.7174; genotype: P=0.020, F (1, 39) = 5.928; interaction: P=0.858, F (1, 39) = 0.03253. For GFAP, treatment: P=0.794, F (1, 40) = 0.06894; genotype: P=0.009, F (1, 40) = 7.487; interaction: P=0.782, F (1, 40) = 0.07757). (C) A trend towards an increased number of Iba1-IR cells in CA1 was present in the Tg-sal compared to the controls wt-sal (P=0.084, Df 38.00) and Tg-AF710B showed a significant reduction of recruitment compared to the Tg-sal (P=0.012, Df 38.00); (2x2 ANOVA: treatment: P=0.016, F (1, 38) = 6.343; genotype: P= 0.161, F (1, 38) = 2.043; interaction: P=0.061, F (1, 38) = 3.719).

(E) Tg-AF710B showed a significant reduction of recruitment of GFAP-IR cells compared to the Tg-sal ($P=0.011$, $Df=40.00$) and the Tg-sal had more GFAP-IR cells recruited compared to the wt-AF710B group ($P=0.017$, $Df=40.00$); (2x2 ANOVA: treatment: $P=0.002$, $F(1, 40) = 10.48$; genotype: $P= 0.183$, $F(1, 40) = 1.836$; interaction: $P=0.161$, $F(1, 40) = 2.043$). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test.

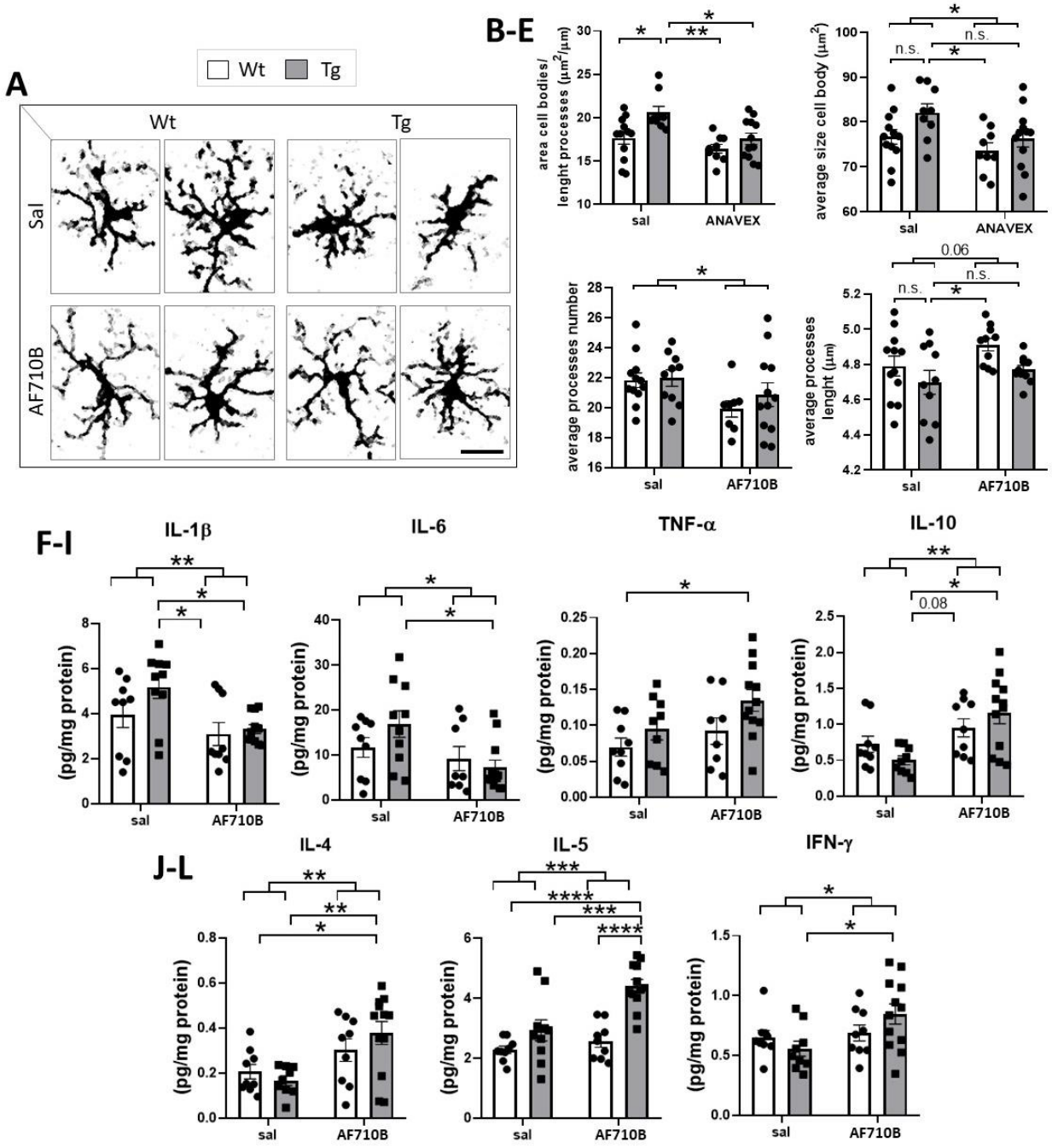


Figure 4-5. AF710B rescued morphological changes of microglia recruited towards hippocampal neurons and altered the cytokines profile.

(A) Representative images of microglia cells in proximity of NeuN from wt and Tg rats treated with saline or AF710B (scale bar 20 µm). (B) Tg-sal showed a significant increase in the ratio "cell bodies area/length processes" compared to the wt-sal (P = 0.019, Df =38.00), which was significantly reduced in the Tg-AF710B (P = 0.015, Df =38.00) and the wt-AF710B (P = 0.001,

Df =38.00) compared to the Tg-sal; (2x2 ANOVA: treatment: P=0.003, F (1, 38) = 10.04 ; genotype: P=0.005, F (1, 38) = 9.044 ; interaction: P=0.187, F (1, 38) = 1.808). (C-E) Treatment with AF710B showed to have a significant treatment effect in 2x2 ANOVA towards microglia body size (P=0.024, F (1, 38) = 5.510), in reducing the average number of processes (P= 0.023, F (1, 38) = 5.656) and a trend towards increasing processes length (treatment: P=0.063, F (1, 38) = 3.667). (F) IL-1β was significantly increased in the Tg-sal compared to the wt-AF710B (P = 0.013, Df = 35.00) and to Tg-AF710B (P = 0.022, Df = 35.00) groups; (2x2 ANOVA: treatment: P= 0.005, F (1, 35) = 9.116 ; genotype: P= 0.117, F (1, 35) = 2.587 ; interaction: P=0.267, F (1, 35) = 1.271). (G) IL-6 in the Tg-sal did not show a significant difference compared to the wt-sal (P = 0.415, Df = 35.00), but was significantly reduced in the Tg-AF710B (P = 0.020, Df = 35.00). (2x2 ANOVA: treatment: P=0.015, F (1, 35) = 6.598; genotype: P=0.492, F (1, 35) = 0.4819; interaction: P=0.138, F (1, 35) = 2.300). (H) TNFα in the Tg-AF710B showed a significant increase compared to the wt-sal (P = 0.019, Df = 34.00). (2x2 ANOVA: treatment: P=0.054, F (1, 34) = 3.990; genotype: P=0.037, F (1, 34) = 4.705; interaction: P=0.577, F (1, 34) = 0.3175). (I) IL-10, showed a significant increase in the Tg-AF710B group compared to the Tg-sal (P = 0.003, Df = 35.00) and a trend towards an increase compared to wt-AF710B (P = 0.087, Df = 35.00) and. (2x2 ANOVA: treatment: P=0.002, F (1, 35) = 12.41; genotype: P=0.958, F (1, 35) = 0.002864 ; interaction: P=0.095, F (1, 35) = 2.942).

(J) IL-4 was significantly increased in Tg-AF710B compared to wt-sal (P = 0.031, Df = 35.00) and to the Tg-sal (P = 0.005, Df = 35.00). (2x2 ANOVA: treatment: P=0.001, F (1, 35) = 12.63; genotype: P=0.680, F (1, 35) = 0.1727; interaction: P=0.189, F (1, 35) = 1.796).

(K) IL-5, was significantly increased in Tg-AF710B compared to wt-sal (P<0.0001, Df = 36.00); sal:Tg (P<0.001, Df = 36.00) and wt-AF710B (P <0.0001, Df = 36.00); (2x2 ANOVA: treatment: P<0.001, F (1, 36) = 12.97; genotype: P<0.0001, F (1, 36) = 25.40; interaction: P=0.021, F (1, 36) = 5.877). (L) INFγ was significantly increased in the Tg-AF710B group compared to the Tg-sal (P = 0.031, Df = 35.00). (2x2 ANOVA: treatment: P=0.032, F (1, 35) = 4.982; genotype: P=0.674, F (1, 35) = 0.1799; interaction: P=0.093, F (1, 35) = 2.987). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test. Additional statistical analyses. Cell body; genotype: P=0.036, F (1, 38) = 4.726; interaction: P=0.471, F (1, 38) = 0.5308; Tukey's multiple comparisons test (Tg-sal vs. wt-AF710B, P = 0.024, Df = 38.00). Average number processes, genotype: P=0.395, F (1, 38) = 0.7411; interaction: P=0.523, F (1, 38) = 0.4151. Average processes

length: genotype: P=0.025, F (1, 38) = 5.479; interaction: P=0.625, F (1, 38) = 0.2425. Tukey's multiple comparisons test (Tg-sal vs. wt-AF710B, P = 0.027, Df = 38.00).

4.3.5 AF710B restored the ratio proBDNF/mBDNF

To address the status of BFCNs, VAChT-IR presynaptic boutons were measured in the hippocampal CA1 region. As expected, the Tg-sal group showed a significant decrease in VAChT-IR synapses compared to the Wt-sal group (**Figure 4-6 A, B**). However, AF710B treatment did not produce significant changes in VAChT-IR synapses in the Tg- AF710B compared to Tg-sal (**Figure 4-6 A, B).** Similarly, the density of glutamatergic synapses, assessed through vesicular glutamate transporter 1 (vGluT1) immunoreactivity, which is required for exocytic glutamate release at presynaptic terminals (Fremeau Jr et al., 2004), remained unchanged between groups (**Figure 4-6 C, Supplemental figure 4-6 A**).

Additionally, we investigated levels of NGF and BDNF. These neurotrophins are both secreted in an activity-dependent manner (Mowla et al., 1999, Bruno and Cuello, 2006) from cortical and hippocampal neurons as precursors, respectively proNGF and proBDNF, and converted in the extracellular space into the bioactive mature NGF (mNGF) (Bruno and Cuello, 2006, Bruno et al., 2009a) and mature BDNF (mBDNF) (Lee et al., 2001, Seidah et al., 1996a, Pang et al., 2004). No differences in the protein levels of proNGF in the cortex and in the levels of mNGF receptors (TrkA) in the hippocampus, were found across the four groups (**Figure 4-6 D-E, Supplemental figure 4-6 B**). Subsequently, protein levels mBDNF and proBDNF were assessed. Although levels both mBDNF and proBDNF were unchanged (**Figure 4-6 F-G, I),** the ratio proBDNF/mBDNF was significantly increased in the Tg-sal compared to the Wt-sal, indicating less conversion of proBDNF into its mature form (**Figure 4-6 H-I).** This ratio was rescued in the Tg-AF710B compared to the Tg-sal (**Figure 4-6 H-I**), indicating that more mBDNF was converted from proBDNF following AF710B treatment.

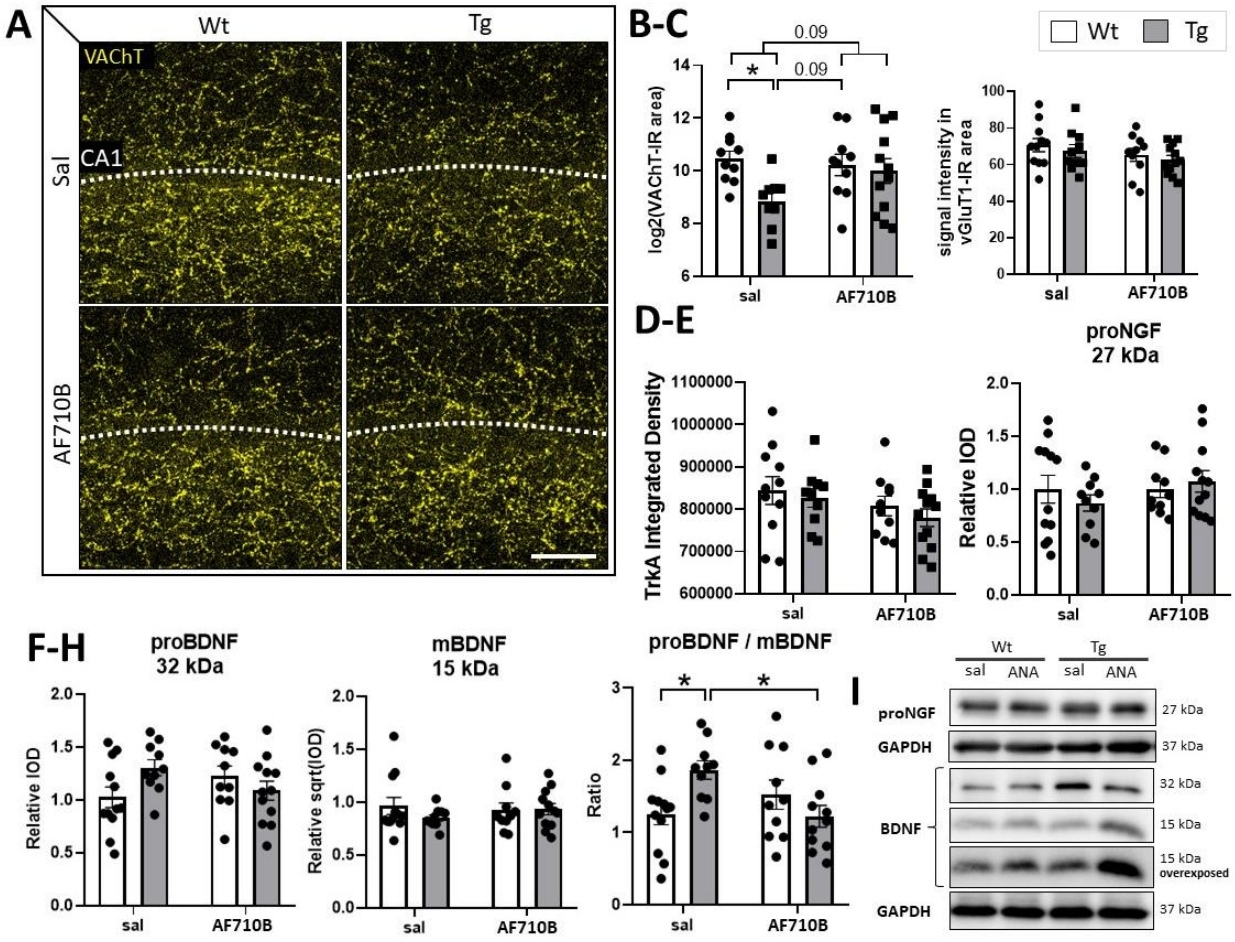


Figure 4-6. AF710B rescued proBDNF conversion to mBDNF.

(A) Representative images of IF for VAChT-IR synapses in CA1 in wt and Tg rats treated either with saline or AF710B (scale bar 25 µm). (B) VAChT-IR area was significantly decreased in the wt-sal compared to the Tg-sal (P = 0.041, Df = 37.00) and Tg-sal showed a trend towards a decrease compared to the wt-AF710B (P = 0.010, Df = 37.00); (2x2 ANOVA: treatment: P=0.255, F (1, 37) = 1.338; genotype: P=0.026, F (1, 37) = 5.377; interaction: P=0.089, F (1, 37) = 3.055). (C, D) No differences between the four groups were detected in the CA1 for vGluT1-IR and TrkA-IR (2x2 ANOVA; vGluT1, treatment: P = 0.130, F (1, 39) = 2.388; genotype: P = 0.373, F (1, 39) = 0.8123; interaction: P = 0.930, F (1, 39) = 0.007837. TrkA, treatment: P = 0.112, F (1, 39) = 2.651; genotype: P = 0.380, F (1, 39) = 0.7874; interaction: P = 0.841, F (1, 39) = 0.04094). (E-G) No differences for proNGF, proBDNF and mBDNF were detected between the four groups (2x2 ANOVA, for proNGF, treatment: P=0.337, F (1, 40) = 0.9444; genotype: P=0.798, F (1, 40) = 0.06645; interaction: P=0.323, F (1, 40) = 0.9996. For proBDNF: treatment: P=0.925, F (1, 40) =

0.009098; genotype: P=0.437, F (1, 40) = 0.6163; interaction: P=0.029, F (1, 40) = 5.155. For mBDNF, treatment: P=0.743, F (1, 40) = 0.1090; genotype: P=0.427, F (1, 40) = 0.6435; interaction: P=0.333, F (1, 40) = 0.9612). (H) the ratio pro/mBDNF was significantly increased in the Tg-sal compared to the wt-sal (P = 0.041, Df = 39.00) and was reported at wt-sal levels in Tg-AF710B group (P = 0.035, Df = 39.00) (2x2 ANOVA: treatment: P=0.249, F (1, 39) = 1.373 ; genotype: P=0.325, F (1, 39) = 0.9922; interaction: P=0.006, F (1, 39) = 8.301). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test was performed. Y values transformed for VAChT, with y=log2(y), and for mBDNF, with Y=sqrt(Y).

4.4 Discussion

The first-generation muscarinic agonists induced adverse peripheral cholinergic effects due to the lack of true subtype specificity decreasing their therapeutic potential and clinical interest (Fisher, 2012). A first-generation compound with similar characteristics, called AF267B, attenuated cognitive deficits and reduced amyloid pathology by activating the PKC pathway, resulting in a downstream effect on metalloproteinase domain 17 (ADAM17) enhancement and a decrease in β-secretase 1 (BACE-1) (Caccamo et al., 2006). The new muscarinic partial agonist AF710B targets M1 muscarinic and Sig-1Rs simultaneously without any agonistic activity on M2–M5 mAChRs (Fisher et al., 2016). The advantage of the selectivity for M1 receptors and the efficacy of this compound at a low dose (10 μg/kg) should contribute to avoiding or minimizing peripheral side effects (Fisher et al., 2003, Hall et al., 2018). We previously showed that AF710B could reduce amyloid pathology and neuroinflammation thereby ameliorating cognitive outcomes at late stages of the AD-like amyloid pathology. Given the increased potential of preventive treatment, we applied the same AF710B treatment paradigm to test whether AF710B could represent a therapeutic option to prevent amyloid hallmarks of AD. Our findings showed that the drug could prevent cognitive decline, reduce amyloid pathology, neuroinflammation and have increase BDNF availability as proposed in (**Figure 4-7**).

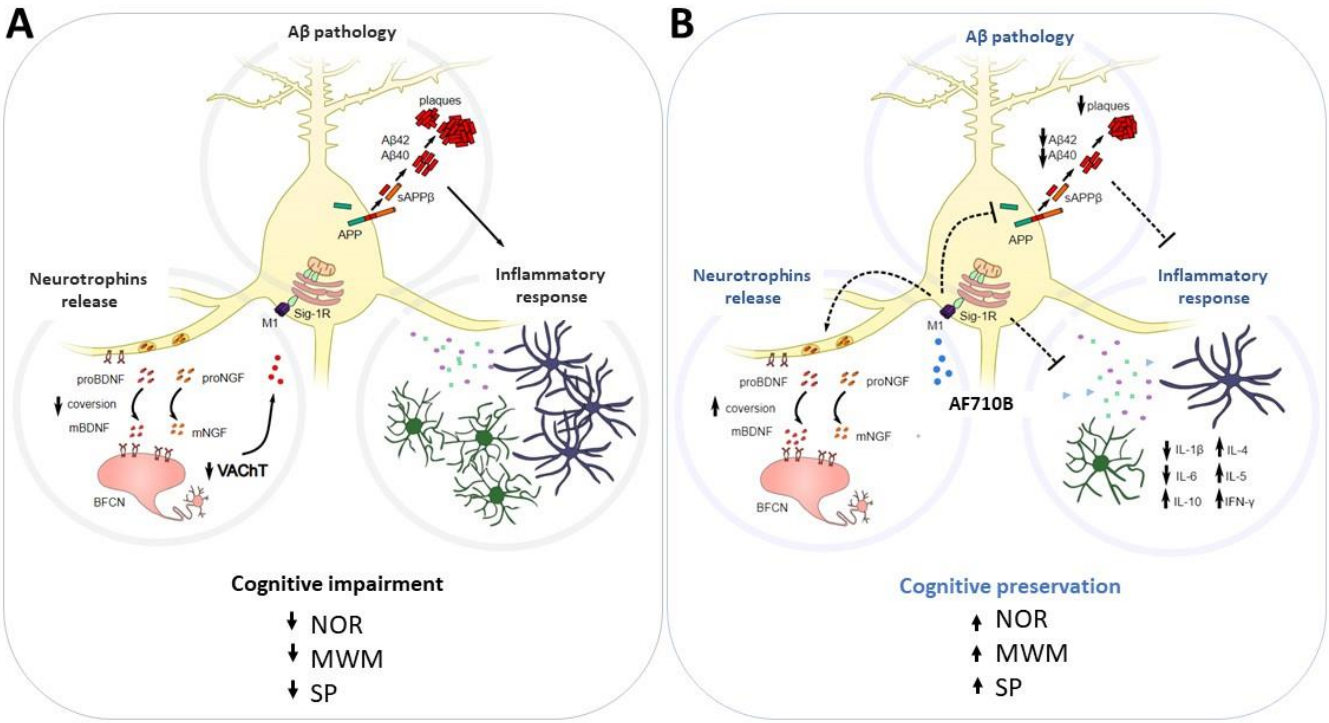


Figure 4-7. Schematic of the pathological mechanisms affecting cognitive impairment in the transgenic rats and the preventive effect of AF710B.

(A) The cognitive impairment in Tg animals results from multiple pathological processes in the brain. Firstly, the production of Aβ40 and Aβ42, induces the formation of dense and cored plaques in the cortex and hippocampus (Aβ pathology). Secondly, microglia and astrocytes are recruited towards Aβ-burdened neurons with morphological changes compatible with an active state (inflammatory response). Thirdly, a decrease in the VAChT-IR synapses and reduced conversion of mBDNF towards the proBDNF is present (neurotrophins release). (B) Following 7 month-AF710B treatment and 1 month of drug wash-out, Tg rats showed cognitive preservation compared to the Tg controls. M1 mAChRs stimulation induced a reduction of Aβ pathology, with less production of toxic Aβ42 and Ab40 and fewer plaques deposition. Additionally, less recruitment of astrocytes and microglia was accompanied by an increased release of anti-inflammatory cytokines and decreased pro-inflammatory mediators. Furthermore, more conversion of mBDNF from proBDNF was detected. The effect of AF710B on BDNF and inflammation could be mediated indirectly, by a reduction of Aβ pathology, or directly by stimulation of Sig-1Rs, as described in the text.

4.4.1 Decreased cognitive decline

In line with our previous work (Hall et al., 2018) AF710B restored cognitive capabilities in the NOR test performed to assess working memory (Antunes and Biala, 2012), and in the MWM, which investigates spatial navigation and reference memory (D'Hooge and De Deyn, 2001).
In addition, we found a preventive effect of AF710B on the decline in social interaction. While social engagement has a protective effect against age-related cognitive decline (Hikichi et al., 2017, Bassuk et al., 1999, Seeman et al., 2011), social withdrawal (Zava et al., 2021) represents one of the early signs in the AD continuum being present in individuals with MCI and subjective cognitive decline (Lopez et al., 2005, Rotenberg et al., 2020). McGill-APP rats have previously been shown to be less motivated to establish social interaction (Petrasek et al., 2018).
Interestingly, the drug was able to restore the cognitive performance in the NOR and SP to wild-type level but not fully recover the acquisition in the MWM test. This suggests that AF710B could have a stronger effect on complex cognitive functions resulting from the association/interaction of multiple brain regions targeted by M1 muscarinic receptors. Indeed, NOR and SP require the intervention of the hippocampus, and frontal cortex (Buckmaster et al., 2004, Clark et al., 2000, Aggleton et al., 2010, Hammond et al., 2004, Smith et al., 2021, File et al., 1998) and integration between these areas (Qi et al., 2018, Phillips et al., 2019). On the other hand, learning in the MWM test is solely dependent on the hippocampal function (Brandeis et al., 1989) rather than cortical (de Bruin et al., 1994, Neave et al., 1994, Warburton et al., 1998, D'Hooge and De Deyn, 2001).

4.4.2 Reduction of Aβ pathology

AF710B reduced plaque deposition in the cortex and hippocampus as well as the soluble and insoluble fraction of Aβ42 and an insoluble Aβ40. In agreement with our results, it was previously demonstrated that M1 selective agonists are capable of reducing the production of toxic amyloid-β species by shifting the APP processing toward a non-amyloidogenic pathway (Caccamo et al., 2006), and levels of cortical soluble Aβ42 in McGill-APP rats in an advanced stage of the pathology (Hall et al., 2018).

4.4.3 Reduction of neuroinflammation

AF710B reduced inflammation in McGill-APP rats, as shown by a decreased recruitment of microglia toward Aβ-burdened neurons in the hippocampus (Hall et al., 2018). The decrease in neuroinflammation could be the consequence of reduced intracellular processing of toxic Aβ but could also be directly mediated by M1 and Sig-1Rs.

Our results confirm that AF710B can prevent neuroinflammation, not only by reducing microglia recruitment but also astrocytes. Astrocytes, have M1-muscarinic receptors (Murphy et al., 1986), and can be modulated by ligands targeting Sig-1R sigma receptors (Prezzavento et al., 2010, Ruscher et al., 2011, Robson et al., 2014), which has been shown to ameliorate reactive astrogliosis in a rodent model of stroke and Amyotrophic lateral sclerosis (Ajmo Jr et al., 2006, Peviani et al., 2014). In addition, Sig-1R agonists inhibit lipopolysaccharide-stimulated activation of microglia and the release of pro-inflammatory molecules (Wu et al., 2015, Hall et al., 2009, Jia et al., 2018, Maurice and Su, 2009).

Furthermore, AF710B treatment increased cytokines typically considered anti-inflammatories, such as IL-4, which are associated with a reduction of cognitive impairment and microglia clearance of Aβ (Kawahara et al., 2012, Shimizu et al., 2008), and IL-10 (Strle et al., 2001), additionally promoting cognitive function (Kiyota et al., 2012). Interestingly, IL-4 and IL-10 suppress microglia secretion of pro-inflammatory IL-1β and IL-6 (Szczepanik et al., 2001, Ledeboer et al., 2002, Pousset et al., 1999), well-known to be associated with AD (Strauss et al., 1992, Yucesoy et al., 2006, Hedley et al., 2002), which were found to be down-regulated following AF710B treatment.

Additionally, AF710B treatment has been shown to increase levels of TNF-α and IFN-γ classically considered pro-inflammatory but recently recognized to play a dual role in AD (Zheng et al., 2016). Indeed, TNF-α is shown to be neuroprotective, playing a role in improving AD-like pathology in AD Tg mice through Aβ clearance (Montgomery et al., 2011). IFN-γ plays a similar but weaker function to IL-4, up-regulating glial major histocompatibility complex (MHC) class II (Butovsky et al., 2005), and its overexpression decreases Aβ deposits and infiltration of peripheral monocytes (Chakrabarty et al., 2010, Monsonego et al., 2006).

4.4.4 Could Sig-1Rs activation be responsible for cognitive impairment reduction?

AF710B did not prevent cognitive impairment by increasing cholinergic function, as there were no changes in the number of hippocampal cholinergic synapses in the Tg-AF710B compared to Tg-Sal. Additionally, since NGF is involved in the phenotypic maintenance of BFCNs (Sofroniew et al., 1990, Hefti, 1986, Cuello et al., 1992, Cuello, 1993), levels of the proNGF, the precursor of mNGF, were measured. No changes in proNGF protein levels nor TrkA receptors in the Tg-AF710B compared to Tg-Sal were found.

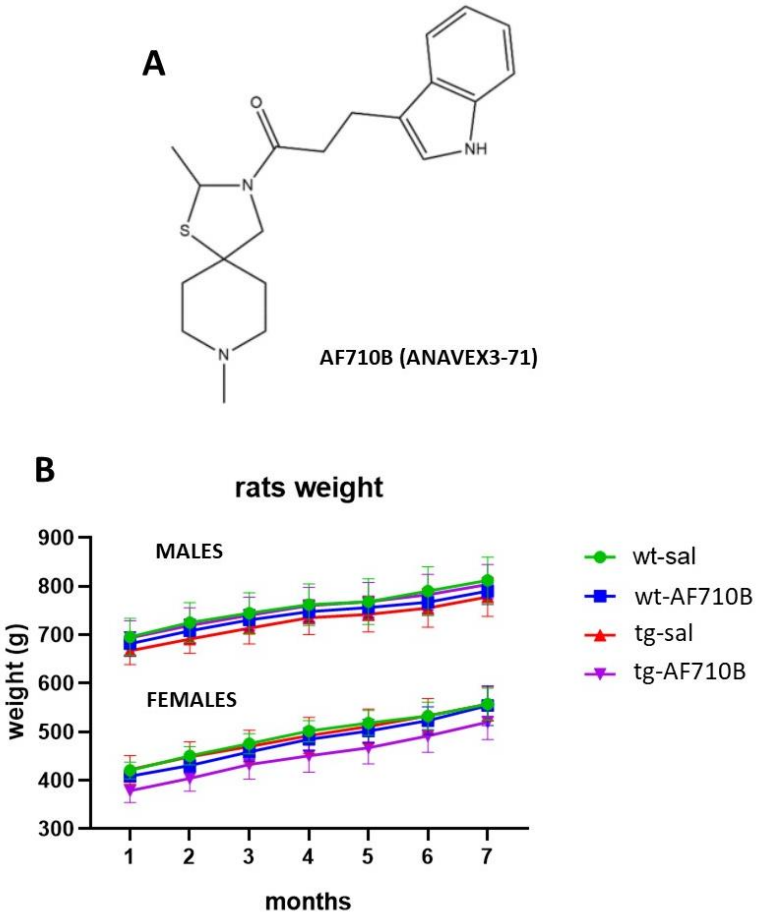
Interestingly, it was shown that anti-amnesic properties of Sig-1Rs agonists occur even in animals with complete cholinergic depletion (Antonini et al., 2011). Cognitive preservation in the Tg-AF710B group could be mediated by BDNF, as a reduction in the conversion from pro to mBDNF was found in the Tg-sal, and it was rescued by AF710B treatment. BDNF sustains both early and late phases of long-term potentiation (LTP) (Figurov et al., 1996, Korte et al., 1995, Korte et al., 1998, Tartaglia et al., 2001) and is markedly involved in learning and memory (Kang and Schuman, 1995, Korte et al., 1998, Broad et al., 2002, Hariri et al., 2003). Changes in BDNF have been shown to be very subtle in McGill-APP rats (Iulita et al., 2017), as only a reduction in BDNF expression in 3-6 months old has been reported, without changes in protein levels of pro- and mBDNF even at later stages (Iulita et al., 2017). As well, a slight but significant 0.4-fold reduction in mBDNF levels was measured in the cortex of wild-type rats following extensive nucleus basalis immunolesion (with approximately an average loss of 43% in VAChT-IR synapses) (Orciani et al., 2022). In support of our findings, stimulation of Sig-1Rs has been shown to affect mBDNF release *in vitro* but did not alter BDNF expression (Fujimoto et al., 2012). *In vivo*, Sig-1R stimulation increased BDNF, consequently rescued cognitive impairment (Kikuchi-Utsumi and Nakaki, 2008, Xu et al., 2015) and promoted neurite outgrowth (Kimura et al., 2013).

In closing, this preclinical study, initiated at an early stage of pathology, provides a solid rationale for the clinical trials of AF710B in AD therapy. Importantly, the sustained recovery in cognition and robust decrease in AD-like pathological markers was observed following a month-long wash-out phase supporting a disease-modifying effect of AF710B.

4.5 Acknowledgments

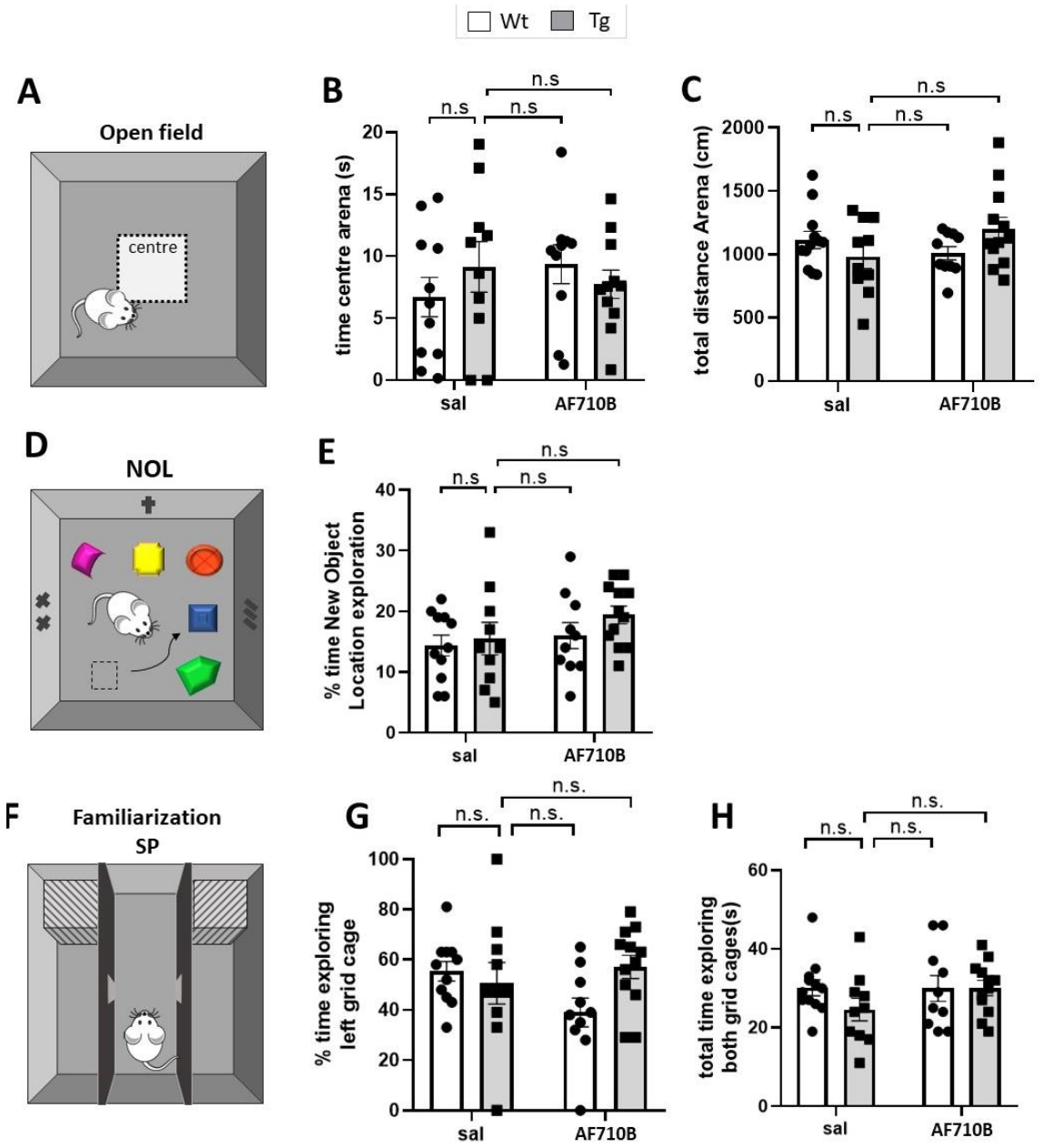
We acknowledge the McGill microscope facility Advanced BioImaging Facility (ABIF) in particular, Dr. Joel Ryan for the technical support and data analyses. CO was the holder of the Associazione Levi-Montalcini fellowship. SDC is the holder of the Charles E. Frosst/Merck Research Associate position. MKF was the recipient of a CIHR Doctoral Award. HH was a recipient of a Fonds de Recherche du Quebec-Sante postdoctoral fellowship. ACC is the holder of the McGill University Charles E. Frosst/Merck Chair in Pharmacology and a member of the Canadian Consortium of Neurodegeneration in Aging.

4.6 Supplemental figures



Supplemental figure 4-1. AF710B and effect on the rat weight.

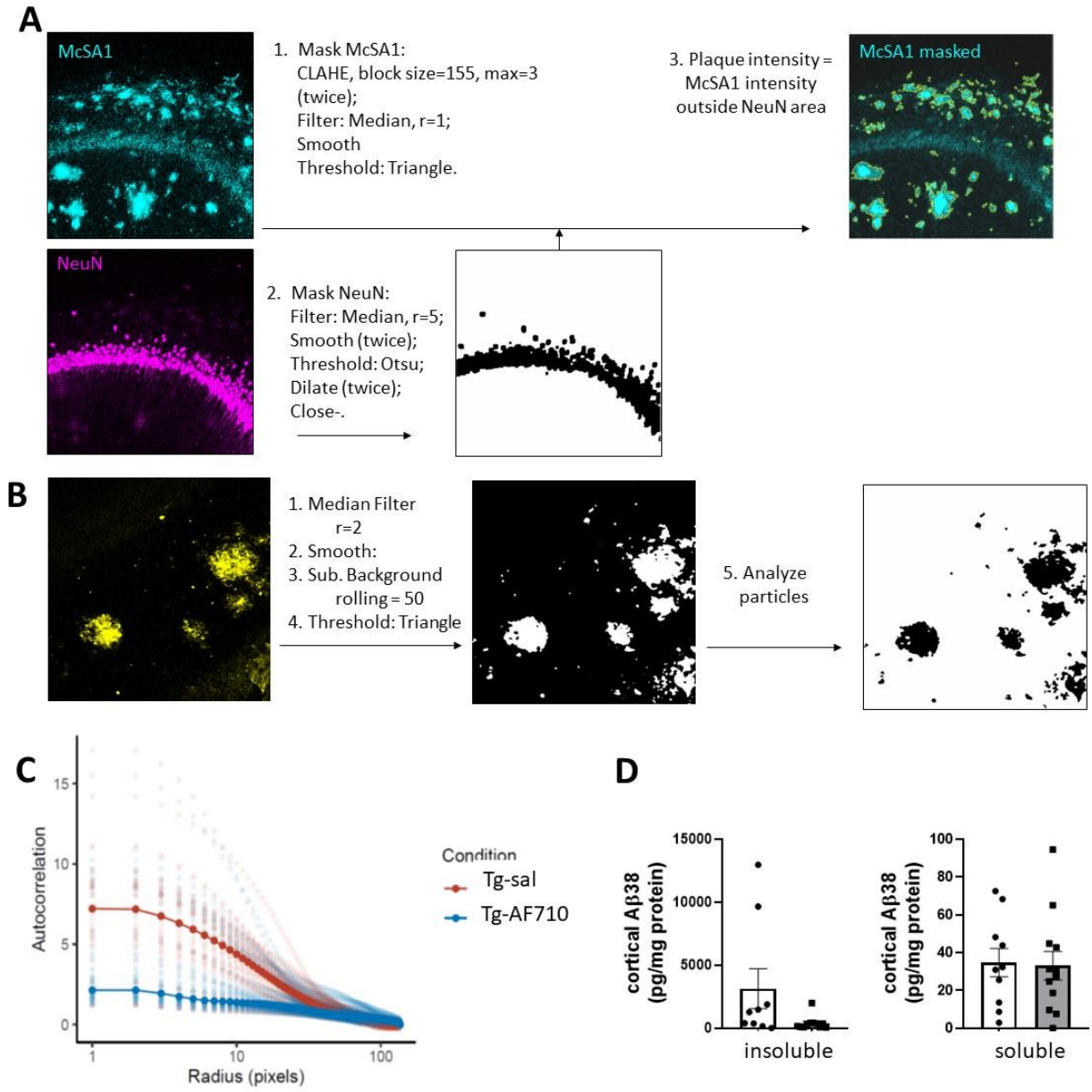
(A) Molecular structure of AF710B, 1-(2,8-Dimethyl-1-thia-3,8-diazaspiro[4.5]dec-3-yl)-3-(1H-indol-3-yl)propan-1-one. AF710B has 0.99.5% chemical purity; 0.99.5% enantiomeric excess; and specific rotation [α] = -56° [C = 0.303, methanol]). The chemical structure of the compound (US Patent 8673931B2), has been previously published (Fisher et al., 2016). (B) rat weight over the 7 months of AF710B treatment divided by sex (top males and bottom females).



Supplemental figure 4-2. Open field, NOL performances and SP habituation.

(A) Representation of the OF arena. (B, C) No differences between the four groups were detected in the OF, for the time spent in the arena centre and the total distance (2x2 ANOVA, for the time in the centre, treatment: P=0.701, F (1, 38) = 0.1495; genotype: P=0.799, F (1, 38) = 0.06588; interaction: P=0.213, F (1, 38) = 1.602. For the total distance, treatment: P=0.480, F (1, 40) = 0.5083; genotype: P=0.695, F (1, 40) = 0.1555; interaction: P=0.047, F (1, 40) = 4.192). (D) Representation of the NOL arena. (E) Tg-sal were not impaired in the NOL and no differences

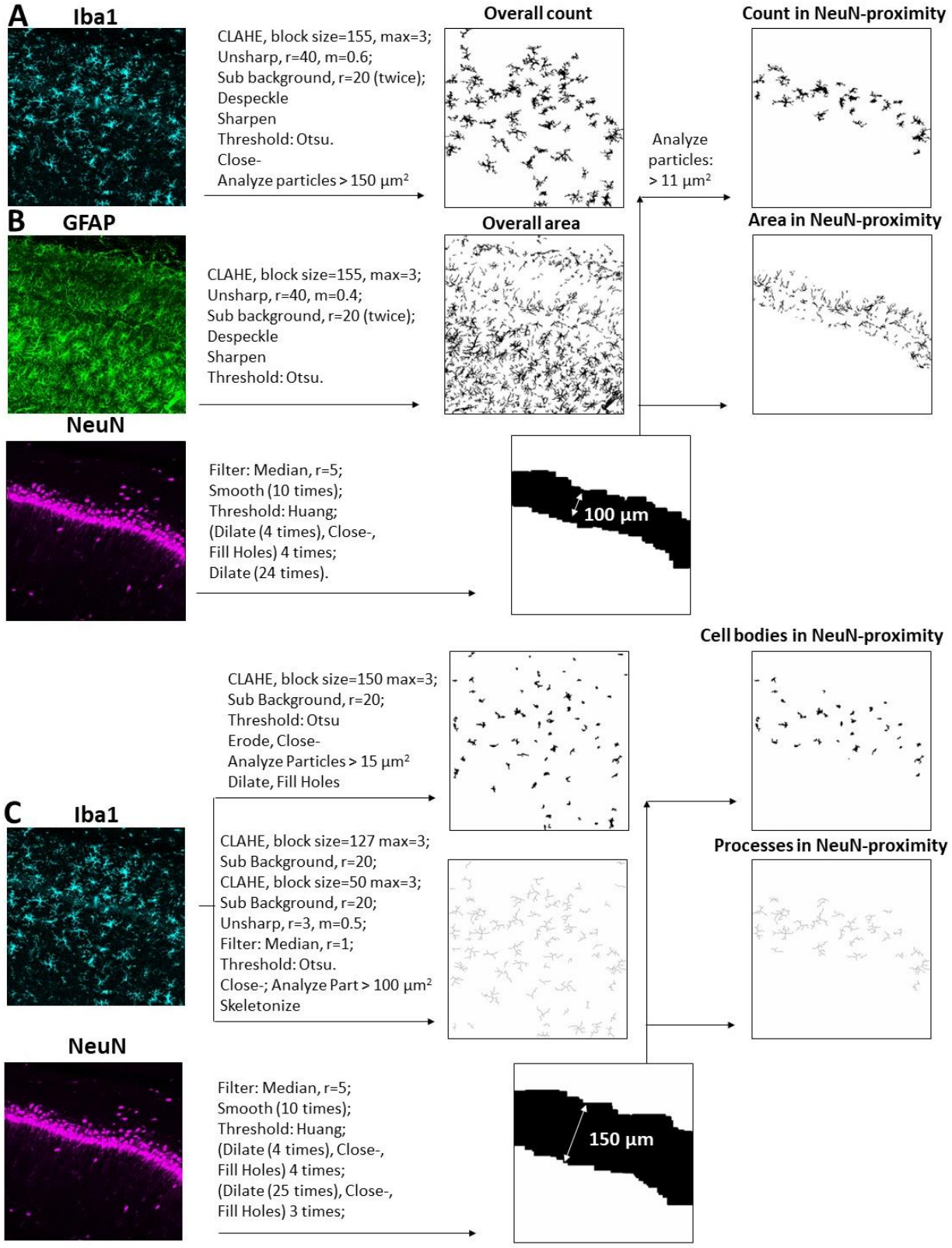
across the four groups were found (2x2 ANOVA: treatment: P=0.172, F (1, 39) = 1.936; genotype: P=0.261, F (1, 39) = 1.301; interaction: P=0.571, F (1, 39) = 0.3264). (F) Representation of the SP arena. (G,H) No differences between the four groups were detected in the SP, for the % time spent exploring the left grid and the total time exploring the two grids (2x2 ANOVA, for the % time spent exploring the left grid, treatment: P=0.397, F (1, 39) = 0.7341; genotype: P=0.248, F (1, 39) = 1.374; interaction:. P=0.052, F (1, 39) = 4.022. For the total time exploring 2 grids: treatment: P=0.295, F (1, 40) = 1.128; genotype: P=0.280, F (1, 40) = 1.200; interaction: P=0.266, F (1, 40) = 1.274). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test.



Supplemental figure 4-3. Flowchart illustrating the major steps employed in the quantification of McSA1 and Thiof-S.

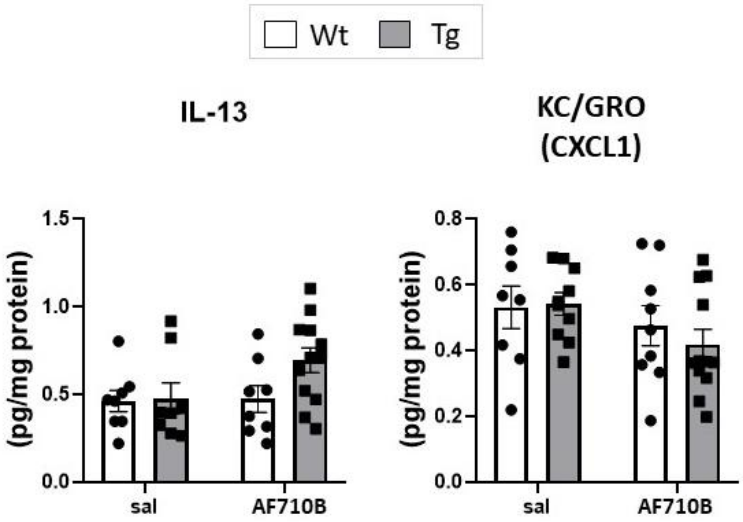
(A, B) Quantification of diffuse plaques with McSA1 and NeuN-IR and mature plaques with Thiof-S staining. (C) A two-dimensional spatial autocorrelation of McSA1 was calculated for each image using the Stack FFT ICS plugin (J. Unruh) in Fiji/ImageJ, and the resulting autocorrelation plot was averaged over multiple angles at sequential length scales, to generate the averaged autocorrelation plot shown in. Each transparent dot represents the averaged autocorrelation at its radius for one image, and the solid-coloured dots and lines represent the median autocorrelation

for all images. (D) No significative changes in the level of insoluble ($P = 0.095$, $U = 27$) and soluble Aβ38 ($P = 0.881$, $t=0.1523$, $df=20$) were found between Tg-sal and Tg-AF710B. Statistical analysis: insoluble Aβ38 with Mann-Whitney test and soluble Aβ38 with unpaired t-test.



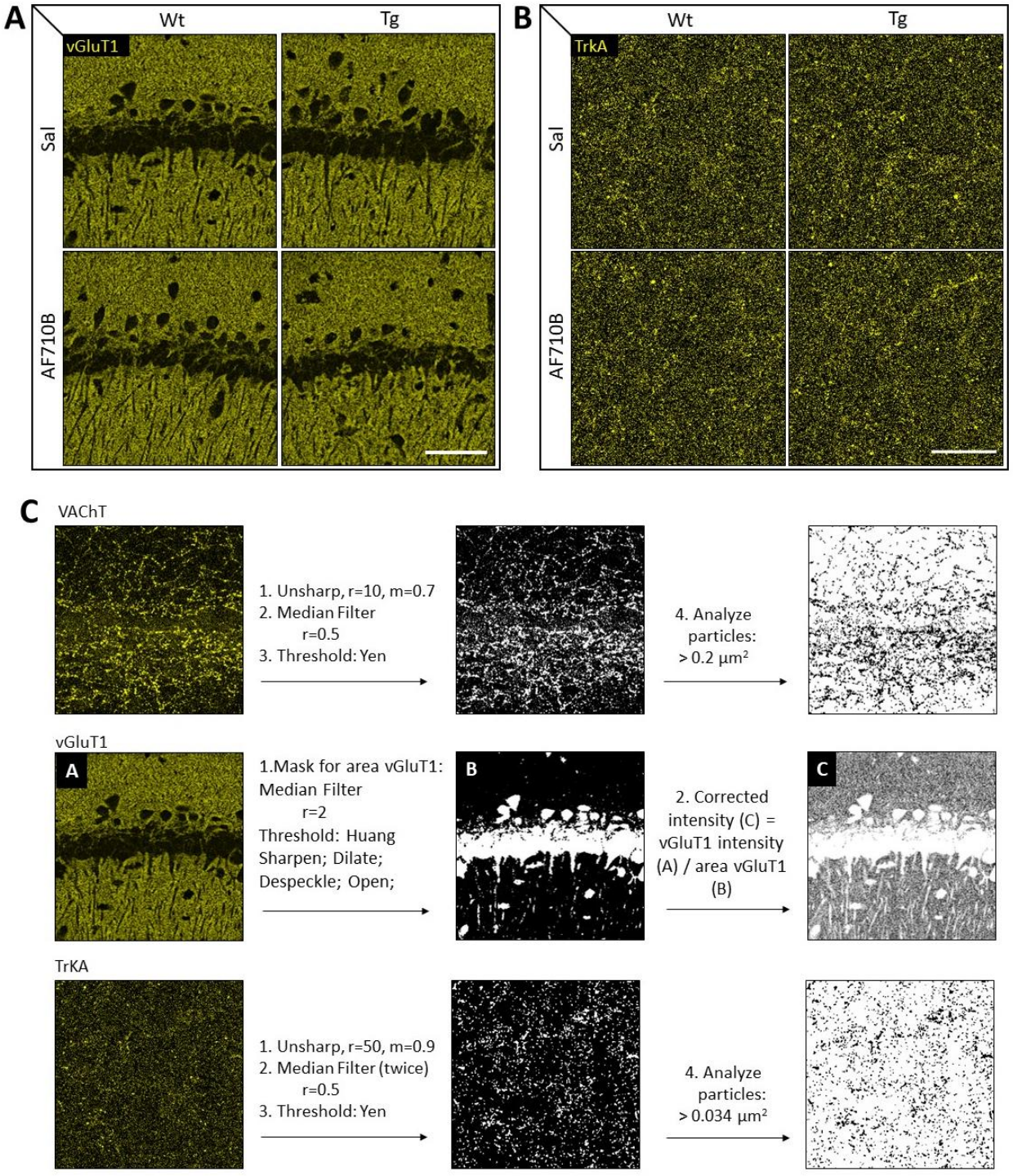
Supplemental figure 4-4. Flowchart illustrating the major steps employed in the quantification of Iba1 and GFAP-IR cells.

(A,B) Flowchart of the steps required for the overall quantification of Iba1 and GFAP-IR cells and the one in the proximity of CA1 neurons (100 µm). (C) Flowchart of the steps required for the quantification of Iba1 morphology in the proximity of CA1 neurons (150 µm). The thickness (150 µm) was increased to include the processes of the recruited microglia.



Supplemental figure 4-5. Inflammatory mediators.

Levels of IL-13 and of KC/GRO did not differ between the four groups (2x2 ANOVA, for IL-13, 2x2 ANOVA, treatment: F (1, 32) = 2.272 P=0.142; genotype: F (1, 32) = 2.370 P=0.134; interaction: F (1, 32) = 1.810 P=0.188. For KC/GRO, treatment: F (1, 35) = 3.040 P=0.090; genotype: F (1, 35) = 0.2117 P=0.648; interaction: F (1, 35) = 0.4183 P=0.522). Statistical analysis: 2x2 ANOVA followed by Tukey's multiple comparisons test.



Supplemental figure 4-6. Representative images of vGluT1 and TrkA in CA1 and Flowchart illustrating the major steps employed in the quantification of VAChT, vGluT1 and TrkA.

(A,B) Representative images of IF for vGluT1 and TrkA in CA1. (C) Flowchart of the steps required for the quantification of VAChT, vGluT1 and TrkA.

Summary main findings

The main objective of this thesis is to understand the role played by basal forebrain cholinergic neurons input in a healthy brain and test whether an experimental cholinergic therapeutical approach can prevent AD-like pathology. Towards this goal, we investigated neurotrophins and the vasculature following a long-term specific immunolesion of the nucleus basalis (nb), one of the main basal forebrain cholinergic nuclei projecting to the cortex. Finally, we assessed whether cholinergic input through one of the muscarinic acetylcholine receptors M1, could have prevented cognitive impairment and other hallmarks-amyloid related in a transgenic APP rat model at the earliest stages of amyloidosis.

Our investigations revealed that following a 6 month-immunolesion of the nb, wild-type rats showed an attentional deficit accompanied by a reduction of cortical markers of glutamatergic (vGluT1), and GABAergic (GAD65) neurons. Furthermore, for the first time, we assessed, following a cholinergic immunolesion, the level of precursor and mature of the two most important neurotrophins in AD: NGF and BDNF. As a result, we found that the cholinergic nb loss produced differential effects on neurotrophin availability, affecting BDNF but not NGF levels. In particular, we found a reduction in BDNF expression and mature BDNF levels. Additionally, cholinergic markers correlated with attentional deficit and BDNF. Finally, we proposed that BDNF dysregulation, consequent to the nb loss, could be responsible for the loss of neuronal markers and cognitive impairment.

Subsequently, to further assess the role importance of basal forebrain cholinergic neurons following the vascular-cholinergic hypothesis, we explored the effect of cholinergic nb loss on the neurovascular unit. Indeed, BFCNs are known to regulate vascular tone and cerebral blood flow, making synapses with multiple cell types. Our findings demonstrated that cholinergic nb loss reduced the average diameter of cortical vessels and the expression of the vascular endothelial growth factor A (VEGF-A). In addition, a long-term nb loss increased the density of astrocytes and microglia in the cortex. Astrocytic end-feet showed higher colocalization with arterioles following cholinergic immunolesion and microglia cells presented morphological changes

indicative of an intermediate state of activation. Overall, these results indicate that cholinergic input affects cell types belonging to the neurovascular unit.

Thirdly, we explored whether a new cholinergic therapy has the potential to prevent an AD-like pathology in transgenic rats. The selective allosteric M1 muscarinic and sigma-1 receptor agonist AF710B takes advantage of that acetylcholine postsynaptic M1 muscarinic brain receptor levels to remain unchanged in AD. We tested whether this compound by daily oral administration (10 μg/kg) for seven months, followed by one month of drug interruption (wash-out), attenuated AD hallmarks in McGill-R-Thy1-APP transgenic rats at early amyloid pathology stages. We found that AF710B prevented cognitive decline, even after a wash-out period, reduced plaque deposition and neuroinflammation and facilitated conversion from precursor to mature BDNF. These results suggest some preventative, disease-modifying properties of the compound over AD-like amyloid pathology.

Overall, our results show the importance of BFCNs in cognition, neurotrophin regulation, neuronal markers and neurovascular unit structure, providing a foundation for studies on BFCNs in AD. Additionally, we provided evidence that new cholinergic therapies can prevent AD-like pathology and could represent a possible therapeutical strategy to prevent disease progression.

CHAPTER 5 - General discussion

5.1 Hypotheses of cholinergic dysfunction in Alzheimer's disease

5.1.1 Hypothesis 1: basal forebrain cholinergic dysfunction is consequential to changes in neurotrophic receptor levels

The maintenance of the phenotype of BFCNs is dependent on NGF signalling through Tropomyosin receptor kinase A (TrkA) receptors and on the effects of proNGF signalling, as previously introduced in Chapter 1.

It was hypothesized that cholinergic degeneration might result partly from changes in the relative levels of mNGF/proNGF receptors instead of changes in ligand levels. Indeed, while cortical levels of p75ntr and its co-receptor sortilin are maintained across the continuum of AD (Mufson et al., 2010), levels of TrkA are reduced in AD but not in MCI (Counts et al., 2004). Furthermore, in contrast with cortical levels, fewer TrkA (and p75ntr) positive neurons are observed in the NbM in MCI (Mufson et al., 2000). Since deficits in receptor levels become evident in patients with established AD, it seems unlikely that they could cause cholinergic pathology before MCI. For this reason, changes in receptor levels are most likely downstream consequences of changes in ligand levels. Indeed, NGF signalling through TrkA induces transcription of TRKA itself, as well as the cholinergic gene locus (Venero et al., 1994, Gibbs and Pfaff, 1994, Figueiredo et al., 1995, Holtzman et al., 1992), and these transcripts are under-expressed in the absence of NGF (Venero et al., 1994). Therefore, an imbalance in mNGF/proNGF levels caused by NGF dysmetabolism may disrupt NGF-TrkA signalling.

5.1.2 Hypothesis 2: basal forebrain cholinergic dysfunction is the product of tauopathy and/or the disruption of axonal retrograde transport

The compromise of BFCNs has been proposed to depend on either tau pathology and/or deficient axonal transport. Tau pathology in NbM neurons correlates with decreased p75ntr expression, as well as with the cognitive decline in MCI and early AD (Vana et al., 2011). Additionally, NFT and pretangle pathology have been observed in BFCNs of cognitively normal people (Mesulam et al., 2004). Vulnerability of BFCNs to tau pathology could happen due to a change in some proteins. Indeed, while the directionality of this relationship has not been established, single-cell profiling

has demonstrated the loss of calcium-binding proteins in BFCNs which are burdened with tau pathology (Ahmadian et al., 2015, Riascos et al., 2014). In addition, an upregulation of rab proteins, implicated in endosomal and retrograde transport pathways, and rac1, a regulator of microtubule stability, has been demonstrated in BFCNs of MCI and AD individuals (Perez et al., 2012, Ginsberg et al., 2011).

The disruption of retrograde transport can be tau dependent. Particularly, *in vitro* evidence demonstrated that tau mediates an amyloid-induced disruption of axonal transport (Vossel et al., 2010) and transgenic mice overexpressing tau have general deficits in axonal transport, although BFCNs were not specifically examined (Ishihara et al., 1999). Furthermore, impaired retrograde transport of NGF has been demonstrated to be also Aβ dependent in mouse models of DS and mice bearing the Swedish APP mutation (Cooper et al., 2001, Salehi et al., 2006). Lastly, impairments of NGF retrograde transport have also been observed *in vitro* in aged neurons and in rats, independently of any amyloid or tau pathology (Shekari and Fahnestock, 2019, Cooper et al., 1994).

The notion that tauopathy in BFCNs drives a disruption of microtubule integrity and, subsequently, a disruption of the retrograde transport of NGF has been advanced. Tauopathy can indeed disrupt axonal transport, as demonstrated with pS422 tau in the squid giant axon (Tiernan et al., 2016). The above mentioned upregulation of rab and rac1 proteins can be interpreted as a compensatory response to re-establish normal axonal transport. The axonal transport disruption would affect the NGF-mediated trophic support to BFCNs. Whether a such event is a primary or secondary cause of BFCNs atrophy has not been resolved.

On the other hand, NGF metabolism could be the leading mechanism for inducing BFCNs atrophy, and the disruption of retrograde transport and tau pathology could be a consequence. It has been shown that NGF dysmetabolism induces atrophy of BFCNs (Allard et al., 2018) and withdrawal of NGF to BFCNs causes axonal degeneration *in vitro* and *in vivo* (Campenot, 1982, Deckwerth and Johnson Jr, 1994, Raff et al., 2002, Tuszynski et al., 1990). There is also abundant *in vitro* evidence that NGF deprivation in PC12 cell lines, mimicking cholinergic neurons (Wiatrak et al., 2020), can induce tauopathy (Nuydens et al., 1997, DAVIS and JOHNSON, 1999a, Davis and Johnson, 1999b, Shelton and Johnson, 2001). As such, tauopathy, axonal degeneration, and the disruption of retrograde transport are likely downstream consequences of the trophic disconnection

by the NGF dysmetabolism. A view reinforced by early studies in preclinical AD already showing evidence of basal forebrain atrophy applying MRI (Grothe et al., 2014, Schmitz et al., 2018). Such findings are in accordance with studies in our lab showing that post-mortem brain samples of cognitively normal individuals have a compromised NGF metabolic pathway accompanied by a loss of cortical cholinergic synapses (Pentz et al., 2020).

5.2 The status of the BFCNs does not affect the NGF metabolic pathway in wild-type rats

Our investigations, highlighted in Chapter 2, would indicate that atrophy/death of BFCNs does not affect the levels of NGF. Since NGF was shown to be released in response to carbachol (a cholinergic agonist) (Bruno and Cuello, 2006)**,** we hypothesized that the selective demise of cholinergic neurons of the nucleus basalis (nb), should have a significant effect on the cortical expression of both the precursor and mature forms of NGF following a reciprocal interaction relationship. Several studies previously showed an up-regulation of total NGF, which returns to physiological levels following a fimbria-fornix transaction, hippocampal lesion, and selective partial nb lesion (Gu et al., 1998, Hellweg et al., 1997, Korsching et al., 1986, Yu et al., 1996). The results of these studies were explained in light of the temporary disruption of retrograde axonal transport, which induced NGF accumulation in the BFCNs target tissue simply because it cannot be transported to the BFCNs as a result of the axotomy experimental model.

When we investigated pro and mNGF after a 6-month cholinergic immune-toxin lesion of the nb, we did not find changes in NGF expression nor the proteins levels. As expected, the partial immunolesion of the nb, involving 75% of cholinergic neurons, was consistent with the loss of cholinergic fibers reported for the corresponding areas in human AD brains (Geula et al., 2021, Geula and Mesulam, 1989, Geula and Mesulam, 1996). We also found morphological changes of VAChR-IR varicosities in the remaining cholinergic synapses, suggesting two possible explanations. The first (a) is that the VAChT morphological changes could be abnormal terminals and synapses analogous to AD brains. Such dystrophic neurites (Masliah et al., 1991, Geddes et al., 1986, Bell et al., 2006), and the long-term reduction of ACh release alone does not impact the NGF metabolic pathway in the absence of additional disruptive mechanisms (such as accumulation of intracellular Aβ). Studies in animal models support our model; indeed, no changes in NGF

metabolism were found in aged cognitively impaired rats with reduced brain ChAT activity (Bruno and Cuello, 2012).

The second possible explanation (b) is that VAChR-IR terminals compensate for their density reduction with a synaptic enlargement. The remaining cholinergic synapses bearing TrkA receptors would have less competition to extracellular available mNGF. The relatively increased access to the NGF could have elicited synaptic hypertrophy, and thus single pre-synaptic could be able to release more ACh per unit.

Since the immunolesion did not eliminate all the cholinergic boutons and TrkA receptors were found, the axonal retrograde transport of mNGF bound to the TrkA receptor should still be functional. Interestingly, in MCI and AD brains, a significant decrease in synaptic number is compensated by an enlargement of synaptic size (Scheff et al., 1990, Scheff et al., 2007).

5.3 NGF changes could be specific to Alzheimer's disease: NGF as biomarker

NGF studies in rat models showed that NGF protein levels were not affected following a nb immunolesion (Chapter 2) nor in aged cognitively impaired rats with decreased ChAT activity (Bruno and Cuello, 2012). On the other hand, Aβ brain injection and transgenic models with amyloid pathology recapitulate the NGF dysregulation (Bruno et al., 2009a, Iulita et al., 2017). These results align with many studies conducted in MCI, AD and Down Syndrome brains, as introduced in Chapter 1. The above body would support a relationship between the dysregulation of the NGF metabolic pathway and the occurrence of Aβ amyloid pathology.

Consequently, several studies have been conducted to test the hypothesis that the status of the NGF metabolic pathway in the brain should be reflected in the body fluids and serve as a biomarker for prodromal AD pathology (Florencia Iulita and Claudio Cuello, 2016, Pentz et al., 2020). CSF from patients with MCI and AD showed altered levels of metallo-proteases and the plasminogen activating system, both critical elements of the NGF metabolic pathway (Hanzel et al., 2014a). These results align with reports that CSF proNGF is increased in AD and correlates with disease staging and cognitive impairments (E Counts et al., 2016). In addition, analysis of plasma from the general population showed significant associations between various markers of NGF metabolism, such as MMP9 and the risk of dementia onset (Iulita et al., 2019).

Few studies were also conducted on individuals with Down Syndrome, representing the largest genetically determined population at risk for AD. Concerning the NGF metabolic pathway, an increase in proNGF MMP-1, MMP-3, and MMP-9 levels was measured in the plasma of AD-asymptomatic individuals with Down syndrome, and they were effective predictors of cognitive decline across two years of follow-up (Iulita et al., 2016). In addition, NGF pathway protein levels in matched CSF/plasma samples from Down Syndrome individual AD-symptomatic and AD-asymptomatic were examined (Pentz et al., 2021a). As a result, CSF from Down Syndrome individuals showed elevated proNGF, neuroserpin, MMP-3, and MMP-9 compared to controls; and proNGF and MMP-9 levels differed in CSF from AD-symptomatic and AD-asymptomatic individuals (Pentz et al., 2021a). Such circumstances, it will be important to validate proNGF and related markers in other studies. If confirmed, developing a reliable ELISA assay capable of distinguishing proNGF from mNGF will be essential.

5.4 History of NGF as a therapeutic target in Alzheimer's disease

Direct infusion of NGF into the brain of rodents (Williams et al., 1986, Kromer, 1987, Venero et al., 1994, Figueiredo et al., 1996, Garofalo et al., 1992, Kolb et al., 1997, Cuello, 1996, Debeir et al., 1999) and primates (Tuszynski et al., 1990, Tuszynski et al., 1991) was able to prevent cholinergic degeneration. In an initial trial, an AD patient was intraventricularly infused with 6.6 mg of NGF. Following three months, the patient showed positive effects on nicotinic receptor expression, cerebral blood flow, and verbal memory tests (Olson et al., 1992). Subsequently, the trial was extended to a further three patients with AD and the positive effects were recapitulated (Jönhagen et al., 1998). However, all three subjects showed significant pain and weight loss (Jönhagen et al., 1998). These side effects, later demonstrated to be mediated by off-target effects in the spinal cord and hypothalamus (McKelvey et al., 2013), led the authors to investigate alternative routes for NGF therapy (Jönhagen, 2000).

An alternative approach has been the intraparenchymal expression of NGF, implanting autologous fibroblasts genetically modified to express human NGF into the forebrain (Tuszynski et al., 2005). This approach passed a phase I clinical trial with no indication of pain or other adverse effects, and at the same time, reduced cognitive decline and increased cortical FDG-PET (Tuszynski et al., 2005).

In subsequent trials, NGF was delivered with an adeno-associated virus (CERE-110) stereotaxically injected into the nb of rodents (Bishop et al., 2008). This treatment was also shown to be safe and well-tolerated in AD patients (Rafii et al., 2014) and post-mortem analyses revealed that cholinergic neurons exhibited hypertrophy in response to the treatment (Tuszynski et al., 2015). However, a randomized clinical trial in 49 patients with AD showed no effect on multiple tests of cognitive decline, on FDG-PET, or on hippocampal atrophy (Rafii et al., 2018). These results brought to abandon this approach (Rafii et al., 2018), although a post-mortem analysis of the brains of 15 patients revealed that, because of mistargeting and a lower-than-expected spread of the vector, not one of the CERE-110 injections reached any cholinergic neurons (Castle et al., 2020).

Another related experimental approach to deliver NGF has been the implantation of inserts containing encapsulated cells genetically modified to release mature NGF (Eriksdotter-Jönhagen et al., 2012). Initial trials demonstrated improved cognition, reduced brain atrophy (Ferreira, Westman et al. 2015), and the ability to alter AD biomarkers, including soluble ChAT (Ferreira et al., 2015, Karami et al., 2015). However, these changes were largely restricted to patients subsequently defined as responders and some changes to biomarkers were negative (Ferreira et al., 2015, Karami et al., 2015).

Furthermore, an NGF therapeutical approach was inspired by observation in patients with type IV hereditary sensory and autonomic neuropathy, who have a point mutation in the NGF gene (an R100W mutant NGF protein) that does not enhance nociception but instead has full neurotrophic properties (Capsoni et al., 2011). The mutant NGF delivered intranasally for two months to ten-month-old APP and PSEN1 transgenic mice led to reduced brain plaque load and preserved cognitive performances (Capsoni et al., 2012). However, these results have not been validated in humans since this mutant NGF is believed to primarily act through glial modulation and cytokine induction (Capsoni et al., 2017, Cattaneo and Capsoni, 2019). This situation makes it unclear how this NGF therapy would affect the course of human AD pathology.

A common feature of all these approaches has been the use of exogenous NGF, which always entails the risk of side effects on nociceptors (McKelvey et al., 2013) or on glia (Cattaneo and Capsoni, 2019), as well as potentially having additional targets inside and outside the CNS.

Therefore, we believe that intervening in proNGF maturation or degradation would be a more promising therapeutic avenue to investigate. Such a strategy would prevent the "off-target" effects of exogenously applied mNGF.

5.5 BFCNs immunolesion affects BDNF and neurotransmitter markers

Another key neurotrophin of interest is BDNF. In Chapter 2, we showed that 192-IgG-immunolesioned rats in the nb have reduced cortical BDNF expression and mBDNF protein levels. In addition, we proposed that the reduction of expression and protein levels of mBDNF in 192-IgG-immunolesioned rats may be responsible for the impact of neuronal markers. Some evidence suggested cooperation of glutamatergic and cholinergic input may be responsible for BDNF regulation (Navakkode and Korte, 2012) in cortical and hippocampal neurons. Indeed, ACh has the potential to increase the excitability of glutamatergic neurons (McCormick and Prince, 1985, Desai and Walcott, 2006, Metherate et al., 1988) and it is well-known that neuronal activity increases BDNF expression (Ernfors et al., 1991, Isackson et al., 1991, Castrén et al., 1998, Zafra et al., 1990).

BDNF transcription is initiated with the increase of intracellular calcium and mediated by the cAMP Response Element-Binding Protein (CREB)-mediated pathway (Shieh and Ghosh, 1999). After transduction, proBDNF packed into vesicles is secreted in response to neuronal activity (Mowla et al., 1999, Poo, 2001) in the extracellular space, where it is converted into mBDNF (Lee et al., 2001, Seidah et al., 1996a, Pang et al., 2004). mBDNF binding to autocrine and paracrine TrkB receptors (Horch et al., 1999, Horch and Katz, 2002) exerts effects on the glutamatergic system (reviewed in (Leßmann, 1998)), including the expression and distribution of vGluT1 (Melo et al., 2013). In addition, BDNF enhances GABA release from interneurons (Ohba et al., 2005, Sakata et al., 2009) and regulates the transcription of presynaptic GAD65 by a Ras-ERK-CREB signalling pathway (Sanchez-Huertas and Rico, 2011). Interestingly, a study comparing changes in an APP transgenic animal model showed that the reduction of BDNF mRNA expression precedes NGF dysmetabolism (Iulita et al., 2017).

5.6 Could BDNF in the body fluids be a suitable biomarker of Alzheimer's disease?

In Chapter 2, we presented our findings showing that BDNF levels are reduced following a lack of BFCNs input. Also, transgenic rats showed less conversion from precursor to mature BDNF compared to wild-types, as discussed in Chapter 4. The above findings align with studies showing decreased BDNF in AD brains (discussed in the Introduction).

CSF and blood investigations were conducted to determine whether BDNF could represent a possible biomarker for early AD detection. Conflicting studies reported either decreased levels of BDNF in CSF from AD patients compared to non-demented controls or other types of dementia (Li et al., 2009, Laske et al., 2007), or found no significant difference (Blasko et al., 2006). However, none of these studies differentiated between mature and proBDNF.

A few studies have investigated the association between levels of BDNF and the risk of progression of cognitive decline and development of AD. A longitudinal study assessed whether serum BDNF levels were associated with 10-year risk of developing dementia (Weinstein et al., 2014). It was found that MCI patients with lower BDNF levels in the serum had progressed to AD dementia (Weinstein et al., 2014). Similar results were obtained in a 3-year study assessing BDNF levels in CSF, in which lower BDNF levels seemed to correlate with a higher risk of conversion from MCI to AD (Forlenza et al., 2015). Other reports described higher BDNF levels in serum (Angelucci et al., 2010) and plasma (Faria et al., 2014) in MCI and early AD patients compared to healthy controls, suggesting a compensatory mechanism in response to AD pathology progression.

5.7 BDNF as a possible therapeutic strategy for Alzheimer's disease

Few studies have focused on rescuing the levels of BDNF by gene delivery. BDNF gene delivery into the entorhinal cortex ameliorated lesion-induced neuronal death and improved hippocampus-dependent memory in rodents and primate models without adverse effects (Nagahara et al., 2009). However, since gene delivery is a very invasive technique, other indirect ways to increase brain BDNF expression have been investigated, such as antidepressants and lithium delivery (reviewed in (Nagahara and Tuszynski, 2011)). Interestingly, a 10-week study of lithium treatment in mild AD patients showed a significant increase in BDNF serum levels and cognitive improvement (Leyhe et al., 2009).

Other strategies, such as caloric restriction and physical exercise, were shown to be effective in increasing BDNF levels (Lee et al., 2000, Molteni et al., 2002). Physical exercise increased BDNF levels in the brains of animal models (Vaynman et al., 2004, Gómez-Pinilla et al., 2002) as well as in human blood and serum samples (Dinoff et al., 2017, Szuhany et al., 2015). Increased BDNF levels in the blood during exercise were likely derived from different tissues such as lungs, bladder, vascular endothelial cells, skeletal and cardiac muscle, etc. Indeed, BDNF is not only a neurotrophic factor but also a smooth muscle tone modulator and an energy regulator (Walsh and Tschakovsky, 2018).

5.8 Differences between BDNF and NGF in the context of human mutations

Our results in Chapter 2 outlined a different regulation of NGF and BDNF by BFCNs. The dysregulation of these neurotrophins in AD, raised questions about the presence of polymorphisms and mutations of these genes in the human population.

Studies of NGF mutations showed that NGF is a crucial regulator of pain (reviewed in (Capsoni et al., 2011)). In particular, a mutation on the NTRK1 gene, coding for TrkA (Indo et al., 1996), induced a human sensory and autonomic neuropathy type IV (HSAN IV) in which patients show pain insensitivity and mental retardation (Indo et al., 1996). This has been interpreted to result from a lack of trophic support during development, with consequences in the brain and sensory neurons (Indo et al., 1996). Furthermore, patients with a mutation of the NGF gene (HSAN V) (Einarsdottir et al., 2004, Carvalho et al., 2011) display a congenital insensitivity to pain but without mental retardation or cognitive deficits (De Andrade et al., 2008). The single nucleotide missense mutation in the NGF gene found in a family of HSAN V patients, results in an amino acid substitution in the mNGF (Einarsdottir et al., 2004), which in cell culture provokes reduced processing of proNGF to mNGF (Larsson et al., 2009). However, it remains to be clarified whether the major impact of the mutation is on the biological function of proNGF or mNGF. Interestingly, one polymorphism in the NGF gene (rs6330) has also been associated with an increased risk of late-onset AD (Di Maria et al., 2012, Nagata et al., 2011).

On the other hand, studies on BDNF focused on a frequent, nonconservative polymorphism (SNP) at nucleotide 196. This SNP produces an amino acid substitution (valine to methionine) at codon 66 (V66M) responsible for memory deficits, anxiety and depression (Egan et al., 2003, Chen et

al., 2006, Castrén and Rantamäki, 2010). The mutation is located in the precursor region of BDNF, altering its interaction with sortilin, which controls BDNF sorting to the regulated secretory pathway (Chen et al., 2005). Consequently, this SNP does not affect the biological activity of the mature protein (Egan et al., 2003). An *in vivo* study, using homozygous mice carrying this BDNF mutation (BDNF Met/Met), showed increased anxiety-related behaviors and even if the mutated BDNF was expressed at normal levels, its secretion from neurons was defective (Chen et al., 2006). Interestingly, although cognitive deficits are present in individuals with the BDNF V66M SNP, this SNP seems to be not associated with an increased risk of late-onset AD (reviewed in (Shen, You et al. 2018)).

5.9 Are the studies on cholinergic neurons relevant for other dementias?

AD does not represent the only dementia showing atrophy and loss of cholinergic neurons. Our studies on the effect of BFCNs loss (Chapter 2 and 3) and a therapeutical approach targeting M1-muscarinic receptors (Chapter 4) could be relevant for other dementias.

In particular, dementia with Lewy bodies (DLB) represents the third most common cause of dementia. Lewy bodies are neuronal inclusions composed of abnormally phosphorylated neurofilament proteins aggregated with ubiquitin and α-synuclein (McKEITH, 2002). DLB has a more profound loss of cholinergic neurons and ChAT activity than AD, which extends to other brain areas, such as the striatum and the pedunculopontine nucleus, which are not affected in AD (reviewed in (Francis and Perry, 2006)). In these brains, M1-AChRs were shown to be preserved or up-regulated in the temporal cortex but appeared to be reduced in the hippocampus (Ballard et al., 2000, Shiozaki et al., 1999, Shiozaki et al., 2001). The pharmacological management of the DLB is the administration of AChEIs, which improves cognition, global function, and overall living activities (McKeith et al., 2017).

Frontotemporal lobe dementia (FTLD) accounts for 20% of dementia cases (Snowden et al., 2002) and comprises several clinical syndromes. From a neuropathological point of view, FTLD is characterized by atrophy of the frontal and anterior temporal lobes and degeneration of the striatum (Mann, 1998, Ferrer, 1999). Interestingly, these patients have a strong alteration in character, speech and social conduct, indicative of frontal lobe dysfunction, although spatial skills and

memory are relatively preserved (Francis and Perry, 2006). Furthermore, in FTLD, a reduction in M1 receptors and an elevation of M2 receptors were reported (Odawara et al., 2003).

Vascular dementia is the second most common dementia after AD (Román, 2005), which is characterized by microvascular brain damage, mainly in the subcortical white matter and multiple infarcts. Vascular dementia is also associated with dysfunction of the basal forebrain cholinergic system (Román, 2005, Román and Kalaria, 2006). This impairment may be absent in cases of "classical" vascular dementia (Perry et al., 2005), but may be an important feature of subcortical ischemic vascular dementia (SIVD) (Kim et al., 2013).

Overall, the dementias listed above, particularly DLB and vascular dementia, could benefit from therapeutic approaches correcting the compromised cholinergic functions.

5.10 Could the vascular-cholinergic hypothesis explain the "non-responders" to AChEIs?

Vascular disease accompanies AD since its early stages and may also be a causal pathway (Sweeney et al., 2019). In fact, vascular risk factors are associated with lower FDG-PET, cerebrovascular disease, higher cerebral Aβ and tau burden, which promote cognitive decline (Vemuri et al., 2017, Rabin et al., 2018). Structural changes in the arteries are responsible for functional changes in cerebral blood flow (Avolio et al., 2018), and they are associated with the accumulation rate of cerebral Aβ (Hughes et al., 2014).

Currently, the most successful symptomatic therapeutic approach to AD consists in increasing ACh levels with AChEIs. However, investigations aiming to evaluate the therapeutic potential of AChEIs disclosed the existence of two subsets of patients, defined as "responders" and "non-responders" to this therapy. It has been hypothesized that a polymorphism in the Paraoxonase-1 (PON-1) gene (Pola et al., 2005), a potent endogenous AChEI involved in multiple biological activities, influences responsiveness to treatment with AChEIs in subjects affected by AD. Another explanation for this individual response follows the *vascular-cholinergic hypothesis* (Claassen and Jansen, 2006). Following this hypothesis, certain brains of AD patients present severe microvascular deformity (De la Torre, 1997) and endothelial dysfunction from vascular disease (Rosenblum, 1997), which could reduce the capability to respond to vasodilatation under cholinergic input. Conversely, it has been shown that "responders" to AChEIs, have improved or stabilized cerebral blood flow (Ceravolo et al., 2004). In contrast, "non-responders", who

demonstrated cognitive deterioration, had a progressive decline in cerebral blood flow (Venneri et al., 2002).

The brain vasculature is a complex system affected by multiple factors, in which Aβ and tau seem to play a key role in AD pathology (Schaeffer and Iadecola, 2021). Additionally, the cholinergic input has a significant physiopathological role. Therefore, it is possible that AChEI administered at preclinical AD stages or other therapeutical approaches preventing amyloid-like pathology (as suggested by our experimental work in Chapter 4), could be beneficial to reduce vascular deformity, which could avoid the presence of "non-responders" to AChEIs.

5.11 Novel vascular biomarkers in Alzheimer's disease

Vascular dysfunction appears early in AD. Measures of BBB integrity (Montagne et al., 2015, van de Haar et al., 2016), cerebrovascular reactivity (Montagne et al., 2016, Suri et al., 2015), and increased cerebrovascular resistance (Yew et al., 2017) have been proposed as biomarkers of vasculature structure and functionality. Additionally, brain imaging (reviewed in (Sweeney et al., 2019)) and CSF or blood biomarkers of vascular damage seem to represent the convenient tools for early AD detection.

5.11.1 Vessels diameter following BFCNs depletion and a possible biomarker in Alzheimer's disease

Emerging evidence indicates cerebral blood flow reductions at large and medium-sized arteries in individuals with AD risk (Clark et al., 2017) and AD models (El Tannir El Tayara et al., 2010). Interestingly, our results in Chapter 3, pointed out the change of diameters of capillaries and arterioles following BFCNs immunolesion, indicating that these neurons contribute to the neurovascular unit (NVU).

New methods of evaluating angiography of three-dimensional vascular anatomy (time-of-flight MRI sequences) provide quantitative parameters such as the number and order of branches, branch artery lengths, volumes and tortuosity (Chen et al., 2018). As such, an MRI of the anatomy of the vessels could represent a non-invasive method to detect early vasculature changes for populations at high risk of AD and other neurodegenerative conditions affecting the brain vasculature.

5.11.2 VEGF following BFCNs depletion and a possible biomarker for Alzheimer's disease

Other non-invasive methods for early AD detection have been exploring biomarkers in CFS and blood. Along with soluble platelet-derived growth factor receptor-b, fibrinogen and albumin (Sweeney et al., 2015, Craig-Schapiro et al., 2011), VEGF is a vascular factor gaining interest in AD biomarker research.

VEGF is reduced in the brains of AD patients (Provias and Jeynes, 2014) and is associated with lower hippocampal volume (Hohman et al., 2015). In addition, VEGF reduction in AD patients has been detected in serum (Huang et al., 2013, Mateo et al., 2007) and CSF (Paterson et al., 2014). Additionally, experimental studies in which AD transgenic mice were treated with VEGF improved cognitive performance and reduced Aβ and p-tau load (Spuch et al., 2010, Wang et al., 2011, Religa et al., 2013, Yang et al., 2005). Our results in Chapter 3 confirmed that a long-term cholinergic immunolesion reduced cortical VEGF-A expression and protein levels. Interestingly, experiments *in vivo* showed that VEGF-A expression depends on cholinergic function (Kimura et al., 2018).

On the other hand, contradicting results on VEGF have been reported, such as an increase of VEGF in CSF samples from AD patients (Tarkowski et al., 2002) was negatively associated with global cognition (Mahoney et al., 2021). The increase in CSF VEGF levels observed in some studies could represent a protective response given by the anti-angiogenic properties of Aβ42. Indeed, Aβ42 antagonizes VEGF binding to VEGF receptor-2 (VEGFR-2) on endothelial cells (Patel et al., 2010) and binds VEGF-165 with high affinity, resulting in its sequestration into senile plaques (Yang et al., 2004, Yang et al., 2005).

In line with our results, VEGF could represent a biomarker indicating the status of BFCNs, although longitudinal studies would be required. Additionally, stimulation of VEGF synthesis or its release, given its neuroprotective functions, may prevent neurovascular dysfunction and other pathological processes in AD.

5.12 Other experimental approaches to study the neurovascular unit

The ability to study the molecular and cellular properties of the cerebrovascular system is crucial to better characterize its contribution to brain physiology and physiopathology. Lack of knowledge of the NVU in a healthy brain and the techniques that allow studying the entire NVU and

maintaining its morphology is one of the biggest challenges to improving our knowledge of the vasculature in AD. In Chapter 3, we studied the NVU by applying biochemical and immunohistochemical approaches following cholinergic immunodepletion. This allowed us to detect morphological changes in the vessels, astrocytes and microglia after partial depletion of cortical cholinergic input.

Vessel isolation, a technique adapted for rodents (Yousif et al., 2007), currently allows the separation of brain vessels into fractions enriched in vessels of different diameters (Boulay et al., 2015b). Such preparations might be used for studying gene activity, protein synthesis and regulation at the BBB (Boulay et al., 2015a) since endothelial cells keep their metabolic properties (Dallaire et al., 1991) and transporter functionality (Boado and Pardridge, 1990). In addition, pericytes and smooth muscle cells (SMCs) remain attached to the endothelial layer; however, astrocytes, microglial cells and neurons are lost.

New data have been recently gathered in rodent vasculature (Vanlandewijck et al., 2018) and AD brain vasculature (Yang et al., 2022) from single-cell transcriptome studies. With this approach, the tissue is digested into single cells after microvascular isolation, and large amounts of RNA are converted into cDNA libraries and consequently sequenced.

Another way to investigate the functionality of the vessels is the cerebral vasodilatory response. This can be measured in individual cortical vessels following a challenge with various pharmacological or physiological agents known to induce vasodilation or vasoconstriction (Bales et al., 2016). First, rodents are anesthetized and placed in a stereotaxic frame. A cranial window is performed on the skull and then they are transferred to the microscope stage. Then the vasodilator or vasoconstrictor is infused into the cranial window for 5 min. The diameter of the exposed arteriole is recorded with a microscope system (Han et al., 2008). This technique has been applied to test the vascular reactivity in preclinical studies following immunotherapy targeting Aβ and APOE (Xiong et al., 2021, Bales et al., 2016). The procedure could be implemented to investigate the cholinergic mechanisms affecting the brain vasculature; such as to study the morphological changes of perivascular astrocytes and microglia following vasodilation (with ACh) or vasoconstriction (with a muscarinic antagonist).

5.13 Cholinergic experimental therapeutics

5.13.1 Targeting cholinergic neurons

The results of Chapters 2 and 3 outlined the importance of BFCNs in cognition and vasculature. Therefore, future experimental approaches could aim to preserve the physiology and function of BFCNs by targeting NGF dysmetabolism. This approach has two facets: favouring the conversion of proNGF to mNGF and reducing the excessive degradation of mNGF. Both occur early, before MCI (Pentz et al., 2020) and could serve as a source of therapeutic targets in AD.

5.13.1.1 Increase conversion of the precursor to mature NGF

The serine protease inhibitor neuroserpin, involved in the disruption of the conversion of proNGF to mNGF, has certain properties that would make it an attractive therapeutic target. Neuroserpin has a more restricted expression in the CNS than other enzymes involved in the pathway and could be expected to have a limited side-effect profile (Osterwalder et al., 1996, Krueger et al., 1997). Furthermore, neuroserpin downregulation has been shown to restore cognition in AD mouse models (Fabbro et al., 2011).

5.13.1.2 Reduce degradation of mature NGF

On the other hand, targeting the degradation of mNGF and, consequently, metalloproteinases is an equally viable approach. MMP-9 could represent a target since the infusion of MMP-9 inhibitor GM6001 (100 µg/kg) into the cerebral cortices of young wild-type rats caused a 2- to 3-fold increase in NGF levels (Bruno and Cuello, 2006). In addition, an MMP-2/9 inhibitor led to increases in mNGF levels, higher densities of cholinergic boutons, and higher TrkA expression when continuously infused for two weeks into the rat cerebral cortex (Allard et al., 2012). Furthermore, few studies investigating the modulation of MMP-9 levels in AD models have reported a neuroprotective effect of MMP-9 (Fragkouli et al., 2014, Kaminari et al., 2017). Like other members of the pathway, MMP-9 has a wide variety of actions (Vafadari et al., 2016), diminishing its possibility of being administered as a therapeutical target to improve brain function.

5.13.2 Targeting ACh receptors

5.13.2.1 Targeting nicotinic receptors

Nicotinic receptors, previously introduced in Chapter 1, were proposed to be an interesting therapeutical target, and particularly α-7 nicotinic acetylcholine receptors (α7-nAChRs) were proposed to improve cognition (reviewed in (Thomsen et al., 2010)). Indeed, Encenicline, a partial agonist of α7-nAChR, was effective in restoring memory function in preclinical studies (Prickaerts et al., 2012) in the absence of gastrointestinal side effects, otherwise present with Varenicline, a drug targeting α4β2 nicotinic receptors (Varenicline-Alzforum). Unfortunately, Encenicline did not meet the primary endpoint of cognitive improvement in phase 3; the compound was discontinued, and the company shut down (Encenicline-Alzforum).

5.13.2.2 Targeting muscarinic receptors

Drugs targeting muscarinic receptors have gained much attention. We showed that a therapeutical approach stimulating M1 muscarinic and sigma-1 receptors (Chapter 4) reduced amyloid-like pathology and neuroinflammation. In addition, it showed disease-modifying properties, as cognitive decline was prevented after a period of drug wash-out. In the following paragraphs, I will describe why they are considered a valid therapeutical approach in AD.

5.14 Why is the M1 receptor considered the best therapeutic target amongst the mAChRs ?

Muscarinic M1 and M3 stimulation with carbachol increased APP production within minutes (Nitsch et al., 1992). However, it was later shown that, unlike selective M1 agonists, M3 muscarinic agonists are not free of peripheral side effects, so they became a less interesting target for therapeutic approaches (Fisher, 2008). The other mAChR subtypes, M2 and M4 mAChRs were also shown to be ineffective in activating α-secretase, responsible for the non-amyloidogenic pathway (Müller et al., 1997), and they may have an inhibitory effect on releasing neuroprotective αAPPs (Müller et al., 1997). On the other hand, post-synaptic M1 mAChR is prevalently expressed in brain areas involved in cognition (Levey, 1993), and is relatively unchanged in AD brains (Araujo et al., 1988). Therefore, many approaches focused on studying the impact of the cholinergic input on the APP pathway through the M1 mAChR-type knocking-out for M1, or performing a BFCNs immunolesion in combination with pharmacological approaches (Haring et

al., 1998, Jones et al., 2008, Müller et al., 1997, Shirey et al., 2009, Potter et al., 1999, Beach et al., 2003). As introduced in Chapter 1 and further discussed, the results of these studies pointed to the role of M1 mAChR in regulating cognitive function, the amyloidogenic pathway and tau phosphorylation (Fisher, 2008).

5.15 M1-agonists from the "AF series"

Dr. Abraham Fisher and colleagues developed conformational analogs of ACh to be used as muscarinic agonists. One of the first compounds produced was **AF102B** ((±)-cis-2-methyl-spiro-(l,3-oxathiolane-5,3')quinuclidine)) (Fisher et al., 1989). The selectivity of this compound for the M1 receptor was assessed by its capacity to displace the M1-selective antagonist [3H]pirenzepine and the non-selective antagonist (mixed M1 and M2 antagonist) [3H]quinuclidinyl benzilate and [(+)-[3H]cis-dioxolane) from receptors of rat forebrain homogenates (Fisher et al., 1989). Furthermore, the selectivity of AF102B for the M1-type was shown by its capability to induce depolarization in the isolated rabbit superior cervical ganglion, typically associated with M1 receptors; and did not induce hyperpolarization, which is associated with M2 receptors (Mochida et al., 1988).

A more recent *orthosteric* M1 muscarinic *agonist* was named **AF267B** [(S)-2-ethyl-8-methyl-1thia-4,8-diazaspiro[4.5]decan-3-one]. This compound attenuated cognitive deficits and decreased amyloid pathology by activating the PKC pathway, resulting in a downstream effect on enhancing metalloproteinase domain 17 (ADAM17) and decreasing BACE-1 (Caccamo et al., 2006). In addition, AF267B decreased tau hyperphosphorylation mediated by the activation of PKC and inhibition of glycogen synthase kinase 3 (GSK-3) (Caccamo et al., 2006, Forlenza et al., 2000). Interestingly, treatment with AF267B decreases tau phosphorylation in ApoE deficient mice showing memory deficits associated with synaptic loss of BFC projections and tau hyperphosphorylation (Genis et al., 1999). Furthermore, AF267B prevented neurotoxic effects through Wnt signalling, which is compromised in AD (De Ferrari and Inestrosa, 2000, Farías et al., 2004). Wnt signalling induces the activation of PKC and blockage of GSK-3 (Farías et al., 2004). Altogether, these results indicate that the M1 mAChR is an important regulator of amyloidogenesis and tau phosphorylation in the brain and provide strong support for targeting the M1 mAChR as a therapeutic candidate in AD.

5.16 AF710B prevents cognitive decline and amyloid-like pathology

AF710B (aka ANAVEX3-71) is the last compound of the "AF series", showing both high potency and selectivity for the M1 mAChR and the sigma-1 receptor.

The specificity of AF710B to bind M1 mAChR was tested by its capacity to displace an M1 mAChR radioligand agonist (tritiated pirenzepine) and a sigma-1 receptor radioligand agonist (tritiated pentazocine) incubated with guinea pig cortical homogenates (Fisher et al., 2016). In addition, AF710B was found to display no significant off-target activity when screened at 83 other GPCRs, ion channels and transporters known to mediate human side effects. Furthermore, it did not bind to human α4β2 - and α7 -nicotinic receptors, sigma-2 receptors, nor M2–M5 mAChR (Fisher et al., 2016). Compared to the previous compounds, this compound is an allosteric agonist instead of an orthosteric. This property was demonstrated using brucine, an M1-positive allosteric modulator that potentiates M1 orthosteric but not M1 allosteric agonists (Fisher et al., 2016). Brucine showed modest cooperativity on M1 mAChR with the carbachol and AF267B (orthosteric agonists), but it failed to potentiate AF710B.

In vivo, AF710B was shown to attenuate cognitive deficits, decrease Aβ pathology, rescue synapses and decrease neuroinflammation in transgenic animal models (Fisher et al., 2016, Hall et al., 2018). Our preclinical trial (Chapter 4) confirmed the same finding but at earlier stages. Indeed we found that AF710B can prevent cognitive decline, reducing soluble and insoluble Aβ42, and insoluble Aβ40, and decreasing plaque deposition in the cortex and hippocampus. This suggests that AF710B shifts APP processing towards the non-amyloidogenic pathway. Additionally, this compound showed disease-modifying properties since the effect lasted after one month of drug interruption (wash-out) performed before behavioral testing and brain perfusion.

5.17 AF710B has an affinity for the sigma-1 receptor

As previously mentioned, AF710B has an affinity for the sigma-1 receptor, which could explain additional protective and preventive properties of the compound over AD-like hallmarks of AD. Sigma-1 receptor is considered a non-G-protein coupled, non-ionotropic intracellular chaperone at the endoplasmic reticulum (ER) that modulates calcium signalling. The CNS, particularly the hippocampal neurons and oligodendrocytes, appears to be the primary site of sigma-1 receptor activity and effects, although it is also present in peripheral tissues (Pabba, 2013). It has been

involved in many functions, such as cardiovascular function, drug abuse, schizophrenia, clinical depression, cancer, mood disorders, amnesia and cognitive deficits (Su et al., 1988, Penke et al., 2018), which depend on the capacity of sigma-1 receptor to bind ligands with very diverse structures. sigma-1 receptor and the less known sigma-2 receptor were wrongly classified for years as opioid receptors (reviewed in (Penke et al., 2018)).

Sigma-1 receptor, under homeostatic conditions, is concentrated at mitochondrion-associated membrane junction sites located between ER and mitochondria (Hayashi et al., 2011). Upon ER calcium depletion or via ligand stimulation, sigma-1 receptor leads to prolonged calcium signalling into mitochondria via type 3 inositol triphosphate receptors (IP3Rs) (Hayashi and Su, 2007). When cells undergo prolonged stress, sigma-1 receptors translocate from the mitochondrion-associated membrane to the ER reticular network and plasma membrane (Su et al., 2010). There it regulates a variety of functional proteins, including ion channels, receptors and kinases (Su et al., 2010), such as phospholipase C and protein kinase C (Morin-Surun et al., 1999). This mechanism allows sigma-1 receptor to mediate the excitotoxic effect of glutamate by promoting calcium entry into mitochondria and reducing oxidative stress, probably via the activation of reactive oxygen species (ROS)-neutralizing proteins (Pal et al., 2012). Some of the above mechanisms likely participated in the beneficial and preventive effects of early AD-like amyloid pathology, as shown in Chapter 4.

5.18 AF710B has anti-amnesic and anti-inflammatory effects mediated by the Sigma 1 receptor

Stimulation of sigma-1 receptors has been shown to alleviate depressive symptoms (Sabino et al., 2009, Moriguchi et al., 2015), cognitive deficits in aged animals (Maurice, 2001, Tottori et al., 2002) and AD mouse models (Jin et al., 2015, Maurice and Su, 2009). Along with calcium modulation and anti-oxidative stress function, anti-amnesic properties of sigma-1 receptors could be partly explained by facilitating ACh release in the forebrain (Matsuno and Mita, 1992, Matsuno et al., 1993). Indeed, stimulation of sigma-1 receptors attenuated learning impairments induced by scopolamine (Matsuno et al., 1997, Senda et al., 1997, Senda et al., 1996). Since we did not find a significant difference between transgenic treated with AF710B and transgenic control in the number of hippocampal cholinergic terminals, proNGF and TrkA receptors, we inferred that the cognitive improvement is probably mediated by mechanisms mediated by direct stimulation of

sigma-1 receptors. Interestingly, anti-amnesic properties of sigma-1 receptor agonists occurred even in animals with complete cholinergic depletion (Antonini et al., 2011) and were accompanied by an increase in BDNF.

Interestingly, we found that transgenic control had a decreased conversion from precursor to mature BDNF, and this deficit was rescued in transgenic treated with AF710B. This suggests that the anti-amnesic properties could be modulated by sigma-1 receptors affecting BDNF directly. In support of our findings, stimulation of sigma-1 receptors showed *in vitro* to affect mBDNF release, but not its expression (Fujimoto et al., 2012) and *in vivo*, to affect BDNF and consequently rescue cognitive impairment (Kikuchi-Utsumi and Nakaki, 2008, Xu et al., 2015) and promote neurite outgrowth (Kimura et al., 2013).

Additionally, AF710B treatment significantly reduced neuroinflammation of astrocytes and microglia recruited towards hippocampal neurons and modulated pro- and anti-inflammatory cytokines (Chapter 4). In agreement with our results, not only have astrocytes been shown to have M1-muscarinic receptors (Murphy et al., 1986), but sigma-1 receptor ligands have been found to modulate the activity of these cells (Prezzavento et al., 2010, Ruscher et al., 2011, Robson et al., 2014) improving reactive astrogliosis in rodent models of stroke (Ajmo Jr et al., 2006) and amyotrophic lateral sclerosis (ALS) (Peviani et al., 2014). Furthermore, sigma-1 receptor ligands inhibit lipopolysaccharide (LPS)-stimulated microglia activation, decreasing ROS production and the release of pro-inflammatory molecules (Wu et al., 2015, Hall et al., 2009, Jia et al., 2018, Maurice and Su, 2009).

5.19 Agonists of muscarinic receptors in clinical trials: the past and the present

Past clinical experiments aiming to treat AD with muscarinic agonists were disappointing. Despite some muscarinic agonists such as xanomeline (agonist for M4 > M1) improved cognition and reduced psychotic episodes in AD patients, it has very low bioavailability in humans (<1%) (reviewed in (Fisher, 2006)). Another compound, Alvameline, was a very weak M1 agonist reported to be ineffective in Phase II/III (Fisher, 2006).

Overall, the compounds that failed in clinical trials had major side effects, were not strong M1 agonists or required multiple daily dosing, which may be inadequate in cognitive studies (Clader and Wang, 2005, Fisher, 2008). In spite of the problems encountered with the first generation of

nonselective muscarinic agonists, a new generation of M1 agonists could represent a valid therapeutic strategy to treat AD.

It has been proposed that the ideal M1 agonist should have the following major characteristics (reviewed in (Fisher, 2006)):

(1) Potential to improve cognition and delay disease progression.
(2) Selectivity for M1 to avoid adverse effects due to stimulation of other muscarinic receptors.
(3) Being a partial agonist not to cause M1 down-regulation.
(4) Good pharmacodynamic, pharmacokinetic and bioavailability profile.
(5) No adverse effects in the peripheral nervous system and CNS.
(6) Easy passage through BBB.
(7) Potential to be preventive.
(8) Low production cost.

Many of the listed characteristics have been shown to be present in AF710B, as preclinical studies of our Lab demonstrated to be preventive (Chapter 4) and delay disease progression (Hall et al., 2018) in a transgenic rat with amyloid-like pathology. Recently, AF710B (aka ANAVEX3-71) entered a phase I clinical trial to evaluate its safety, tolerability, and pharmacokinetics (clinicaltrials.gov, Identifier: NCT04442945). In addition, a similar compound with mixed ligand properties for sigma1/muscarinic receptors (ANAVEX3-72), has been reported to have memory-preserving and neuroprotective effects in mice treated with the muscarinic receptor antagonist scopolamine and with synthetic Aβ oligomer injection, (Villard et al., 2011). ANAVEX3-72 compound is currently in phase IIb of a clinical trial (clinicaltrials.gov, Identifier: NCT04314934).

Future studies will be needed to confirm whether agonists of sigma-1 and M1-muscarinic receptors could represent a therapeutic avenue to prevent or delay AD in humans.

5.20 The way forward

We have shown (in Chapters 2 and 3) the importance of BFCNs in attention, BDNF regulation, neuronal markers and neurovascular unit structure. These studies provide a framework for understanding early cognitive deficits in AD.

Additionally, we provided evidence (Chapter 4) that new cholinergic therapies can prevent AD-like pathology and could represent a possible therapeutical strategy to prevent disease progression. A great deal of work remains to be done before any of these possibilities are fully realized. Nevertheless, we hope that we have contributed to additional knowledge on the BFNCs and therapeutical strategy that future investigators may build upon and their work will one day make a difference for people living with AD.

Original contributions

1. I demonstrated that a long-lasting immunolesion of the nucleus basalis (nb) depleting significantly the cortical cholinergic input results in reduced BDNF expression and mature BDNF levels; without affecting the expression of NGF.
2. I have established that the diminished levels of cortical cholinergic synapses elicits attentional deficits.
3. I found that the loss of nb neurons results in the reduction of glutamatergic (vGluT1), and GABAergic (GAD65) markers in the cerebral cortex.
4. I proposed a mechanism by which the BDNF dysregulation provoked by the deletion of cholinergic nb neurons results in diminished glutamatergic and GABAergic cortical markers and cognitive impairments.
5. I demonstrated that the cholinergic neuronal nb loss is sufficient to alter cortical vasculature, in particular the diameter of cortical capillaries and arterioles as well as a downregulation of the expression of the vascular endothelial growth factor A (VEGF-A).
6. I demonstrated that a long-term loss of cholinergic nb neurons increased the density of activated astrocytes and microglia in the cerebral cortex.
7. I found that the partial depletion of the basal forebrain cholinergic input to the cerebral cortex is sufficient to provoke an intermediate-activated astrocytic cell population with a repolarization of end-feet with a higher colocalization with arterioles and microglia cells.
8. I demonstrated that a new cholinergic therapy, the selective allosteric M1 muscarinic and sigma-1 receptor agonist (AF710B), has the potential to prevent an AD-like pathology in transgenic rats when delivered at early stages of amyloid pathology.
9. I showed that 7 months of AF710B administration and an additional 1 month of a wash-out, prevented impairment of learning, memory and social interaction capabilities in transgenic rodents.
10. I established that AF710B treatment reduced toxic cortical Aβ42 and Aβ40, and plaque deposition in the cortex and hippocampus.
11. I identified that AF710B reduced neuroinflammation, rescued microglial cell morphological changes and changed profiles of inflammatory mediators in transgenic rats.

12. I detected that AF710B treatment rescued the ratio of precursor/mature BDNF, which was increased in transgenic rats, favouring more conversion and availability of the mature isoform compared to the precursor.
13. I proposed a mechanism in which AF710B prevented cognitive deficits by reducing amyloidosis, neuroinflammation and rescuing the conversion from precursor to mature BDNF.

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