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Impact of Arterial Stiffness on the Development of Alzheimer's Disease

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Impact of Arterial Stiffness on the Development of Alzheimer's Disease

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Résumé

La maladie d'Alzheimer (MA) se caractérise par une détérioration de la mémoire, une protéine tau hyperphosphorylée et par l'accumulation et l'agrégation progressives de peptides β amyloïdes neurotoxiques (Aβ) dans le parenchyme cérébral et les vaisseaux sanguins (angiopathie amyloïde cérébrale). Un dysfonctionnement cérébrovasculaire peut expliquer l'accumulation d'Aβ, et son implication dans l'apparition et la progression de la MA est de plus en plus reconnue. Cependant, la cause de l'insulte cérébrovasculaire dans la MA est encore inconnue. La rigidité artérielle est un suspect très stratégique de détérioration cérébrovasculaire. La rigidité artérielle augmente la pulsatilité de la pression artérielle dans les petits vaisseaux sanguins et a été identifiée comme un important facteur de risque de démence chez l'homme. Nous émettons l'hypothèse que la rigidité artérielle augmente la pulsatilité du débit sanguin cérébral (DSC), ce qui modifie la clairance de l'Aβ, la régulation du DSC et la fonction de barrière hémato-encéphalique. Ceci contribuerait à l'accumulation de peptides Aβ dans le cerveau et à l'accélération de l'apparition/la progression de la MA. Pour tester cette hypothèse, nous avons étudié les effets de la rigidité artérielle sur les fonctions cognitives ainsi que sur la clairance cérébrale de l'Aβ dans un modèle murin de MA. Nous avons utilisé le modèle de souris triple transgénique (3xTg-AD) pour reproduire les neuropathologies Aβ et tau (enchevêtrements neurofibrillaires). Dans ce modèle, nous avons induit la rigidité artérielle par calcification carotidienne. Nous avons montré que les souris 3xTg-AD sont plus vulnérables que les souris de type sauvage au stress mécanique de la raideur artérielle, qui se caractérise par une déficience cognitive. Le dysfonctionnement de la clairance Aβ en tant que mécanisme possible du déclin cognitif est en cours d'investigation dans notre étude; cependant, l'autre mécanisme potentiel tel que la rupture de la barrière hémato-encéphalique et le découplage neurovasculaire mérite d'être exploré plus avant. Dans l'ensemble, notre étude a démontré qu'en présence d'une rigidité artérielle, le déclin cognitif apparaît plus tôt dans le modèle murin de la MA, ce qui a mis en évidence l'importance des facteurs de risque vasculaires dans la progression de la MA.

Mots-clés : rigidité artérielle, maladie d'Alzheimer, dysfonctionnement cognitif, 3xTg.

Abstract

Alzheimer's disease (AD) is characterized by memory deterioration, hyper-phosphorylated tau protein, and by the progressive accumulation and aggregation of neurotoxic amyloid β-peptides (Aβ) in the brain parenchyma and blood vessels (cerebral amyloid angiopathy). Cerebrovascular dysfunction may explain Aβ accumulation, and its involvement in the onset and progression of AD is increasingly recognized. However, the cause of the cerebrovascular insult in AD is still unknown. Arterial stiffness is a very strategic suspect for cerebrovascular deterioration. Arterial stiffness increases blood pressure pulsatility in small blood vessels and had been identified as an important risk factor for dementia in humans. We hypothesize that Arterial stiffness increases cerebral blood flow (CBF) pulsatility, which alters Aβ clearance, CBF regulation and the blood-brain barrier function. This would contribute to Aβ peptides accumulation in the brain and to the acceleration of the onset/progression of AD. To test this hypothesis, we investigated the effects of Arterial stiffness on cognitive functions as well as on cerebral Aβ clearance in a murine AD model. We used the triple transgenic mouse model (3xTg-AD) to reproduce Aβ and tau (neurofibrillary tangles) neuropathologies. In this model, we induced Arterial stiffness by carotid calcification. We have shown that 3xTg-AD mice are more vulnerable than wild-type mice to the mechanical stress of the arterial stiffness, which is characterized by cognitive impairment. Aβ clearance dysfunction as a possible mechanism in cognitive decline is under investigation in our study; however, the other potential mechanism such as blood brain barrier breakdown and neurovascular uncoupling worth further explorations. Altogether, our study demonstrated that, in the presence of arterial stiffness cognitive decline appears earlier in mouse model of AD, which highlighted importance of vascular risk factors in AD progression.

Keywords: arterial stiffness, Alzheimer disease, cognitive dysfunction, 3xTg.

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AD: Alzheimer's disease

Aβ : amyloid beat

APP: amyloid precursor protein

BBB: blood brain barrier

BSA: bovine serum albumin

CAA: cerebral amyloid angiopathy

CBF: cerebral blood flow

CSF: cerebrospinal fluid

CTF: C-terminal fragment

DG: dentate gyrus

EC: endothelial cell

GMP: Gla matrix protein

LDLR: low-density lipoprotein receptor

LRP-1: low-density lipoprotein-related protein 1

MRI: Magnetic Resonance Imaging

MMP: matrix metalloprotease

NFT: neurofibrillary tangles

NVC: neurovascular coupling

NO: nitric oxide

NGS: normal goat serum

PFA: paraformaldehyde

PBS: phosphate-buffered saline

PET: positron emission tomography

PP: Pulse Pressure

PSD-95: post synaptic-density-95 **ROS:** reactive oxygen species

RAGE: receptor for advanced glycation end products

SEM: standard error mean

VaD: vascular dementia

WT: wild-type

This thesis is dedicated to my family who have been supporting me for every single second.

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Chapter 1: Introduction

1. Alzheimer disease

1.1. AD: Symptoms, progression, epidemiology

Alzheimer's disease (AD) is a chronic neurodegenerative disease and the leading cause of cognitive impairment and dementia in the elderly (aged ≥ 65 years) [1]. According to the Alzheimer's Society, AD constitutes 50–75% of dementia cases throughout the world. Approximately more than 50 million individuals worldwide are suffering from dementia, a number that is projected to reach > 115 million by 2050 [2] and the estimated total cost of care for AD treatment in 2020 was about $305 billion [3]. Given the significant economic and social costs of AD, researchers are focusing their efforts on understanding the disease's pathophysiology and finding early diagnostic and therapeutic options.

AD is a long-term, progressive disease that begins with pathophysiological changes in the brains of those who are affected years before any clinical symptoms appear. Individuals with such changes may be asymptomatic or show clinical symptoms ranging from minor memory lapses to severe and debilitating memory and cognitive function loss [4]. Impairment in cognitive function is occasionally preceded by deterioration in emotional control and social behavior [5]. The earliest clinical features of Alzheimer's disease is usually a mild memory loss with otherwise intact cognition. Difficulty recalling recent conversations, names, or events are common early symptoms, as are apathy and depression. Additional neuropsychiatric symptoms appear as the disease advances, practical abilities diminish, and personality and mood changes are common. Patients with advanced Alzheimer's disease will exhibit a deterioration in language, executive function, delusion/hallucination, and visuospatial abilities, as well as severe autonomic and motor dysfunction [2, 6, 7]. Age, genetics, biological sex, and other factors determine the duration of each part of the progression [8].

1.2. Vascular risk factors for AD:

1.2.1. Large artery stiffening

One of the consequences of vascular aging is arterial stiffness, which is an important risk factor and a predictor of cognitive impairment and dementia in the elderly [9, 10]. It is well known that increasing arterial stiffness result in Pulse Pressure (PP) increase during aging, and it has been suggested that this may be a triggering event in the development of atherosclerosis [11]. Increase in arterial stiffness and pulse pressure with age are predictive of poor cognitive performance in humans, particularly with a decrease in executive functions and an accelerated cognitive decline [12-15]. In patients with AD a correlation between AD and the increase in pulse pressure (PP) and aortic arterial stiffness has been reported, supporting that functional changes in the arterial system may play a role in the AD pathogenesis [16]. Related vascular diseases such as chronic hypertension also could cause arterial stiffness. However, independent impact of central arterial stiffness, evaluated by pulse-wave velocity between carotid and femoral arteries, has been indicated in aging population [15, 17, 18]. Different cohort studies also showed the link between large arteries stiffness and cognitive dysfunction [9, 19, 20]. Collectively, studies suggest that microvascular damage caused by increased PP is predictive of poor cognitive performance and precipitates cognitive decline. Findings of these studies are thus the basis of the vascular "biomechanical hypothesis" illustrating that cognitive decline is caused by the destructive power of high pulse pressure on cerebral blood vessels [21].

Reduction in elastin protein, increase in collagen crosslink formation and elastin fragmentation contribute to the onset of arterial stiffness during aging [22]. In an experimental model of arterial stiffness by carotid calcification, an increase in the production of superoxide anion in the hippocampus was reported and leaded to cerebral gliosis [23, 24]. Although some different mechanisms were suggested for arterial stiffness effects on cognitions like decreased cerebral blood flow (CBF) [25], improper Aβ clearance through the blood brain barrier (BBB) and the perivascular and paravascular (glymphatic) pathways [26], oxidative stress and inflammation [23, 27], and endothelial dysfunction [28], the exact mechanism by which arterial stiffness affects cognition is not fully understood and needs further exploration.

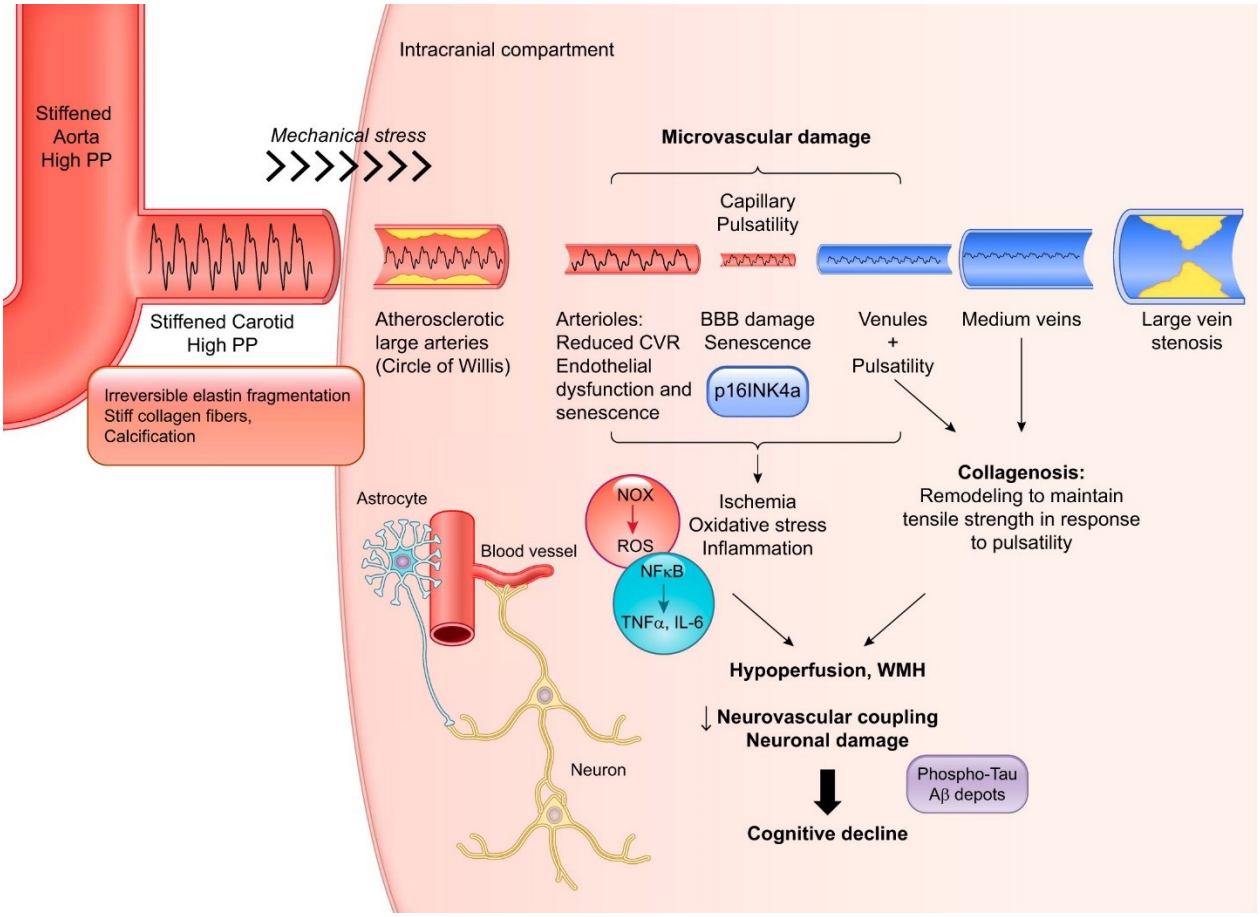


Figure 1. Schematization of the known possible physiological and molecular processes that connect penetration of pulse pressure into cerebral microcirculation to the development of cognitive impairment. Large arteries stiffening are caused by irreversible elastin fragmentation induced by the arterial wall's lifelong exposure to the mechanical stress generated by heartbeats. The amplitude of the pulse pressure (PP) reaches fragile low-resistance cerebral microcirculation. Reduced cerebrovascular reactivity, disruption of the blood-brain barrier (BBB), endothelial nitric oxide synthase failure, and endothelial senescence (p16INK4a expression) are all proposed as the consequences of capillary pulsatility. Infiltration of inflammatory cells and harmful chemicals following BBB breakdown leads to inflammation (via NF-B), oxidative stress (through activation of NADPH oxidase (NOX)), and ischemia. Pulsatility encourages collagenosis in the venules and medium-sized veins, which results in cerebral hypoperfusion. Together, this harmful ischemia and inflammatory milieu promotes parenchymal damage, neurovascular uncoupling, and neuronal damage, which ultimately results in cognitive decline and dementia. ROS, Reactive oxygen species. Figure adopted from Thorin-Trescases et al. [14].

1.2.2. Hypertension

Hypertension had been suspected more than a century ago as a vascular cognitive impairment risk factor. However, recently, it was linked to AD which commonly co-exist with vascular

dementia which account for 85 percent of AD cases [29]. Hypertension contributes to dementia and cognitive impairment through different pathways such as chronic brain injury, brain atrophy acceleration and neuroinflammatory processes [30]. Midlife hypertension has been reported as a risk factor for late-life dementia [31, 32]. Indeed, hypertension is the most important modifiable risk factor for lots of cerebrovascular diseases like stroke, vascular dementia, lacunar infarction, white matter damage, microhemorrhages, etc. [33].

Cerebrovascular injury is one of the well-known consequence of hypertension. This seems to be mostly mediated by oxidative stress pathway [34]. Hypertension cause brain blood vessels alterations both structurally and functionally. Structurally speaking, hypertension cause hypertrophy, stiffening and remodeling of vascular wall, atherosclerosis, small-vessel disease and microvascular rarefaction which could resulting in white matter damage, microinfarcts, and microhemorrhages [35]. The main determinants of hypertension induced vascular changes are renin-angiotensin-aldosterone system and angiotensin II. Angiotensin II play a role in promotion of Aβ cleavage by activating related enzymes, β- and γ-secretase [36, 37]. Moreover, it can induce vascular remodeling by increasing free radicals production, and cause vascular hypertrophy by activation of epidermal growth factor receptor [38, 39]. Loss of endothelial nitric oxide synthase–derived nitric oxide has been showed to be the possible mechanism involved in microvascular rarefaction [40, 41]. Regarding hypertension induced small-vessel disease, a wide variety of mechanisms have been reported such as BBB disruption, endothelial injury, vascular inflammation and microtheombi [35]. Functionally, chronic hypertension affects brain vascular circulation mainly by disrupting autoregulation, neurovascular coupling (NVC), and endothelium-dependent mechanisms. The curve of autoregulation (a curve illustrating relationship between blood pressure and CBF) shifted to the right in hypertensive subjects, meaning constant level of CBF occurs at higher pressure [42]. This could precipitate brain hypoperfusion during sudden reduction in blood pressure and “watershed” infarcts in susceptible brain structures [43], or augment the risk of transient ischemic attack, stroke and even mortality in the context of systolic hypertension as well as high pulse pressure [14, 44]. Hypertension also affect NVC through mismatch of energy demands and blood flow, along with reduction in basal level of CBF [34, 43]. Furthermore, hypertension cause cerebral endothelial cell injury leading to disability of

endothelial cells to regulate microvascular flow as well as performing the antithrombotic and antiatherogenic effects [45].

Despite the possible beneficial effects of antihypertensive therapy in dementia and AD, their efficiency in preventing incidence of AD is not prominent [46]. Therefore, the exact biological relationship between AD and hypertension is not clear. Experimental and human studies have demonstrated increase amyloid accumulation in hypertension, which might be the result of APP cleavage augmentation [47, 48]. However, some investigations have mentioned vascular injury and disruption in vascular Aβ clearance as the main cause of hypertension induced amyloid accumulation [34]. Supporting this idea and considering the fact that Aβ is cleared through perivascular space partially, pathogenic role of perivascular macrophages in hypertension has been also indicated [49]. However, definition of the precise contribution of hypertension in AD needs further explorations.

1.3. AD-associated vascular alterations in the brain

1.3.1. Atherosclerosis

Atherosclerosis (ATH) is a chronic inflammatory disease defined by the accumulation of lipids in the arterial vessel wall. It is a major cause of death and disability globally [26]. As the disease advances, atherosclerotic plaques accumulate and the artery lumen narrows. Plaques that have been stable for years can suddenly become unstable, rupture, and cause thrombus development. As a result, in addition to narrowing the vascular lumen, atherosclerotic plaques increase the risk of acute cardiovascular events such as myocardial infarction and stroke [50, 51]. When atherosclerosis develops in the cerebral blood vessels, reduced blood flow can impair the supply of oxygen and energy to the brain, which can lead to detrimental neurological consequences. Atherosclerosis is characterized by different and complex vascular injury process such as: lipid deposition within macrophage foam cells; inflammation, with monocyte adhesion to endothelial cells (ECs); neointimal hyperplasia, involving vascular smooth muscle cells (VSMCs); and extracellular matrix remodeling [52, 53]. Atherosclerotic plaques are most commonly observed in the aorta, coronary arteries, carotid and cerebral arteries [54].

Cerebrovascular atherosclerosis has been found as a major pathological characteristic of AD. A wide number of studies illustrated the link between atherosclerosis and AD. Substantial atherosclerosis in the arteries of the circle of Willi was demonstrated in patients with AD [55]. In another study, extensive atherosclerotic lesions were exhibited in Leptomeningeal arteries which were correlated with tangle and plaque load [56]. Furthermore, Arvanitakis et al. [57] discovered that atherosclerosis and arteriolosclerosis were associated to lower cognitive performance and an elevated risk of Alzheimer's disease. In this regard, another study indicated that coronary artery calcification is associated with an increased risk of dementia in the elderly [58]. Impaired Aβ clearance, nonresolving inflammation, and ApoE4 vascular effects may all play a role in the link between atherosclerosis and AD [36, 59]. Hypoperfusion and hypoxia as well as severe atherosclerosis may also increase Aβ production , which promotes the creation of atherosclerotic lesions through inflammation, vascular oxidative stress, and endothelial dysfunction [26, 36, 59]. However, further explorations are needed to improve the insights into possible mechanisms of atherosclerosis on the brain, and how these contribute to AD and dementia.

1.3.2. Capillary rarefaction

Vascular rarefaction is reduction of microvascular density through aging. This phenomenon is exacerbated by AD progression [60]. String vessels, which are the remnant of microvessels that become collapse and acellular, could be seen widely in autopsy of AD patients [61]. In 90% of AD brains these vascular alterations are visible and reported to be worse in ApoE4 carriers [62]. An experimental study on mouse model of AD, increased rarefaction of pial collaterals was reported [63]. Multitude factors may play a role in vascular rarefaction in AD such as toxicity of Aβ to vascular cells [43]. Hypertension as a vascular risk factor also contributes to vascular rarefaction [31].

1.3.3. Cerebral Amyloid Angiopathy (CAA)

Cerebral Amyloid Angiopathy (CAA) is the deposition of Aβ into the walls of cerebral arteries and capillaries [64, 65]. There is more susceptibility of CAA development in AD patients because of the increase in amount of amyloid plaques [64]. In 85% to 95% of AD individuals CAA was reported as a cerebrovascular pathology [26]. Impairment in Aβ clearance or increase in Aβ production

probably initiate Aβ deposition in the basement membrane of capillaries and media and adventitia of arteries resulting in CAA [26, 66]. As this pathological process advance, vascular cells could be degenerated due to the wide accumulation of Aβ [26]. Vascular structural changes in CAA lead to reduced blood flow and increase of vessels fragility, which finally manifested by ischemic lesions and cerebral microhemorrhages [67]. Gradual deposition of Aβ in perivascular spaces induces stagnation of interstitial fluids and results in alteration of cerebral microcirculation. In addition, vascular reactivity alteration and compromised vasodilation have been detected in early stage of CAA, even prior to the onset of symptoms appearance suggesting possibility of their role in neurodegeneration [67, 68]. Moreover, CAA seems to deteriorate the neurodegenerative processes by increasing oxidative stress, and neuroinflammation, in which promoting accumulation of Aβ [69]. CAA also could affect the integrity of leptomeningeal and cortical small and medium-sized arteries and arterioles [70]. Thus, the deleterious effects of CAA on vessels walls might contribute to reduction in resting CBF, and blood brain barrier (BBB) disintegration [65, 71]. These pathological phenomena lead to the exacerbation of neurodegenerative and cognitive decline resulting in cognitive impairment and dementia [65, 72].

1.3.4. The Blood Brain Barrier

Central nervous system circulation is separated from the peripheral blood circulation by a specialized vascular structure called the BBB. BBB not only delivers nutrients and oxygen according to current neuronal needs by controlling the passage of molecules and ions, but also protects the brain from toxins and pathogens, which provide an environment that allows all brain structures to function properly [73]. BBB consists of several cells types collaborating to maintain brain homeostasis. Endothelial cells (ECs), forming the inner layer of cerebral vessel walls, are the core anatomical element of BBB (Figure. 2) [74]. Uniqueness of BBB ECs compare with other tissues are the continuous intercellular tight junctions (TJs), lack fenestrations and extremely low rate of transcytosis. This significantly restricts both the paracellular and transcellular movement of molecules through the EC layer [75]. Pericytes and vascular smooth muscle cells are other components of the BBB which are also key components of vascular tone regulation [74]. Astrocytes with end-feet covering much of the vasculature are the other part of the BBB.

Astrocytes also provide nutrition for neurons, perform neurotransmitter clearance and recycling, and regulate extracellular potassium balance among other functions [76].

ECs in concert with other cells of the neurovascular unit, such as pericytes, astrocytes, and perivascular cells contribute to normal BBB maintenance, function, and integrity [77]. However, disrupted BBB function and integrity is common in advance age and AD. Occurrence of BBB disruption in early AD course raise the possibility of BBB role in the mechanisms underlying cognitive dysfunction in AD [78]. Disruption of the BBB is in correlation with pericyte and EC degenerations, astrocytic end-feet and basement membrane alterations, which altogether could result in Aβ clearance disruption, and infiltration of circulating factors into the brain [78]. Some experiments with murine animal models have demonstrated that BBB disruption leads to the accumulation of blood-derived neurotoxic products including hemoglobin, fibrin, thrombin, iron-containing hemosiderin, and free iron and/or plasmin that are able to damage neurons especially within the hippocampus [79-82]. Interestingly, a study in ApoE transgenic mouse model of AD have shown that cyclosporine A could improve BBB integrity through inhibiting an inflammatory pathway of TJ degradation and some neurotoxic serum proteins extravasation in pericytes [83]. Further studies in this subject may reveal new therapeutic approaches for cerebral pathologies related to BBB disruption.

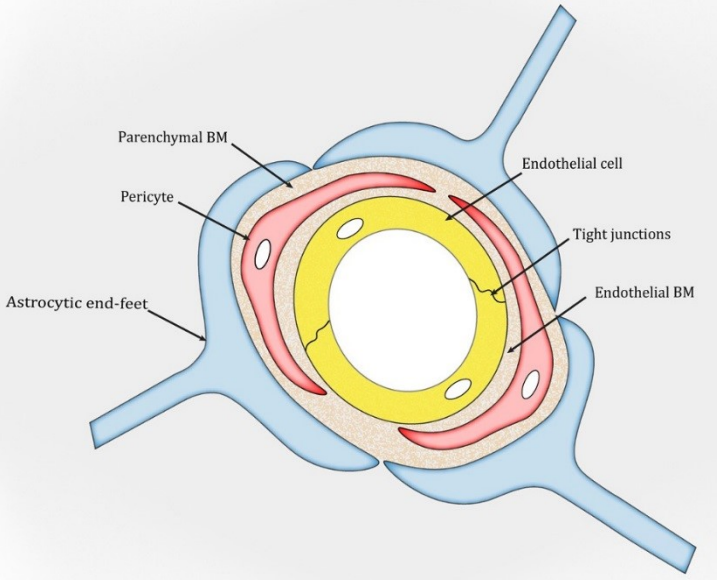


Figure 2. The schematic diagram of the blood brain barrier (BBB). Endothelial cells, astrocyte end foot, and pericytes are key elements composing the structure of BBB. Tight junctions, which prevent influx of most materials from blood to brain, link endothelial cells together. Figure adopted from Xu et al. [84].

1.3.5. Cerebrovascular dysfunction

CBF is very critical for a proper brain function. CBF decline and cerebrovascular reactivity impairment have long been described in early stage of AD [74]. Several studies in humans have demonstrated alterations of the BBB and of cerebrovascular reactivity, a decrease in resting CBF or increased cerebrovascular resistance in patients at a very early stage of AD [85-89]. Some studies illustrated the CBF decrease in the hippocampus, amygdala, and entorhinal cortex, regions, which are altered in AD and involved vividly in cognition. These characteristic appear prior to clinical symptoms [90]. In a parallel manner, early studies using transcranial Doppler measurements in the middle cerebral artery showed that individuals with a higher CBF velocity are at lower risk of dementia or atrophy of the hippocampus and the amygdala. It has been showed that cerebral hypoperfusion occurs earlier than Aβ accumulation, brain atrophy and cognitive dysfunction [91, 92].

Not only AD patients autopsy have demonstrated evidence of vascular pathology[93], clinical imaging and cerebrospinal fluid (CSF) analysis studies have indicated a CBF decline and BBB disruption 10 to 20 years before clinical stage of disease [82, 94]. Therefore, characterization of these cerebrovascular dysfunctions raised the need for the development of new techniques to identify early vascular biomarkers and thus predict cognitive decline [95, 96]. Measurement of changes in cerebral perfusion in certain brain regions, by neuroimaging techniques such as single photon emission tomography (SPECT), Magnetic Resonance Imaging (MRI), Diffusion tensor imaging (DTI) and positron emission tomography (PET) made preclinical detection of AD more feasible [97]. Utilization of different MRI imaging sequences have provided the detection possibility of disruption of the BBB [96], assessing white matter hyperintensities [96], microhemorrhages [98], as well as decreased cerebrovascular reactivity [99], which allows early vascular diagnosis of AD.

Although CBF alteration may follow neuronal activity reduction in AD, a wide range of evidences raise the prospect of early contribution of vascular dysfunction in disease progression. Regarding this possibility, some experimental studies in transgenic mouse model of AD demonstrated *in vivo* and *in vitro* vasoactive effects of Aβ in brain blood vessels [100, 101]. Impairment of CBF

responses to vasodilators and neurovascular coupling dysfunction are some of Aβ vasoactive effects [102, 103]. Another study by Zhang et al. showed that Aβ affect cerebral endothelial function [63]. They also found that CBF increases in response to acetylcholine application as an endothelium dependent vasodilator is significantly attenuated in a mouse model of AD, even prior to cognitive impairment or Aβ deposition [63]. This idea was expanded by other studies indicating that endothelial dysfunction is a consequence of vascular oxidative stress, and that ROS scavengers could reverse this process [102]. Aβ appears to be detrimental to both endothelium and pericytes where Aβ induces toxicity and cellular degeneration [104, 105]. Accordingly, early CBF reduction in both white matter and grey matter was observed in pericyte-deficient mutant mice with no Aβ pathology [106, 107]. Pathologic effects of Aβ on cerebrovascular autoregulation and neurovascular coupling also have been illustrated [103, 108, 109]. These investigations, collectively, can demonstrate independency of CBF reduction and dysregulation to Aβ pathology and occurrence before neuronal dysfunction.

1.4. Vascular intervention for AD primary prevention

Because AD commences decades before cognitive impairment appears, early detection and primary prevention is essential to delay the onset and therefore decline the future prevalence of Alzheimer's disease and minimize disease burden [110]. Several observational studies have discovered a number of modifiable risk factors for AD. The US National Institutes of Health highlighted diabetes mellitus, smoking, midlife hypertension, midlife obesity, depression, mental inactivity, physical inactivity, poor diet, and low educational attainment as being associated with increased risk of cognitive decline, AD, or both [111, 112]. A recent study discovered that 12 potentially modifiable risk factors were linked to 41% of dementia cases in the US [113]. Paying more attention to these risk factors by a healthy lifestyle, a high cognitive reserve, and the control of modifiable cardiovascular risk factors may all help to lower the risk of dementia, including AD [113, 114]. Up to a third of AD cases are estimated to be attributable to mentioned modifiable risk factors suggesting capability of reduction in AD incidence by early interventions and managements of these factors [115, 116]. Several intervention studies focusing on primary dementia prevention are now underway with the goal of lowering disease incidence. It is worth

highlighting the FINGER (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability) study, which looked into whether a multidomain intervention may help older adults in order to declining cognitive dysfunction [117]. This study illustrated that 2-year intervention (social stimulation, nutritional counseling, physical and cognitive training, and management of vascular risk factors) reduced the risk of cognitive decline in the intervention group of at-risk elderly individuals (60–77 years old) compare to the general population at risk of dementia [118]. Accordingly, other multidomain intervention studies yielded good results, the findings of which will reaffirm beneficial effects of primary prevention [119].

1.5. Hypotheses of AD pathogenesis

The early 1900s were the first time that AD was recognized by a German physician. Researchers have been continually exploring the pathogenic mechanism of this neurodegenerative disorder and effective disease-modifying therapies in the over 100 years [112]. The collection of a vast number of research in recent years has greatly enhanced our understanding of AD; nevertheless, this understanding is still superficial. Several hypotheses on the pathogenesis of AD have been proposed which amyloid hypothesis, vascular hypothesis and tau hypothesis are of the most known hypotheses.

1.5.1. The Amyloid Hypothesis

The amyloid cascade hypothesis, proposed over 20 years ago by John Hardy and David Allsop, is one of the most well-known theories for AD pathogenesis [120]. According to this hypothesis, the deposition and accumulation of the Aβ peptide in the brain is the main cause of the neurodegenerative process. Aβ is a transmembrane protein synthesized via the amyloidogenic pathway through a proteolytic process of the amyloid precursor protein (APP) [121]. The non-amyloidogenic pathway leads to production of some neurotrophic and neuroprotective products such as the C-terminal fragment (CTF)-α, the soluble ectodomain of APP-α (sAPPα), and other smaller fragments. This pathway generally occurs under normal circumstances and α- and γ-secretases are the enzymes that mostly involved in it. The second pathway is the amyloidogenic pathological pathway in which at the first step APP is cleaved to soluble β-APP fragments (sAPPβ) and CTF-β by β-secretase followed by CTF-β cleavage to Aβ and APP intracellular domain through

γ-secretase (Figure. 3) [121]. According to amyloid hypothesis, AD pathogenesis is because of early production or reduced/improper clearance of Aβ [122]. The accumulation of the Aβ peptide is toxic and leading to initiation of a multistep cascade such as deregulation of cellular homeostasis by activation of a number of kinases, result in abnormal phosphorylation of tau and its aggregation [123].

In order to study the pathogenesis of AD and in particular the amyloid hypothesis, a number of genetically modified mouse models have been produced, leading to the deposition of Aβ in the brain [124]. Despite the existence of senile plaques in the parenchyma of these mice, neither the accumulation of tau in the form of neurofibrillary tangles (NFT), nor neuronal death are observed in these mice, or seen very late in disease progression [124]. A reconsideration of amyloid hypothesis then suggested that tau is the main factor underlying the development and progression of AD, and the extracellular accumulation of Aβ is not intrinsically cytotoxic and that Aβ does not induce tau accumulation [125]. The amyloid hypothesis therefore evolved into the proposition that the soluble Aβ oligomers Aβ40 and Aβ42 represented to be much more toxic than fibrillar amyloid plaque cores or Aβ monomers and would thus be the cause of synaptic function disruption in the AD [126]. Accordingly, Ferreira proposed the "Aβ oligomer pathogenic theory," which suggests that soluble Aβ oligomers are the initial components in a succession of pathological changes in Alzheimer's disease [127]. Another concept regarding amyloid hypothesis propose that amyloid plaques are a defense mechanism to sequester excessive and harmful levels of soluble Aβ peptides and inactivate them in the form of fibrillar core structures surrounded by glial cells [128]. A human monoclonal antibody called aducanumab binds to soluble oligomers and amyloid fibrils selectively [129]. Reanalyzed data from the aducanumab trials showed significant positive findings leads to US Food and Drug Administration (FDA) approval of this drug for Alzheimer disease on June 7, 2021 [130]. However, this drug's approval is controversial despite being the first approved disease-modifying therapy for AD [131]. Some members of FDA Peripheral and Central Nervous System Drugs Advisory Committee expressed their opposition to the aducanumab in an article explaining the contradictory results of the two related trials and aducanumab's potential safety hazards [132].

Although the available data strongly support the central role of pathologic Aβ accumulation in mediating pathogenesis of AD, the amyloid hypothesis still faces several obstacles; including the acknowledgment that disrupted Aβ homeostasis is insufficient to cause the AD pathophysiologic process. Failure of multiple clinical trials targeting Aβ by using related antibodies in prevention of cognitive impairment or improvement of cognitive parameters suggesting other possibilities in AD pathogenesis [133]. Lack of association between Aβ and cognitive impairment, and equivocal findings on the clinical efficacy of Aβ-targeting therapy are of the more challenging issues regarding this hypothesis [134].

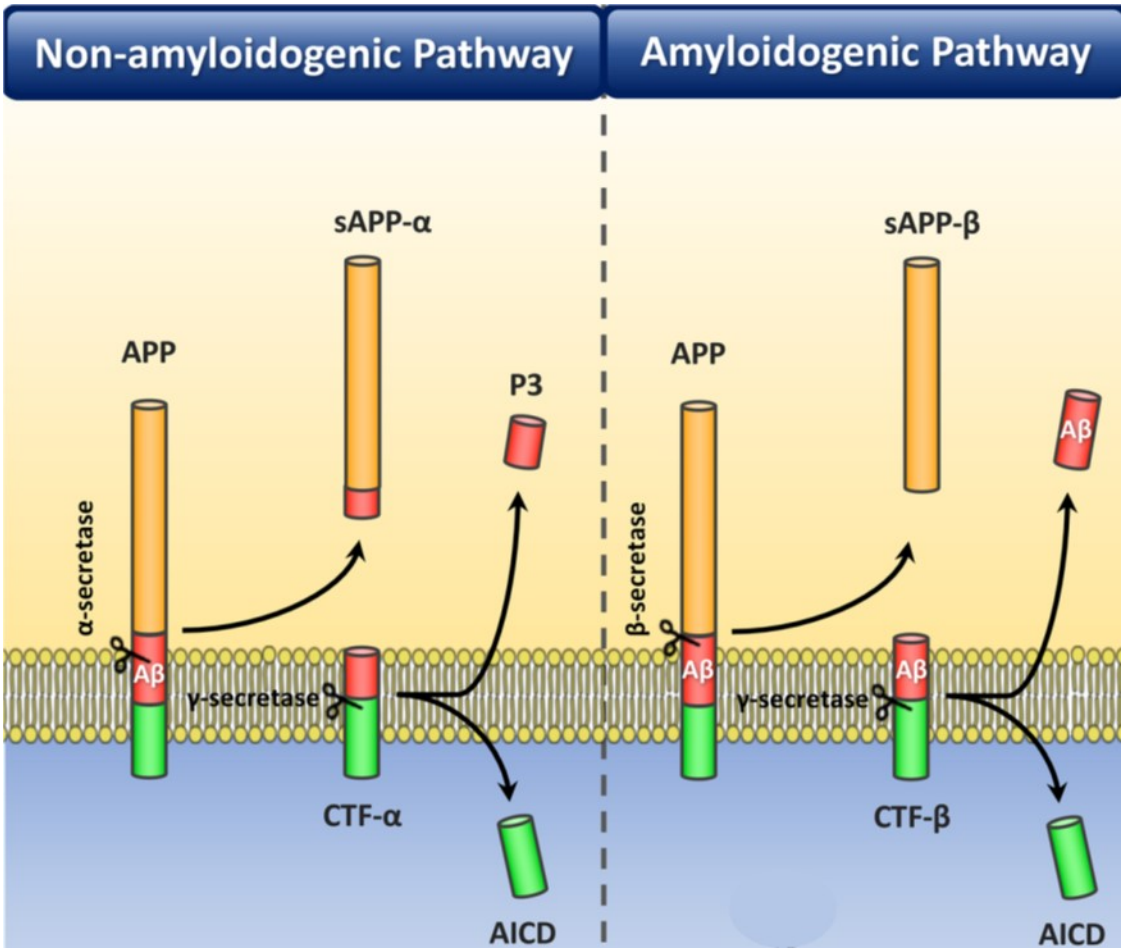


Figure 3. Amyloidogenic and non-amyloidogenic pathways of the amyloid precursor protein (APP). In the non-amyloidogenic pathway (left), APP cleavage by α-secretase leads to the production of sAPPα and the membrane-tethered CTF-α. At the next step, CTF-α is cleaved by γ-secretase that resulting in release of the P3 peptide and APP intracellular domain (AICD). In the amyloidogenic pathway (right), APP is cleaved by β-secretase and leads to the formation of sAPPβ and CTF-β, which in turn CTF-β cleavage by γ-secretase resulting in production of the Aβ peptide and AICD. Figure adopted from Coronel et al. [121]

1.5.2. Tau Hypothesis

Tau is a microtubule-associated protein that regulate the stability of tubulin assemblies. Claude Wischik extracted tau protein from plaques in the brains of AD's patients in 1988, revealing the possibility of tau protein role in dementia for the first time [135]. Tau is mostly present in the brain's neuronal axons, along with microtubules (MTs) [136]. The physiological role of tau in the CNS is not completely clear, however, some experiments demonstrated involvement of Tau in maintaining microtubule structure and cytoplasmic transport function, synaptic structure and function, and neuronal signaling regulation [137-139].

A complex multifactorial process develops tau pathology. In the brains of AD's patients, hyperphosphorylated tau causes configuration alterations and tubulin polymerization capacity reduction [140, 141], leading in microtubule dysfunction [142]. Increased cytosolic tau levels cause tau–tau interactions and polymerization, resulting in insoluble paired helical filaments and straight filaments, which form intraneuronal fibrillar deposits known as NFTs [140]. NFTs promotes cell dysfunction, reduce the number of synapses, and cause neurotoxicity [143]. However, the pathogenic role of tau still needs further explorations.

Evaluating multiple lines of evidence indicate that hyperphosphorylated, aggregated tau is a key cause of neurodegeneration in AD [144]. According to Braak staging, tau pathology in the AD brain propagate along a neuroanatomically connected network in a stereotypical pattern which initially appears in the transentorhinal region and then spreads to the limbic region and neocortical areas [145]. In a recent PET study of AD patients, a close link between the spatial patterns of tau tracer binding and the patterns of neurodegeneration and the clinical presentation was reported [146, 147]. However, another recent cross-sectional and longitudinal tau-PET imaging studies in amyloid-positive individuals whom their cognitive function were normal showed widespread and continuous accumulation of tau pathology outside of the entorhinal cortex, implying that tau propagation may not be as spatially restricted as previously thought [148, 149]. In contrary to Aβ, the stage of tau pathology is closely linked to the evolution of cognitive impairment in AD [150, 151]. In neuropathological studies, cognitive impairment in AD is only observed when tau extends from the entorhinal cortex to the neocortex [152]. Although only the presence or accumulation

of tau was reported as the predictor of cognitive impairment in longitudinal and cross-sectional studies of tau- and amyloid-PET imaging combined with structural MRI, the presence or accumulation of amyloid was predicting the severity of tau-associated cognitive impairment [153, 154]. These observations have led to the theory that the onset and spread of tau pathology is the primary cause of cognitive loss and neurodegeneration in Alzheimer's disease.

Tau has received a lot of attention recently, due to the failure of several Aβ-targeting therapies in clinical trials, as well as the fact that tau pathology correlates better with cognitive deficits than Aβ lesions. Recent discovery by Park and colleagues have indicated that mutated tau in mice leads to selective suppression of NVC that precedes tau pathology and cognitive dysfunction. The neurovascular uncoupling was restored by suppressing tau production [155]. They proposed that tau binding to post synaptic-density-95 (PSD-95), results in decoupling of neuronal nitric oxidase synthase from PSD-95 [155]. Alongside, some studies investigated beneficial effects of tau kinase and aggregation inhibitors, microtubule stabilizers, and immunotherapeutic medications in AD . However, most of them, such as the tau aggregation inhibitor LMTM (TRx0237), are toxic and ineffective [156]. In a mouse model of tau lesions, the antifungal compound epothilone D increased the number of microtubules, decreased the number of aberrant axons, and improved tau-related pathologies [157, 158]. However, because of the adverse effects of epothilone D, its clinical trial could not be continued. In Alzheimer's disease patients, AADvac1 as a tau vaccination demonstrated positive outcomes in terms of safety and immune response [159]. However, more research is needed to prove its clinical effectiveness.

1.5.3. Vascular Hypothesis

Nowadays, a wide range of evidences strongly support the vascular origin of AD [26, 43, 74, 96, 160]. The “vascular hypothesis” originally proposed as the “CATCH hypothesis” (Critically attained threshold of cerebral hypoperfusion) by de la Torre, proposes that cerebrovascular dysfunction, and more specifically chronic cerebral hypoperfusion, is an early event in the incidence of AD [161]. More recently, this hypothesis has been updated under a new hypothesis called "the two-hit vascular hypothesis of Alzheimer's disease". According to “two-hit hypothesis” cerebrovascular damage ("hit 1"), in particular via hypoperfusion and/or dysfunction of the BBB,

is a self-sufficient first insult to initiate neuronal injury and neurodegeneration, but may also promote accumulation of Aβ peptide in the brain (“hit 2”) [162, 163]. In support of the vascular hypothesis, there is also a causal relationship between vascular pathologies and the development of sporadic AD. The vascular hypothesis explains AD primarily develops from sustained cerebral hypoperfusion that could reach a critical threshold where brain cells can no longer cope with the dwindling energy supply being delivered [95]. Following chronic brain hypoperfusion delivery of oxygen and glucose will decline, thereby slowly creating a neuronal energy crisis featuring reduced ATP synthesis, increase of oxidative stress and an ischemic-hypoxic state, result in abnormalities in protein synthesis (disassembly, misfolding, aberrant cleavage) affecting cell structure and function [95]. This reduction in ATP impair the normal action of neurotransmitters, and affect cleavage of APP leading to overproduction of Aβ, an upregulation of BACE-1, and the hyperphosphorylation of tau protein and its deposition in form of NFT [160, 164]. Damage to microtubules due to hyperphosphorylation of tau protein declines retrograde axonal transport of trophic and growth factors (such as brain-derived neurotrophic factor) that are essential for neuronal survival [165]. In vivo studies in the ischemic rat by reversible multiple occlusions of the middle cerebral artery and by bilateral occlusion of the carotids, used to investigate neuronal energy crisis where the hypoperfusion led to the accumulation of the Aβ peptide [166]. Some experimantal studies on aged rats demonstrated mechanically reducing CBF in the aged experimental animals for 12 months, led to a chronologic set of cognitive deficits that initiated with mild memory loss, learning disability, diminished protein synthesis, and neurometabolic slowdown. These cognitive dysfunctions were followed by absence of grooming behavior, neuronal loss in the posterior cingulate cortex and hippocampus, severe memory loss, gliosis, and extensive posterior parietal cortex atrophy [167-170]. Ischemic conditions would also be likely to modulate the expression of presenilin genes, and accelerate the formation of free radicals [171]. The neuropathologic effects of chronic brain hypoperfusion in aged rats appeared to establish a model that resembled the progressive clinical course of AD. More interestingly, most of these pathological processes occurred in hypoperfused rats are in the absence of neuritic plaques, and the pathologic process could be reversed if CBF was restored within 2-3 weeks [172].

Furthermore, vascular damage seems to influence the amyloidogenic pathway, by reducing the clearance of Aβ and its accumulation in the brain [173]. The consideration of the vascular component as the primary pathological insult in AD not only does not negate the neurodegenerative mechanisms characteristic of AD, but also imply the fact that the vascular and degenerative mechanisms are interconnected and developed in parallel [174]. According to this vascular hypothesis as the neuropathological changes progress, cerebrovascular and cognitive function continues to deteriorate in parallel [164]. This sequence of events was recently confirmed by a multifactorial data-driven analysis of brain images and biomarkers (in elderly subjects with AD compared to elderly subjects without dementia), in which a chronological classification of disease progression was performed [91]. These imaging data suggest that intracerebral vascular dysregulation is an early pathological event during the development of Alzheimer's disease, resulting in neurodegeneration process [91].

The similarity of AD to vascular dementia (VaD) is one of the strongest indicators that AD is a vascular disorder. Both dementias have essentially identical symptoms and signs, as well as microstructural alterations [175, 176]. Moreover, their detrimental effects on cognitive function, and common relationship to over two dozen vascular risk factors are further clues supporting this idea [93, 177]. Described new insights need further investigations and can introduce prospective potential for therapeutic interventions in AD.

1.6. Clearance of Aβ

Aβ is generated from amyloid precursor protein (APP), a membrane protein that acts as a signalling receptor, during neuronal activity [178, 179]. Under normal conditions, APP hydrolyze via alpha-secreted enzymes, which precludes formation of Aβ, and gamma-secreted enzymes that cleave the resulting carboxy-terminal fragment [180]. The products of this pathway do not aggregate [181]. In case of APP cleavage by β-secretase 1 (also known as BACE1) instead of α-secretase, the subsequent γ-secretase cleavage will result in soluble monomeric Aβ, and the most common subtypes of Aβ in human body are Aβ1–40 and Aβ1–42 [182]. Aβ1–40 is predisposed to be deposited in the vasculature, as obseved in CAA [183, 184]. Aβ1–42 is more hydrophobic than

Aβ1–40 because of two additional amino acids, thus, it could produce insoluble aggregates forms [185].

Various clearance processes can remove soluble Aβ from the brain. Cellular uptake from the interstitium by neurons, microglia, and astrocytes, or uptake from the perivascular space by smooth muscle cells, perivascular macrophages, and astrocytes, have a main role in Aβ clearance via extracellular and intracellular degradation pathways [186]. Aβ efflux into the blood is performed through BBB clearing system. Interstitial fluid bulk flow clearance mainly transport Aβ into CSF sink (ventricles and subarachnoid space), via perivascular drainage, or via glymphatic drainage. CSF absorption clearance remove Aβ into the circulatory system via the arachnoid villi and blood–CSF barrier, or into the cervical lymph nodes from the perivascular and perineural spaces by lymphatic clearance [187]. Majority amount of Aβ in periphery is cleared by blood components, such as red cells (RBCs) and monocytes, however, liver and kidney also have important role in this process [186].

Transport of Aβ from brain into the peripheral circulation is thought to facilitate the most of physiologic Aβ clearance [188]. Removal of Aβ through the BBB requires specific transporter proteins. Specifically, low-density lipoprotein-related protein 1 (LRP-1), members of the LDL receptor (LDLR) family, and receptor for advanced glycation endproducts (RAGE), are of important primary receptors for Aβ efflux and influx, respectively [189].

Among Aβ scavenger receptors, LRP1 has been investigated extensively in numerous studies. LRP-1, which is found predominantly on the abluminal surface of brain endothelial cells, binds either ApoE-Aβ complexes or Aβ alone, causing endocytosis of either species [190, 191]. Furthermore, LRP-1 facilitate cellular Aβ uptake and lysosomal degradation within neurons, microglia, and astrocytes [192]. Superiority of LRP1 to other receptors in clearing Aβ may be due to its ability in endocytosis, which could be up to 16 times more rapid than that of other Aβ receptors, particularly at higher concentrations [190, 193]. For endocytosis performance, LRP1 binds to Aβ directly or indirectly through its coreceptors or ligands [194]. Blocking LRP-1 expression in wild type mice led to impaired Aβ clearance across the BBB, and consequently, increased Aβ

deposition and cognitive dysfunction [195]. Accordingly, LRP-1 expression reduction reported in transgenic mouse model of AD as well as AD patients, and aging adults [183, 191, 196].

RAGE belongs to the immunoglobin supergene family and is expressed on the surface of vascular endothelial cells, glial cells, pericytes, and neurons [197]. RAGE located on the luminal membrane of capillary endothelial cells and transport free Aβ from the circulation into the interstitial fluid of the brain [198, 199]. Advanced glycation endproducts, S100 calcium-binding protein B (S100B), and high mobility group box 1 (HMGB1) activate RAGE, causing accumulation of Aβ [190, 200]. Under normal physiological conditions, expression of RAGE is at low levels in endothelial cells [197]. However, such inflammation-related pathological conditions like vascular diseases and chronic neurodegenerative diseases lead to over expression of RAGE [201, 202]. RAGE also lead to neuronal damage and apoptosis by exacerbating the inflammatory response inside the brain through cytokines release. One experimental study by Dean et al. demonstrated that systemic injection of Aβ is endocytosed by vascular endothelial cells in a RAGE-dependent manner [203]. It also has been reported that Aβ uptake and transport could be reduced by RAGE-specific immunoglobulin and soluble RAGE, which illustrates well the role of RAGE in Aβ transport into the brain [204].

1.7. Alzheimer disease mice models

In order to study the pathological mechanisms of AD, researchers has largely relied on the use of transgenic mouse models that overexpress mutated human genes associated with familial forms of AD. AD-related mutations led to the creation of transgenic mice, which feature key aspects of AD neuropathology, including in particular the extracellular deposits of the Aβ peptide in the form of extraneuronal amyloid plaques and at the level of the blood vessels, as well as the clinical signs associated with this disease, such as cognitive deficits (Table 1). The cerebrovascular abnormalities observed in humans such as CBF reduction, impaired functional hyperemia, and endothelium-dependent vasodilation responses are mainly found in most of trangenic models. Therefore, these models provide the possibility of investigating micro-hemorrhages, endothelium dependent vasodilatory responses, cerebral hypoperfusion, and BBB breakdown. In some of transgenic models, vascular abnormalities could be indicated before the deposition of neuritic

plaques and/or the development of cognitive dysfunction, supporting the concept that vascular alterations are involved initially in the incidence of AD pathogenesis [43]. Although none of trangenic models imitates human pathology exactly, however, each model provides valuable information about its potential mechanisms. The table below (Table 1) describes some trangenic models mostly used in research, such as 3xTg-AD, 5xFAD, PD-APP, Tg2576, and TgCRND8. This table is not exhaustive; for a complete list of all available models, please refer to the ALZOFORUM.org website.

Table 1. Transgenic models of AD over express mutated human genes associated with familial forms of AD. These transgenic mouse models thus reproduce the vast majority of the neuropathological lesions characteristic of AD, in particular the extracellular deposits of the Aβ peptide in the form of extraneuronal amyloid plaques and in the blood vessels, and hyperphosphorylation of the tau protein. Moreover, these transgenic models also develop the clinical signs associated with this disease, such as cognitive dysfunction.

Transgenic mouse model of AD		
	Gene mutations	**Phenotype characterization**
3xTg-AD	APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V	- Extracellular Aβ deposits by 6 months - By 12 months extensive tau immunoreactivity in CA1 - Cognitive impairment manifests at 4 months as a deficit in long-term retention [205]
5xFAD	APP KM670/671NL (Swedish), APP I716V, APP V717I, PSEN1 M146L (A>C), PSEN1 L286V	- Extracellular amyloid deposition begins around 2 months - Gliosis begins at 2 months - Impaired spatial working memory by 4 to 5 months of age [206].

PD-APP	APP V717F (Indiana)	- Deficits in working memory from 3 months before the appearance of histological amyloid lesions, which appear at 6 months and whose quantity increases with age (extraneuronal Aβ plaques and CAA). - Glial activation and neuritic involvement, but no NFT [207]
Tg2576	APP KM670/671NL (Swedish)	- Numerous parenchymal Aβ plaques by 11-13 months - Increase in microglial density and size in plaque-forming areas in 10-16 month old hemizygotes - Impaired spatial learning, working memory, and contextual fear conditioning at <6 months [208]
TgCRND8	APP KM670/671NL (Swedish), APP V717F (Indiana)	- Amyloid deposits at 3 months; dense cored plaques and neuritic pathology by 5 months - Early impairment in acquisition and learning reversal in the reference memory by 3 months [209]

Investigating the contribution of cerebrovascular risk factors in AD is generally carried out using classic transgenic mouse models of familial AD. For instance, the study of the contribution of hypertension (induced by infusion of Ang II), which is very prevalent in the incidence of AD, has been particularly studied in the APP/PS1 model [210, 211]. All of these data support the fact that vascular pathology play a critical role in development of AD.

1.8. AD and VCID Comorbidity

The co-occurrence of AD and VaD, often known as mixed dementia, is a possibility because vascular pathology not only facilitate but also accelerate vascular impairments [212]. Cognitive decline and neuropsychiatric symptoms are common clinical features of both AD and VaD. Only 9% of people with cognitive abnormalities had pure AD pathology, according to a clinical investigation by Boyle et al. Nonetheless, the pathogenesis of Alzheimer's disease is frequently associated with vascular dysfunction or other neurodegenerative diseases [213]. A clinical study reported that only 28% of 63 patients with mild cognitive impairment had pure Alzheimer's disease, whereas the 24% of them had mixed dementia [214]. These findings suggest that there are significant commonalities between AD and VaD. Rather than focus on distinguishing AD from VaD, more study should be done to better understand dementia's etiology and create therapeutic solutions.

Early neurovascular dysfunction, neuronal loss, increasing neurodegeneration, and Aβ and NFT accumulation in the brain are all the pathologies seen in AD [173, 215]. Synergistic effect of vascular damage and Aβ accumulation exacerbate tau hyperphosphorylation, and neuronal loss [216]. AD affects all cell types of the neurovascular unit, including endothelial cells, astrocytes, glia, and neurons, depending on the stage of the disease [173]. Furthermore, vascular pathologies can be caused by genetics, vascular risk factors, environmental variables, and lifestyle patterns [173]. The neurovascular theory of Alzheimer's disease also proposes that cerebrovascular dysfunction and neurovascular integrity disturbances have a role in the onset and progression of cognitive decline, and converge of these events at cerebral vasculature leads to AD [173].

2. Arterial Stiffness

2.1. Introduction

The functions of arterial system are delivering blood from the heart to peripheral tissues, depending on metabolic activity, and damping the oscillations of blood pressure caused by pulsatile ventricular ejection [217]. The walls of the large arteries transform the pulsatile flow into a more constant flow by vascular recoil during systole that serves as a reservoir to attenuate decreased blood pressure during diastole, which is called "Windkessel effect" [218]. Compliance of the arterial system ensures that the high-pressure blood flow is captured to preserve a stable perfusion in vulnerable vascular beds with high and low resistance [219]. The stiffness of the arteries gradually rises from the heart to the periphery under physiological conditions, which is partly related to the proximodistal constriction of the arterial diameter. This stiffness gradient generates an impedance mismatch, resulting in partial wave reflections that limit pulsatile energy transmission and protect microcirculation from injury [220].

Although with normal aging the compliance of the arteries decreases, certain pathologies will accelerate this process. The term "arterial stiffness" illustrate the alteration of these properties of the vascular walls [221]. An increase in arterial stiffness result in a decrease in the Windkessel effect of the arteries, an increase in pulse pressure (the difference between systolic and diastolic blood pressure), and the pulse wave velocity, which cause increase in systolic pressure by early return of the reflected waves to the heart [222]. Consequently, the left ventricle need an additional workload to overcome the increase in arterial pressure. This could result in left ventricular hypertrophy and heart failure [223]. Arterial stiffness impairs damping function and transmits pulsatile blood pressure deeper into the microvascular structure, which damages distal organs, especially the kidney and brain [21]. Although arterial stiffness is increases rapidly with high blood pressure, it also counts an independent predictor of cardiovascular events. Arterial stiffness exhibits a two-way causal relationship with hypertension. While high blood pressure promotes stiffness by damage to the arterial wall, arterial stiffness is also the main cause of increased systolic blood pressure [224].

2.2. Vascular alterations characteristics in arterial stiffness

2.2.1. Structural and mechanical alteration

The vascular wall undergoes dynamic physiological changes during life [225]. Characteristics of vascular remodeling in large arteries associated with old age is very similar in humans, non-human primates and rodents and includes luminal dilation, intimal and medial thickening, vascular stiffness, and decreased endothelial function [226]. The proportional contributions of two main scaffolding proteins, collagen and elastin, determine the vascular wall's stability and compliance. A dynamic process of production and degradation keep the balance in the content of these proteins. Inflammation disrupts this balance, resulting in an increase in collagen formation and a decrease in elastin levels, contributing to vascular stiffness [227]. Aging also alters the balance between catabolic matrix metalloproteases (MMPs) and their inhibitors (the tissue inhibitors of metalloproteinases (TIMPs)), which regulate collagen and elastin remodeling. Experimental studies in aged rats and non-human primates demonstrate increased expression and activity of MMP-2 in their vessels compared to younger counterparts [228, 229]. Hypertension also stimulates excess collagen production, which results in increased intima-media thickness, and hypertrophy of the vascular smooth muscle layer [230, 231]. Arterial remodeling is associated with endothelial cell disorganization, increased collagen, MMPs, transforming growth factor β, intracellular cell adhesion molecules and cytokines, degradation of elastin fibers and infiltration of smooth muscle cells, macrophages and mononuclear cells [232]. The increase in collagen and the breakdown of elastin lead to mechanical changes characterized by a reduction in compliance, elasticity and distensibility and therefore arterial stiffness [233]. The breakdown of elastin fibers predisposes these fibers to mineralization with deposition of Ca2+ and phosphorus, thus increasing arterial stiffness [234]. Arterial stiffness is also caused by the formation of advanced glycation end products (AGEs) which result from the non-enzymatic glycation of proteins to form irreversible cross-links with collagen. Together with AGEs, collagen forms a molecule that is more rigid and less sensitive to hydrolytic turnover [235]. AGEs also reduce the elastic matrix of the vascular wall and can affect endothelial cell function by deactivating nitric oxide (NO) and increasing the generation of reactive oxygen species (ROS) such as peroxynitrite [227, 236].

2.2.2. Endothelial Dysfunction and functional alteration

The endothelium not only play an important role in maintaining vascular homeostasis, but also is involved in many physiological functions, including promotion of angiogenesis, blood pressure regulation, and control of the coagulation process [237, 238]. Arterial stiffness and endothelial dysfunction are of the major contributors to age-related vascular alterations [239]. Impairment of endothelial function is also associated with important risk factors for cardiovascular disease, including smoking, hypertension, diabetes, high cholesterol, high blood sugar, obesity, chronic inflammation, and a family history of cerebrovascular diseases [240]. Increased oxidative stress is a common pathologic finding in many of these risk factors [241]. Imbalance between the oxidative and antioxidant systems results in increased ROS production leading to NO inactivation and increased nitrosative stress thereby impairing endothelial function [242]. NO bioavailability Reduction because of its interaction with ROS to form peroxynitrite is the primary mechanism responsible for the deterioration of endothelial function [242].

Endothelial dysfunction, arterial remodeling, and decreased CBF have been linked to central nervous system inflammation and early vascular cognitive impairment in humans [243]. However, animal studies explain better the underlying mechanisms in this process. For example, experimental studies in transgenic mice with severe dyslipidemia (LDLr−/−; hApoB100+/+), which characterized by carotid stiffening and mild hypertension demonstrated cerebrovascular endothelial dysfunction, cerebral artery wall remodeling associated with MMP activation, CBF reduction, and early cognitive impairment [244-246]. Pulse pressure increase for one week disrupted the BBB and induced inflammation and production of ROS in the mouse brain [247]. Cerebral endothelial dysfunction is also associated with neurovascular coupling alteration [248]. In aging and in atherosclerotic mice cerebrovascular endothelial dysfunction was indicated which was in parallel with declined resting CBF and cognitive impairment [249, 250]. More interestingly, prevention of endothelial dysfunction restricts CBF maladaptation and slows the cognitive impairment [254]. This sequence of events is still theoretical and needs to be proven more thoroughly.

2.3. Animal model of arterial stiffness

Arterial stiffness models used for the animal studies categorized mainly in two groups: models where vascular stiffness is induced by genetic, chemical or surgical interventions; and those where stiffness is secondary to other conditions, such as aging, atherosclerosis, or hypertension [251].

2.3.1. Spontaneous models

In the spontaneous models, arterial stiffness appears often secondary to another condition such as aging, hypertension, atherosclerosis, etc. [252]. Throughout aging, dynamic physiological changes in the vascular wall are occurring [253, 254]. Increase in the thickness of the intima-media layer is one of the structural alterations, which could be observed in the aged vasculature. Not only subclinical intima-media thickening is strongly associated with aging, but also it is a predictor of future cardiovascular events [255]. Mechanical changes characterized by reduction in arterial compliance, elasticity decline and increased stiffness [256]. Stiffness can also develop alongside or because of hypertension, which is also closely associated with aging. One of the consequence of high blood pressure is vascular remodeling. Spontaneously hypertensive rat is the animal model most widely used to study essential hypertension and left ventricular hypertrophy [257]. This model also exhibits stiffer arterial walls, reduced elastic, providing the possibility of using this model to study arterial rigidity in the context of hypertension [258].

Rigidity can occur in the context of atherosclerosis, which is characterized by plaque deposits composed of triglycerides and cholesterol. These depositions are observable at the level of the vascular wall and cause alteration of media layer by calcifying it and increasing the intima-media thickness. Although calcification and intima-media thickness are some characteristics of arterial stiffness, however, formation of plaques does not seem to correlate directly with the arterial stiffness [259]. Diabetes and chronic inflammatory diseases are other pathological conditions associated with the development of arterial stiffness [260, 261]. In diabetes, it is suggested that hyperglycemia, dyslipidemia and hypertension lead to some pathological processes such as insulin resistance, oxidative stress, endothelial dysfunction as well as the formation of pro-inflammatory cytokines and advanced glycation end products, all of which lead to increased

vessel wall stiffness [261]. Although all of these models display some stiffness features in the arteries, however, they affect throughout the body generally and could not be used as an appropriate model for investigating contribution of arterial stiffness as an individual parameter on the brain.

2.3.2. Surgical models

Arterial stiffness can be surgically induced by ischemia, replacing part of the artery with a rigid tube, or aortic constriction model [252]. Ischemia model replicate the conditions of age-related cardiovascular pathologies such as myocardial infarction and stroke by blocking the arteries, such as the aorta or the carotid artery in order to prevent blood flow from going to the heart or the brain. The procedure consists of inducing an interruption of the circulation of the vasa vasorum irrigating the smooth muscle cells of the vascular wall leads to adventitia degeneration, reduction of medial conductance, alteration of elastin and collagen fibers, phenomena that are associated with increased arterial stiffness [262].

Another model is the replacement of the artery, such as the aorta or carotid, by a rigid tube. This surgical procedure results in an increase in arterial pulse pressure with minimal change in blood pressure and mean flow [263]. Pulse pressure increasing also perform by murine constriction model of the transverse aorta. It consists of isolating the transverse aorta between the trunk, right, and left carotid arteries following by ligating between truncus anonymous and the left carotid, which leads to an increase in blood pressure. The resulting hypertension leads to increase pulse pressure in right common carotid artery [264]. Despite of advantageous of these models in arterial stiffness induction, they have still some limitations. Ischemia and tube replacement model of surgery cause substantially decrease CBF during the surgery, which may thus affect the brain. Moreover, constriction of aorta result in cardiomyopathy and heart failure, which is a confounding factor in the studies, and also restrict the study of long term effect of arterial stiffness individually on brain.

2.3.3. Genetic models

The most common manipulations for genetic models are mutations of genes controlling the components of the vascular wall. For example, gene coding for elastin is modified and heterozygous mice (Eln +/-) are used in this model. Eln +/- mice show a significant alteration in the elastic properties of the arteries, leading to their stiffness and hypertension [265]. Another genetically modified mouse model is Marfan syndrome model with a homozygous hypomorphic mutation in the fibrillin-1 gene, a protein involved in the formation of elastic fiber. This pattern shows a significant increase in aortic stiffness, as well as aortic dilation [266]. Deletion of the gene encoding the ApoE is another interesting model. Mice with a deletion of this gene develop calcification of the aorta and coronary arteries and atherosclerosis [267, 268]. Finally, an important model is the mouse with a deletion of the gene encoding the Gla matrix protein (GMP). GMP is a calcification regulatory protein, which its mutation cause development of significant arterial calcification leading to arterial stiffness [269-271]. However, these models are not specific to blood vessels or to a precise segment of the arterial tree, therefore simultaneously affecting many organ functions.

2.3.4. Chemical models

Chemical models represent the most used models to study arterial stiffness. The most commonly used are those, which consist in administering vitamin D and nicotine to animals or even vitamin K and warfarin [272, 273]. In one hand, toxic levels of vitamin D induce arterial calcification, and in the other hand, nicotine could stimulate release of catecholamines or directly cause vasoconstriction of the arteries [274, 275]. The combination of vitamin D and nicotine induce calcification of the elastic fibers of the arterial media leading to their fragmentation [272]. Furthermore, vitamin K and warfarin inhibit the maturation of the GMP, which is necessary to prevent calcification of the arterial media [273]. These conditions all lead to a decrease in the elastic capacity of the arteries [270]. However, these models cannot be used to study the effects of arterial stiffness on the brain since compounds such as vitamins D and K are known to directly interact with the central nervous system [276-278].

2.3.5. Calcification and arterial stiffness

Vascular calcification is a potential contributor to increased rigidity of large arteries with aging [279, 280]. A new model for carotid artery calcification has been developed by Dr. Girouard's team [24]. In this model, direct application of sterile $CaCl_2$ to the adventitia of the carotid artery leads to arterial stiffness. The application of $CaCl_2$ to the carotid artery leads to the development of Ca2+ deposits on the artery, thickening of the intima-media layers with an increased deposition of collagen, elastin fragmentation, and macrophage infiltration in the calcified carotid artery 2 weeks after $CaCl_2$ application. There is also a decrease in carotid compliance accompanied by an increase in the β index. All of these changes occur without altering systolic blood pressure or carotid radius. This model also provide researchers the possibility of stiffening one single artery, which is a distinct advantage over transgenic models that genetic manipulation potentially affect most of large and non-large arteries. Furthermore, the cerebral-related outcomes could be investigated more extensively in this carotid artery calcification model compared with the transgenic models.

Therefore, there are several models for studying arterial stiffness which some of the models were described. In these models vascular stiffness arises as a result of other conditions such as atherosclerosis or hypertension or is induced by genetic, chemical or surgical manipulations. Unlike most of these models, which present confounding factors such as hypertension or ischemia, the carotid calcification model makes it possible to study the effects of arterial stiffness on the brain specifically.

2.4. Arterial stiffness and cognitive dysfunction

Alterations of arterial structure and function through aging are associated with a marked increase in the rigidity of the central arteries [281, 282]. Because of its probable participation in cerebrovascular disease, hypertension, vascular dementia, and Alzheimer's disease pathology, arterial stiffness may contribute to the pathogenesis of cognitive impairment [283]. Arterial stiffness exposes the cerebral vasculature to the potentially damaging forces resulting from increase in arterial pulse pressure [284]. It is now defined as a predictor of cognitive impairment including memory, executive function and processing speed as well as dementia [15, 285, 286].

Several studies in older population not only demonstrated the link between arterial stiffness and structural brain damage as well as poorer cognitive performance, but also indicated increase risk of mild cognitive impairment progression toward dementia [287, 288]. Association between aortic stiffness and early signs of brain aging in young adults was examined in Framingham study. An association between aortic stiffness and subclinical cerebrovascular injury has been reported in this study; however, cognitive impairment was not evident until midlife. The outcomes of this study suggest that initial structural brain injury due to vascular stiffness may begin early in the life course, prior the onset of cognitive dysfunction [289]. Furthermore, the literature agrees on the link between arterial stiffness and different domains of cognition such as executive functions, processing speed as well as episodic, visual and spatial memories [290, 291].

Increased downstream blood flow pulsatility caused by arterial stiffness leads to cerebral microvascular remodeling, white matter damage, and decreased gray matter [287, 292, 293]. High pulse pressure of intracranial arterial circulation leads to cerebrovascular remodeling and impairs cerebrovascular and hippocampal function, which contributes to cognitive decline [243, 294, 295]. While development of cerebral capillary damage and BBB degradation in the hippocampus of individuals with early cognitive impairments have been reported, these pathologies were independent of changes in the Aβ and/or tau biomarker of AD [94]. Furthermore, BBB disruption, a pathology involved in cognitive dysfunction, is exacerbated by the increased penetration of pulsatile pressure waves into the cerebral microcirculation [14, 296]. Hughes et al. [297] demonstrated a link between arterial stiffness and Aβ deposition in the brain that was independent of blood pressure. Arterial stiffness lead to dysfunction in perivascular amyloid clearance system by affecting structure and function of penetrating arteries. This may cause vascular dynamics to be disrupted, complicating Aβ perivascular flow and, as a result, decreasing Aβ clearance, leading to plaque development [298]. Another mechanism implicated in cognitive impairment is neuroinflammation characterized by the activation of microglia in response to plasma constituents entering the brain [299]. Indeed, the rupture of the BBB allows toxins, proteases or other substances present in the blood to enter the interstitial space of the brain and cause damage to neurons and surrounding glial cells [300]. Therefore, arterial stiffness may contribute to the pathogenesis of cognitive dysfunction mostly through its potential role in

cerebrovascular impairments such as the BBB disruption, vascular alteration, neuroinflammation, etc.[14].

3. Scientific background, Hypothesis, and Objectives

Cerebrovascular involvement in the pathogenesis of many types of dementia is increasingly recognized, affecting both the incidence and course of cognitive decline [74, 96, 301]. In the concept that alterations in the vascular system precede neurodegenerative disorders, the pathological events and mechanisms that occur in the brain with aging are only partially characterized. Arterial stiffness seems to be the perfect candidate to initiate both cerebrovascular diseases and AD, regarding incidence of cognitive disorders in function of the degree of arterial rigidity [291]. Indeed, arterial stiffness increases cerebral blood flow pulsatility, which is believed to be very detrimental to endothelial cells [14, 20, 302]. Accordingly, the biomechanical hypothesis defines the role of arterial stiffness and and the consequent increase in pulse pressure in the incidence of cognitive decline via cerebrovascular damage. In addition, the presence of cardiovascular disorders present additional or potentiating effects on the vascular damages associated with aging, leading to an early incidence of cognitive disorders. In particular, the increase in arterial stiffness with age could thus initiate or accelerate the incidence and/or development of dementia (in particular AD) in susceptible individuals [34, 160, 303]. However, clear precipitative and/or aggravative effect of arterial stiffness in AD progression and cognitive function deterioration remains to be demonstrated.

We hypothesize that arterial stiffness contributes to the onset/progression of AD via altered BBB integrity and Aβ clearance, resulting in the accumulation of Aβ peptides in brain and blood vessels (CAA), which together lead to neuronal and cognitive dysfunctions.

To test this hypothesis, we propose to answer the following specific question:

Does chronic AS precipitate and/or aggravate cognitive deficits in a mouse model of AD? And if so, is it characterized by decreased clearance of Aβ peptides.

The objective of this thesis was to determine the effects of arterial stiffness on the cognitive functions using the carotid calcification model of arterial stiffness. We sought to determine the

deleterious effect of arterial stiffness on cognitive functions in wild-type and 3xTg mouse model of AD.

Chapter 2: Materials and Methods

1. Animals

All animal experiments were performed in accordance with the guidelines of the *Canadian Council for Animal Care* and the *Animal Research Reporting of In Vivo Experiments* and was approved by the Montreal Heart Institute Ethics Committee. The male and female 3xTg mouse model of AD used in this study (generous gift from Dr. Frédéric Calon, Université Laval) was developed by Oddo and colleagues [205]. Three mutant genes with human mutations for the human beta-amyloid precursor protein (APPswe), tau (tauP301L), and presenilin-1 (PS1M146V), are present in these animals. The 3xTg-AD mimics many aspects of AD, including as Aβ and tau pathology, as well as cognitive abnormalities. Previous research has indicated that 3xTg-AD mice develop gross neuropathological and cognitive alterations around the age of 12 months [304, 305]. The non-transgenic wild-type (WT) controls utilized in this project were obtained from backcrosses of our 3xTg-AD colony with B6126SF1/J animals (C57BL6/129Svj). Mice were housed individually under standard conditions (24°C; 12-h:12-h light/dark cycle) with ad libitum access to water and a regular chow (Envigo #2018 Teklad global 18% protein rodent diet). Following acclimation, WT and 3xTg mice were randomly assigned to two groups receiving either a carotid periarterial application of NaCl (control group) or $CaCl_2$ (arterial stiffness group). Arterial stiffness and sham surgeries were performed at the age of 5 months and cognitive tests were performed 3 weeks later, at 6 months of age.

2. Surgical procedure

Carotid artery stiffness was performed in mice using the method described [24].

In brief, 5 months-old mice were anesthetized with a mixture of ketamine/xylazine (80 mg/Kg and 8 mg/Kg respectively; CDMV, Saint-Hyacinthe, Canada). Through a midline neck incision, right common carotid artery was gently isolated following by sliding small piece of sterile triangle shaped parafilm underneath of the artery. At the next step, a sterile gauze (5×5 mm) presoaked in 0.3 M $CaCl_2$ solution (Sigma-Aldrich, Oakville, Canada) was applied on top of the carotid artery

for 20 minutes. Having removed the gauze and parafilm, the incision was sutured using 5-0 silk stitches. In Control (sham) group, animals underwent the same surgical procedure, but a sterile gauze soaked in 0.9% NaCl was applied. Post-surgery, anti-inflammatory analgesic, Carprofen (Rimadyl, 5 mg/kg; CDMV, Saint-Hyacinthe, Canada) was injected subcutaneously every 24 hours during two consecutive days. To prevent infection, mice also received trimethoprim sulfadiazine (Tribrissen, 30 mg/kg subcutaneous injection; CDMV, Saint-Hyacinthe, Canada) for 3 days at 24-hour intervals. Animals' weights were monitored weekly following the surgery to assure good recovery. In all groups, there was no mortality event resulting from the surgery complications. For confirmation of the carotid artery calcification, carotid samples were dissected for Von Kossa staining (performed at the Institute for Research in Immunology and Cancer, Université de Montréal), to visualize calcium deposits [306].

3. Neurobehavioral tests

Behavioral experiments were performed three weeks after the surgery for carotid calcification. To decrease the stress induced by the experimental manipulations, mice were handled three consecutive days before behavioral experiments. In all behavioral experiments, mice movements were first video-tracked and subsequently analyzed by a position tracking system (Smart 3.0; PanLab). The experiments began with the open-field test first. The Barnes maze test occurred the next day after the end of the open-field test.

3.1. Open-field test

Open-field tests were performed as previously described [307]. Each mouse was allowed to freely explore a home-made squared open field made of gray polyvinyl chloride (45 cm × 45 cm) for 5 min. Data were then analyzed automatically using a custom-made zone pattern (Smart 3.0, Panlab) consisting of three concentric squares defining central, intermediate, and peripheral regions. Anxiety was evaluated from the time spent in the center versus the periphery. The intermediate part does not bring information of interest for the present study. Locomotor activity was evaluated by measuring the total distance traveled and zone transitions using a pattern

divided into 16 squared OF zones of equal size. The test chamber completely cleaned (Versaclean 10%) between every single experiment.

3.2. Barnes maze

Barnes maze is designed to assess hippocampus-dependent spatial learning [308] using an elevated platform (PanLab). The experimental protocol was similar to that described by Sunyer and coworkers [309] with slight modifications. The Barnes maze consists in a circular surface (92 cm in diameter) standing about three feet above the floor with 20 peripheric holes in it. Mice were trained to use spatial cues to find the target hole connected to a small dark escape chamber under the platform termed “escape box”. To eliminate the use of the intra-maze odor cues, while the target is retained at the same position relative to the room, the platform is rotated with each trial. Moreover, after each trial, the platform, the starting chamber, and the escape box were thoroughly cleaned (Versaclean 10%) to decline any possible scent trails. An aversive pulsed noise (85 dB) was used during each trial and turned off when the mouse entered in the escape box.

On the familiarization period (day 1), the mouse was placed in the middle of the maze in a dark colored cylindrical start chamber (starting chamber). After 30 seconds have elapsed, the starting chamber was lifted, simultaneously the buzzer was turned on, and the mouse was allowed to explore the maze for three minutes. In case of disability of the mouse to find the escape box, it was gently guided to the escape box and remaining there for two minutes.

One day following the familiarization period, mice were trained four trials per day with an inter-trial interval of 15-20 min for 4 days. At the beginning of each training trial, mice were placed in the same starting chamber in the center of the maze. After 10 seconds, the starting chamber was lifted and the buzzer was started. The mice were free to explore the maze for a maximum duration of 3 min. The trial ended when the mouse enters the escape box or after 3 min have elapsed. The buzzer was turned off immediately after the mouse enters the escape box, and the mouse was allowed to stay in the escape box after each trial for 1 min. If a mouse failed to reach the target hole at the end of the 3 min period, it was gently guided to it. Video tracking and data analysis were carried out using Smart 3.0 (PanLab). The following parameters were collected for training sessions: number of errors, and the latency and the distance traveled to escape through the target

hole. Errors were defined as nose pokes and head deflections over any hole other than the target hole.

One day after the acquisition phase, mice performed a probe trial in which the escape box was closed. Mice freely explored the maze for 3 min. The number of errors, the latency and the distance traveled to reach the target hole for the first time (primary search) were recorded. Furthermore, the time spent in the quadrants (target, left, right and opposite, excluding a 15 cm diameter central zone) and the number of visits for each hole during the total probe test search (90 s), were collected. Target hole and two of its adjacent holes were considered as target holes, and the rest 17 holes were defined as non-target holes. The selective search ratio was calculated as the number of hole visits in (target)/(target + non-target). Accordingly, a ratio of 1 illustrates that a mouse visited only the target holes, and a ratio of 0.5 means that it visited target and non-target holes equally.

4. Tissue Preparation for Immunostaining

At the end of the experimental period and four weeks following surgery, mice were anesthetized with sodium pentobarbital (100 mg/kg body weight; CDMV, Saint-Hyacinthe, Canada). The inferior vena cava was exposed using an abdominal incision. For vascular labeling, 0.1 mL of DyLight 594 conjugated Lycopersicon Esculentum (Tomato-Lectin, Biolynx, VECTDL1177) was injected through inferior vena cava into general circulation system. After five minutes, the mice were transcardially perfused with 40 mL of phosphate-buffered saline (PBS), pH 7.4. The mice were then decapitated and brains were removed and kept in -80 °C until sectioning. The right side of the brain was identified, and 20 µM coronal sections were cut and collected with a cryostat.

4.1. LRP-1 and RAGE Immunostaining

For immunohistochemical analysis, brain slices were fixed in 100% cold methanol for 10 min. The sections were washed three times with PBS. To block unspecific binding sites of antibodies, the sections were incubate for 90 min with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA), and 10% NGS without BSA for LRP-1 and RAGE, respectively. Sections were then

incubated with antibodies against LRP-1 (1:100, abcam, ab92544) or RAGE (1:100, abcam, ab37647) overnight at 4°C, and an autofluorescence was incubated with same blocking solution without primary antibody. Next day sections were washed three times with PBS (5 min each time), incubated with Alexa 488-conjugated secondary antibodies (Thermo Fisher Scientific, A11008) for 90 min at room temperature. Following three PBS washing, the slides were prepared for imaging. Images were acquired using Olympus laser-scanning microscope (model FV1000MPE) and analyzed with ImageJ software. Imaging was performed in at least 3 brain sections per mouse from the right and left primary somatosensory cortex as well as from the hippocampal regions (CA1 and CA3), and dentate gyrus (DG).

5. Statistical analysis

No statistical method was used to establish the sample size, but our sample size is the one generally used in the similar studies [310]. Data analysis of the behavioral and probe data was performed with GraphPad Prism software (version 8, La Jolla, USA). T test, One-wat ANOVA and repeated two-way ANOVA followed by a Tukey's least significant difference posttest for multiple group comparisons. Data are displayed as mean ± SEM. Asterisks denote statistical significance as calculated by the specified statistical tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$;****$p < 0.0001$; ns, not significant.).

Chapter 3: Results

In our model of arterial stiffness, calcification of carotid artery was induced by $CaCl_2$ application and was confirmed by calcium deposits formation in the carotid artery through Von Kossa staining. Cognitive dysfunction induced by our model of arterial stiffness was previously demonstrated using Morris water maze [27]. Consequently, as the first step, to confirm our data obtained with water maze, we investigated effects of arterial stiffness on cognitive function using Barnes maze, in 6-months old C57BL/6 male mice. Afterward, to assess the impact of arterial stiffness on cognition in a mouse model of Alzheimer disease, 3xTg and their WT, mice were also subjected to Barnes maze.

1. Carotid stiffness affect memory function in Barnes maze

Mice were first subjected to open-field test to evaluate the level of anxiety and assess locomotion normality in each group. Both groups showed similar anxiety level (Mann–Whitney tests, time in center: $p < 0.93$; periphery: $p < 0.93$; center/periphery ratio: $p < 0.93$; Fig. 4 A,B,C). No significant change in locomotion was detected in arterial stiffness group (total distance traveled: Mann–Whitney test, $p < 0.77$; number of zone transitions: $p < 0.49$; Fig. 4 F,G).

Then, we examined the mice's cognitive function using the Barnes maze, which is a hippocampus-dependent spatial learning and memory task. At 3 weeks after the induction of carotid calcification, both mice with a calcified artery and their controls were successful in learning. This was exhibited by progressive reduction in escape latencies and distances. Except the latency of first acquisition phase, latencies and distances between the two groups did not differ at any day during training (Figure 6; A,B), indicating absence of obvious learning impairments at this time point.

Following the acquisition phase, spatial memory was measured with a probe test 24 hours later. During the memory probe test, $CaCl_2$ mice performed poorly with increased number of errors, traveled distance, and latency in the primary search for the target, however, just the later one was statistically significant ($p < 0.01$), demonstrating detrimental effects of arterial stiffness in

long-term spatial memory at the single trial level, relative to control NaCl mice (Fig. 7; A-C). During the total search period, $CaCl_2$ mice spent less time in the target quadrant than NaCl mice (two-way ANOVA, NaCl-target vs NaCl-nontarget $p < 0.0001$, NaCl-target vs $CaCl_2$-target: $p < 0.05$; Fig. 7D), confirming again an impairment in long-term spatial memory. Furthermore, $CaCl_2$ mice visited less the target hole, relative to NaCl mice, indicating a deficit in spatial memory precision (Number of visits: two-way ANOVA, NaCl-target vs NaCl-nontarget $p < 0.0001$ Fig. 7F; selective search ratio: t test NaCl vs $CaCl_2$: $p < 0.05$; Fig. 7E). Together, these results reveal that arterial stiffness impairs long-term spatial memory.

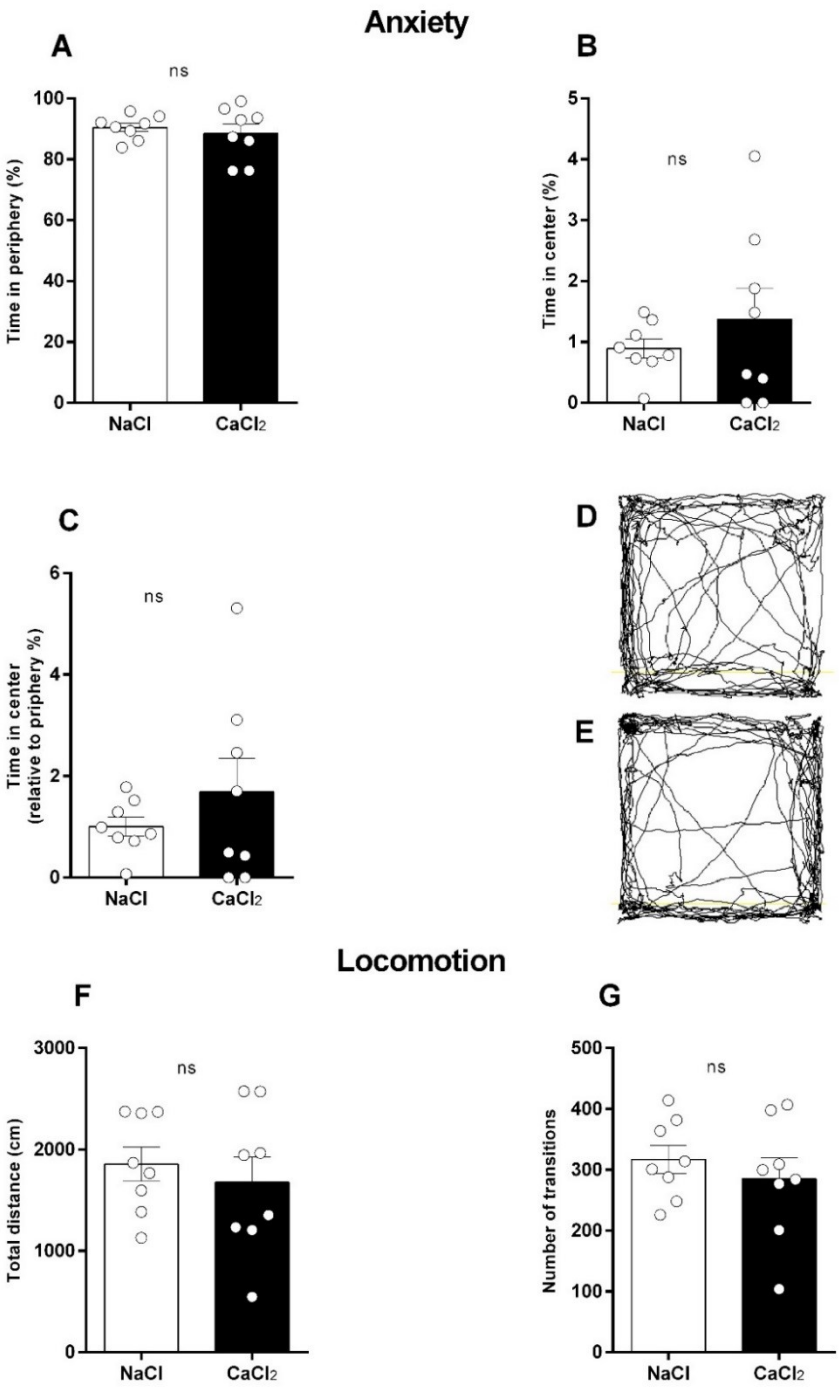


Figure 4. Open field test results for anxiety and locomotion. (A-C) Anxiety properties of NaCl and $CaCl_2$ mice: percentage of time in the periphery (A) and in the center (B), and time in center/periphery ratio (C). (D,E) Representative paths traveled by sham surgery (D) and carotid stiffness (E) mice during 5 min free exploration in a square open-field. (F,G) Locomotion properties of NaCl and $CaCl_2$ mice: total distance traveled (F), and number of zone transitions (G). Data are compared using Mann–Whitney test, and presented as mean ± SEM (n = 8). **ns**, not significant.

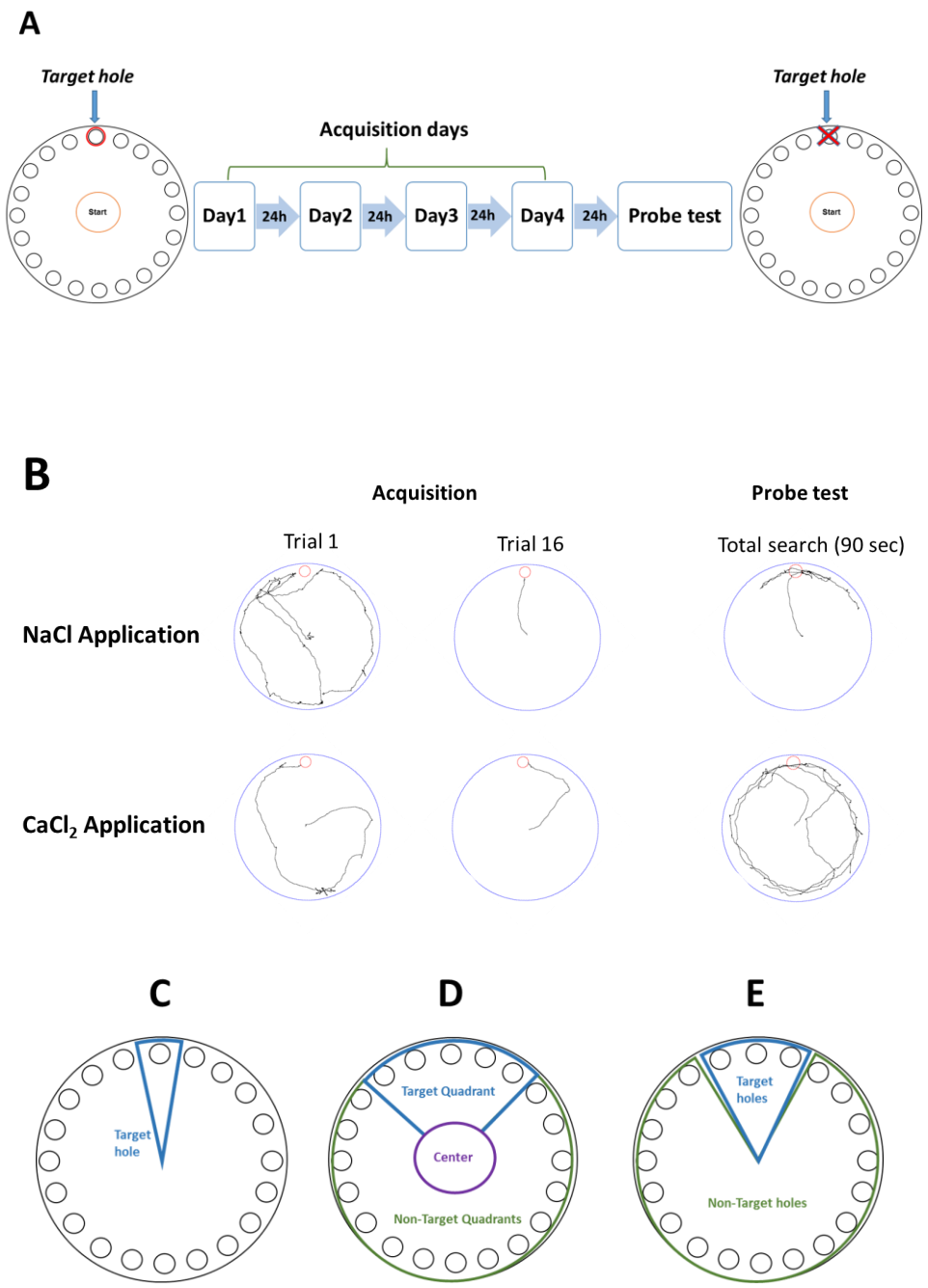


Figure 5. Barnes maze protocol description. A, Schematic diagram of the spatial learning and memory protocol in the Barnes maze. B, Representative paths traveled by right carotid application of NaCl and $CaCl_2$ mice during the first and last (16th) acquisition trials, as well as during total search of the probe test for 90 seconds. C, Target hole location for primary search of probe test (time, distance, and number of errors. D, Zone description of Target quadrant vs non-Target quadrant. E, Holes description of Target holes vs non-Target holes.

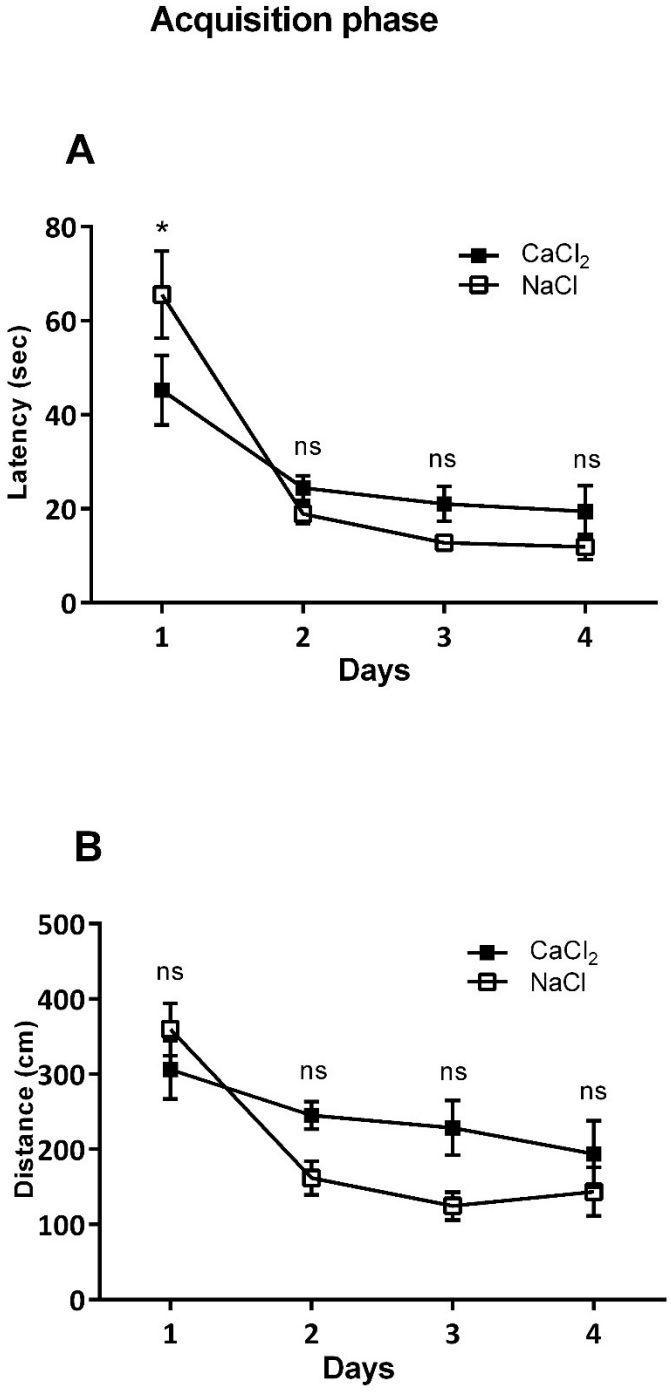


Figure 6. Performance in the Barnes maze during the four days training phase of NaCl and $CaCl_2$ mice. A, The time mice spent to reach the target hole. B, The distance mice passed to reach the target hole. Data were compared using two-way ANOVA and Sidaks's method for multiple comparisons. Data are presented as mean ± SEM (n = 8). ***p < 0.05; **ns**, not significant.

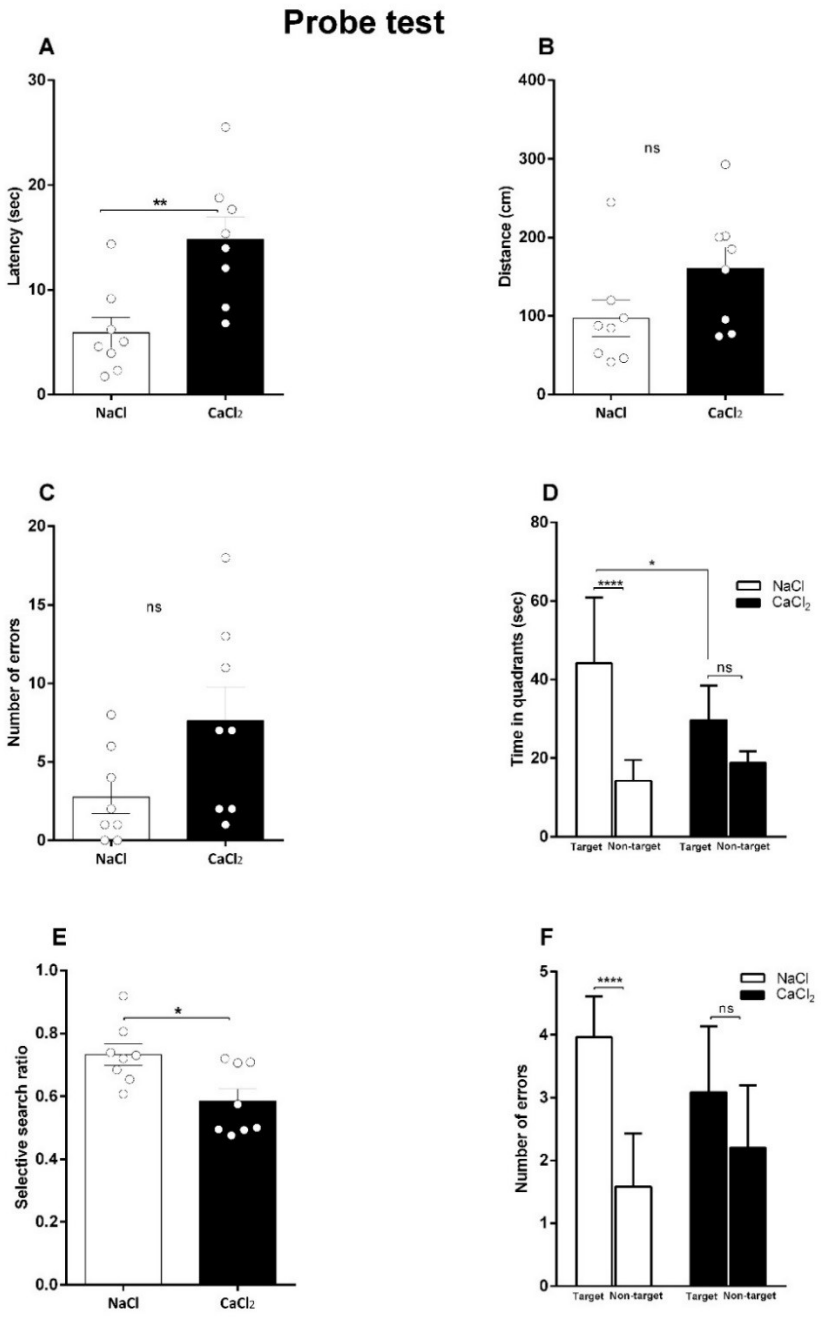


Figure 7. Spatial memory performance of NaCl and $CaCl_2$ mice during the probe test (90 sec). A, Latency before the first visit of the target hole. B, Distance before the first visit of the target hole. C, Number of errors before the first visit of the target hole. D, Amount of time spent in Target and average of non-Target quadrants of the maze during the total search period of the probe test. E, Selective search ratio, which is the number of hole visits in (target)/(target + non-target). F, Number of visits expressed as target vs average of all non-Target holes. Data are compared using Mann–Whitney test (A, B, C, E) and two-way ANOVA and Tukeys's method for multiple comparisons (D, F). Data are presented as mean ± SEM (n = 8). *****$p < 0.05$; ******$p < 0.01$; ********$p < 0.0001$; **ns**, not significant.

2. Carotid stiffness induced by $CaCl_2$ application surgery is associated with cognitive impairment in 3xTg mice

Three weeks after arterial stiffness surgery, anxiety and locomotion of the four groups were evaluated using open-field test. The analysis demonstrated that although WT-NaCl group spend less time in center, however, this difference was just significant compare to 3xTg-NaCl in terms of time in center relative to periphery (Fig. 8; A-C). The same pattern of anxiety behavior could be seen in locomotion, however, it is not statistically significant in any group (Fig. 8; H,I).

To assess the impact of arterial stiffness on cognition, WT and 3xTg mice were subjected to the Barnes maze. All groups of mice were successful in learning. This was exhibited by progressive reduction in escape latencies, errors numbers, and distances (Fig. 10).

To investigate spatial reference memory, we performed the probe test 24 h after the last training day. During probe test, Tg-$CaCl_2$ mice spent significantly more time to reach the target hole for the first time revealing memory deficiencies compare to WT-NaCl group (Fig. 11; A). Although the number of errors as well as the distance passed by the mice to reach the target hole showed the same trends, but they were not significant between groups (Fig. 11; B,C). During the total search period, Tg-$CaCl_2$ mice could not differentiate properly the target quadrant versus non-target quadrants, illustrating long-term spatial memory dysfunction. While comparing the time spent in the target quadrant versus non-target quadrants were not statistically significant in Tg-$CaCl_2$ group, other groups spent more time in target quadrant versus non-target quadrants (Fig. 11; D). Accordingly, in terms of numbers of target holes and non-target holes visits, which illustrate spatial memory precision, all groups except Tg-$CaCl_2$ visited target holes more than non-target holes significantly (Fig. 11; F). Selective search ratio which defined by the number of hole visits in (target holes)/(target + non-target holes) indicated less selective search ratio of Tg-$CaCl_2$ group compare to all other groups (Fig 11; E). The insufficient number of mice per group precluded examinations of sex differences it this study, however, this variable will be considered as a part of a separate study.

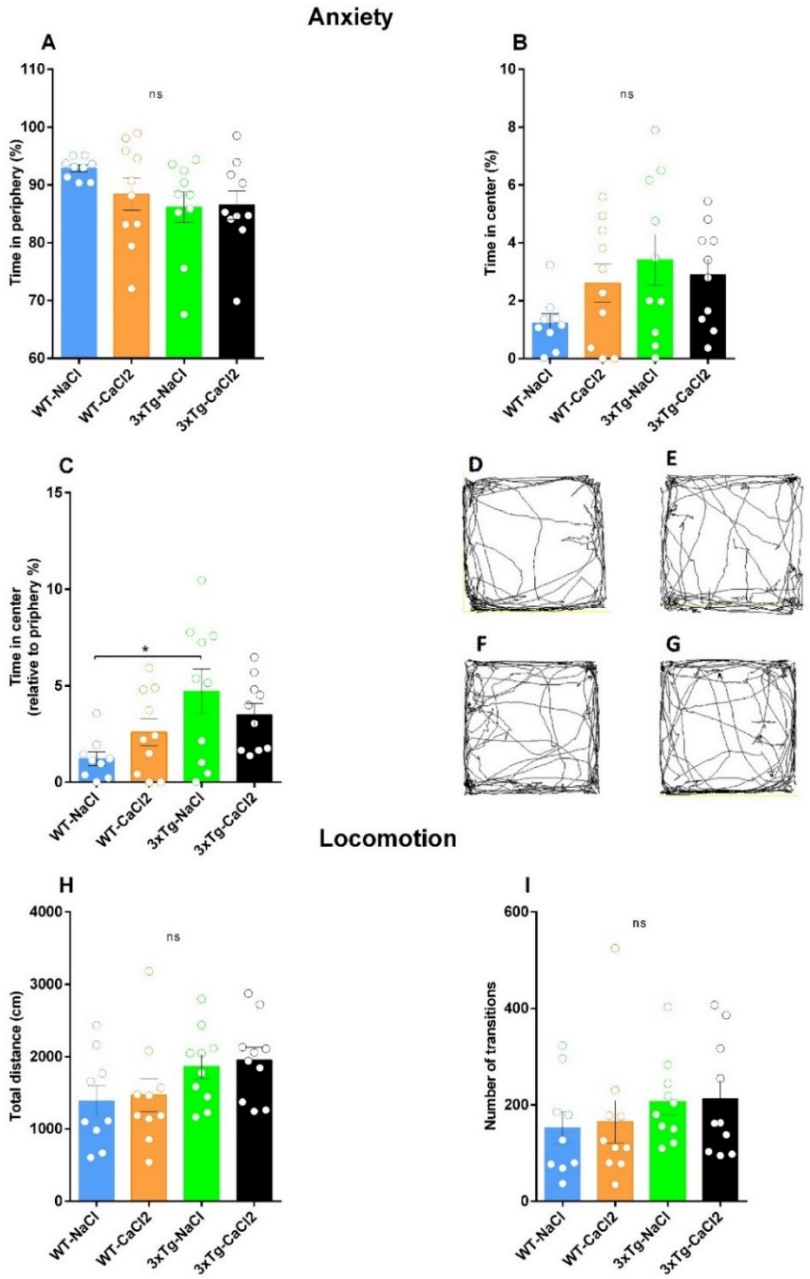


Figure 8. Open field test results for anxiety and locomotion. (A-C) Anxiety properties of four groups of mice: WT-NaCl, WT-$CaCl_2$, 3xTg-NaCl, and 3xTg-$CaCl_2$. Percentage of time in the periphery (A) and in the center (B), and time in center/periphery ratio (C). (D-G) Representative paths traveled by WT-NaCl (D), WT-$CaCl_2$ (E), 3xTg-NaCl (F), and 3xTg-$CaCl_2$(G) mice during 5 min free exploration in a square open-field (H,I) Locomotion properties WT and 3xTg mice: total distance traveled (C), and number of zone transitions (D). Data were compared using one-way ANOVA followed by Tukey post-hoc analysis. Data are presented as mean ± SEM (n = 9–10). ***p** < 0.05; **ns**, not significant.

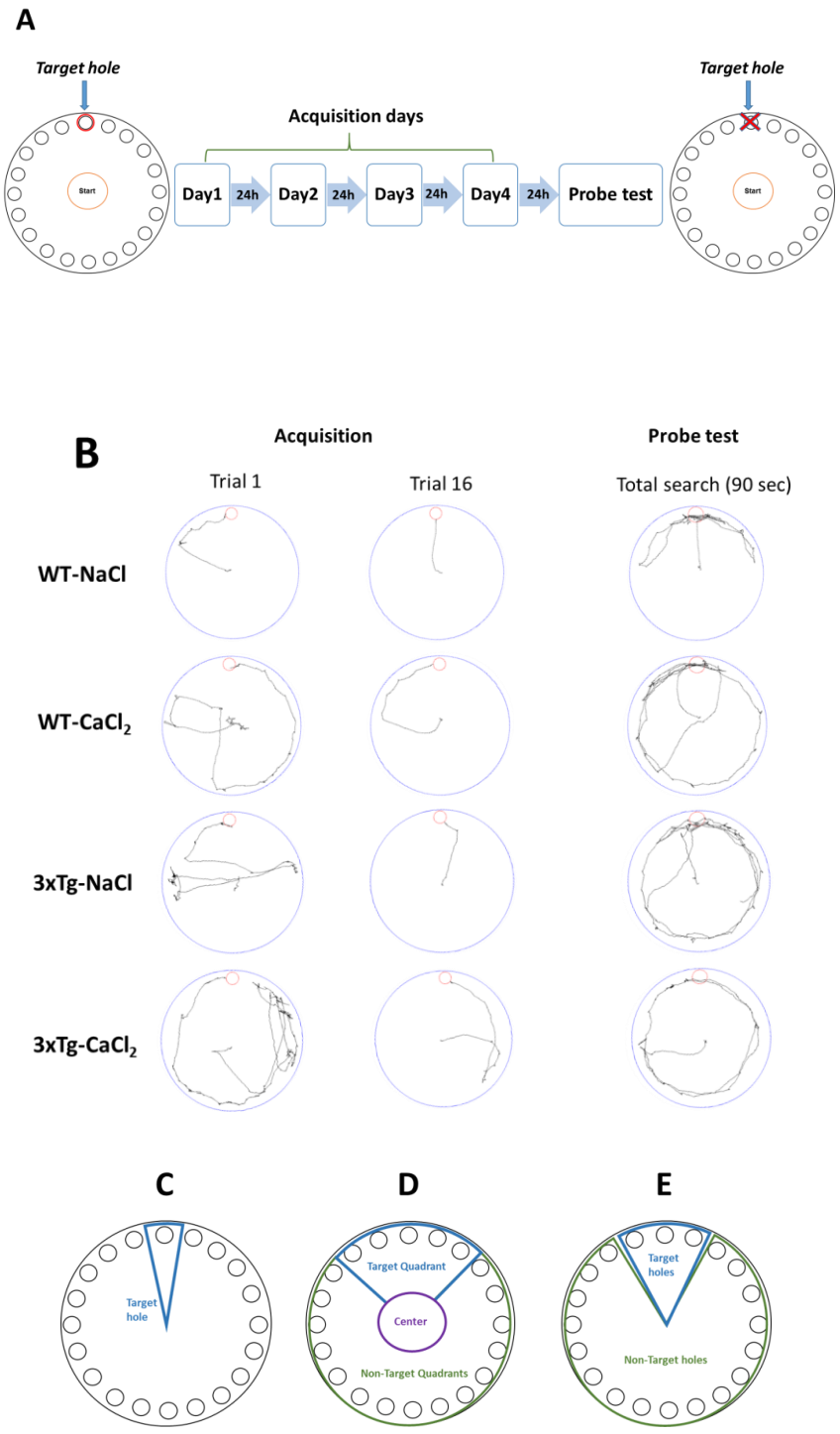


Figure 9. Barnes maze protocol description. A, Schematic diagram of the spatial learning and memory protocol in the Barnes maze. B, Representative paths traveled by right carotid application of NaCl and $CaCl_2$ in WT and 3xTg mice during the first and last (16th) acquisition trials, as well as during total search of the probe test for 90 seconds. C, Target hole location for primary search of probe test (time, distance, and number of errors. D, Zone description of Target quadrant vs non-Target quadrant. E, Holes description of Target holes vs non-Target holes.

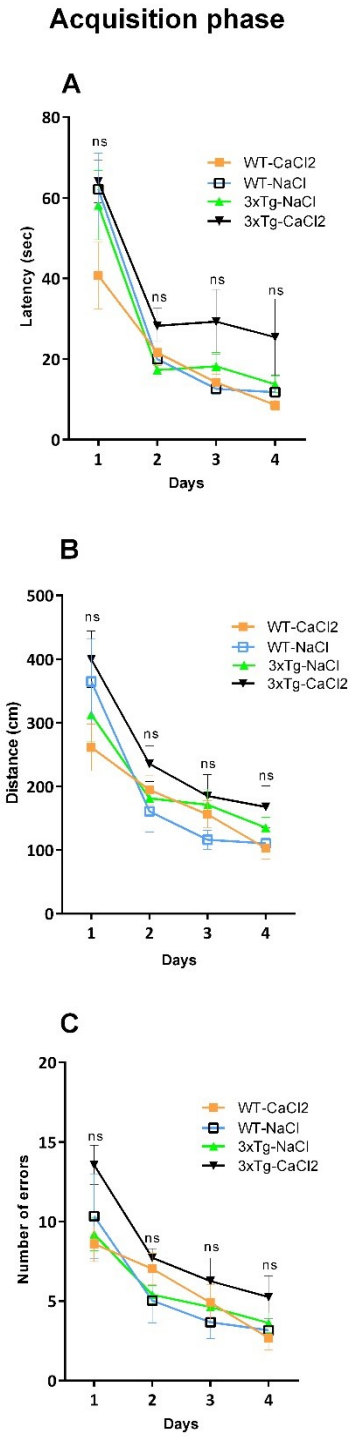


Figure 10. Performance in the Barnes maze during the four days training phase of the four groups of mice. A, The time mice spent to reach the target hole. B, The distance mice passed to reach the target hole. C, Number of errors before first visit of target hole. Data were compared using two-way repeated-measures ANOVA. Data are presented as mean ± SEM (n = 9–10). **ns**, not significant.

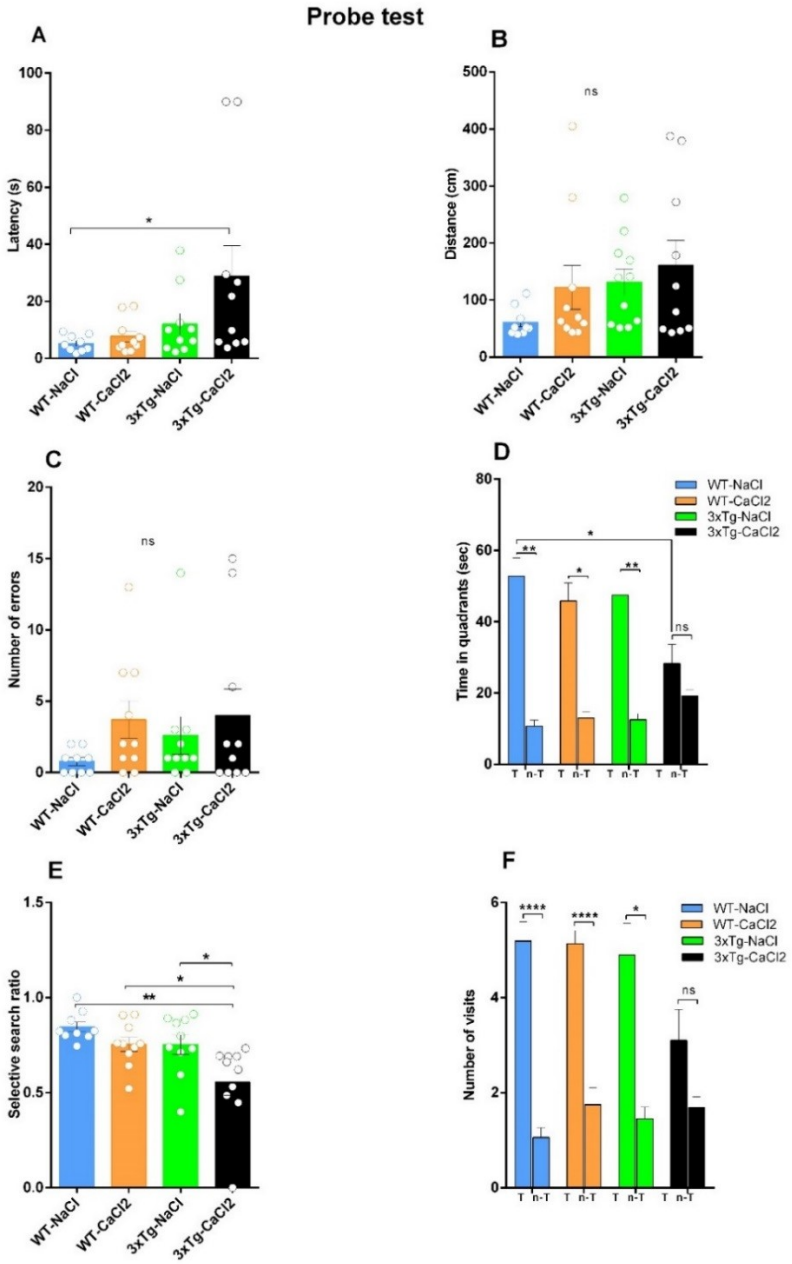


Figure 11. Spatial memory performance of the four groups of mice during the probe test (90 sec). A, Latency before the first visit of the target hole. B, Distance before the first visit of the target hole. C, Number of errors before the first visit of the target hole. D, Amount of time spent in Target and average of non-Target quadrants of the maze during the total search period of the probe test. E, Selective search ratio, which is the number of hole visits in (target)/(target + non-target). F, Number of visits expressed as target vs average of all non-Target holes. Data are compared using one-way ANOVA followed by Tukey post-hoc analysis (A, B, C, E) and two-way ANOVA and Tukeys's method for multiple comparisons (D, F). Data are presented as mean ± SEM (n = 9–10). *p < 0.05; **p < 0.01; ***p < 0.001;****p < 0.0001; **ns**, not significant.

Chapter 4: Discussion and conclusion

Arterial stiffness, which increase pulse pressure in smaller arteries as well as pulse wave velocity, is associated with cognitive decline in clinical studies [15, 311]. However, whether arterial stiffness, as an individual parameter accelerate the progression of AD needs further explorations. The vascular biomechanical hypothesis in humans suggests that increase in large arteries stiffness during aging and increased PP in the brain have a destructive effect on cerebral normal functions. This hypothesis that arterial stiffness and high PP cause functional and structural brain damages would establish the causal link between the deterioration of cerebrovascular structures and the incidence of cognitive impairment [14, 303, 311-313]. In order to improve the understanding of the impact of arterial stiffness on cognitive functions, the objective of this thesis was to characterize deleterious effects of arterial stiffness in a mouse model of AD that could lead to early cognitive decline.

We chose to use the mouse model of arterial stiffness based on carotid calcification that was developed in our lab [24]. The usual problems of the other models such as brain injury by parameters other than the stiffness itself or blood pressure alteration have been resolved in our model, thus, makes it feasible to study the effects of arterial stiffness, as an independent parameter on the brain. Previous results of our model of arterial stiffness indicated that it impairs CBF regulation and cerebral vasculature integrity, damaging the BBB and leading to cognitive deficits [27]. Indeed, arterial stiffness affects the mechanisms regulating CBF such as cerebral autoregulation, NVC, and endothelium-dependent dilation [27]. More interestingly, decreased resting CBF has been seen in the hippocampus and entorhinal cortices, anatomical areas that are critically important for memory [314, 315]. CBF reduction was followed by cognitive impairment at 3 weeks after arterial stiffness induction.

1. Summary of results

Our laboratory have already explored the link between arterial stiffness and vascular damage in a couple of studies [23, 24, 27]. In one of these studies by Muhire et al. [27], pathologic effects of arterial stiffness on cognitive function were investigated. The behavioral test for spatial memory

evaluation used in their study was Morris Water maze. They showed that arterial stiffness impaired learning and memory function in C57BL/6J mice. Our lab got accessed to Barnes maze, which is a very sensitive test for spatial memory investigations, through a collaboration with Dr Jean-Claude Lacaille. Therefore, to see whether Barnes maze could replicate the Morris Water Maze results in our model of arterial stiffness, we first studied the deleterious effects of carotid stiffness in cognitive function using Barnes maze. We find out that our model of arterial stiffness caused significant spatial memory impairment in Barnes maze (Fig. 7).

Thus, our model of arterial stiffness results in cognitive dysfunctions. This was preceded by cerebral autoregulation impairment, diminished cerebrovascular reactivity, BBB breakdown, and cerebral gliosis [23, 27] suggesting that these parameters may participate in the cognitive decline. We therefore wished, in the design of our study, to better characterize the impact of the arterial stiffness on exacerbation of cognitive decline in AD progression. Our results demonstrated that arterial stiffness caused spatial memory impairment in 6-months old 3xTg model of AD. Normally, Barnes maze detects the spatial memory dysfunction at least at 6.5-month old 3xTg mice [316]. However, in our study 3xTg mice with arterial stiffness displayed significant cognitive dysfunction at age of 6 months, indicating the aggregative effects of arterial stiffness in chronological phenotypic pattern of AD (Fig. 11). At the next step, we will investigate the Aβ clearance dysfunction as a possible mechanism of arterial stiffness related cognitive decline in mouse model of AD.

2. Barnes maze, a sensitive test to assess spatial learning and memory

Spatial learning and memory is an important parameter used for cognitive function evaluating in AD [317, 318]. In this regards, researchers utilize various methods to investigate spatial learning and memory such as the radial arm maze test, Y mazes, novel object recognition test, Morris water maze and Barnes maze test [318]. Barnes maze is of the best choice for testing learning and memory. The acquisition phase of Barnes maze is followed by the probe trial permits an assessment of spatial learning and spatial memory, which is believed to be linked with hippocampal functions [308, 319, 320]. Barnes maze was successful in detection of cognitive impairment in our model. The results obtained from Barnes maze in our model of arterial stiffness

illustrated a similar pattern of cognitive impairment detected with the Morris water maze [27]. In both tests, the mice with carotid calcification spent more time to reach the target zone and spent less time in target quadrant in probe test. However, some parts of our results obtained by Barnes maze do not confirm our hypothesis. For instance, despite of significant difference in probe test latency between investigated groups of our two behavioral studies (Figure 7 and 11), there are no significant differences for probe test distance as well as number of errors. The first reason is probably the sample size of our two studies (8 for the first study and 9-10 for the second study), which is much lower than the number of 12 estimated by a G power analysis for sample size. These results might also be explained by different exploratory behaviors in mice. This kind of behavior are particularly more prominent in the first day of acquisition phase, when the stress level is high. Low exploratory behavior and high level of stress at day one could lead a delay in exploration of the holes to reach target hole. These types of behaviors are usually resolved at the next days of acquisition phase. Selective search ratio, time in quadrants and number of visits of target and non-target holes in probe test are the most precise way to analyze memory performances.

Although the Morris water maze is still more popular than Barnes maze, the latest has some advantages, which make it a good choice. The flexibility of the Barnes maze test allows evaluation of diverse aspects of spatial learning and memory, using the same group of animals, in a relatively short time period. Furthermore, performance of Barnes maze is much easier relative to other types of mazes, e.g., radial arm maze or Morris water maze [317]. Since there is no water immersion in Barnes maze, the level of animal stress is much less that Morris water maze. Water immersion is very stressful for animals and increase level of corticosterone, which could affect animal performance [321, 322]. Moreover, Morris water maze is more physically demanding for rodents than Barnes maze. The most often stated disadvantageous of the Barnes maze task versus Morris water maze is its sensitivity to genetic alternations. For instance, Stewart et al. [323] reported the lower ability of Barnes maze in detection of spatial memory impairments in Tg2576 mice model of AD, compared to the T maze or Morris water maze. A study in Sprague–Dawley rats receiving 3,4-methylenedioxymethamphetamine (MDMA) to induce memory deficits reported similar results. However, they propose the improper configuration of the Barnes maze

test in detection of cognitive dysfunctions [324]. They used both Morris water maze and Barnes maze in same group of mice with different orders such that half the litters received the Barnes maze first and the other half received the Morris water maze first. Moreover, rats received two trials per day (versus four trials per day in our study) and number of errors and time in quadrants were not analyzed. These variabilities could affect the accuracy of Barnes maze in memory impairment detection. However, inability of Barnes maze in few models of cognitive impairment could not be extended to all models. Finally, lots of non-cognitive factors such as anxiety, motivation, and exploration activity could influence the Barnes maze task. However, by properly controlling and testing these variables, Barnes maze could be used as an appropriate and sensitive test for cognitive function detection.

3. Anxiety and locomotion in 3xTg-AD and wild-type mice

There is conflicting evidence about the presence of anxiety-like behaviors in mouse models of AD, with some findings pointing to either an increase, a decrease, or no change between transgenic animals and wild-type controls [325]. Three tests are mostly used for anxiety evaluation in mice: open-field, elevated plus maze, and light-dark box. Among the mentioned tests, the open-field test is frequently used to examine anxiety-like behaviors in mice, which is based on mice's aversion to open spaces and light. This task typically scored according to the time spent in periphery versus center area (the center area is anxiogenic for the mice), how many times a mouse enters different zones, and total locomotion activity [326-328].

In our study, the time in center relative to periphery in 3xTg-NaCl was higher compare to WT-NaCl mice (Fig. 8). Whether these levels of anxiety were sex-associated is not clear. According to the literature, 3xTg-AD females do not seem to experience any increases in anxiety throughout the elevated plus maze and light-dark box [329, 330]. However, increasing time spent in center area of open-field test had previously been indicated in female 3xTg-AD mice [331]. In accordance with the latter findings, Gloria et al. demonstrated decline of anxiety behaviors in female 12-month-old 3xTg mice using open-field test [332]. While 4-month-old 3xTg mice showed similar locomotion and anxiety level compare to control wild-type mice. This study proposed contribution of increase in $D_{2/3}R$ in anxiety reduction of 3xTg mice [332]. Conversely, another

study indicated increased anxiogenic phenotype in female 6 and 12 months old 3×Tg-AD mice in the open field test [333]. The most probable reason in these controversial results could be the different timeline and methods of behavior analysis in these studies. While in one of these studies the exploration time was one hour and the anxiety-like behavior was evaluated for the time spent within as well as the number of entries into the central area (13 × 13 cm) of the open field test [333], the other study recorded mice's behaviors for one hour for two consecutive days, and evaluated the time spent in the central (15 × 15 cm), intermediate and corner squares [332]. The motor functions and anxiety-related behaviors using open-field and elevated plus-maze tests showed no change of these parameters in 18-month-old 3×Tg-AD male mice compare to wild-type controls [334]. Furthermore, there are conflicting reports for exploratory activity of 3xTg-AD mice illustrating hyperactivity [335, 336], hypoactivity [337-339], and no alteration [340] in open-field test. In order to rule out alterations in anxiety, activity, or motor abilities as the probable cause of Barnes maze performance deficits, anxiety and/or locomotor assessments, such as elevated plus maze or open field test are used before Barnes maze [341]. We performed open field test prior to Barnes maze in our behavioral tests. However, our results show that the level of anxiety is mostly increased in the NaCl-3xtg mice while cognitive impairment is prominent in CaCl2-3xtg mice suggesting that anxiety could not totally explain our observations in the cognitive performances in CaCl2-3xtg mice. The age that was used in our study was 6 months, and we used male and female mice. As mentioned previously, the number of mice per group was not enough for investigating sex difference in our study. Thus, the precise changes of anxiety and locomotor activity of 3xTg mice in different ages as well as male and female diversity need further explorations.

4. Aβ clearance impairment as a possible mechanism involved in pathological effects of arterial stiffness in AD development

Cerebrovascular damage and degeneration develop progressively throughout the early presymptomatic stage of Alzheimer's disease [93]. The vascular-mediated clearance of Aβ is reduced as cerebrovascular damage and degeneration occur. This important phase most likely indicates the transition from the presymptomatic to the symptomatic stages of AD. Because the

vasculature clears around 85 percent of Aβ from the brain, a loss of efficiency in this process will likely result in a rapid increase of Aβ in the brain parenchyma, triggering Aβ plaque development [342]. Rapid rise of Aβ levels in the brain parenchyma increases cerebrovasculature damage simultaneously, which result in a vicious cycle that drives the degenerative alterations in AD [342]. Damage to the brain microvasculature may also alter Aβ perivascular elimination, encouraging the formation of cerebrovascular Aβ aggregates, and ultimately limiting angiogenesis and causing vascular dysfunction [189]. Aβ can be found in small concentrations in the normal brain and in the cerebral arteries of young people [343, 344]. In more than 95 percent of AD individuals, failure in Aβ clearance reported to be a key component in the pathophysiology of late onset sporadic AD [71].

At 6 months of age, 3xTg mice showed a high amount of vascular adhesion molecules and multiple inflammatory mediators in the hippocampus and cortex [345]. Deposits of Aβ may promote the inflammation of BBB as a self-defense strategy in the early stages of the 3xTg AD mouse model to prevent future CNS damage. It has been suggested that this form of self-protection against Aβ accumulation in the brain, resulting in partial clearance/degradation of Aβ by macrophages or immune cells [346]. Extravagated macrophages, monocytes, and T lymphocytes have been shown to play a protective role in the brain parenchyma, facilitating the removal of Aβ during the early stages of AD [347]. During disease progression, Aβ alters the function of cerebral vasculature through inflammation and could lead to neuronal damage in late AD. Vascular risk factors such as hypertension and arterial stiffness increase the inflammation through transferring pathological mechanical stress into cerebral microcirculation [14]. This could accelerate the AD progression and leads to earlier manifestation of cognitive impairment. In our study, we found out that the cognitive function in sham surgery group of 3xTg mice were normal at 6 months of age, but that cognitive dysfunction was detected in $CaCl_2$-3xTg mice at the same age. These results could illustrate the aggravative effects of arterial stiffness in AD progression. Direct effects of arterial stiffness in Aβ clearance is a suggested mechanism involved in its pathogenesis. Cerebral arterial pulsatility has been proposed to contribute in glymphatic exchange of Aβ. Arterial stiffening and cerebrovascular disease might cause failure of the Aβ clearance by altering arterial pulsatility [71]. One of our main objective in this project is to study the role of LRP-1 and RAGE (the two important

transmembrane proteins in efflux and influx of Aβ from the brain, respectively) in deleterious effect of carotid stiffness in AD progression. Our preliminary results in this regard have been reported in Appendix section (Figures 12-15); however, the related experiments are going on.

5. Impact of age and sex in AD progression

One of our main objective in this study was to observe the accelerative role of arterial stiffness in AD progression. Thus, we performed our experiments in 6-month-old male and female 3xTg-AD and wild-type mice. In 3xTg-AD mice, deposition of Aβ reported to first appear at the age of 6 months while the first definitive manifestation of tauopathy detects around 12 months of age [205, 305]. It is widely recommended that tauopathy has a stronger relationship to cognitive dysfunction compare with Aβ [348]. Therefore, the beginning of cognitive deficits expected to appear between 8 and 12 months of age [205, 305, 349]. However, an experimental study in this mouse model of AD indicated starting of detectable deficits in spatial learning and memory function in male and female mice at 6.5 months of age using Barnes maze [316]. We thus selected the 6 months as the closest age to see the probable deteriorative effects of arterial stiffness on AD development. This will display the importance of prevention and resolving the vascular risk factors of AD in susceptible individuals more clearly.

Although we observed obvious cognitive decline in male C57BL/6J mice exposed to carotid calcification in our first study, the cognitive impairment following arterial stiffness in wild-type strain of transgenic mice (C57BL6/129Svj) of combined sexes were not significant. While the effects of different mice strains in cognitive test performance could be a possible reason related to these findings, however, effects of sex on arterial stiffness-induced cerebral injury has a higher probability. In some unpublished data of our lab, it has been demonstrated that arterial stiffness leads to impaired NVC and endothelium-dependent vascular reactivity in male mice but not in reproductive female mice. The female protection to arterial stiffness detrimental effects was removed following ovariectomy and restored after Estradiol therapy in ovariectomized mice. A comparative study by Girouard et al. proposed that ANG II- induced cerebrovascular dysregulation was less prominent in female mice, which was related to estrogen [350]. Estrogen dramatically inhibits superoxide anion production in cerebral blood vessels and endothelial cells

[351-353]. Since increased oxidative stress has been reported in arterial stiffness-induced brain damage [23], estrogen can have protective effects on brain function by suppressing the generation of free radicals. Increased endothelial nitric oxide synthetase expression [354], decreased astrogliosis and release of inflammatory cytokines [355], cerebral vascular inflammation reduction [356], antiapoptotic action [357], and glucose transport improvement [358] are other protective effects of estrogen in brain. Our first study was performed in male mice, while the second study in 3xTg-AD and their wild-type mice were carried out in mix of male and female mice. As mentioned previously, the number of mice were not enough for sex differences analysis; however, we expect to see the same kind of results related to sex, at least for wild-type mice. Susceptibility of the female 3xTg mice to arterial stiffness would determine whether female mouse model of AD are protected to arterial stiffness brain damages or not.

In addition to age and sex, the number of animals in each group, the number of experimental sessions, and the groups' number used for statistical analysis might be the reason of the observed discrepancies. Our first study was performed in two mice experimental sessions, while in our second study in transgenic mouse model of AD; we did our experiments in five experimental sessions. Increase of the number of experimental sessions could cause some discrepancies in the results particularly behavioral tests. We had two and four groups in our first and second studies, respectively. Increasing the number of groups led to a decreased of statistical discrimination. Because of capacity limitation of mice's numbers in behavioral test performance, and to avoid circadian effects on performance, it is optimal to perform the test in a select window of time on a given day. Therefore, we had limitations for the number of mice for each series of experiments, which leads to increasing the number of experimental sessions in the second part of the study that included 3xTg mice. In addition, since the number of transgenic mice was limited and that we performed our experiments at a specific age, equalizing number of mice for each group and sex for every experimental session was not possible.

6. Unilateral effects of arterial stiffness on brain

Memory and spatial navigation are both attributed to the hippocampus [359]. Although the function of left and right hippocampi have been reported to be identical in rodents widely, some recent studies have revealed asymmetries in neuronal connections in brain hemispheres [360]. To investigate dissociation between the right and left hemispheres in short-term and long-term memory, Shipton et al. displayed that silencing of the left hippocampus CA3 alone leads to impairment of long-term memory performance [361]. On the contrary, unilateral silencing of CA3 in left or right side resulted in short-term memory dysfunction. Another study also illustrated important role of left CA3 lateralization in spatial working memory [362]. In our study, since the stiffness was only performed in the right carotid artery, the right hemisphere is mostly affected. Whether the left carotid calcification would cause similar, more, or less complications is an interesting topic, which could be investigated in a separate study.

7. Translation of mouse model of AD and arterial stiffness in humans

The presenilin-1, tauP301L, and transgenic amyloid-protein precursor genes are all mutated in the 3xTg-AD mouse model [305]. Both Aβ and tau development exhibit age- and region-dependence, similar to the human AD pathophysiology, with Aβ plaques appearing prior to the buildup of NFTs [363]. In comparison to P301L and Tg4510 transgenic AD mouse models, 3xTg-AD mice display characteristics that are closer to human levels for the familial forms of AD [364]. Therefore, 3xTg-AD model is a proper model regarding our objective, since the pathology observed in this model resembles human patients. The main advantage of this model is that it is well characterized. The next step will be to study the effect of arterial stiffness on models of sporadic AD. We showed an early development of cognitive decline in 3xTg-AD mice following carotid calcifications. A large body of evidences raise the prospect of early contribution of vascular dysfunction in AD progression [63, 91]. This could imply that not only individuals with vascular risk factor are at greater risk to involve with AD, but also the progression of disease is faster than usual in this population.

8. Conclusion

In conclusion, as suggested by other researchers [365, 366], it is apparent that the vascular component of AD should be better considered. A large body of evidences imply that brain damage in humans and especially cerebrovascular damages precede the clinical manifestations of neurological pathologies by years, indicating that prevention of cognitive decline should go through the management of preserving normal vascular function. Midlife control of vascular pathologies like hypertension reduce risk of cognitive decline in elderly [119]. Accordingly, timely control of arterial stiffness at earlier ages may confer greater cognitive protection than in older patients. We observed deleterious effects of arterial stiffness in cognitive impairment of young mouse model of AD. These results could illustrates that arterial stiffness accelerate disease progression in AD-susceptible individuals. Thus, the prospect of pharmacological or non-pharmacological preventive approaches targeting arterial stiffness will diminish its deleterious impact throughout aging and AD progression, which helps in protection of brain health and predict a better quality of life.

9. Study limitations

The limitations of our study are mainly related to restriction in the number of 3xTg-AD mice used due to the time it takes to produce the transgenic mice. Moreover, the mice used in our main study were both males and females. The number of mice was not enough to study the sex differences; however, it is one of future objective. Although the Aβ clearance impairment as a probable mechanism in arterial stiffness-induced cognitive dysfunction in 3xTg-AD mice is under investigation currently, the possible other mechanisms such as BBB breakdown, cerebral blood flow dysregulation, and mitochondrial dysfunction worth further explorations.

10. Perspectives

This study provide a better understanding of the effects of arterial stiffness on AD deterioration. However, several questions remain to be explored. For example, what is the exact mechanisms involved in arterial stiffness-induced cognitive impairment? Does the high pulse pressure induced by carotid calcification could result in collagen crosslink formation and elastin fragmentation in

cerebral blood vessels? What is the long-term effects of arterial stiffness on AD progression? Does sex differences affect the cerebral injury following arterial stiffness in AD model? When individuals at risk of AD incidence should be screened for vascular risk factors such as arterial stiffness and what is the treatment starting point in susceptible individuals? Since the majority of AD cases belong to sporadic form of disease, does arterial stiffness could exacerbate AD progression in sporadic model of AD?

Appendix

AD is characterized by pathological Aβ accumulations in the brain. The processes that control the production and clearance of Aβ, regulate its homeostasis in the brain [189]. While there are several physiological ways that Aβ can be eliminated, one important method is the passage of Aβ across the BBB via cerebrovascular means [186]. We plan to explore the function of LRP1 and RAGE, the main protein involved in the clearance of Aβ through cerebral efflux and influx of Aβ, respectively.

LRP1 is abundantly expressed in the central nervous system and speeds up the metabolism of amyloid and decreasing accumulation of it [367]. Lower expression of LRP1 in neurons and capillary endothelial cells in the brain of AD patients results in reduction of Aβ clearance and amyloid accumulation [368]. Because high cholesterol reduces LRP1 expression, patients with high cholesterol have a higher risk of developing AD than the general population [369]. RAGE is a member of the immunoglobulin superfamily, which detects a wide variety of ligands, including amyloid and advanced glycosylation end products. Aβ crosses the BBB into the brain via RAGE [203]. Increased ligand concentrations have the potential to increase RAGE expression, which in turn causes toxic proteins to build up in the brain and cause pathologic neurological reactions. RP1, a RAGE antagonist peptide, was utilized by Huang et al. in APP/PS1 animal model of AD. According to their findings, RP1 decreased the expression of APP and β-secretase, which decreased the production and buildup of Aβ, improved memory impairment, and decreased the burden of amyloid plaque in the APP/PS1 mice [370]. Thus, LRP-1 and RAGE are important contributors in AD pathogenesis and possible therapeutic target to prevent the course of AD and regulate aberrant APP metabolism.

To explore the mechanism involved in deleterious effects of arterial stiffness in AD progression, we tried to measure the cerebral LRP-1 and RAGE expression. Owing to the fact that 85 percent of Aβ is cleared through brain vasculature [342], we were looking for the total amounts of LRP-1 and RAGE as well as their expression in cerebral vasculature. To reach this goal, we needed to do co-staining of LRP-1 and RAGE with cerebral blood vessels staining. We tried and mixed many

protocols for optimization of the best protocol for immunostaining of these proteins, which prolonged more than six months. At the beginning, we tried to do co-staining of LRP-1 and RAGE with CD31 (a marker for vessels configuration). However, LRP-1 staining was working well with methanol tissue fixation, while CD31 was working well with Ethanol fixation. We switched to Collagen IV for vascular staining, but we needed to perfuse the mice with paraformaldehyde (PFA) and fix the brain with PFA for Collagen IV. We saw that LRP-1 do not work well with PFA fixation either. We also tried different types of LRP-1 antibodies; however, the best LRP-1 immunostaining was with methanol fixation. Finally, we used tomato lectin for vasculature staining, as described in methods sections. Lectin was very awesome for vascular staining and used for blood vessels staining in our optimized protocol. Although RAGE staining was working with both unfixed or fixed sections with PFA, the background of the pictures was better with Methanol fixation. Finally, we completed the protocol optimization, and below are some preliminary results and some images of our RAGE immunostainings. The number of samples was not high enough to complete the analysis; however, the project is in progress.

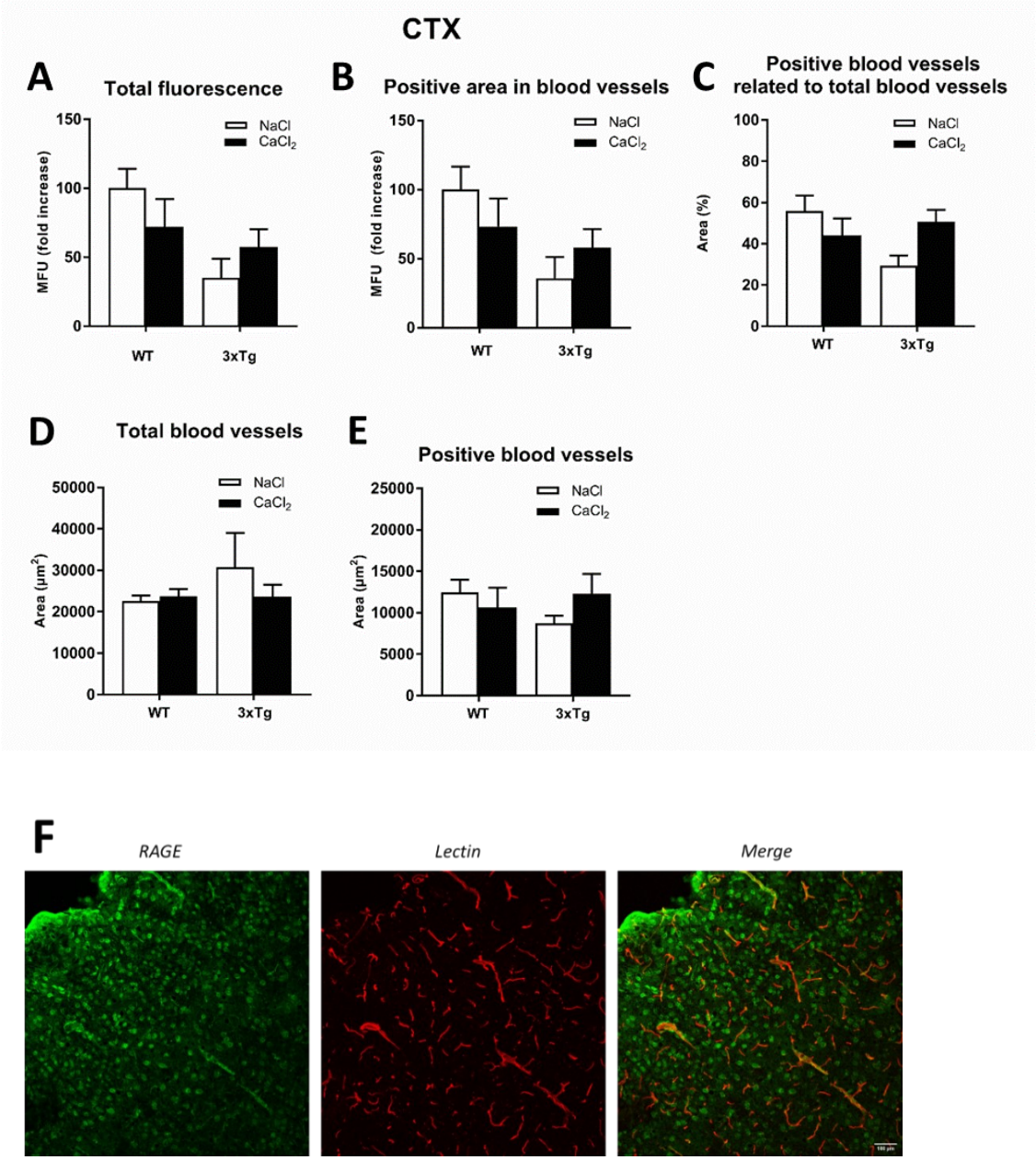


Figure 12. Immunostaining of RAGE-positive cells in somatosensory cortex (CTX) of the affected side of WT and 3xTg mice. A: Total fluorescence represents mean intensity ratio of total RAGE fluorescence to control of each day of imaging, B: Positive area in blood vessels represents mean intensity ratio of RAGE fluorescence in blood vessels to control of each day of imaging, C: Positive blood vessels related to total blood vessels represents area of RAGE in blood vessels divided by total area of blood vessels (in Percentage), D: Total blood vessels represents total area of blood vessels (in μm^2), E: Positive blood vessels represents total area of RAGE in blood vessels (in μm^2). F: representative images of RAGE staining in blood vessels in somatosensory cortex of 3xTg mice (scale bar 100 µm). Analysis was done by ImageJ with semi-automatic macro. Data are mean ± SEM; (n=3-4).

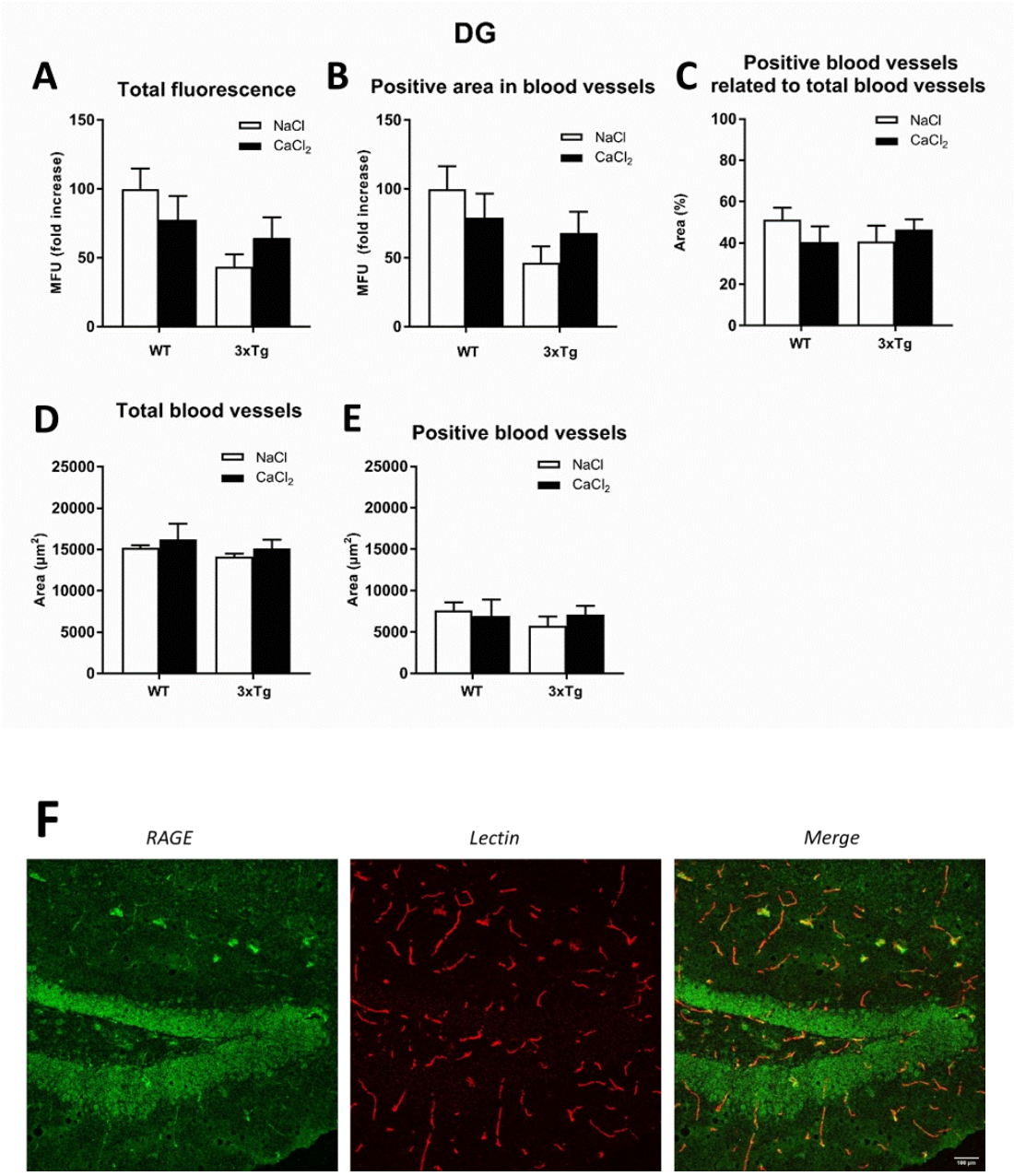


Figure 13. Immunostaining of RAGE-positive cells in DG of the affected side of WT and 3xTg mice. A: Total fluorescence represents mean intensity ratio of total RAGE fluorescence to control of each day of imaging, B: Positive area in blood vessels represents mean intensity ratio of RAGE fluorescence in blood vessels to control of each day of imaging, C: Positive blood vessels related to total blood vessels represents area of RAGE in blood vessels divided by total area of blood vessels (in Percentage), D: Total blood vessels represents total area of blood vessels (in μm^2), E: Positive blood vessels represents total area of RAGE in blood vessels (in μm^2). F: representative images of RAGE staining in blood vessels in DG of 3xTg mice (scale bar 100 µm). Analysis was done by ImageJ with semi-automatic macro. Data are mean ± SEM; (n=3-4).

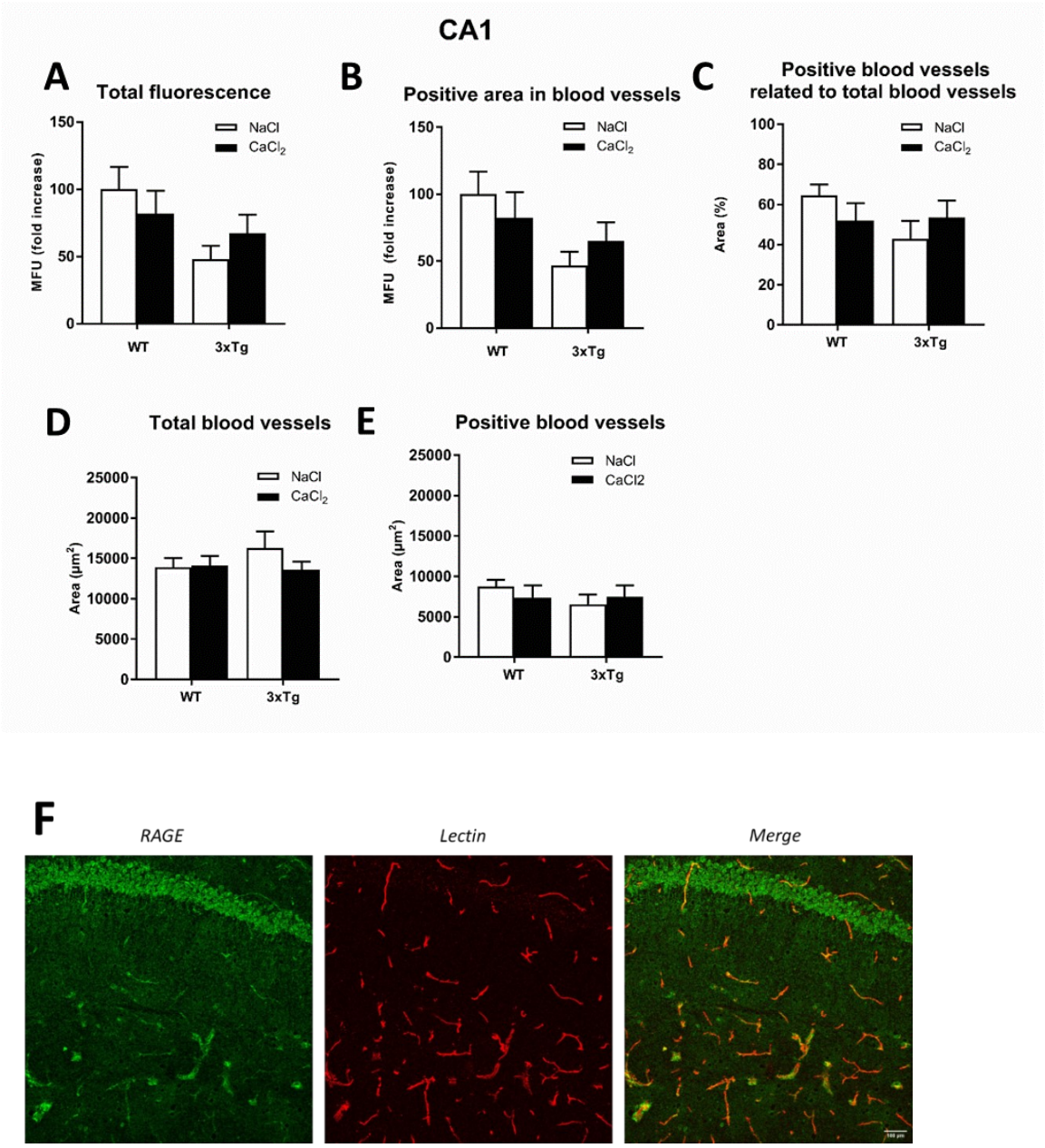
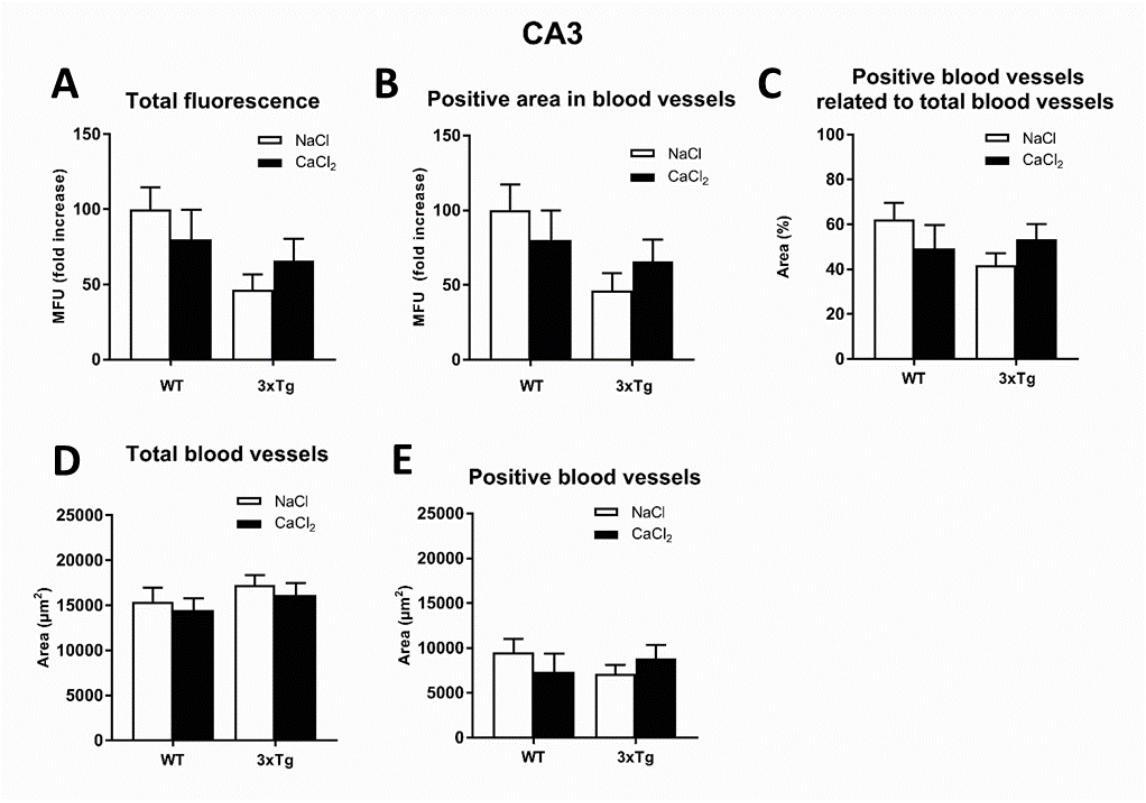


Figure 14. Immunostaining of RAGE-positive cells in cornu ammonis area 1 (CA1) of the affected side of WT and 3xTg mice. A: Total fluorescence represents mean intensity ratio of total RAGE fluorescence to control of each day of imaging, B: Positive area in blood vessels represents mean intensity ratio of RAGE fluorescence in blood vessels to control of each day of imaging, C: Positive blood vessels related to total blood vessels represents area of RAGE in blood vessels divided by total area of blood vessels (in Percentage), D: Total blood vessels represents total area of blood vessels (in μm^2), E: Positive blood vessels represents total area of RAGE in blood vessels (in μm^2). F: representative images of RAGE staining in blood vessels in CA1 of 3xTg mice (scale bar 100 µm). Analysis was done by ImageJ with semi-automatic macro. Data are mean ± SEM; (n=3-4).



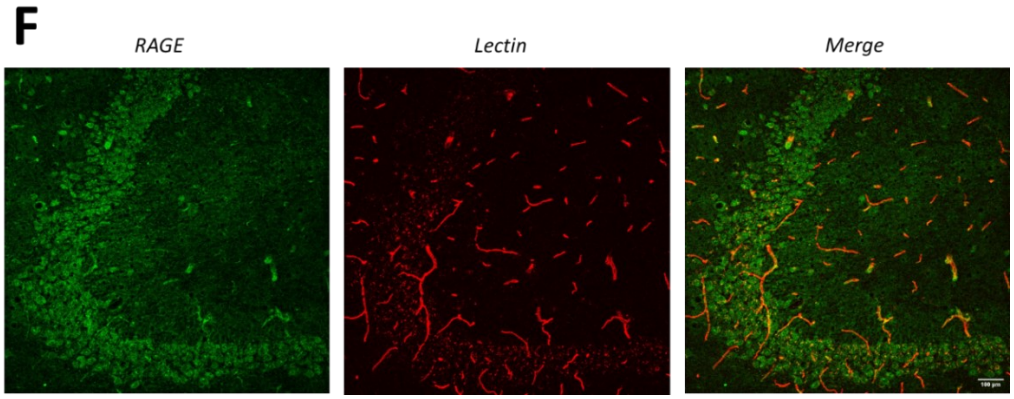


Figure 15. Immunostaining of RAGE-positive cells in cornu ammonis area 3 (CA3) of the affected side of WT and 3xTg mice. A: Total fluorescence represents mean intensity ratio of total RAGE fluorescence to control of each day of imaging, B: Positive area in blood vessels represents mean intensity ratio of RAGE fluorescence in blood vessels to control of each day of imaging, C: Positive blood vessels related to total blood vessels represents area of RAGE in blood vessels divided by total area of blood vessels (in Percentage), D: Total blood vessels represents total area of blood vessels (in μm^2), E: Positive blood vessels represents total area of RAGE in blood vessels (in μm^2). F: representative images of RAGE staining in blood vessels in CA3 of 3xTg mice (scale bar 100 µm). Analysis was done by ImageJ with semi-automatic macro. Data are mean ± SEM; (n=3-4).

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