Early Defects in Neurogenesis in the 3xTg Mouse Model of Alzheimer's Disease

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder leading to dementia. Interestingly, AD is more prevalent in women than men, where two third of the diagnosed AD population is female. The underlying neuropathology is characterized by extracellular Aβ plaques and intracellular tau tangles leading to neuronal cell death. The hippocampus, the main site for learning and memory exhibits the most significant neuronal loss in AD. It is also one of the primary neurogenic niches in the adult brain. A decline of hippocampal neural stem cells is a common feature of AD indicating a defect in neurogenesis. To model AD neuropathology, a triple transgenic model of AD (3xTg) was used. We hypothesize that the defects associated with adult neurogenesis precede the onset of AD hallmarks. Our data showed that neurogenesis defects are present as early as post-natal day 5 of age in the 3xTg model of AD, well before the development of the neuropathology. The early defects were also observed in the TgCRND8 model of AD at 3 months of age. Moreover, no statistically significant difference was detected between male and female mice at 3 months and 9 months of age when investigating NSC populations. This could indicate that sex may not need to be taken in consideration in research design when investigating NSC decline in 3xTg mice. These findings are of clinical relevance, as they may identify early changes that may open opportunities for therapeutic interventions aiming at preventing or delaying neurodegeneration.

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LIST OF ABBREVIATIONS

^{14}C Carbon-14

^{3}H-thymidine Tritiated thymidine

3xTg Triple Transgenic Model

A$\boldsymbol{\beta}$ Amyloid beta

AD Alzheimer's Disease

ANOVA Analysis of variance

APP Amyloid Precursor Protein

Ascl1 Achaete-Scute homolog 1

BrdU 5-bromo-2'-déoxyuridine

CA1/2 Cornu Ammonis 1/2

CA3/4 Cornu Ammonis 3/4

CBIA Cell Biology and Image Acquisition Core

CIHR Canadian Institutes of Health Research

Cre Cyclization Recombinase

CSF Cerebrospinal Fluid

DAPI 4',6-diamidino-2-phenylindole

DCX Doublecortin

DG Dentate Gyrus

DNA Deoxyribonucleic acid

EdU 5-ethynyl-2'-deoxyuridine

ERT2 Estrogen Ligand-Binding Domain

GCL Granular Cell Layer

GFAP Glial Fibrillary Acidic Protein

GFP Green Fluorescence Protein

HopX Homeodomain-Only Protein Homeobox

IF Immunofluorescence

LoxP Locus of X-over P1

MCI Mild Cognitive Impairment

MSc Master of Science

Nestin Neuroepithelial Stem Cell Protein

NFTs Neurofibrillary Tangles

NTG Non-transgenic

NSCs Neural Stem Cells

OHRI Ottawa Hospital Research Institute

PBS Phosphate-Buffered Saline

PCR Polymerase Chain Reaction

PET Positron Emission Technology

PFA Paraformaldehyde

PrP Prion Protein

PSEN1 Presenilin1

PSEN2 Presenilin2

RGL Radial Glial-Like

ROS Reactive Oxygen Species

SEM Standard Error of the Mean

SGZ Subgranular Zone

Sox2 SRY (sex determining region Y)-box 2

SVZ Subventricular Zone

TAM Tamoxifen

TAPs Transit-Amplifying Progenitors

Tbr2 T-box brain protein 2

Type I cell SGZ Neural Stem Cells

Type II cell SGZ Intermediate Progenitor Cells

Type III cell SGZ Neuroblasts

YFP Yellow Fluorescent Protein

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INTRODUCTION

1.1 Overview of Alzheimer's disease (AD)

1.1.1 Dementia and clinical presentation of AD

Dementia is characterized by a progressive but severe decline in memory and other cognitive abilities, such as learning (van der Flier & Scheltens, 2005). While the syndrome of dementia can be caused by many brain diseases and injuries, Alzheimer's disease (AD) remains the most common cause; estimated to account for 60-70% of cases. (Bekris et al., 2010a; Cunningham et al., 2015; Holtzman et al., 2011; van der Flier & Scheltens, 2005). It was first characterized by Dr. Alois Alzheimer in 1906, and is now considered to be the most commonly diagnosed neurodegenerative disorder (Dauer & Przedborski, 2003; Katzman, 1976). The onset and the symptoms associated with AD reflects a selective loss of neurons involved in the hippocampal and neocortical circuitry, which are regions that control language, decision-making, and memory (Anand & Dhikav, 2012a; Bird & Burgess, 2008; Fu et al., 2018; Treves et al., 2008). The early clinical symptoms of AD therefore include difficulty remembering recent conversations, names or events (Mattson, 2004a; Neugroschl & Wang, 2011). Deficits in problem solving abilities and changes in attention are also observed (Holtzman et al., 2011). As the dementia progresses, impaired communication, confusion, disorientation, behavior changes and poor judgment are also common (Atri, 2019; Bekris et al., 2010a; Förstl & Kurz, 1999; Goedert & Spillantini, 2006; Holtzman et al., 2011; Mattson, 2004a). At this point, patients meet the criteria for what is termed mild cognitive impairment (MCI) (Petersen et al., 2018). It is important to note that the most common cause for MCI is AD, but other brain diseases and injuries, each characterized by a set of signs and symptoms that are combined with an underlying

substrate of neuropathology can also lead to MCI (Holtzman et al., 2011; Kapasi et al., 2017). The progression of the cognitive decline in AD is gradual but constant and can average between 7-10 years (Holtzman et al., 2011). In the later stages of the disease, where memory deficits are more apparent and affect long term memory, patients are completely dependent on caregivers for every aspect of daily life. In advanced disease, patients ultimately have difficulty speaking, swallowing, controlling bladder and bowel function, and often become non-ambulatory (Braak et al., 2011; Holtzman et al., 2011) .

Because of the amount of care required over many years and the increasing prevalence of the disease in an aging society, AD has been marked as one of the major challenges of this century as it is likely to become an important global public health concern (Cunningham et al., 2015; Livingston et al., 2017; Prince et al., 2016). To this day, acetylcholinesterase inhibitors and memantine are the only medications licensed for treating the cognitive symptoms associated with AD (Cunningham et al., 2015; Neugroschl & Wang, 2011; Weller & Budson, 2018). However, these medications are not effective in delaying the progression of neurodegeneration in individuals with AD (Weller & Budson, 2018).

One of the proposed reasons behind this absence of disease-modifying treatment is that intervention only happens after patients experience symptoms of dementia, by which time brain damage has occurred and is very difficult to reverse (Villemagne et al., 2013). Intervention at an earlier stage and even before the onset of clinical symptoms is therefore desired.

1.1.2 Pathogenesis of AD

The symptoms of AD such as progressive memory loss and learning difficulties are consistent with synaptic impairment eventually leading to extensive neuronal death (Chong et al., 2018). AD is characterized by heavy cortical and hippocampal shrinkage, and severe ventricle enlargement on a macroscopic scale while the neuropathology hallmarks of the disease of a microscopic level include accumulation of toxic proteins: extracellular plaques composed of amyloid-beta (Aβ) and neurofibrillary tangles of misfolded hyperphosphorylated tau (Chong et al., 2018; Irvine et al., 2008; Lane et al., 2018; Mattson, 2004a; Shahani & Brandt, 2002; Zhou et al., 2011). Altogether, the amassing of these plaques and tangles adversely affect vital processes for neurons and their networks such as metabolism, communication, and repair (Bekris et al., 2010a; Mattson, 2004a). This causes widespread damage as neurons stop functioning, lose connections with other neurons and eventually die (Mattson, 2004a).

1.1.2.1 Aβ plaques

The Aβ peptide, a key component of AD neuropathology, is 40 to 42 amino acids in length and is formed by the proteolytic cleavage of a transmembrane protein called amyloid precursor protein (APP). The function of this protein remains uncertain (Tiwari et al., 2019). The N terminus of the Aβ peptide is found in the extracellular domain of the APP while the C terminus is located in the transmembrane region. Through the activity of the β and γ-secretases the N and C termini of the Aβ peptide are generated, while the non-pathological processing of APP via a third enzyme, the α-secretase, prevents the formation of Aβ peptide altogether (Goedert & Spillantini, 2006).

In the AD brain, a naturally occurring form of the Aβ peptide, Aβ1-42, is prone to aggregation and clumps together to form plaques that collect between neurons (Bekris et al., 2010a; Irvine et al., 2008; Mattson, 2004a). "Diffuse plaques" contains Aβ peptides that are aggregated in a non-fibrillar conformation and can be detected using various immunohistochemical techniques. When Aβ peptides deposits in a fibrillar form with a β-sheet conformation, they are defined as "neuritic plaques" and can be detected using fluorescence microscopy with stains such as Thioflavin-S.

Axons and dendrites surrounding these neuritic plaques are swollen and degenerating, therefore disrupting synaptic function, which demonstrates Aβ toxicity (Bloom, 2014; Ribeiro et al., 2019). Moreover, reactive astrocytes and microglia around the plaques induce neuroinflammation that increase the presence of harmful reactive oxygen species in the brain which may contribute to brain injury (Bekris et al., 2010b; Haass et al., 1992; Mattson, 2004b).

1.1.2.2 Neurofibrillary Tangles

Tau belongs to the family of highly soluble proteins contained in the nerve cells (Chong et al., 2018; Hirokawa et al., 1988; Shahani & Brandt, 2002). They play a critical role in normal synaptic function by promoting the assembly of tubulin and the stabilization of microtubules, which guide nutrients and molecules from the soma to the axon and dendrites (Baas et al., 2016; Conde & Cáceres, 2009; Duan et al., 2017; Hirokawa et al., 1988; Kapitein & Hoogenraad, 2015).

In AD, abnormal post-translational modifications of the tau protein, such as hyperphosphorylation, leads to conformational changes in the tau monomer to tau

oligomers. The accumulation and aggregation of tau oligomers disrupt microtubule assembly and eventually drives the formation of neurofibrillary tangles (NFTs) inside neurons. By affecting the neuronal microtubule cytoskeleton, these NFTs can harm the synapses, therefore causing dysfunctional synaptic communication between neurons leading to synaptic loss, which can be observed in AD (Bekris et al., 2010b; Mattson, 2004b).

1.1.2.3 Amyloid cascade hypothesis

The most widely accepted model for the root cause of AD is called the amyloid cascade hypothesis (Zhou et al., 2011). It proposes that the disease starts with the accumulation of Aβ oligomers over the course of age either through increased production or reduced clearance mechanisms (Hardy & Higgins, 1992; Zhou et al., 2011). This accumulation causes aggregate stress that induce the change in tau hyperphosphorylation resulting in the formation of the NFTs (Zhou et al., 2011). Over the last 20 years, drug development research for AD focused on this hypothesis, leading to many amyloid-targeted programs. However, the failed attempts to demonstrate the clinical benefit of targeting Aβ led many to the reevaluate the central and early role of Aβ in the onset of AD (Tolar et al., 2019). This remains controversial considering; (1) the genetic data supporting the causative effect of Aβ (Bateman et al., 2011; Hartley et al., 2015; I. E. Jansen et al., 2019; Tolar et al., 2019); (2) the strong correlation between the levels of Aβ oligomers, the progression of the disease and the severity of the clinical symptoms of AD (Blessed et al., 1968; Cline et al., 2018; Esparza et al., 2013; Sengupta et al., 2016; Tai et al., 2013); (3) synaptic toxicity of Aβ oligomers (Gaspar et al., 2010; Hong et al., 2018; Koffie et al., 2012; Savage et al.,

2014; T. Yang et al., 2017); (4) the target of many failed phase 3 clinical trial was Aβ monomer despite strong genetic evidence indicating that neurotoxic soluble Aβ oligomers are a key player in AD pathogenesis (Egan et al., 2019; Honig et al., 2018; Ostrowitzki et al., 2017; Panza et al., 2019; Salloway et al., 2014); (5) limited blood-brain barrier penetration for several anti-amyloid immunotherapy (Cummings et al., 2018; Honig et al., 2018; Logovinsky et al., 2016; Ostrowitzki et al., 2017; Salloway et al., 2014; Sevigny et al., 2016); and (6) the heterogeneity of AD populations contributing to the high variability in AD trials (Abushakra et al., 2016; Nelson et al., 2019; H.-S. Yang et al., 2018).

To develop efficient therapeutic strategies for AD, future drug development programs will need to investigate agents that factor a high selectivity for Aβ oligomers and an effective blood-brain barrier penetration (Tolar et al., 2019). The aim would be to reduce brain levels of Aβ oligomers sustainably to prevent synaptic toxicity and neurodegeneration.

One other factor to consider when investigating the failures of the drug programs targeting Aβ is to determine if the drugs were administered early enough to obtain the desired therapeutic effects. Interestingly, Aβ accumulation begins as up to 15-20 years prior to the onset of clinical dementia signs and symptoms (Dubois et al., 2014; Fan & Wang, 2020; Villemagne et al., 2013). Advances in biomarker and positron emission technology (PET) imaging demonstrated that Aβ deposition was estimated to become abnormal around 17 years before the onset of dementia, then followed by substantial changes in hippocampal volume and severe cognitive impairments 3–6 years before the onset of dementia (Villemagne et al., 2013). Establishing the rate of AD-related pathology allows for a better insight in the sequence of events leading to AD dementia. By understanding the pathological threshold associated with AD pathology, this potentially permits intervention

within a therapeutic window that could allow for a better prognosis for individuals prone to developing AD (Crous-Bou et al., 2017; Cummings et al., 2007; Fan & Wang, 2020; Villemagne et al., 2013).

For optimal success and benefits, the identification of high-risk individuals earlier, but also the ability to distinguish AD patients from patients presenting different pathologies mimicking the clinical presentation of AD is critical. Future research for diagnostic markers of AD will therefore be imperative to consider. The development of methods able to identify presymptomatic populations of AD patients in a timely manner would permit optimal therapeutic intervention aiming at preventing or delaying neurodegeneration before the onset of clinical symptoms.

1.1.3 Classification of AD

AD can either be classified as sporadic or familial. Both sporadic and familial AD follow the same trajectory of neuropathological changes in the brain yet have different underlying causes. Familial AD, also known as early-onset AD, is directly caused by mutations to the amyloid precursor protein (*APP*) gene or either one of the genes affecting APP processing: presenilin1 (*PSEN1*) and presenilin2 (*PSEN2*) (Bekris et al., 2010a; Lane et al., 2018; Piaceri et al., 2013; Tanzi & Bertram, 2005), resulting in increased production of the pathological Aβ1-42 peptide. Familial AD represents approximately 5% of AD diagnoses and has a typical onset of less than 65 years of age (Mendez, 2017). Considering that familial AD is inherited as an autosomal dominant mutation, most patients have a family history of the disease (Bateman et al., 2011). Sporadic AD, also known as late-onset AD, can occur naturally with advanced age from a complex combination of genetic and

environmental risk factors. Sporadic AD primarily develops in later life with an onset greater than 65 years of age and is the most common form of AD (Mendez, 2017; Piaceri et al., 2013). Age is the greatest risk factor for sporadic AD, with the prevalence of the disease doubling every 5 years after 65 years of age (Lane et al., 2018). Approximatively 1/3 of sporadic AD cases could be attributed to modifiable risk factors including obesity, diabetes, physical inactivity, smoking, low levels of education, and depression (Livingston et al., 2020; Norton et al., 2014).

Despite these classifications, patients diagnosed with AD will develop similar neuropathology and no effective treatment for either form has yet to be discovered. To ameliorate prognostic outcomes for AD, research should focus on the development of a reliable and non-invasive biomarker that could detect pathogenic changes in cognitively normal people. That way, at-risk individuals could undergo amyloid therapy or lifestyle changes within a therapeutic window before they present symptoms of memory loss and potentially delay the onset of dementia or prevent it entirely.

1.1.4 Sex differences in AD

The identification of presymptomatic populations of AD patients could also potentially lead to the emergence of male-specific or female-specific preventions or treatments. Interestingly, AD is more prevalent in women than men, where two thirds of the diagnosed AD population is female. For a long time, this was attributed to the well-established fact that women lived longer than men, reaching ages when AD is more prevalent (WHO | World Health Statistics 2011, n.d.). The cause and underlying mechanism behind this sex

difference remains unknown. Recent studies of living human AD populations found similar Aβ burden in cerebrospinal fluid (CSF) when comparing males to females (Mielke, 2020). However, the sex differences in pathology only become notable in advanced age where the burden becomes greater for female (Buckley et al., 2018, 2019; Hohman et al., 2018; Jack et al., 2015; W. J. Jansen et al., 2015).

Post-mortem studies, which are useful to gain insight on the pathology of the disease at terminal stages, showed that women had greater global AD pathology than men only after age 75 years. Prior to this age, the levels of brain pathology remain equivalent between sex (Hohman et al., 2018). However, sex could have an impact on AD diagnosis by influencing the brain's resilience to similar levels of pathology (Dubal, 2020). It is well established that during development sex-specific hormones lead to divergent neuronal circuitry between females and males (Fester & Rune, 2021; Rocca et al., 2014). These differences may also affect the ability of these neuronal circuits to cope with damages later and therefore lead to different disease trajectory (Dubal, 2020; Zhu et al., 2021).

Sex hormones, such as estrogen, also has crucial neuroprotective properties for the brain and plays an important role in the maintenance of hippocampal functions (Correia et al., 2010; Merlo et al., 2017; Uddin et al., 2020; Xu et al., 1998). Therefore, when studying sex differences in AD it is important to factor the sudden drop of estrogen that occurs in women after menopause (Uddin et al., 2020). When looking at AD patient subgroups, women who underwent natural or surgically induced menopause it was observed that they presented higher levels of NFTs and amyloid burden than men (Oveisgharan et al., 2018)

Societal and environmental factors, such as access to education, risk factor exposure and daily activities, could also explain the observed sex difference (Mielke et al., 2014a, 2018; Rocca, 2017). Their influence on the maturing brain could lead to increased vulnerability to AD pathology.

Sex differences in the pathology of AD reveal that men and women could benefit from personalized medicine strategies or therapies. Therefore, further studies aiming towards understanding the mechanisms behind sex differences are of great importance to potentially ameliorate the prognostic outcomes of this disease.

1.1.5 AD and Adult Neurogenesis

The progressive memory impairments observed in AD is associated to the fact that one of the regions severely affected by AD in the brain is the hippocampus. By working with other medial lobes, the hippocampus plays a critical role in the acquisition of episodic and semantic memories (Anand & Dhikav, 2012b; The Hippocampus and Memory: Insights from Spatial Processing | Nature Reviews Neuroscience). This role is possible because the hippocampus contains a neurogenic niche called the sub granular zone (SGZ) of the DG where new neurons are generated throughout life from self-renewing cells known as neural stem cells (NSCs)(Kuhn et al., 1996a; Lazarov et al., 2010; G. Ming & Song, 2011; Urbán & Guillemot, 2014; J. Zhang & Jiao, 2015). These self-renewing NSCs can proliferate and differentiate into neural progenitors that can get integrated in the local neuronal network and modify how learning and memory are encoded and retrieved in the hippocampus (Akers et al., 2014; Urbán & Guillemot, 2014). This process is called adult neurogenesis.

Determining the extent in which decreased neurogenesis occurring in the hippocampus contributes to the cognitive decline in AD, could be interesting for both diagnostic and therapeutic purposes.

1.2 Adult Neurogenesis in the Mammalian Brain

1.2.1 History of Adult Neurogenesis

In the field of neuroscience, a concept that remains controversial among the scientific community is adult neurogenesis. It is defined as the formation of neurons *de novo* throughout life from a pre-existing population of self-renewing, multipotent cells described as neural stem cells (NSCs) (Reynolds & Weiss, 1992; Richards et al., 1992). Prior to 1965, it was a long-held belief that neurogenesis only occurred during embryonic development and early postnatal stages of mammalian development (Gross, 2000). The idea that this process could also happen in later stages in life emerged when ^{3}H-Thymidine labelling experiments identified the presence of newly born neurons in the hippocampus and the olfactory bulb of adult rats (Altman, 1969; Altman & Das, 1965). Further studies led to the discovery of two distinct niches: (1) the subventricular zone (SVZ) of the lateral ventricle and (2) the subgranular zone (SGZ) of the dentate gyrus (C et al., 1996; C & A, 1993, 1994; Eriksson et al., 1998; Fh et al., 1995; Palmer et al., 1997). These pools of NSCs remain quiescent until activation occurs via cell-intrinsic and cell-extrinsic factors where they can either replicate or terminally differentiate into mature post-mitotic neurons (Kempermann et al., 2018; G.-L. Ming & Song, 2011; Spalding et al., 2013; Sung et al., 2020; Toda & Gage, 2018).

The discovery and understanding of adult neurogenesis in the mammalian brain are important in order to investigate how the generation of new circuitry in the brain occurs and how this impacts behavior. Generating new circuits in the brain is key for memory, mood, learning, cognition and forgetting (Aimone et al., 2011; Frielingsdorf & Kuhn, 2007).

1.2.2 Neurogenesis in the Subgranular Niche

The SGZ neurogenic niche is located in the hippocampus, a region greatly affected in AD. Adult hippocampal neurogenesis relies on a pool of self-renewing radial glial-like (RGL) NSCs that differentiate into mature neurons (Seri et al., 2001). This process is implicated in the regulation of different cognitive processes such as learning, memory, pattern differentiation and cognitive flexibility (Toda et al., 2019). Integration of newborn neurons in the dentate gyrus (DG) allows for flexibility in the hippocampal circuit both structurally and functionally. The hippocampal circuit is described as a tri-synaptic pathway where excitatory connections from the entorhinal cortex project to the DG, then from the granule cells of the DG to the CA3/4 pyramidal cell region, and then finally from the CA3/4 pyramidal cell on to the CA1/2 pyramidal cell region (Yeckel & Berger, 1990). The current model for neurogenesis in the SGZ niche describes a population of NSCs (Type I cells) comprised of two different subpopulations with different morphologies: RGL or horizontal (Suh et al., 2007). The RGL subpopulation is found to be quiescent while the horizontal subpopulation presents both quiescent and activated cells (Lugert et al., 2010). Activated cells will then differentiate into progenitor cells (type II cells) eventually leading to neuroblasts (type III cells) (Seri et al., 2001). The newly formed neuroblasts travel from

the SGZ to the granule cell layer (GCL) of the DG to integrate with the CA3 region of the hippocampus. Neurons recently integrated demonstrate similar electrophysiological properties to older mature neurons present in these circuits demonstrating they are functionally similar to the neurons already present in the local neuronal circuitry (Toni et al., 2007; van Praag et al., 2002). The hippocampus is known to be an important part of the limbic system and has significant implications in the acquisition of episodic and semantic memories (Anand & Dhikav, 2012a; Bird & Burgess, 2008; Squire, 1992). Hippocampal neurogenesis is key in different cognitive tasks such as spatial memory retention, navigation and pattern discrimination as well as in stress response and emotional behavior (Bird & Burgess, 2008; Deng et al., 2010; Kheirbek & Hen, 2011; G.-L. Ming & Song, 2011; Takada et al., 2015). Lesion studies demonstrated that the dorsal hippocampus was involved in spatial memory and learning while the ventral hippocampus was concerned with emotions (Altman & Das, 1965; Fanselow & Dong, 2010; Henke, 1990; Huckleberry & Shansky, 2021; Jurkowski et al., 2020; Kheirbek & Hen, 2011; Morris, 1984).

1.2.3 Identification of NSC lineage

Identifying stem cell lineage and investigating the stages of stem cell development using specific markers is a common technique used to study neurogenesis. In the SGZ niche, type I cells are the quiescent population that exhibit a RGL morphology and that are marked by Nestin, SRY (sex determining region Y)-box 2 (Sox2) and glial fibrillary acidic protein (GFAP) (G.-L. Ming & Song, 2011). These cells have the potential to regenerate NSCs through symmetric division, a process known as self-renewal, as well as the capacity to form specialized cell-types through differentiation, via asymmetric division. Upon

activation, type I cells will transition to progenitor cells and lose their RGL morphology. Neuroblast cells are progenitor cells that committed to the neural lineage, and they express factors such as T-box brain protein 2 (Tbr2) and Achaete-Scute homolog 1 (Ascl1) (Kempermann et al., 2018; Kronenberg et al., 2003; Seri et al., 2001). Compared to progenitor cells, neuroblasts cells divide at a slower pace. Neuroblasts cells may also start to express the factor doublecortin (DCX) but the expression levels will increase as the differentiation process is well underway and that these cells migrate to the granule layer as immature neurons (Kempermann et al., 2004).

Recent constructions of neural cell lineages revealed that non-radial cells or TAPs could undergo several divisions and switch between asymmetric or symmetric divisions (Pilz et al., 2018). Previous models thought that adult neurogenesis in the SGZ niche involved little self-renewal of the TAP population (Götz, 2018a). The findings that these TAPs present some stem cell characteristics prompts revision to current model and demonstrates that the study of stem cells is still novel and this gives new insight on how to define a stem cell itself (Götz, 2018a; Pilz et al., 2018).

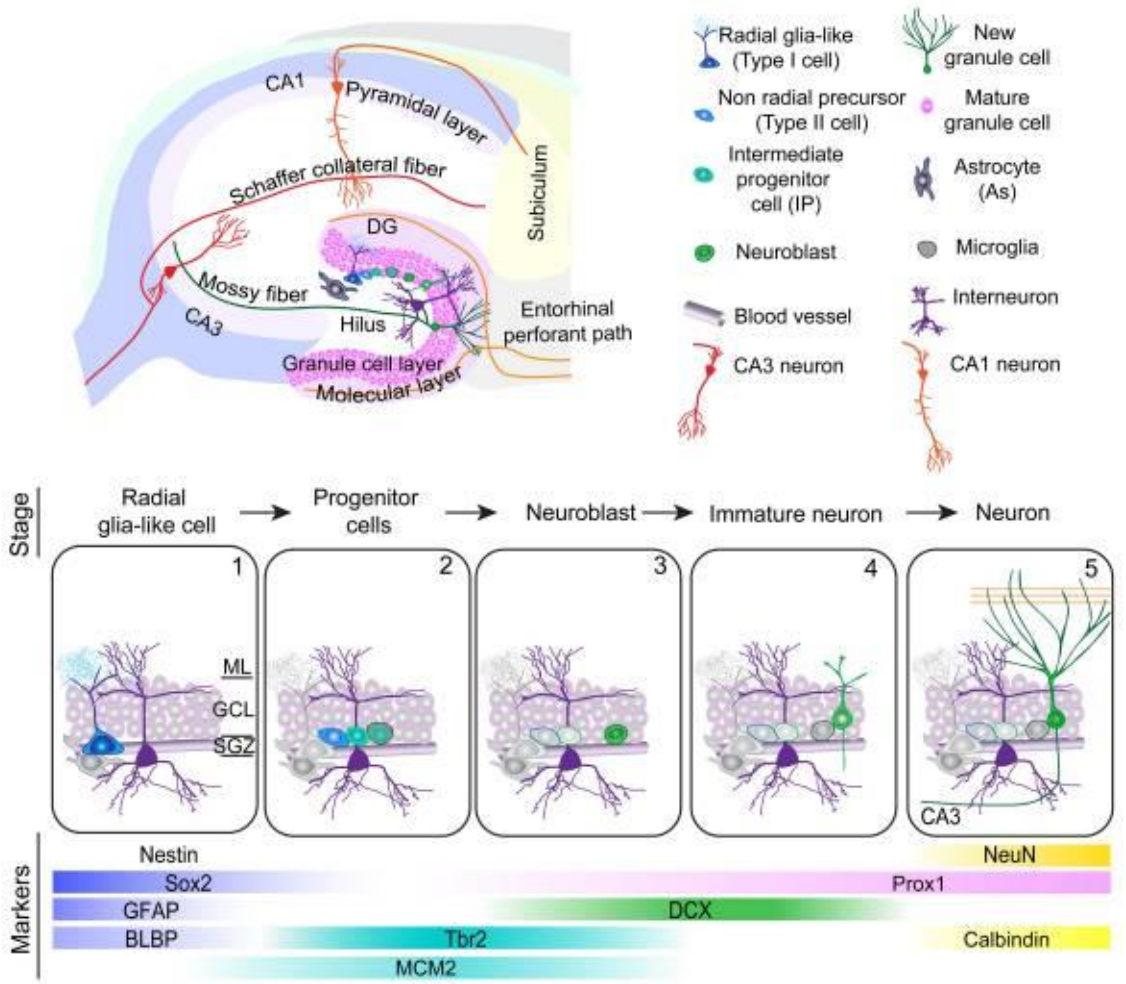

Figure 1. Adult Neurogenesis in the Dentate Gyrus of Hippocampus. Summary of five developmental stages during adult hippocampal neurogenesis: (1) activation of quiescent radial glia-like cell in the subgranular zone (SGZ); (2) proliferation of non radial precursor and intermediate progenitors; (3) generation of neuroblasts; (4) integration of immature neurons; (5) maturation of adult-born dentate granule cells. Also shown are expression of stage-specific markers. ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone; GFAP: glial fibrillary acidic protein; BLBP: brain lipid-binding protein; DCX: doublecortin; NeuN: neuronal nuclei. Adapted from (Ming and Song 2011).

1.2.4 Neurogenesis in the Human Brain

While evidence of adult neurogenesis in the mammalian brain is well established and accepted in the field of neuroscience, the phenomenon remains controversial in human brains. The first evidence that adult neurogenesis happened in humans was obtained by post-mortem studies of BrdU (5-bromo-2'-déoxyuridine) integration in the brains of cancer patients (Eriksson et al., 1998). Further studies were later published demonstrating that in the adult human brain about seven hundred newborn neurons were added in the GCL of the DG and that the rate of turnover, approximately 1.75% annually, was comparable between middle-aged humans and mice. This was quantified by measuring the concentration of nuclear-bomb-test-derived ^{14}C in genomic DNA (Spalding et al., 2013). Some discrepancies remain between research regarding the numbers of neuroblast generated daily depending on the methods of quantification (Dennis et al., 2016; Eriksson et al., 1998; Knoth et al., 2010). Moreover, additional studies suggest that adult neurogenesis is in fact highly reliant on various factors such as age, stress, environmental factors, and exercise leading to the understanding that it is a more dynamic process than what is currently thought(Livingston et al., 2020; Norton et al., 2014). Additionally, dysregulation of hippocampal neurogenesis and decreased hippocampal volumes is suggested to be strongly involved in the cognitive impairment observed in neurodegenerative disorders but also linked to the psychological symptoms observed in psychiatric disorders (Bonne et al., 2008; Crews et al., 2008; Feng et al., 2001; Frey et al., 2007; Haughey et al., 2002; Kuhn et al., 1996; Spalding et al., 2013; Winner et al., 2012; Zhang et al., 2007).

The study of adult neurogenesis in humans is difficult due to several technical issues, such as post- mortem tissue sample handling (Boekhoorn et al., 2006) and a lack of reliable cell markers for the quantitative study of human adult neurogenesis (Mathews et al., 2017). In fact, two recent studies published in high impact journals obtained opposite conclusions on the longevity and significance of adult neurogenesis in the human SGZ (Boldrini et al., 2018; Sorrells et al., 2018) because of differences between their methodologies (Lee and Thuret, 2018). Future studies will need to focus on resolving technical issues involved with studying adult neurogenesis in humans, to obtain meaningful results that can contribute to the development of novel therapeutic strategies for the brain (Kempermann et al., 2018). However, to study the alterations in neurogenesis it is perceived as advantageous to study the process in murine models. At a fundamental level, the process of adult neurogenesis is relatively conserved amongst species (Ernst and Frisén, 2015).

1.3 Adult neurogenesis and AD

Adult neurogenesis offers great opportunities in regenerative medicine for neurodegenerative diseases such as AD (Bond et al., 2015). The observed neuron loss in AD happens in two regions of the brain: the cerebral cortex and the hippocampus. The latter is an important region for learning and memory and exhibits the most significant neuronal loss in AD (Mattson, 2004b). The hippocampus also contains the SGZ neurogenic niche where new neurons are generated throughout life from self-renewing NSCs (Kuhn et al., 1996a; Lazarov et al., 2010; G. Ming & Song, 2011; Urbán & Guillemot, 2014; J. Zhang & Jiao, 2015).

A decline of hippocampal NSCs is a common feature of AD indicating a defect in neurogenesis, yet remains unknown whether the decline in hippocampal NSC precedes AD neuropathology (Moreno-Jiménez et al., 2019; Tobin et al., 2019; Unger et al., 2016). Adverse effects of Aβ pathology on NSCs, and subsequent decline in adult neurogenesis, could potentially contribute to the cognitive impairment and the depletion of neurons observed in AD (Haughey et al., 2002b). However, changes in adult neurogenesis could also be an early event in AD neurodegeneration instead of a late consequence of the disease and would therefore precede AD pathology (Tobin et al., 2019). Indeed, in several animal models of AD a decline in NSC population is observed earlier than the apparition of Aβ plaques (Demars et al., 2010).

Identifying early changes in adult neurogenesis could be of clinical relevance for diagnostic purposes (Moreno-Jiménez et al., 2019). It would permit intervention in presymptomatic at-risk individuals before the memory loss begins. Therapeutic interventions aiming at preventing or delaying the onset of dementia could implicate changes in certain health and lifestyle choices since protective factors against dementia include higher education, healthy diet, physical activity, and social networking(Livingston et al., 2020). Moreover, since rejuvenation of neurogenic niche can lead to improved cognition (Navarro Negredo et al., 2020), adult neurogenesis may represent a promising treatment in regenerative medicine for AD. Promoting the growth and integration of new cells from inherent populations of stem cells is a promising strategy for replacing lost or damaged tissues.

1.3.1. Animal models of AD and neurogenesis

To replicate AD neuropathology, over 100 mouse models have been created based on familial human mutations and phenotype. However, no model so far has fully succeeded in mimicking the neurodegeneration of AD. The triple transgenic model of AD (3xTg) is a very popular model that carry three mutations in genes associated with familial AD: β-amyloid precursor protein ($\beta APP_{Swe}KM670/671NL$), presenilin-1 ($PS1_{M146V}$), and MAPT ($tau_{P301L}$) (Oddo et al., 2003a). 3xTg mice develop age-related, progressive neuropathology including plaques and tangles, but also present neuroinflammation and cognitive deficits (Belfiore et al., 2019; Oddo et al., 2003a). Extracellular hippocampal Aβ deposits and tangle formation are apparent by six months and become more extensive with age (Belfiore et al., 2019). Impaired neurogenesis has also been reported in this model as early as 4 months of age when looking at the proliferation of NSCs in both the SVZ of the lateral ventricles and the SGZ of the hippocampus (Hamilton et al., 2010; Rodríguez et al., 2008).

The TgCRND8 model of AD is another interesting model that express a double mutant form of APP ($\beta APP_{Swe}KM670/671NL$; $\beta APP_{Ind}V717F$) (Chishti et al., 2001). This leads to a 5-fold increase in the production of Aβ in this model and plaque formation can be detected as early as 3 months of age (Chishti et al., 2001). At this age, they also demonstrate early impairment in the acquisition and learning reversal when they are undergoing the reference memory version of the Morris water maze (Chishti et al., 2001). Interestingly, there are conflicting reports of adult neurogenesis in TgCRND8 mice. One study found decreased hippocampal neurogenesis at 3 months of age, yet another found an elevated number of proliferating NSCs in the DG at an earlier age prior to the emergence of Aβ

plaques (Fiorentini et al., 2010; Kanemoto et al., 2014). To elucidate the mechanisms contributing to the observed AD-associated defect in neurogenesis, a better knowledge of the dynamics of hippocampal neurogenesis in humans will be crucial.

1.4 Rationale for the Hypothesis and Objectives

Alterations in adult neurogenesis could be a promising non-invasive therapy for early intervention with clinical trial medications or lifestyle changes within a pre-symptomatic time frame. The aim would be to prevent or delay the onset of dementia and the associated neurodegeneration. Adult neurogenesis could also represent a promising treatment in regenerative medicine for AD to replace lost or damaged cells by promoting endogenous stem cell repair responses.

It remains to be determined how early these defects can be detected in the AD brain to better assess the potential of neurogenesis for therapeutic purposes. Moreover, given that there is an observed sex difference in human AD pathology and etiology, we wanted to investigate if there was a sex difference in adult neurogenesis in a mouse model of AD. This will indicate how sex differences impact disease progression and presentation, as well as the need to factor these differences into research design as a biologic variable for future experiments involving adult neurogenesis in the AD brain.

HYPOTHESIS AND OBJECTIVES

Hypothesis: Defects associated with adult neurogenesis in the 3xTg mouse model of AD precedes the onset of AD neuropathology. The depletion of NSCs is greater in females than in males.

Aim I: Characterize NSC populations in the 3xTg mouse model of AD at different time points of brain development using immunohistochemistry and fluorescence microscopy.

Aim II: Characterize sex differences of NSC populations in the 3xTg mouse model of AD.

MATERIAL AND METHODS

2.1 Mice

Three transgenic mouse lines were used for this study: 3xTg (B6.Cg-Tg(APPSwe,tauP301L)1Lfa *Psen1*^{tm1Mpm}/2J), 3xTg crossed onto a Nestin-Cre^{ERT2} background and TgCRND8 (Hybrid C3H/He-C57BL/6; APP KM670/671NL (Swedish), APP V717F (Indiana))(Chen et al., 2009; Chishti et al., 2001; Oddo et al., 2003b). 3xTg mice were examined at post-natal day 5, post-natal day 7, 1 month of age, 3 months of age, 6 months of age and 9 months of age. 3xTg Nestin-Cre^{ERT2} mice were studied at 3 months of age. The purpose was to drive conditional Rosa26-YFP expression in Nestin+ cells for lineage tracing purposes. TgCRND8 mice were examined at 3 months of age. These mice were provided by Dr. Steffany Bennett's lab (University of Ottawa Brain and Mind Research Institute)(Chishti et al., 2001). Littermate controls were used. All experimental protocols involving mice were performed in compliance with University of Ottawa policy concerning the care and use of animals in research (protocol #ME-1765).

Genotyping was conducted by PCR using REDExtract-N-AmpTM PCR ReadyMixTM (MilliporeSigma) and following the guidelines from the manufacturer. Primer sequences for genotyping are shown (Table 1). Genotyping for 3xTg was done using primers specific for the PSEN1^{tm1Mpm} (IPCF & IPCR) & Tg(APPSwe,tauP301L)1Lfa (TF & TR)

2.2 Animal Euthanization

The animals were euthanized by 30ul of 1mg/kg intraperitoneal injection of Euthanyl (Sodium Pentobarbital). By inserting a perfusion needle in the left ventricle and sectioning the right atrium, mice were perfused with 0.9% cold saline followed with cold, fresh 4%

paraformaldehyde (PFA) (pH 7.4). Brains were then dissected and were post-fixed in 4% PFA at 4°C for 24 hours while rocking then incubated in 20% sucrose in 1x PBS, 0,3% sodium azide for minimum 48 hours and kept in solution until processed.

2.3 Tamoxifen Administration

In the Nestin-CreERT2 mice (Chen et al., 2009), the Estrogen-activated Cre-recombinase is localized to Nestin-expressing cells throughout the organism. When Tamoxifen (TAM) is administered it leads to the binding of TAM to estrogen-receptors on the Cre-recombinase protein permitting the Cre to the nucleus where it will cut loxP restriction sites. In our study, adult mice 2 months of age were administered daily 100ul TAM by oral gavage at a concentration of 50mg/mL for 5-consecutive days (Khacho et al., 2016).

Target	Primer Sequences
Cre	GCG GTC TGG CAG TAA AAA CTA TC
	(Transgene Forward A Cre F)
	GTG AAA CAG CAT TGC TGT CAC TT
	(Transgene Reverse A Cre R)
	CTA GGC CAC AGA ATT GAA AGA TCT
	(Internal Positive Control Forward A)
	GTA GGT GGA AAT TCT AGC ATC ATC C
	(Internal Positive Control Reverse A)
R26-YFP	GCG AAG AGT TTG TCC TCA ACC
	(Mutant reverse)
	AAA GTC GCT CTG AGT TGT TAT
	(Common)
	GGA GCG GGA GAA ATG GAT ATG
	(Reverse)
PSEN1^{tm1Mpm}	CTA GGC CAC AGAAT GAA AGA TCT
	(IPCF-Forward)
	GTA GGT GGA AAT TCT AGC ATC ATC
	(IPCR-Reverse)
Tg(APPSwe,tauP301L)1Lfa	AGG ACT GAC CAC TCG ACC AG
	(TF-Forward)
	CGG GGG TCT AGT TCT GCA T
	(TR-Reverse)
Sex Chromosomes	CAC CTT AAG AAC AAG CCA ATA CA (Forward)
	GGC TTG TCC TGA AAA CAT TTG G (Reverse)

Table 1. Genotyping sequences for PCR primers.

2.4 EdU Labelling

In vivo labelling of proliferating cells was achieved by giving an intraperitoneal injection of 10μL/g of a 5 mg/mL EdU (5-ethynyl-2'-deoxyuridine) solution (Clickbase, BCK647-IV-IM- M) to the adult mice, 2.5 hours prior to being sacrificed. Staining for EdU was conducted in accordance with the manufacturer's instructions (Clickbase, BCK647-IV-IM-M) following primary and secondary antibody labelling.

2.5 Tissue Processing

The brain was cut into two hemispheres. One hemisphere was transferred back in the sucrose solution (20% sucrose in 1x PBS, 0,3% sodium azide) and stored at 4°C for future use. The other hemisphere was immersed in -40°C isopentane (Thermo Fisher Scientific) for 60 seconds and then preserved in aluminum foil on dry ice until cryosectioned. Temperature of the Leica CM1850 was set at -22°C and adult brains were mounted with Tissue-Tek® O.C.T. Compound (Sakura Finetek). 30μm thick serial coronal sections of the SGZ were obtained using the Leica CM1850. Sections were serially collected from the beginning of the dentate gyrus to the end of the dentate gyrus into 9 wells of a 24-well plate containing 4mL of PBS 1X + 0.01% sodium azide as free-floating sections. In this regard, each of the 9 wells contained every 9^{th} section from beginning to end. Tissue sections were stored at 4^{o}C.

2.6 Immunostaining

For immunofluorescence staining, all sections of one well were washed 3 times in washing solution (1x PBS) for 5 minutes each wash, rinsed 3 times in washing solution containing 0.1% Triton X-100 and 0.1% Tween-20 for 5 minutes each rinse, then incubated overnight at 4°C with primary antibodies (Table 2) diluted in 1x PBS containing 0.1% Triton, 0.1% and Tween 20. Sections were then rinsed 3 times in washing solution for 5 minutes each rinse, incubated for 2 hours at room temperature in 1x PBS containing 0.1% Triton, 0.1% and Tween 20 with secondary antibodies (Table 2) along with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). Sections were mounted on FisherbrandTM SuperfrostTM Plus Microscope Slides (Thermo Fisher Scientific) and coverslips were mounted onto slides with Immunomount (Genetex). Images for quantifications were taken using a DeltaVision Elite-Olympus IX-71 at a 20X magnification or a ZEISS Axioscan 7 at a 20X magnification and were analyzed using Fiji (Image J). Different antibodies were sometimes used for the same targets depending on antibody combinations and/or commercial availability.

Antibody	Host Specie	Source	Dilution
Primary Antibodies			
DCX	Goat	Santa Cruz Biotechnology (sc-8066)	1: 500
	Rabbit	Cell Signaling Technology (4604S)	1:500
GFP	Chicken	Abcam (ab13970)	1:1000
HopX	Rabbit	Atlas Antibodies (HPA030180)	1:2500
Nestin	Goat	R&D Systems (AF2736)	1:1000
Sox2	Goat	Neuromics, GT15098	1:1000
	Rabbit	Milipore (Ab5603)	1:500
Secondary Antibodies			
Anti-Goat 488	Donkey	Jackson ImmunoResearch (705-545-147)	1:1000
Anti-Goat Cy3	Donkey	Jackson ImmunoResearch (705-165-147)	1:1000
Anti-Goat Cy5	Donkey	Jackson ImmunoResearch (705-495-147)	1:1000
Anti-Rabbit 488	Donkey	Jackson ImmunoResearch (711-545-152)	1:1000
Anti-Rabbit Cy3	Donkey	Jackson ImmunoResearch (711-165-152)	1:1000
Anti-Rabbit Cy5	Donkey	Jackson ImmunoResearch (711-605-152)	1:1000
Anti-Chicken 488	Donkey	Jackson ImmunoResearch (703-545-155)	1:1000

Table 2. Primary and secondary antibodies used for immunohistochemistry protocols.

2.7 Cell Counts and Statistical Analysis

Cells expressing the marker of interest were quantified along the entire SGZ of the DG. The sum of all positive cells from all sections from one well was multiplied by 9 to obtain the estimated total cell number per DG from beginning to end (Khacho et al., 2016). All counts were performed using Fiji (ImageJ). A two-way ANOVA was used for statistical analysis (genotype × sex) and posthoc were also used (NTG x 3xTg). The analysis was performed using GraphPad PRISM software (GraphPad Software, Inc). The numbers of biolological replicates 'n', are shown in figure legends.

2.8 Cresyl Violet Staining

For Cresyl violet staining, sections were mounted on glass slide, dehydrated with ethanol, stained with Cresyl violet dye and mounted with Permount (Fisher Scientific). Refer to (Sirkin, 1983) for complete protocol.

2.9 Volume Measurements and Statistical Analysis

Granular cell layer, dentate gyrus, hippocampus, and whole hemisphere were traced and quantified (Vandenbosch et al., 2016). The sum of all area measurements from all sections from one well was multiplied by 9 to obtain the estimated total volume for every structure. All measurements were performed using Fiji (ImageJ). A two-way ANOVA was used for statistical analysis (genotype × sex) & posthoc were also used (NTG x 3xTg).; The analysis was performed using GraphPad PRISM software (GraphPad Software, Inc).

RESULTS

3.1 Early post-natal volume reduction in hippocampal neurogenic regions of 3xTg mice

The hippocampus, one of the regions affected in AD in humans is also one of the main neurogenic niches in the brain. We first wanted to assess whether there was an observable overall decrease in the volume of brain structures in the 3xTg model and how early these changes could be assessed, potentially before the onset of plaques and tangles. We reasoned that significantly smaller structures in the 3xTg mice, could indicate defects in hippocampal neurogenesis and would be helpful in determining at what age we should investigate for deficits. We wanted to study time points younger than 6 months of age as this is the time where extracellular hippocampal Aβ deposits and tangle formation becomes apparent (Oddo et al., 2003). To assess the impact of AD mutations on the volume of hippocampal structures, the granular cell layer, dentate gyrus, hippocampus, and whole hemisphere were traced and quantified.

First, sections were serially collected from the beginning of the dentate gyrus to the end of the dentate gyrus into 9 wells as free-floating sections. In this regard, each of the 9 wells contained every 9^{th} section from beginning to end. Then, the sum of all area measurements from all sections from one well was multiplied by the thickness of the tissue (30 µm) and by 9 to obtain the estimated total volume for every structure. All measurements were performed using Fiji (ImageJ).

To obtain relative brain measurements, absolute volume of the structure was divided by whole brain volume. This allowed us to determine the ratio of the whole brain size that represents hippocampal structures and compare between genotypes for potential differences.

By using cresyl violet staining on hippocampal sections from NTG and 3xTg, reduced volumes of the GCL, DG, and hippocampus were observed at 3 months of age and as early as post-natal day 7 in the 3xTg model (Figure 2a, 2b). There was no difference in the volume of the whole brain between NTG and 3xTg mice (Figure 2). Post-mortem studies demonstrated that AD patients presented with smaller whole brains when compared healthy individual (Mattson, 2004a). Therefore, the lack of difference in whole brain volume observed in this study could be explained by the fact that this characterization was done at very early stages of life and the reduction in volume is typically observed in late stages of the disease. It is important to note that the decline in volume of hippocampus observed in 3xTg mice is a common observation in human AD patients at early stages of dementia, which could potentially be attributed to changes in human adult neurogenesis (Moreno-Jiménez et al., 2019).

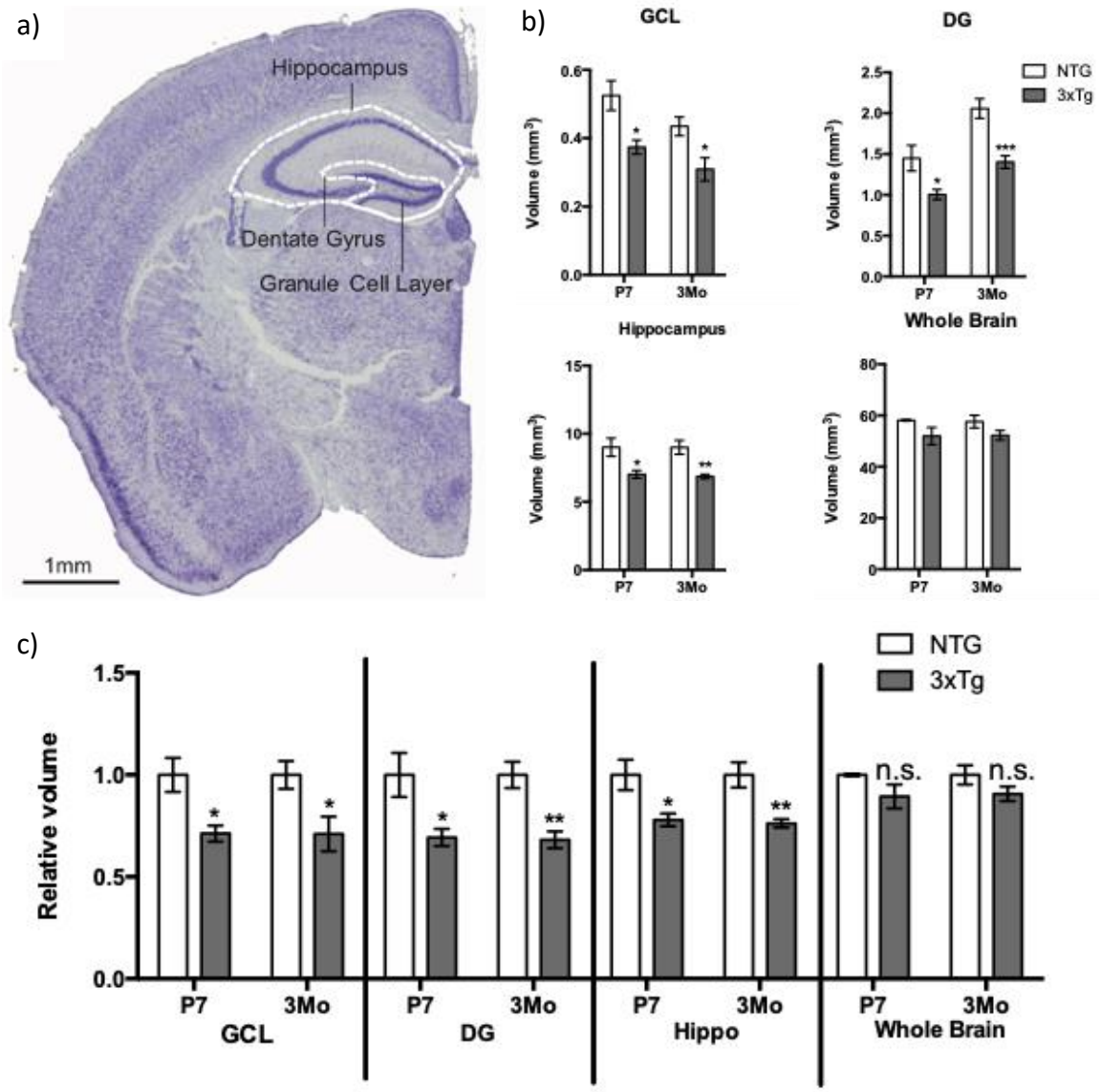

Figure 2. Reduced volume of the granular cell layer (GCL), dentate gyrus (DG) and hippocampus in 3xTg mice at 3 months of age and at postnatal day 7. a) Representative Cresyl violet staining of a coronal section of NTG mice brain. GCL, DG and hippocampus are labelled. Scale Bar 1mm. **b)** Absolute volume measurements of GCL, DG, hippocampus, and whole brain for NTG & 3xTg at post-natal day 7 (P7) and 3 months of age (3Mo). **c)** Relative volume measurements of GCL, DG, hippocampus, and whole brain for NTG & 3xTg at post-natal day 7 (P7) and 3 months of age (3Mo). A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 for each group.

3.2 Early post-natal decrease of adult hippocampal neurogenesis in 3xTg mice

The onset and the symptoms associated with AD reflects a selective vulnerability for cells present in the hippocampal circuitry, which is an important region for learning and memory (Anand & Dhikav, 2012a; Bird & Burgess, 2008; Fu et al., 2018; Treves et al., 2008). Considering the importance of NSCs in the maintenance of these cognitive abilities, a decline in neurogenesis could explain the progressive deficits observed in AD.

Given the evidence that the number of immature neurons found in the brains of human AD patients is greatly reduced compared to age-matched healthy individuals, this supports the relevance of impaired neurogenesis in the mechanism underlying the cognitive deficits associated with AD (Cole et al., 2019; Moreno-Jiménez et al., 2019; Nicaise et al., 2020).

Moreover, the observed reduction in the volume of hippocampal structures in the 3xTg (Figure 2.) supports the potential presence of alterations in hippocampal neurogenesis in this model. Moving forward, considering our results and the published data in human subjects (Moreno-Jiménez et al., 2019), we wanted to determine if neurogenesis was affected in our AD model. The fact that the decrease in volume can be observed as early as post-natal day 7 (Figure 2.) indicated that this problem could be present even before the onset of AD hallmarks. Early time points in brain development were therefore investigated for potential deficient neurogenesis.

Alterations in neurogenesis could be the result of encountered defects in either self-renewal, proliferation, and/or differentiation. To determine where along the process these defects were occurring, we needed to investigate specific cell types at different stages of differentiation. Adult neurogenesis in the SGZ of the DG was measured using

immunofluorescence protocols on serial coronal-cut frozen brain sections. The SGZ was defined as a 2-cell wide layer between the granular zone (marked by DAPI) and the hilus of the dentate gyrus (Kuhn et al., 1996a). Specific antibodies that recognize markers of cells at different stages of differentiation were used.

To determine if the problem is related to self-renewal, we need to quantify the cells with the potential to regenerate NSCs via symmetric division. These are the type I cells and the markers for these cells include Sox2 and Nestin. Sox2 is also expressed in recently activated cells undergoing proliferation (Aimone et al., 2010; G.-L. Ming & Song, 2011). As the cell progresses through the differentiation process, the expression of Sox2 in the progenitor population decreases (G.-L. Ming & Song, 2011). We quantified the number of Sox2+ cells in the SGZ of the hippocampus at post-natal day 5, post-natal day 7, 1, 3, 6 and 9 months of age. (Figure 3a, 3b). Sox2 cells were identified as a bright nuclear fluorescence signal, both in the SGZ of the dentate gyrus and outside the SGZ. The Sox2 cells found outside the SGZ have previously been shown to be radial astrocytes and were not counted (Venere et al., 2012). For the Sox2 analysis, two-tailed unpaired t-tests were conducted. It revealed that there was a statistically significant difference between NTG mice and 3xTg mice, for the number of Sox2+ cells at post-natal day 5, post-natal day 7, 1, 3, 6 and 9 months of age time points (Figure 3c.).

To investigate the possibility that the deficit relates to the cells inability to properly differentiate we quantified: 1) neuroblasts, which are late progenitor cells committed to the neuronal lineage and 2) immature neurons. Both cell types express the cell marker Doublecortin (DCX) and can be identified as a cytoplasmic stain within the SGZ. DCX+ cells were quantified in the SGZ of the hippocampus in the 3xTg and NTG mice at 1, 3, 6

and 9 months of age (Figure 3a, 3b). For the DCX analysis, two-tailed unpaired t-tests were conducted. It revealed a statistically relevant difference between NTG mice and 3xTg mice, for the number of DCX+ cells at the 1, 3, 6 and 9 months of age time point (Figure 3d).

Moreover, we observed with DAPI staining that the 3xTg presented with a smaller GCL than the NTG (Figure 4a). Quantifications of volume with cresyl violet confirmed the observed phenotype (Figure S1). This is in accordance with the data shown previously with the Cresyl violet staining (Figure 2b).

Considering the observed reduction in volume of hippocampal structures at P7, we wanted to further investigate the proliferating NSC populations at this time point by labelling with specific antibodies for RGL and early progenitor cells (Sox2, Nestin, EdU). EdU (5-ethynyl-2'-deoxyuridine) is an alkaline-containing thymidine analog that gets incorporated into cells that are dividing in the S phase and fluoresces in 647nm following a reaction of the terminal alkyne group with fluorescent azides, in a Cu(I)-catalyzed [3+2] cycloaddition (Buck et al., 2008). Interestingly the post-natal day 7 time point has been described as a critical period of brain development, where massive changes and a condensation of proliferating precursor cells starts to occur in the mouse brain (Nicola et al., 2015).

Quantification of labelled cells and analysis with a student t-test revealed a significant decline in Sox2+ (RGL/ Type 2a cells), Sox2+/Nestin+ (RGL), and Nestin+/EdU+ (RGL) cells in the SGZ of 3xTg mice at post-natal day 7. Area measurements revealed a decline of GCL (GZ) volume in 3xTg mice.

The results of these fluorescence microscopy enumerations (Figure 3. & Figure 4.) indicate that 3xTg mice may have a decreased capacity in either (1) the self-renewal potential of NSCs, (2) the proliferation propensity of NSCs and/or (3) the differentiation capacity of those cells.

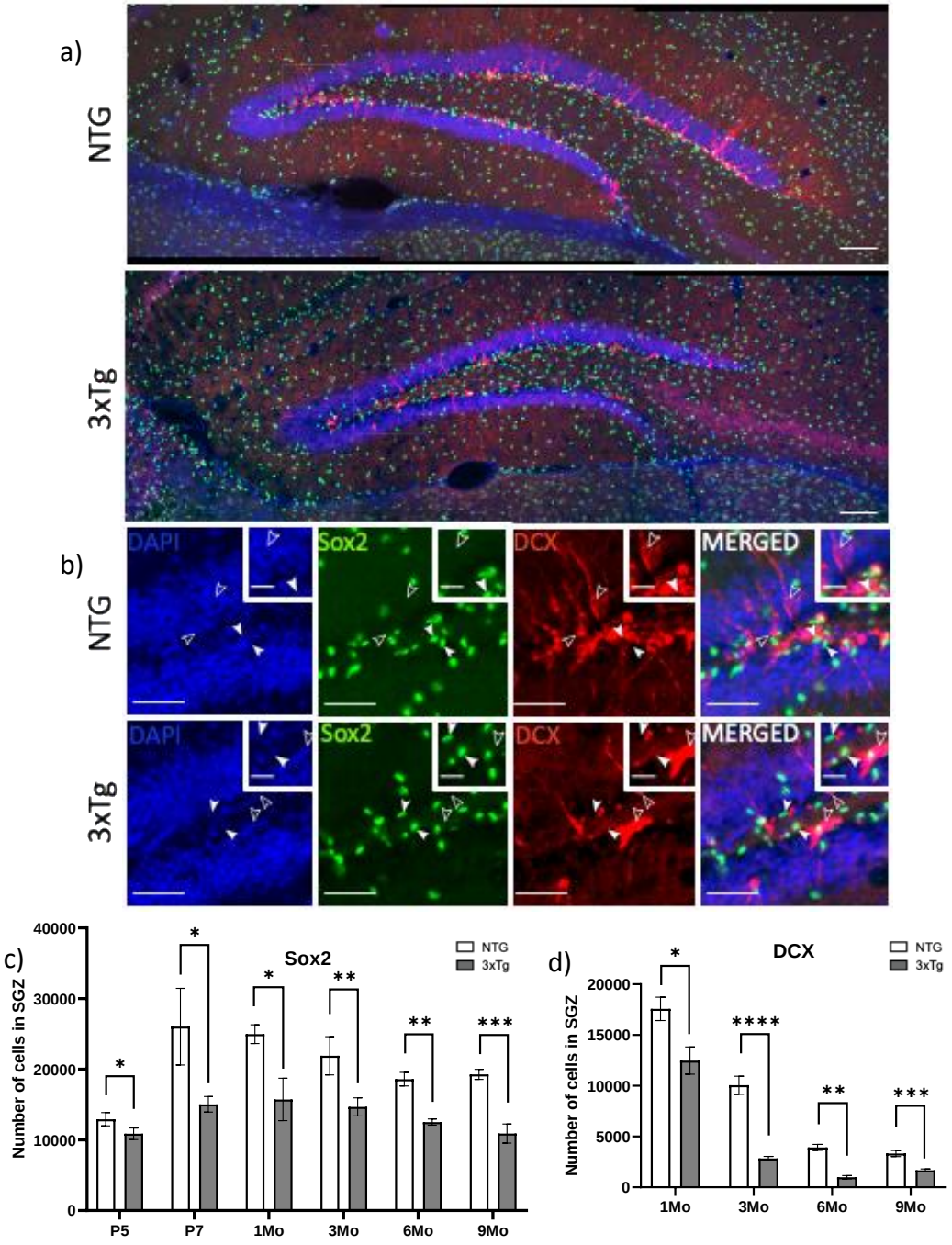

Figure 3. Decline of NSC populations in the SGZ of 3xTg mice at various time point in brain development. a) Representative Fluorescent images of GCL with Sox2 (uncommited cells containing NSCs) and DCX (late committed progenitors) at 3 months of age in coronal sections of NTG (Top) and 3xTg (Bottom). GCL is visualized by DAPI. Scale bar 100µm. **b)** Magnified view of fluorescent images in coronal sections of NTG & 3xTg with DAPI, Sox2, DCX, and Merged staining. Scale bar 25µm. **c)** Quantification of Sox2 cells in the SGZ of GCL for post-natal day 5 (P5); post-natal day 7 (P7); 1 month of age (1Mo); 3 months of age (3Mo); 6 months of age (6Mo); and 9 months of age (9Mo). **d)** Quantification of DCX cells in SGZ of GCL for 1 month of age (1Mo); 3 months of age (3Mo); 6 months of age (6Mo); and 9 months of age (9Mo). A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n*= 3 for P5, P7, 1Mo & 6Mo, *n*= 4 for 9Mo & *n*= 6 for 3Mo.

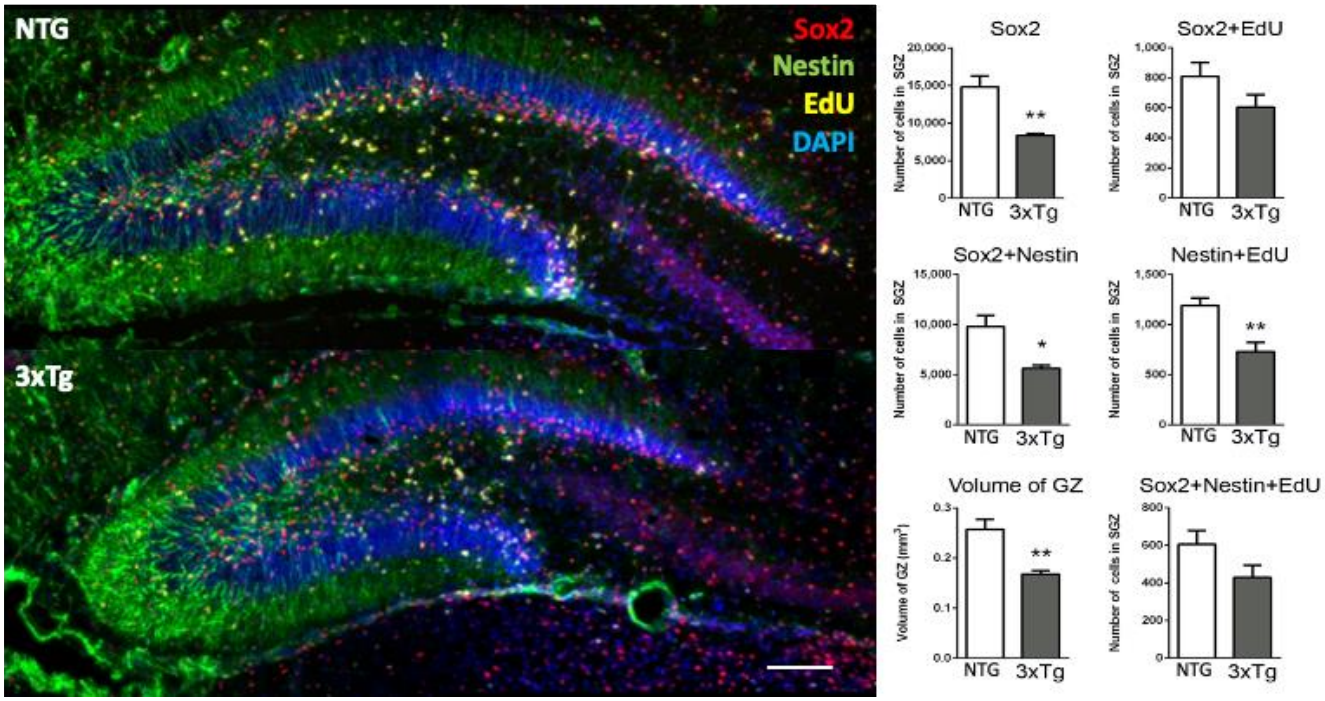

Figure 4. Reduced volume of granular zone and number of proliferating NSCs in SGZ at postnatal day 7. Immunofluorescence microscopy was performed on coronal cryo-sections (30µm) using antibodies for Sox2, Nestin, and fluorescence of EdU incorporation into newly dividing cells was measured 2 hours after injection. Quantification of labelled cells revealed a decline in Sox2+, Sox2+/Nestin+, and Nestin+/EdU+ cells in the SGZ of 3xTg mice. Area measurements revealed a decline of GZ volume in 3xTg mice. Data represented as mean ± SEM. Statistical analysis was performed by Student's t-test, $p < 0.05$*, $p < 0.01$**, Sample size: $n = 4$ female animals for each Wt and 3xTg.

3.3 Decline of immature neuroblast populations in 3xTg hippocampus revealed by lineage tracing

To gain further insight on the defects of neurogenesis and determine if the observed decreased in NSC pool is linked to defects in the self-renewal capacity of the cells or their ability to differentiate, we wanted to label a cohort of stem cells and investigate their differentiation via lineage tracing. By taking advantage of a tamoxifen-inducible Cre-recombinase transgene regulated under the Nestin promoter, this permitted labelling of Nestin+ cells with yellow fluorescent protein (YFP) in a temporally controlled manner. Estrogen-activated Cre-recombinase is localized to Nestin-expressing cells throughout the organism. When Tamoxifen (TAM) is administered it leads to the binding of TAM to estrogen-receptors on the Cre-recombinase protein permitting the Cre to the nucleus where it will cut loxP restriction sites. The cells that expressed Nestin at the time of gavage (2Mo) will permanently express YFP. Therefore, giving the opportunity to conduct lineage tracing experiments at later time points (3 months of age) (Figure. 5).

Lineage tracing experiments in the 3xTg model at 3 months of age was conducted with labelling of Sox2+ cells, that maintain the potential to self-renew, and DCX+ cells, currently undergoing the differentiation process. Overall, the 3xTg model, when compared to non-transgenic mice, showed significantly less Sox2+, EdU+, DCX+ and YFP+ cells (Figure 6e). The number of DCX+YFP+ cells in the YFP population was lower in the 3xTg than in the NTG while no statistically significant difference was found between the number of Sox2+YFP+ cells in the YFP population (Figure 6b, 6d). This indicates that the problem encountered by the NSCs could occur after their activation when they have entered cell cycle.

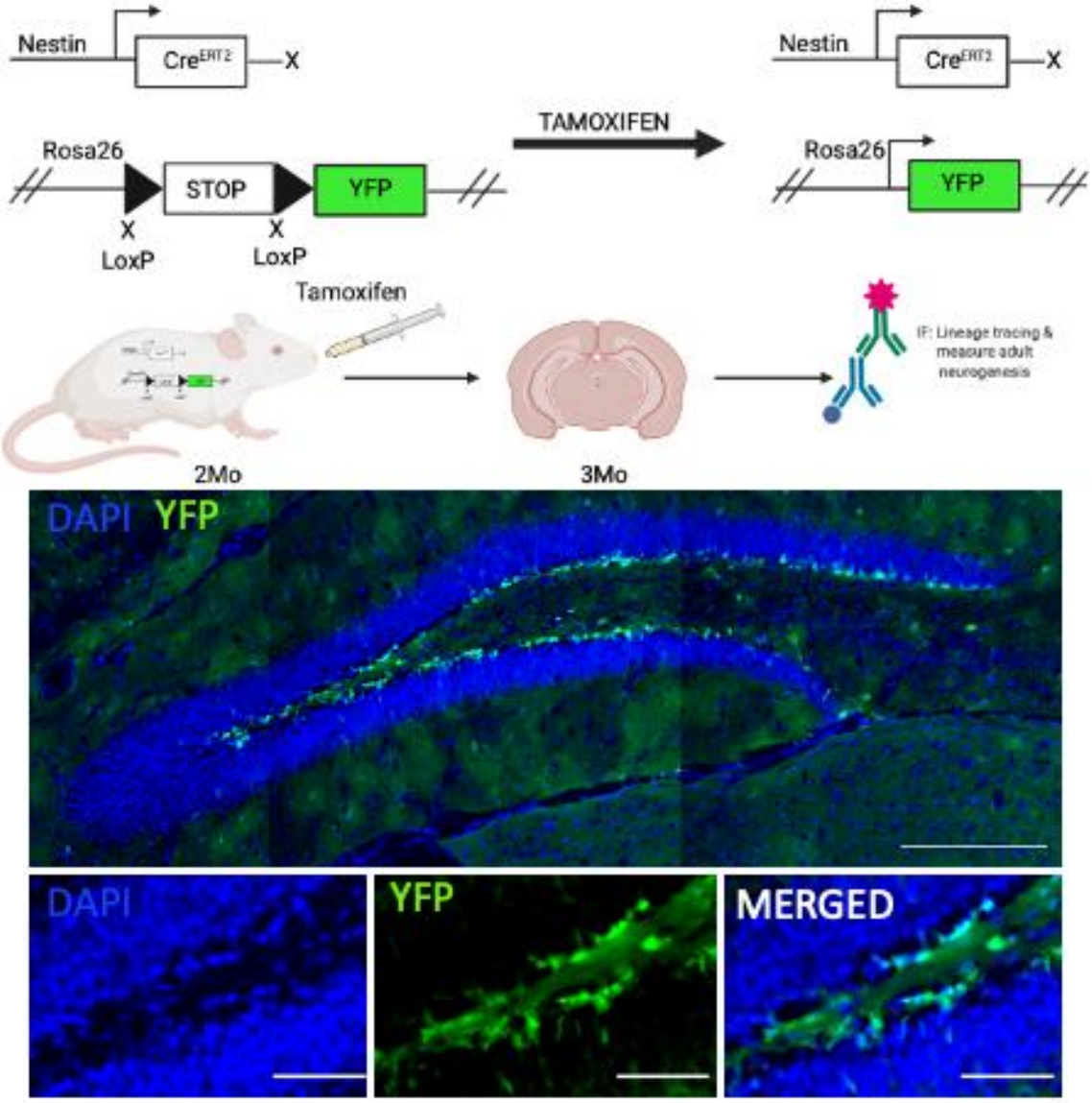

Figure 5. Measuring Adult Neurogenesis in lineage tracing experiments with an inducible Nestin Cre^{ERT2} construct. Image of Nestin Cre^{ERT2} transgenic construct. Mice at 2 months of age were gavaged with TAM for 5 consecutive days and the brains were processed 1 month later (3 months). Binding of TAM allows the Cre-recombinase protein to enter the nucleus and cut loxP restriction sites. This leads to permanent labelling of Nestin+ cells with YFP. Representative fluorescent images and magnified view in coronal sections of NTG Nestin Cre^{ERT2} mice with DAPI and YFP.

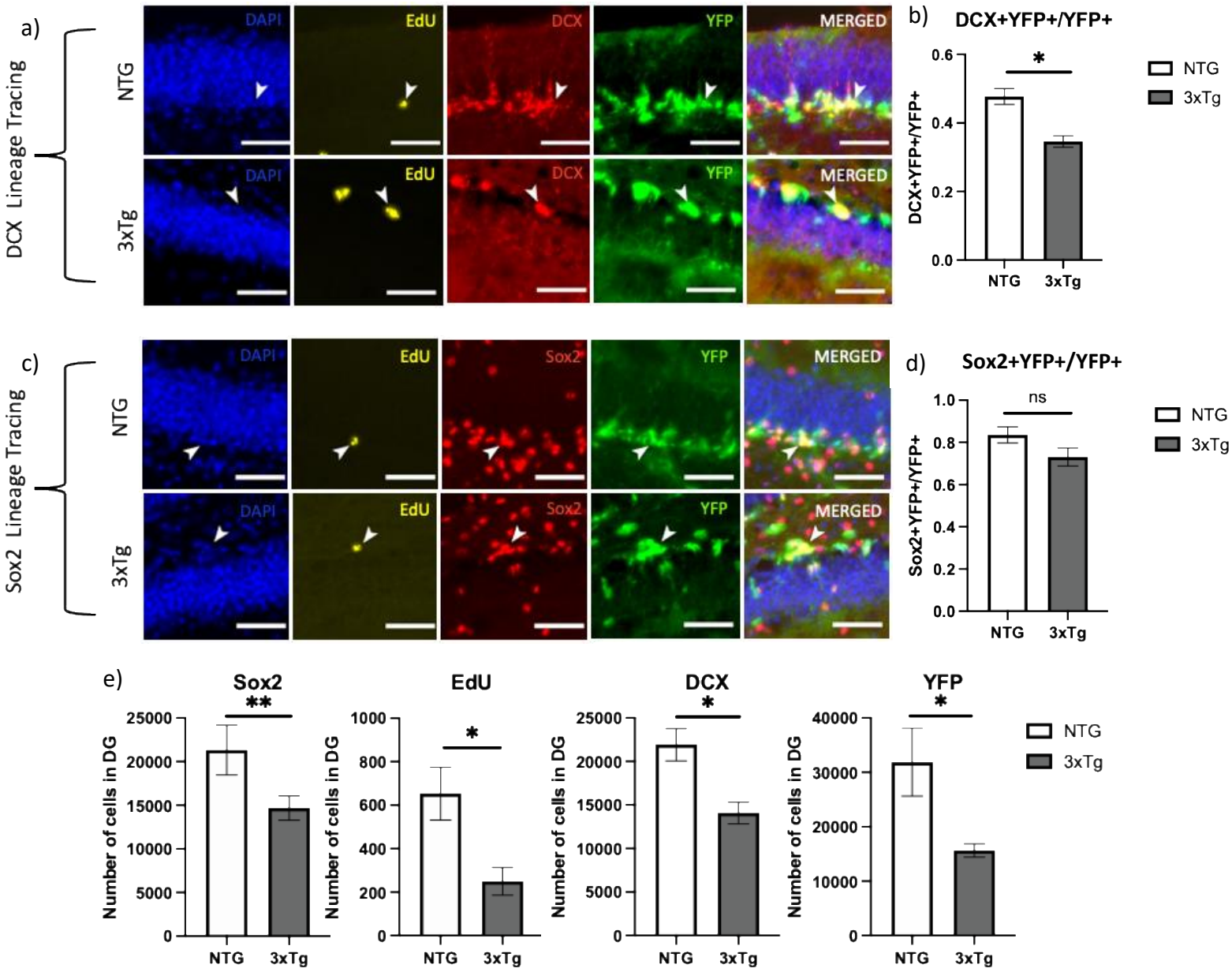

Figure 6. Lineage tracing for Sox2, EdU and DCX in a Nestin Cre^{ERT2} construct reveals no significant decrease of Sox2+YFP+ cells between NTG and 3xTg but demonstrate a significant decrease of DCX+YFP+ cells. a) Magnified view of fluorescent images in coronal sections of NTG & 3xTg with DAPI, EdU, DCX, YFP and Merged staining. NTG (Top) and 3xTg (Bottom). Scale bar 25µm. **b)** Quantification of DCX+YFP+ cells in YFP+ population in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls **c)** Magnified view of fluorescent images in coronal sections of NTG & 3xTg with DAPI, EdU, Sox2, YFP and Merged staining. NTG (Top) and 3xTg (Bottom). Scale bar 25µm. **d)** Quantification of Sox2+YFP+ cells in YFP+ population in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls. **e)** Quantification of Sox2+, EdU+, DCX+ & YFP+ cells in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 each group. Credit: Amaal Abdi & Jacob Cuthbert

To validate the results regarding the Sox2+ cells from the lineage tracing experiment, we wanted to stain for a second marker labelling Type 1 quiescent NSCs (RGL). We stained for Homeodomain-only protein homeobox (HopX) which has been identified as a marker for adult stem cells in several tissues throughout the body: including the intestine and hair follicles (Takeda et al., 2011, 2013). It was also reported that HopX is expressed in NSCs in the DG and is found to be involved in modulating proliferation/neurogenesis (De Toni et al., 2008; Li et al., 2015). Ki67 assessment and BrdU short term labelling demonstrate that Hopx+ cells represents a slowly cycling or quiescent NSC population in the SGZ are not proliferating (Braun et al., 2003; Li et al., 2015). Loss of HopX, leads to an enhancement in neurogenesis that is validated by an increase in the number of BrdU+ cells and DCX+ cells, but also by a reduction in Sox2+ quiescent NSCs (Li et al., 2015). Interestingly, with this marker, it is possible to discriminate DG NSCs from the NSC population found in the second neurogenic niche of the brain; the lateral ventricle (LV) (Li et al., 2015).

Overall, the 3xTg model, when compared to NTG mice, showed significantly less Sox2+, HopX+ and YFP+ cells (Figure 7). However, no significant difference was found between percentage of HopX+YFP+ cells in the YFP population when looking at 3xTg compared to NTG (Figure 7). These results are in accordance with what has been previously investigated with Sox2+ populations (Figure 6) and leads us to believe that the defect in neurogenesis occurs after the activation of NSCs. This points to defects in either proliferation, differentiation or even both. It should also be relevant to indicate that Sox2+ cells that where HopX- were observed. This observation supports the possibility of NSC

heterogeneity where HopX+ cells would represent a subpopulation of NSCs found in the SGZ (Fiorelli et al., 2015).

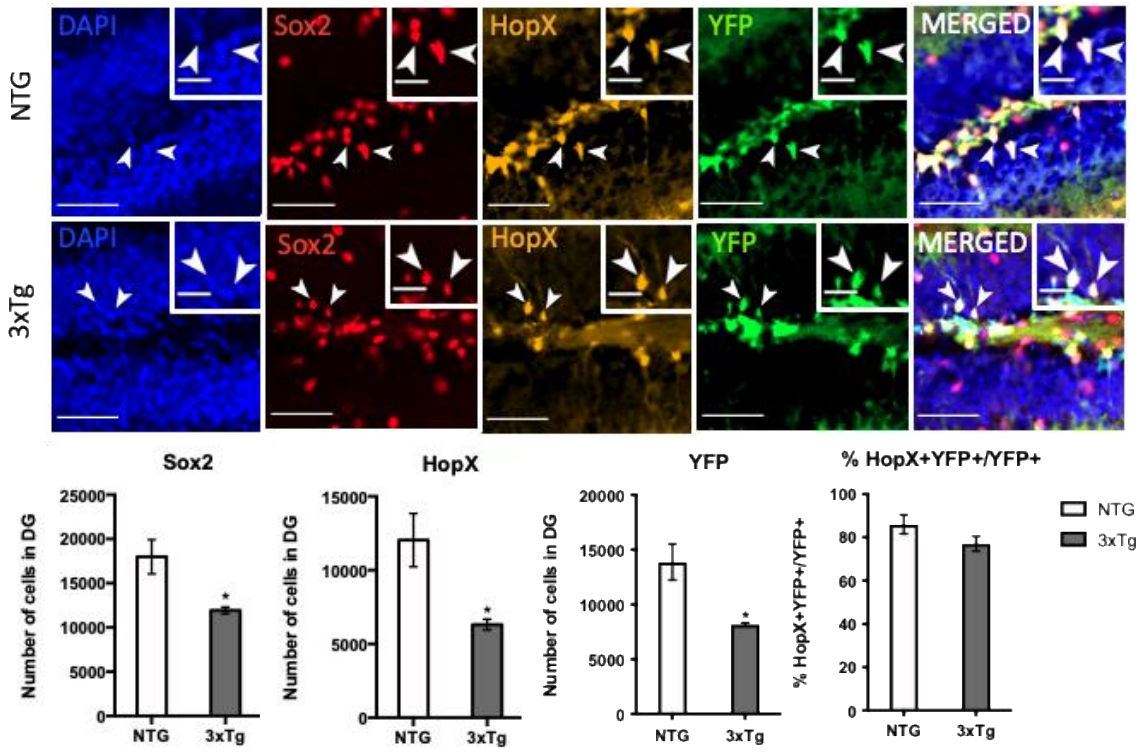

Figure 7. Lineage Tracing Experiment for HopX in an Inducible Nestin Cre^{ERT2} Construct reveals no significant decrease of HopX+YFP+ cells between NTG and 3xTg. a) Magnified view of fluorescent images in coronal sections of NTG & 3xTg with DAPI, Sox2, HopX, YFP and Merged staining. NTG (Top) and 3xTg (Bottom). Scale bar 25µm. **b)** Quantification of Sox2 cells in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls **c)** Quantification of HopX cells in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls. **d)** Quantification of YFP cells in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls. **e)** % of HopX+YFP+ cells in YFP+ population in the SGZ of GCL at 3 months of age for 3xTg with aged-match controls. A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 4 each group.

3.4 Investigating Adult Neurogenesis with TgCRND8 mice

Considering the significant variability that can be observed when comparing studies of adult neurogenesis in various animal models of AD (Götz, 2018b; Lazarov & Marr, 2010; Marlatt & Lucassen, 2010; Mu et al., 2010), we thought it would be important to validate our findings in a different model of AD. In some AD mouse models, adult neurogenesis was decreased and others demonstrated an increase (Lazarov & Marr, 2010; Marlatt & Lucassen, 2010; Mu et al., 2010). The factors that may cause these discrepancies in the literature include: promoters that harbor the mutations, the age of the animals, the age of the onset of the disease, the susceptibility of the animal to the protein, overexpressed transgenes and the level of transgene expression (Lazarov & Marr, 2010; Marlatt & Lucassen, 2010; Mu et al., 2010). We studied the TgCRND8 mice which contains human mutations associated with early-onset familial AD in the *APP* gene (KM670/671NL) and (V717F) under control of the hamster prion (PrP) promoter (Chishti et al., 2001). It is an accelerated model of AD that is generated to induce early development of AD pathology with plaque formation at 5 months of age (Chishti et al., 2001). The TgCRND8 model was studied at 3 months of age, just like the 3xTg model for both cresyl violet staining and immunofluorescence protocols for Sox2 and DCX. Moreover, defects in neurogenesis, as exampled by less Sox2+ and DCX+ cells in the TgCRND8 model, were also observed (Figure 8). The defects in the TgCRND8 are in accordance with the 3xTg data. All of the evidence suggests that defects in neurogenesis occurs before the onset of AD hallmarks, as measured by previous studies.

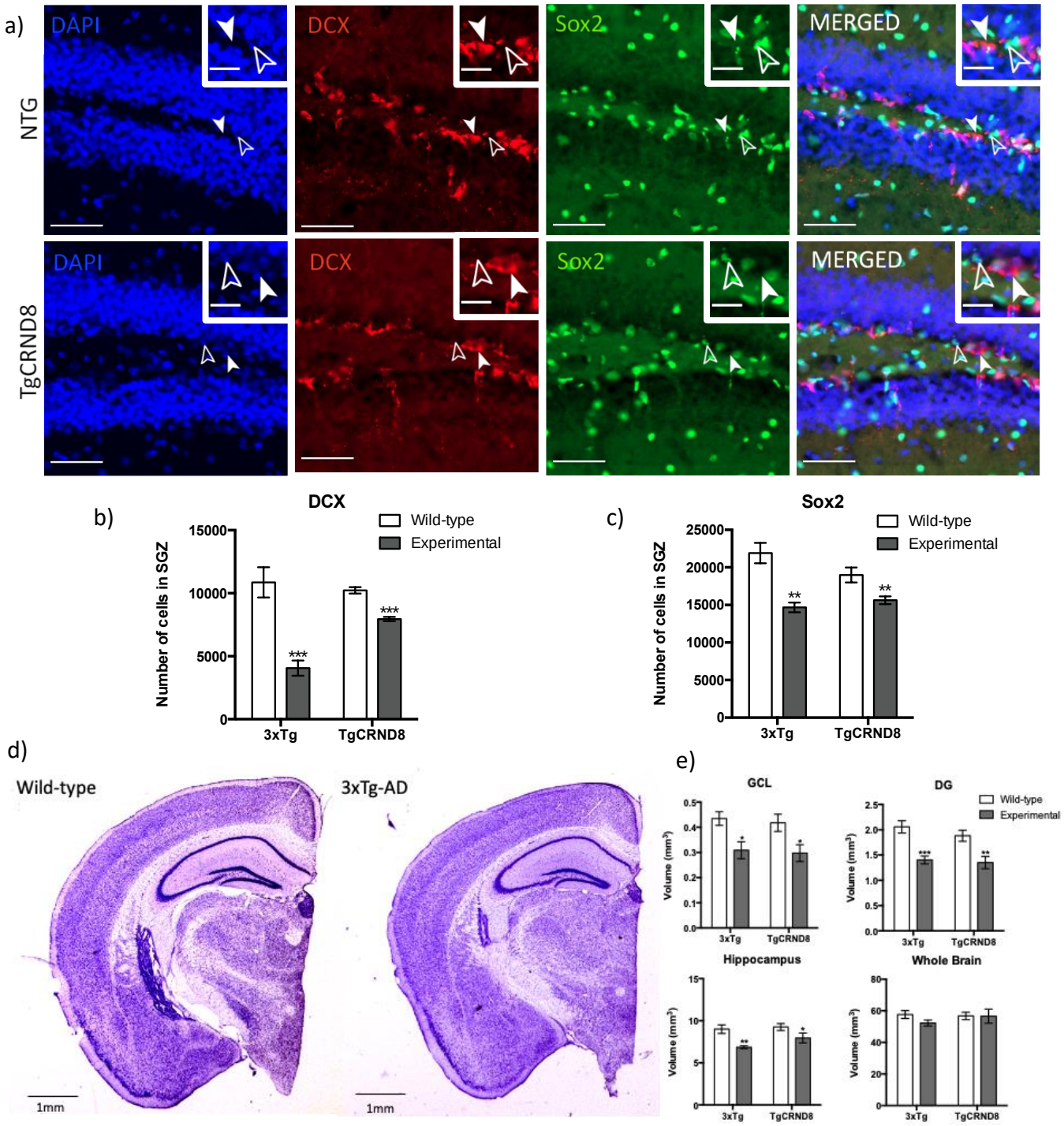

Figure 8. Decline of NSC Populations in the SGZ and Reduced Volume of Hippocampal Structures in the TgCRND8 mouse model of AD at 3 months of age. a) Magnified view of fluorescent images in coronal sections of NTG & TgCRND8 with DAPI, Sox2, DCX, and Merged staining. NTG (Top) and TgCRND8 (Bottom). Scale bar 25µm. Microscope: Axioscanner **b)** Quantification of DCX cells in the SGZ of GCL at 3 months of age for 3xTg and TgCRND8 with aged-match controls **c)** Quantification of Sox2 cells in the SGZ of GCL at 3 months of age for 3xTg and TgCRND8 with aged-match controls. A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 each group. **d)** Representative Cresyl violet staining of a coronal section of NTG & 3xTg mice brain. Scale Bar 1mm. **e)** Volume measurements of GCL, DG, hippocampus, and whole brain for 3xTg and TgCRND8 with aged-match controls at 3 months of age (3Mo). A two-tailed, unpaired t-test was used for statistical analysis (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 each group.

3.5 No sex differences in adult hippocampal neurogenesis of 3xTg mice

Interestingly, AD is more prevalent in women than men, where two thirds of the diagnosed AD population is female (Babapour Mofrad & van der Flier, 2019; Djordjevic et al., 2020; Dubal, 2020; Mayeda, 2019). Sex differences in the pathology of AD reveal that men and women could benefit from personalized medicine strategies or therapies. Therefore, further studies aiming towards understanding the mechanisms behind sex differences are of great importance to potentially ameliorate the prognostic outcomes of this disease.

To investigate the possible sex differences of NSC population decline in the 3xTg mouse model of AD the number of Sox2 cells and DCX cells were quantified in the SGZ of the hippocampus at 3 months of age and 9 months of age. 3xTg mice of both sexes were studied with age- and sex- match controls. The number of Sox2 and DCX cells present in the SGZ were quantified. When conducting Two-Way ANOVA (sex x genotype) the analysis revealed that there was a statistically relevant difference between NTG mice and 3xTg mice, for the number of Sox2+ cells & DCX+ cells (Figure 9a, 9b). No statistically significant difference was detected between male and female mice at 3 months and 9 months of age (Figure 9a, 9b).

The possibility of sex differences in the 3xTg model was also conducted by looking at volume measurements of hippocampal structures. Cresyl violet staining was performed on serial coronal sections to visualize the different brain structures. This analysis revealed a statistically significant decrease in volume of the SGZ, DG, and hippocampus in 3xTg mice (Figure 9c). There was no difference in the volume of the whole brain between NTG and 3xTg mice (Figure 9c). In addition, no statistically significant difference for volume of

neurogenic regions was detected between male and female mice at 3 months and 9 months of age (Figure 9c).

This could indicate that sex may not need to be taken in consideration in research design

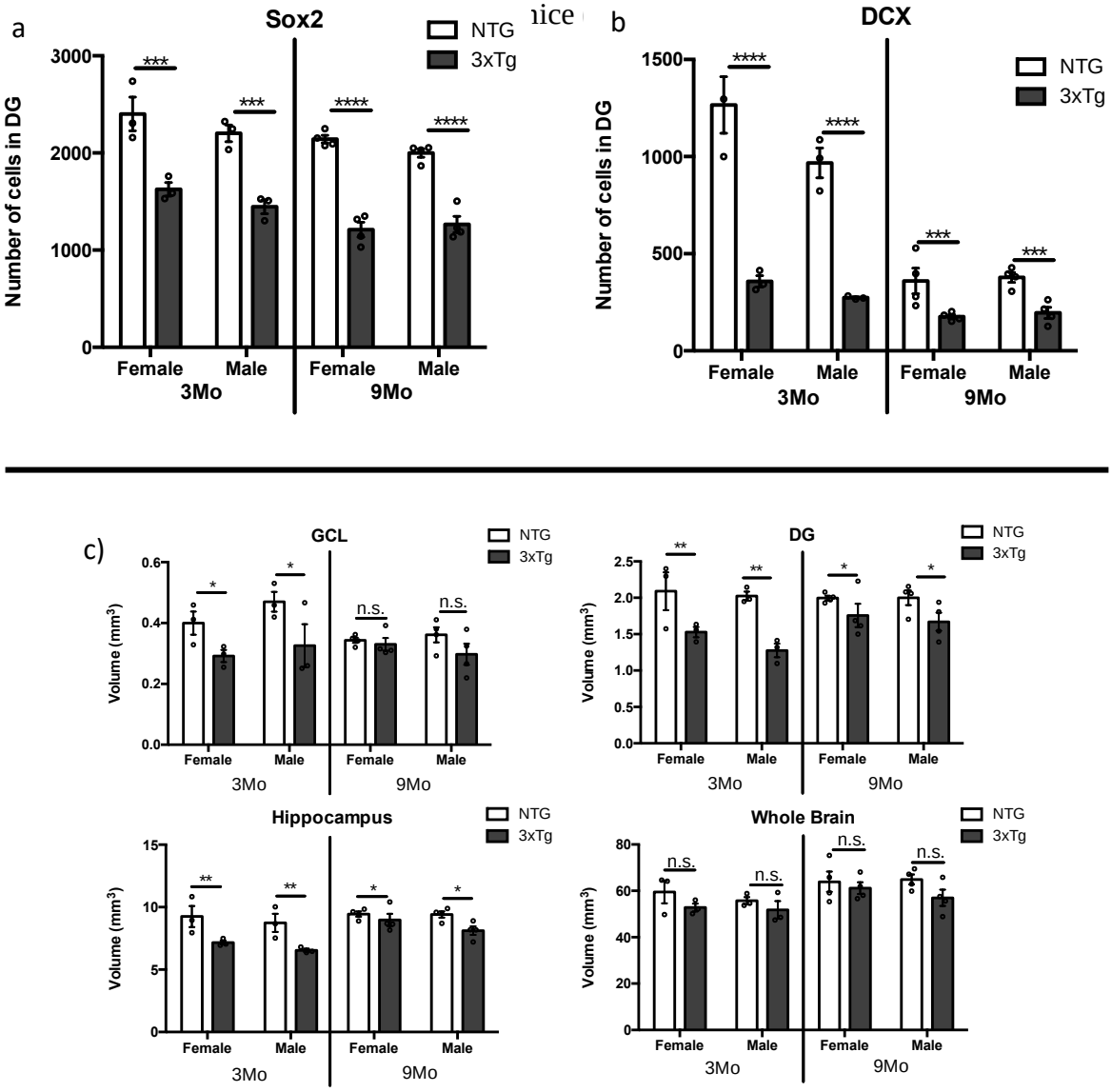

Figure 9. No sex difference in volume and NSC depletion in 3xTg mice at 3 & 9 months of age a) Quantification of Sox2 cells in the SGZ of GCL of 3xTg male and female with aged- and sex-matched controls at 3 months of age (3Mo) and 9 months of age (9Mo) . **b)** Quantification of DCX cells in SGZ of GCL of 3xTg male and female with aged- and sex-matched controls at 3 months of age (3Mo) and 9 months of age (9Mo). A two-way ANOVA was used for statistical analysis (genotype × sex) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 3Mo group; *n* = 4 9Mo group. **c)**Volume measurements of GCL, DG, hippocampus, and whole brain. A two-way ANOVA was used for statistical analysis (genotype × sex) (*p<0.05, **p<0.01 and ***p<0.001), *n* = 3 3Mo group; *n* = 4 9Mo group.

DISCUSSION

4.1 Summary of results

A growing body of literature has recently indicated that defective neurogenesis, due to a decline in hippocampal NSCs, is a common feature in AD and could contribute to the cognitive impairments related to the disease (Moreno-Jiménez et al., 2019; Tobin et al., 2019). However, it remains uncertain if the decline in neurogenesis precedes or follows the accumulation of the main neuropathology hallmarks of AD: Aβ plaques and NFTs of misfolded hyperphosphorylated Tau (Moreno-Jiménez et al., 2019; Tobin et al., 2019; Unger et al., 2016). Understanding the sequence of events leading to AD dementia and determining if alterations in adult neurogenesis is an early event in AD neurodegeneration would be of great relevance for diagnostic and therapeutic purposes. It would permit intervention in presymptomatic at-risk individuals before the onset of dementia symptoms allowing for a better prognosis for individuals prone to developing AD and it could represent a promising treatment in regenerative medicine for AD (Livingston et al., 2020; Moreno-Jiménez et al., 2019; Navarro Negredo et al., 2020; Unger et al., 2016).

This thesis aimed to determine if defects in neurogenesis were observable before the onset of AD hallmarks, including Aβ plaques and Tau tangles. The results conducted in this thesis support the following statements:

> The volume of the GCL, DG and Hippocampus of 3xTg mice is significantly reduced compared to littermate control (NTG) at post-natal day 7 and 3 months of age.

3xTg model presented with depleted pools of Sox2+ cells and DCX+ cells as early as post-natal day 5 and 1 month of age respectively.

Lineage tracing experiments showed a significant decrease in pools of Sox2+, DCX+, EdU+ and YFP+ cells in the 3xTg model compared to littermate controls.

In the 3xTg model, DCX+YFP+ cell population was decreased when compared to littermate controls. However, no significant depletion of Sox2+YFP+ cells was observed in the 3xTg mice compared to littermate controls.

Early defect phenotype: decrease in Sox2+ and DCX+ populations and smaller GCL, DG and hippocampus was observed in a second model of AD (TgCRND8) at 3 months of age when compared to non-transgenic controls.

No sex differences were detected in the Sox2+ and DCX+ populations in 3xTg model of AD at 3 and 9 months of age.

Collectively, these findings support the hypothesis that defects in neurogenesis in the 3xTg model of AD occur early on in brain development and precedes the onset of AD pathology. Based on the literature, in the 3xTg model, the onset of AD pathology occurs at the age of 6 months with extracellular hippocampal Aβ deposits and tangle formation and they become more extensive with age (Belfiore et al., 2019). Therefore, to test this hypothesis, we investigated time points earlier than 6 months of age. The following sections of this thesis discuss the major findings from this study and their implications.

4.2 Volume reduction in hippocampal structures in early post-natal 3xTg mice

Impaired neurogenesis has been reported in the 3xTg model as early as 4 months of age when looking at the proliferation of NSCs in both the SVZ of the lateral ventricles and the SGZ of the hippocampus (Hamilton et al., 2010; Rodríguez et al., 2008). We wanted to assess if defective hippocampal neurogenesis could be observed earlier. We therefore investigated two time point by measuring the volume of hippocampal structures, reasoning that significantly smaller structures would be helpful in determining if these time point would be relevant to study for defects. When compared to control cohorts, the 3xTg model presented with a smaller volume of GCL, DG and hippocampus as determined by Cresyl Violet staining (Figure 2). This observation was made at two different time points: post-natal day 7 and 3 months of age. Structurally the SGZ niche starts forming between P7 and P14 (Nicola et al., 2015). Defective neurogenesis at P7, a critical time point of brain development, could explain the smaller morphological patterns in 3xTg mice when compared to NTG mice which then remains throughout adulthood and can then be observed at later time points such as 3 months of age. This indicate that future diagnostic tests may have to be conducted earlier than expected to identify at-risk individuals. Preferably this would be done before the sharp transition from constituting neurogenesis (embryonic) to activity-dependent neurogenesis (adult) to alleviate the risks of permanent changes in NSC pools.

Interestingly, whole brain volume did not differ between the two genotypes. In post-mortem studies, AD patients present smaller whole brains than healthy individuals (Mattson, 2004b). The lack of difference in whole brain volume observed in this study could be explained by the fact that the characterization was done at a very early stage and

neurodegeneration is typically observed in late stages of the disease. The death of neurons observed in affected regions of AD occurs over a prolonged period of time of many years (Mattson, 2004b).

4.3 Decrease of adult hippocampal neurogenesis in early post-natal 3xTg mice

Our finding that the 3xTg model presented with smaller hippocampal structures as early as post-natal day 7 and the previous literature indicating that human AD patients presented with a significant reduction in the number of immature neurons when compared to age-matched healthy controls (Cole et al., 2019; Moreno-Jiménez et al., 2019; Nicaise et al., 2020) led us to investigate how neurogenesis is affected in our model of AD and how early the potential defects could be observed. The fact that the decrease in volume can be observed at post-natal day 7 and 3 months of age (Figure 2.) indicated that the potential alterations in neurogenesis could be present even before the onset of AD hallmarks, which are apparent by 6 months, according to previous literature. We conducted immunohistochemistry experiments on serial coronal-cut frozen brain sections to characterize Type I (RGL) and Type III (neuroblast) populations.

To study the type I cell population (RGL), that still have a propensity to self-renew, the marker used to label these cells was Sox2 (Aimone et al., 2010; G.-L. Ming & Song, 2011). The Sox2 analysis revealed statistical significance between genotypes at post-natal day 5, post-natal day 7, 1, 3, 6 and 9 months of age.

To investigate Type III (neuroblast) cells, that committed to neural lineage and a currently undergoing the differentiation process, we quantified DCX+ cells. The multiple t-test in the DCX analysis revealed significance at 1, 3, 6 and 9 months of age. Overall interpretation suggests that the 3xTg mice are losing DCX+ cells earlier and faster than NTG.

Previous literature already demonstrated that alterations in neurogenesis was a common feature of the disease and could be the underlying cause behind the observable cognitive defects (Moreno-Jiménez et al., 2019; Tobin et al., 2019). However, this thesis reports that defects related to adult neurogenesis are present very early on in the 3xTg model of AD and that it is even observable prior to the onset of AD pathology. This novel finding is very interesting because it leads to the possibility of eventually looking at hippocampal neurogenesis as a potential diagnostic marker for AD. By identifying presymptomatic populations of AD patients, this would allow therapeutic intervention to be undertaken before the onset of clinical symptoms and therefore leading to a better prognosis in delaying or even preventing neurodegeneration (Dubois et al., 2014; Fan & Wang, 2020; Villemagne et al., 2013).

Further characterizations of the alterations in neurogenesis were conducted at the post-natal day 7 time point. The labelling with specific antibodies for RGL and Type 2a cells (Sox2, Nestin, EdU) was conducted. Compared to control cohorts, 3xTg mice demonstrated a significant reduction in Sox2+ (RGL/Type 2a cells), Sox2+/Nestin+ (RGL), and Nestin+/EdU+ (RGL) cells in the SGZ of 3xTg mice (Figure 4). Area measurements with DAPI staining at P7 revealed a decline of GCL (GZ) volume in 3xTg mice (Figure 4). This concords with the data obtained with the cresyl violet staining (Figure 2).

The results (Figure 3 & Figure 4) indicate that 3xTg mice presents with alterations in neurogenesis. However, it remains uncertain whether it is because NSPCs present decreased capacity in either (1) the self-renewal potential, (2) the proliferation capacity of those cells or (3) their propensity to differentiate into newborn neurons that properly integrate the neuronal network. It is possible that the decline of Sox2+ cells, due to a reduction in self-renewal or increased cell death, reduces the pool of downstream DCX progenitors independent of changes to differentiation capacity. It is also possible that the pool of Sox2+ is inherently smaller in the 3xTg compared to the NTG, because of the smaller structure. Defects in differentiation and/or proliferation capacities of the cells could also be involved in the decreased pool of DCX+ cells.

To gain a better understanding at where along the differentiation process the defects were potentially occurring, we conducted lineage tracing experiments by using a Nestin-Cre^{ERT2} model (Figure 5). 3xTg when compared to control littermates (NTG) had fewer Sox2+ cells, EdU+ cells, DCX+ cells and YFP+ cells (Figure 6). By calculating the total number of Sox2+YFP+ cells over the total number of YFP+ cells and the total number of DCX+YFP+ cells over the total number of YFP+ cells; we observed that despite the smaller NSC pool, the proportion of Sox2+ cells for both genotype remains similar while the proportion of DCX+ cells in the 3xTg decrease when compared to the NTG cohort (Figure 6).

This suggest that the reduction in neurogenesis observed in the 3xTg model may be occurring after the cells are activated, when they are undergoing cell division. However, it is not possible to determine if the problem is related to the proliferation capacities of the

NSC pool, their ability to differentiate or even the survival and integration of the newborn neurons in the local circuitry.

We searched to further investigate the differentiation process by staining with HopX which is expressed in Type 1 NSCs (RGL) in the DG and is involved with the modulation of proliferation/neurogenesis (De Toni et al., 2008; Li et al., 2015). This marker labels primarily NSCs present in the DG. The results revealed a similar conclusion to the Sox2+YFP+/YFP+ labelling where the total number of HopX+YFP+ cells in the YFP+ population was similar in NTG and 3xTg. This leads us to believe that the defect in neurogenesis may occur after the activation of NSCs. Then again, with these results it is not possible to determine if this is caused by defects in proliferation, differentiation or survival and we are not able to fully reject the hypothesis that self-renewal capacities in the 3xTg model is not problematic. It however provides crucial information to guide future experiments.

4.4 Validating the Early Defect Phenotype in Another Model of AD

We studied the TgCRND8 mice which contains human mutations associated with early-onset familial AD in the APP gene (KM670/671NL) and (V717F) under control of the hamster prion (PrP) promoter (Chishti et al., 2001). It is an accelerated model of AD that is generated to induce early development of AD pathology. As early as 3 months of age, in this model, it is possible to detect via thioflavin-S staining the apparition of Aβ plaque (Chishti et al., 2001). Moreover, at 3 months of age, the TgCRND8 mice also demonstrate early impairment in the acquisition and learning reversal when they are undergoing the reference memory version of the Morris water maze (Chishti et al., 2001). When looking

at adult neurogenesis, the TgCRND8 model is controversial: One study found decreased hippocampal neurogenesis at 3 months of age, yet another found an elevated number of proliferating NSCs in the DG at an earlier age prior to the emergence of Aβ plaques (Fiorentini et al., 2010; Kanemoto et al., 2014). Our experiments in the TgCRND8 model were conducted at 3 months of age. Volume measurements of brain structures in the TgCRND8 model were quantified using cresyl violet staining. A reduction in the size of the GCL, DG and hippocampus of the TgCRND8 was observed when compared to control littermates. The reduction in hippocampal structures in the TgCRND8, is consistent with the observed reduction in the 3xTg. We also observed defects in neurogenesis in the TgCRND8 model of AD at 3 months of age. Sox2+ and DCX+ populations were decreased similarly to the 3xTg mice (figure 7). According to previous literature, the TgCRND8 model demonstrate pathology at 3 months, which is the time point we characterized. Considering this, it was not possible to determine if our hypothesis that defective neurogenesis presents earlier than the onset of AD hallmarks is valid in this model. However, our experiments confirm previous studies regarding the early presence of alteration in adult neurogenesis in this model. This warrants further investigation at earlier ages.

4.5 Investigating sex differences in neurogenesis in the 3xTg model of AD

This study was investigating the possible sex differences of NSC population decline in the 3xTg mouse model of AD. Considering that the pathology of AD in humans is greater in females than in males, we hypothesized that females of the 3xTg mouse model of AD would demonstrate a greater depletion in NSCs. This study revealed a strong genotype

effect where the 3xTg mice model presents fewer NSCs, yet there was no difference between females and males.

At 3 months of age and 9 months of age, the 3xTg mice had fewer Sox2 and DCX cells in the SGZ compared to the NTG mice (Figure 8). The 3xTg model also presented with a smaller volume of GCL, DG and hippocampus than the NTG mice, at both 3 and 9 months of age, as determined by Cresyl Violet staining (Figure 8).

Interestingly no sex differences were noted in terms of NSC population decline and volume of hippocampal structures in this mice model at this age. Given these observations, the hypothesis that female 3xTg mice present a greater loss of NSC than male mice were therefore rejected.

Initially we believed that the reason behind the observed absence of sex differences in NSC populations at 3 months of age was likely because the time point we were investigating in the mice correlates to early adulthood in the human (Dutta & Sengupta, 2016). At this age in humans, no signs of neurodegeneration or dementia could have manifested in familial AD. Many studies showed no sex differences in incidence of AD until a very late age where females had the highest incidence (Mielke et al., 2014b). This has mostly been attributed to the fact that later in life, women experience a drop in estrogen levels, a hormone known to have neuroprotective activity (Mielke et al., 2014b; Mofrad & Flier, 2019). Considering the sex differences observed in the pathology and progression of AD in humans, we investigated sex differences in a later time point: 9 months of age, expecting to see a greater loss of NSC in the 3xTg females compared to 3xTg males. However, no sex difference was quantified. A better understanding of the sex differences in AD will lead to improved care and treatment adapted to both genders.

We also observed a reduced volume of GCL, DG and hippocampus in 3xTg mice at an age that is largely before the onset of amyloid plaques. It is important to determine if sex differences need to be considered when we think about the possibilities that adult neurogenesis offers in regenerative medicine and personalized treatments. Considering that AD is more prevalent in females than in males, sex differences should continue to be considered in research design and reporting when studying this disease. The reasons that could possibly explain that we were not able to detect sex difference in NSC decline in the 3xTg model include: mutations in the model and the promoters that harbor the mutations, the age of the animals and also the age of the onset of the disease. Future experiments looking at sex difference in neurogenesis could focus on later time points in the 3xTg model, the assessment of neurogenesis could be done using different methods or characterize different cell populations using other markers than Sox2 and DCX when using the immunofluorescence protocols. It is also possible that sex differences could be observed in other models than the 3xTg. No model so far has fully succeeded in mimicking the neurodegeneration of AD and this thesis indicates that the 3xTg model may not fully accomplish this due to the absence of observed sex difference in neurogenesis.

4.6 Future Directions

To improve this study, quantification of cycling NPCs using a marker for proliferation (Ki67, Tbr2) in lineage tracing experiments should be considered. That way it would be possible to know if the pool of cells able to proliferate is affected in the 3xTg mice model. Using a marker for mature neurons (NeuN) would also indicate if the cells that underwent differentiation are able to integrate in the neural circuit or that despite this process they fail

to survive. This study did not investigate these two crucial populations but should be considered for future studies.

Moreover, considering the following results: decrease in Sox2+ cells (Figure 3, 5 and 6) and the fact that there was no significant difference in Sox2+YFP+ cells (Figure 5), future experiments to determine if the mechanism behind this early phenotype is not caused by changes in NSC properties for self -renewal should be conducted.

It would be interesting to see if the differences in NSC populations can be correlated to memory deficits in 3xTg mice through the use of the Morris Water Maze or other behavioral tests. This may be especially important at 3 months of age, considering that we measured a significant reduction in the size of the hippocampus of 3xTg mice. Characterization of dendritogenesis and arborization in the 3xTg should also be conducted. This would give insight on the newborn neuron's capacity to integrate in the local circuitry and to survive (Ghosal et al., 2010).

One of the most important elements to take in consideration is the mechanism behind this loss in NSPCs. Understanding this could be achieved by conducting single-cell RNA sequencing and RNA velocity experiments to characterize and reveal key molecular targets, which are dysregulated in AD mice. Understanding which gene is differentially expressed in the AD model compared to littermate controls could lead to the discovery of therapeutic targets where manipulation via the use of agonists/antagonists would lead to a successful rescue of neurogenesis. These experiments would also be useful in understanding where along the differentiation process are the defects occurring.

CONCLUSION

Our findings demonstrate that **defects associated with adult neurogenesis precedes the onset of AD hallmarks and that no sex difference could be detected in adult neurogenesis in the 3xTg model of AD.** These information leads to a better understanding of the reason and timeline behind the exhaustion of the NSC pool in AD. These findings show that alterations in adult neurogenesis could be an interesting non-invasive biomarker to identify pre-symptomatic populations of AD patients therefore optimizing therapeutic intervention before the onset of dementia. By having a better understanding of the defects in neurogenesis occurring in AD, this could potentially lead to a possible rescue of this depletion, and could also open the gate to regenerative medicine being a possible treatment for AD.

APPENDICES

Appendix I : Supplemental Data

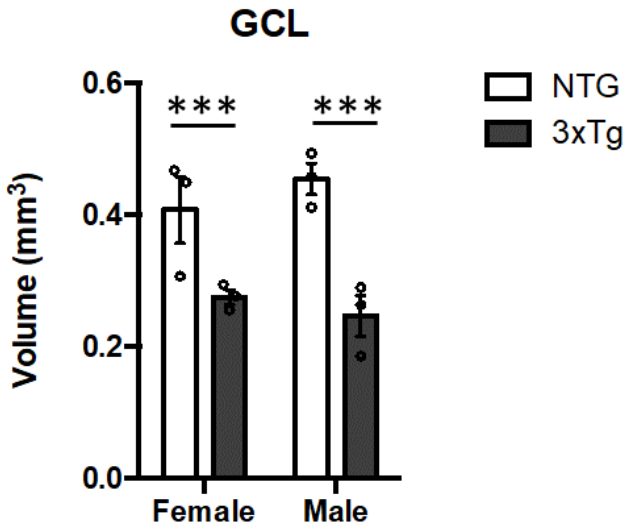

Figure S1. Reduced Volume of GCL, assessed by DAPI staining, in 3xTg mice compared to NTG, independent of sex. Animals were euthanized at 3 months of age. Volume measurements of GCL. A two-way ANOVA was used for statistical analysis (genotype × sex) (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001), *n* = 3 each group.

Appendix II: Permission to Reprint Published Figures

ΕΛΣΕς ΙΕΡ ΛΙΧΕΝΣΕ
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Θυν 06, 2021

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INTRODUCTION

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