Identifying novel targets to restore defects in neurogenesis in the 3xTG mouse model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD), marked by a serious and progressive decline in cognitive abilities, is a severely debilitating disease that is becoming an increasing concern with our aging population. Defects in neurogenesis have been shown to exist in AD and aggravate the neuropathology and cognitive deficits associated with the disease. In this study, I aimed to characterize the cellular and molecular defects of neurogenesis in the triple transgenic mouse model of AD (3xTG). To do so, I first performed a detailed immunohistochemistry characterization using neurogenic markers that were quantified and analyzed in the hippocampus of control and 3xTG mice. This analysis not only revealed an overall decrease in the pool of neural stem and progenitor cells (NSPCs) in 3xTG brains, but also defects in proliferation, differentiation and a loss within the neuroblast, immature neuron and mature neuron populations. Subsequent immunohistochemistry analysis of two molecular targets, Hopx and LPAR1, involved in NSC maintenance and proliferation respectively, revealed their dysregulation in 3xTG brains, providing some indication of molecular defects underlying this loss. The neurosphere assay was next employed to assess cell-autonomous defects and fewer neurospheres were formed from cultured 3xTG NSPCs, suggesting a defect in NSPC pool expansion that is intrinsic to 3xTG NSPC function. Molecular characterization of these cultured NSPCs via qPCR revealed the upregulation of mitochondrial and fatty acid oxidation genes in 3xTG NSPCs, suggesting not only a dysregulation of metabolic functions, but also an acclimation to oxidative stress conditions. Interestingly, 3xTG NSPCs formed larger and more neurospheres when grown in galactose medium – which is used to simulate oxidative stress – relative to the control, confirming an adaptative response to oxidative stress conditions. Further characterization of

these cellular defects and underlying molecular mechanisms can reveal novel therapeutic strategies for AD.

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3xTG – Triple Transgenic Model

Aβ – Amyloid beta

ACh – acetylcholine

ACSF – artificial cerebrospinal fluid

AD – Alzheimer's disease

ANOVA – Analysis of variance

APP – Amyloid Precursor Protein

Ascl1 – Achaete-Scute Family BHLH Transcription Factor 1

CBIA – Cell Biology and Image Acquisition Core

cDNA – complementary DNA

Cpt1 – carnitine palmitoyltransferase 1

Cre – Cyclization Recombinase

DAPI – 4′,6-diamidino-2-phenylindole

Dcx – Doublecortin

DG – Dentate Gyrus

DMEM – Dulbecco's Modified Eagle Medium

EdU – 5-ethynyl-2'-deoxyuridine

EGF - Epidermal growth factor

ER^{T2} – Estrogen Ligand-Binding domain

F12 – F-12 nutrient mixture

FAD – familial AD

FACS – fluorescence-activated cell sorting

FGF – fibroblast growth factor

GFAP – Glial Fibrillary Acidic Protein

GFP – Green Fluorescence Protein

HopX – Homeodomain-only protein (HOP) homeobox

IF – Immunofluorescence

IHC – Immunohistochemistry

IPC – Intermediate Progenitor Cell

loxP – Locus of X-over P1

MAPT – microtubule associated protein tau

Nestin – Neuroepithelial Stem Cell Protein

NFTs – Neurofibrillary Tangles

NTG – nontransgenic (wildtype; control)

NSCs – Neural stem cells

NPCs – Neural progenitor cells

NSPCs – Neural stem and progenitor cells

PBS – Phosphate buffered saline

Pen-Strep – Penicillin and Dihydrostreptomycin

PFA – Paraformaldehyde

Prox1 - Prospero Homeobox 1

PSEN1 – Presenilin1

RGL – radial glial-like

ROS – reactive oxygen species

SEM – standard error of mean

SGZ – subgranular zone

Sox2 – SRY (sex determining region Y)-box2

SVZ – subventricular zone

TAM – tamoxifen

TAP – Transit Amplifying Progenitor

Tbr2 – T-box brain protein 2

YFP – yellow fluorescent protein

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1. INTRODUCTION

1.1 Overview of Alzheimer's disease

Alzheimer's disease (AD) is one of the most prevalent forms of dementia and is a highly progressive and severe neurodegenerative disease (Dos Santos Picanco et al., 2018; van der Flier & Scheltens, 2005). The clinical presentations begin with impaired memory and judgement before the progression of the cognitive decline impairs the patients' behaviours, speech, judgement and motor abilities (Dos Santos Picanco et al., 2018; Förstl & Kurz, 1999; Rathmann & Conner, 2007; van der Flier & Scheltens, 2005). Ultimately, patients struggle to complete basic tasks and eventually lose the ability to swallow, speak and control their bladder (Dos Santos Picanco et al., 2018; Förstl & Kurz, 1999). At the endpoint of the disease, patients are bedridden and depend entirely on their caregivers.

AD pathology is characterized by significant brain atrophy and extracellular lesions of amyloid plaques and neurofibrillary tangles which are believed to be involved in the neuronal loss (DeTure & Dickson, 2019; Dos Santos Picanco et al., 2018; Perl, 2010). The majority of AD cases fall under the classification of sporadic AD (SAD) – of which there is no known genetic basis for the disease. In spite of this, genome wide association studies suggest that risk factors include mutations in genes affecting APP, tau, cholesterol metabolism and immune responses (Bekris et al., 2010; Dos Santos Picanco et al., 2018; Lane et al., 2018). The biggest risk factor for SAD – which affects 90% of AD patients – is age, but it is believed to manifest from a cumulation of genetic and environmental factors. For the less prevalent form of familial AD (FAD), there are some causative mutations identified in amyloid precursor protein (APP), presenilin 1 (PSEN1) and microtubule associated protein tau (MAPT) genes (Bekris et al., 2010;

Lane et al., 2018). FAD tends to occur much earlier than sporadic AD and, because of its strong genetic basis, having a family history of early onset AD or FAD is the greatest risk factor for developing this disease (Bekris et al., 2010).

The emotional toll of witnessing a loved one battle through this disease is particularly severe, not to mention the extraordinary financial burden placed on our society – something that is expected to increase exceptionally with our current aging population (Wong, 2020). Consequently, there is great interest in studying AD to better understand its pathology and discover therapeutic options.

1.2 History of AD research

AD research has an extensive history, with the majority of studies focused on elucidating the mechanisms underlying the loss of brain tissue and gaining insight on biomarkers and the pathological hallmarks of the disease. Despite some breakthroughs, there is still a lack of consensus in the field on the fundamental causes of the disease with several alternative hypotheses and therapeutic approaches being proposed (Du et al., 2018; Liu et al., 2019).

1.2.1 The cholinergic hypothesis

The cholinergic hypothesis was one of the first hypotheses proposed in regard to AD pathology. In 1976, Peter Davies and A.J.F. Maloney remarked a decrease in the function of a specific enzyme – choline acetyltransferase – in AD brains particularly within the amygdala (a region of the brain that functions to process dangerous or threatening stimuli), hippocampus (region of the brain that regulates learning and processes memories) and cortex (responsible for higher order cognitive functions) (Davies & Maloney, 1976). Since the neurodegeneration of AD

begins and primarily occurs in these regions of the brain, this was a particularly striking finding. Choline acetyltransferase is a key enzyme in the synthesis of acetylcholine (ACh), an important neurotransmitter that was subsequently found to be reduced at synapses within the brains of AD patients (Davies & Maloney, 1976; Hardy & Allsop, 1991). As such, AD was proposed to be a disease caused by a deficiency in ACh, which lead to the use of acetylcholinesterase inhibitors that prevent the degradation of ACh as a strategy to prevent the symptoms of AD. While this did yield some improvements with respect to cognitive function, it was not effective at preventing the onset and progression of AD (Hardy & Allsop, 1991). This prompted discussion of whether the cholinergic deficiency is more a symptom rather than the cause of AD. Acetylcholinesterase inhibitors continue to be used, as they are effective at slowing the cognitive decline seen in AD patients (Casey et al., 2010; Yiannopoulou & Papageorgiou, 2020).

1.2.2 The amyloid hypothesis

John Hardy and David Allsop subsequently developed the amyloid hypothesis following their discovery of a mutation of the APP gene which, when combined with the observation of the accumulation of amyloid beta (Aβ) plaques in the brains, provided some evidence of its involvement in AD (Hardy & Allsop, 1991). They postulated that this mutation would result in a cascade beginning with Aβ deposits that would lead to the hyperphosphorylation of the protein tau causing neurofibrillary tangles to form, ultimately resulting in neuronal death (Hampel et al., 2021; Hardy & Allsop, 1991). Further studies into APP revealed that there are two main pathways by which Aβ is produced; the alpha pathway where APP is broken down into soluble Aβ by both α- and γ-secretase and the beta pathway where APP is instead hydrolyzed by β- and γ-secretase resulting in insoluble Aβ (Hampel et al., 2021; O'Brien & Wong, 2011; Y. Zhang et al., 2011). Accumulation of the insoluble Aβ was also shown to cause neuronal death and impair

cognition – adding to the evidence in support of this hypothesis (Hampel et al., 2021; O'Brien & Wong, 2011; Zhang et al., 2011). Several proposed therapeutics for AD are based on this hypothesis as it was viewed as the best explanation for what is triggering the neuronal death and therefore the best strategy to preventing the neurodegeneration seen in AD. These therapeutics include inhibitors of the β- and γ-secretase inhibitors (which generate the insoluble Aβ); drugs that prevent the aggregation of Aβ to avoid the formation of plaques; and other strategies to remove Aβ such as monoclonal antibodies that can target Aβ for elimination via phagocytic microglia (Casey et al., 2010; Yiannopoulou & Papageorgiou, 2020). Unfortunately, many of these therapies – particularly the immunotherapies – have had poor clinical success with off-target effects and failing to improve cognition in patients (Casey et al., 2010; Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020). This has ultimately led to increased calls for their abandonment in clinical trials and caused many to explore other alternative hypotheses.

1.2.3 The tau pathology hypothesis

Similarly, a hypothesis involving tau proteins, which is the main component of neurofibrillary tangles (NFT) – one of the hallmark neurohistopathological markers of AD – has been circulated. Tau proteins bind to and stabilize microtubules, which, in neurons, are involved in intercellular transport, particularly to the dendrites and axons (Frost et al., 2009; K. Iqbal et al., 2010; Probst et al., 2000). In AD, these tau proteins become hyperphosphorylated, resulting in stronger binding with the microtubules and causing their aggregation, ultimately forming NFTs (Frost et al., 2009; K. Iqbal et al., 2010; Probst et al., 2000). After remarking that these pathological NFTs began in specific regions of the brain and spread more broadly throughout different regions, it was suggested that these aggregated tau proteins behave in a prion-like fashion – inducing spread throughout the brains of AD patients (Frost et al., 2009; K. Iqbal et al.,

2010; Probst et al., 2000). This was supported by a study whereby mice from a transgenic mouse line of late-onset tau pathology developed tau pathology more quickly when injected with brain extracts from other transgenic mice with tau aggregation compared to brain extracts from control mice (Bolmont et al., 2007). In addition to the accumulation of toxic deposits, the impaired inter- and intracellular signalling caused by the destabilized microtubules due to these hyperphosphorylated tau proteins was proposed to be neurotoxic(Bolmont et al., 2007). While some drugs that target tau proteins continue to be developed as therapeutic options for AD, there has been limited success clinically and experts anticipate that they will perform similarly to therapies targeting Aβ (Casey et al., 2010; Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020).

1.2.4 The neurovascular hypothesis

Another emerging hypothesis, termed the neurovascular hypothesis, postulates that AD is caused by a failure of the brain vasculature system that maintains nutrient levels and eliminates waste in the brain tissue microenvironment (Iadecola, 2004; Nelson et al., 2016; Soto-Rojas et al., 2021). The vasculature system serves a fundamental role to the functioning of the brain – as evidenced by the fact that 20% of cardiac output is routed to the brain, replenishing it with roughly 20-25% of the bodies oxygen and glucose despite it accounting for only 2% body mass (Iadecola, 2004; Nelson et al., 2016; Soto-Rojas et al., 2021). Any disruptions to the brain's blood supply will result in nearly instantaneous interruption to the function of neurons and can lead to significant damage to the tissue if for a prolonged period of time. Due to this essential role, it was indeed troubling when dysfunction of the vasculature system was observed in AD patients. Damaged neurovasculature has been reported in AD settings where imaging of the brains of AD patients have shown that these impairments of cerebral vasculature predate

neurodegeneration (Iadecola, 2004; Nelson et al., 2016; Soto-Rojas et al., 2021). This included dysfunctional angiogenesis (the formation of new blood vessel) and senescence of cells involved in the vascular unit, which resulted in decreased blood flow and hypoxia in the brain (Nelson et al., 2016; Soto-Rojas et al., 2021). Lifestyle factors can contribute to an increased risk of impaired vascular function – such as having high cholesterol levels from diet, having hypertension or being type 2 diabetic (Iadecola, 2004; Nelson et al., 2016; Soto-Rojas et al., 2021). Hyperlipidemia and hyperglycemia have been correlated with a higher risk of AD and impaired cognitive function, however clinical trials with statins or diabetic drugs were unsuccessful in improving the symptoms of AD, including restoring cognition (Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020).

1.2.5 The neuroinflammation hypothesis

It has also been theorized that AD could potentially be caused by an aberrant neuroinflammatory response (Du et al., 2018; Heneka et al., 2015; Kinney et al., 2018; Liu et al., 2019). Microglia cells – macrophages that function specifically in the brain and spinal cord – have been determined to be hyperactive in AD patients where they tend to aggregate around senile plaques and neurons with NFTs. There, they secrete inflammatory factors, such as TNF-α and a host of pro-inflammatory cytokines like IL-1β, TGF-β and IL-12, to initiate an innate immune response (Heneka et al., 2015; Kinney et al., 2018; McGeer et al., 1988). Elevated levels of these factors were also observed in AD patient's brains, supporting an inflammatory environment in AD brains (Heneka et al., 2015; Kinney et al., 2018). Since a highly neuroinflammatory environment can be neurotoxic, it was proposed that this could worsen the progression of AD. Further studies revealed that Aβ may be involved in the activation of microglia to trigger this response, where it was shown that Aβ binds to a specific receptor

complex on microglia cells to elicit the secretion of the inflammatory factors (Heneka et al., 2015; Kinney et al., 2018; Meda et al., 1995). Consequently, anti-inflammatory drugs have been used to suppress the immunotoxicity observed in AD patient brains and its therapeutic potential continues to be assessed in clinical trials (Casey et al., 2010; Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020).

1.2.6 The mitochondrial hypothesis

The mitochondrial hypothesis is another proposed explanation for the pathology of AD, particularly due to findings of mitochondrial defects in AD brains. Mitochondria play a crucial role in the brain where they supply the high energy demands of the organ. Impaired mitochondrial function, brought on through a combination of genetics and environmental factors, has been linked to aging and AD symptoms (Moreira et al., 2010; Perez Ortiz & Swerdlow, 2019; Wang et al., 2020). A decrease in mitochondrial activity – mainly through reductions in the oxidative phosphorylation, beta-oxidation and Kreb cycle metabolic pathways – has also been observed in aged and AD settings (Moreira et al., 2010; Perez Ortiz & Swerdlow, 2019; Wang et al., 2020). Additionally, enzymes involved in these metabolic functions and mitochondrial respiration were reportedly decreased in AD patients' brains (Moreira et al., 2010; Wang et al., 2020; Yao et al., 2009). Neurons depend on oxidative phosphorylation to supply their energy needs, therefore a reduction in this can impair neuronal function greatly, worsening the symptoms of aging and neurodegenerative diseases like AD.

Mitochondrial dysfunction can also lead to the accumulation of reactive oxygen species (ROS) resulting in oxidative stress conditions – which further induces damage to mitochondria. Intriguingly, increased levels of ROS have been observed in AD brains prior to the onset of Aβ pathology and clinical presentations of AD (Bhatt et al., 2021; Ionescu-Tucker & Cotman, 2021;

Manoharan et al., 2016; Markesbery, 1999). Furthermore, a link between oxidative stress and loss of neuronal function has been established in both *in vitro* and *in vivo* studies (Ionescu-Tucker & Cotman, 2021; Markesbery, 1999; Uttara et al., 2009), supporting the idea that mitochondria contribute to AD pathogenesis.

Furthermore, mitochondria contain their own DNA – termed mtDNA – which code for a variety of key components that are necessary for the metabolic functions of the organelle (Perez Ortiz & Swerdlow, 2019). Due to their lack of histones and proofreading processes, mtDNA is particularly susceptible at obtaining mutations and deletions (Perez Ortiz & Swerdlow, 2019). These mutations accumulate with age and can have detrimental effects on a variety of mitochondrial functions – such as bioenergetics, antioxidation and reduced oxidative metabolism (Hoekstra et al., 2016; Klein et al., 2021; Reid et al., 2022). While specific mtDNA mutations that are linked to AD have yet to be uncovered, there are some indications that alterations in mtDNA may be involved in the symptoms of AD (Hoekstra et al., 2016; Klein et al., 2021; Reid et al., 2022).

Since some mitochondrial metabolites play a role in epigenetic regulation, it has also been proposed that mitochondrial defects underly the abnormal DNA modifications observed in AD brains (Nikolac Perkovic et al., 2021; Wang et al., 2020). These observations lead to the development of the mitochondrial cascade hypothesis where it was theorized that mitochondrial dysfunction through environmental and inherited factors can trigger the onset of AD once exceeding a certain threshold (Liu et al., 2019; Swerdlow et al., 2014; Wang et al., 2020). Studies have looked at stimulating mitophagy or supplementing metabolites of the mitochondrial tricarboxylic acid cycle as a therapeutic strategy for AD, results of which look fairly promising

(Joshi et al., 2017; Liu et al., 2019; Pleen & Townley, 2022; Reddy et al., 2017; Wang et al., 2020; Wilkins et al., 2014; Yiannopoulou & Papageorgiou, 2020).

1.3 Current landscape of the AD research field

The hypotheses summarized above have been among the major developments that have defined the past 50 years of AD research. Approximately 80% of the AD drugs that have made it to clinical trials are based on one of these hypotheses (Du et al., 2018; Liu et al., 2019). However, while these studies have yielded key insights on the cellular and molecular processes of the AD brain, there continues to be a lack of consensus on the fundamental basis of AD pathology. The failure of AD drugs developed over the years to prevent the onset of the disease and improve the cognition of AD patients suggests that the root causes of AD have yet to be elucidated. The inability to decipher what is a cause or consequence of AD has led to no viable treatment options for preventing or eliminating the disease (Du et al., 2018; Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020). As a consequence, current clinical interventions have primarily been to preserve quality of life by delaying the progression of the disease (Du et al., 2018; Liu et al., 2019; Yiannopoulou & Papageorgiou, 2020).

The direction of AD research continues to point towards uncovering the pathophysiology of AD at a more precise level. Having a better understanding of the fundamental basis of the disease will allow for the development of targeted and innovative therapeutic strategies. Additionally, drugs developed over the past few decades that have failed in clinical trials continue to be improved on in the hopes that it will be able to improve some clinical outcomes of patients. Efforts are also being made to enhance diagnostic strategies of the disease which would not only assist with teasing out the disease mechanisms but could improve clinical outcomes by allowing for diagnosis and clinical interventions in the early stages of the disease. Additionally,

identifying biomarkers has been proposed as one way to enhance treatment strategies where a personalized medicine approach can be used to tailor treatment options for each patient – which is likely to result in improved clinical outcomes (Mantzavinos & Alexiou, 2017). Finally, better diagnostic approaches can be used to ensure all preclinical AD patients are removed from control groups in prospective clinical trials – an issue that has been pointed out as a key flaw in AD research that could contribute to the overwhelming failure of recent drug development (Du et al., 2018; Liu et al., 2019; Mantzavinos & Alexiou, 2017; Yiannopoulou & Papageorgiou, 2020).

In sum, while we have some insight into the inner happenings of the brains of these patients, AD largely remains a mystery. However, the future continues to be promising as AD research remains a major field within biomedical and translational research.

1.4 Animal models of Alzheimer's disease

Many of the recent breakthroughs made in our understanding of AD have been achieved through the use of animal models. Several models of AD have been established (Billings et al., 2005; Edler et al., 2021; Head, 2013; LaFerla & Green, 2012; Mhatre et al., 2013; Oddo et al., 2003; Platt et al., 2013; Rivera et al., 2016; Sterniczuk et al., 2010; Wilson-Sanders, 2011) over the past few decades – the majority of which possess mutations associated with amyloid and tau pathology. Invertebrate and distant vertebrate animal models of AD exist – such as the fruit fly, zebrafish and *C.elegans* – and have contributed to some understanding of basic disease mechanisms, however they are used less often in favor of animals more closely related to humans (Mhatre et al., 2013; Wilson-Sanders, 2011). Although some bigger animals, such as canines and nonhuman primates, have been used in some studies, permitting analysis of age-

associated risks of AD, a major challenge to their widespread implementation include the high cost of care for these larger animals which can restrict sample size and therefore statistical power of studies (Edler et al., 2021; Head, 2013; Heuer et al., 2012; Rivera et al., 2016).

Consequently, mouse models remain the most widely used of all animals to study AD. The first transgenic mouse model of AD emerged in 1995, where an overexpression of the human APP mutant resulted in the formation of amyloid plaques and neurodegeneration observed in AD patients (German et al., 2005; Hall & Roberson, 2012; Jankowsky & Zheng, 2017). These mice, referred to as the PD-APP mutants, contain a single mutation in the APP gene. They begin to exhibit deficits in their working memory fairly early on – at 3 months of age – before the amyloid plaques form and further defects in their recognition memory are detected at around 6 months of age (German et al., 2005; Hall & Roberson, 2012; Jankowsky & Zheng, 2017).

Despite the excitement of modeling AD features in mice for the first time, the inability to capture other important aspects of AD in PD-APP mice, such as neurodegeneration, led to the pursuit of better AD mouse models. This was followed by the subsequent development of over 100 transgenic mouse lines all with the goal of establishing models of various aspects of AD (Hall & Roberson, 2012; Jankowsky & Zheng, 2017). Many of these mouse models possess mutations in APP and/or PSEN1 – mutations that have been identified in familial AD patients (Hall & Roberson, 2012; Jankowsky & Zheng, 2017). Some mouse models have combined mutations in these genes so as to better recapitulate the pathology of AD in humans. One such model, the 5xFAD mouse line, express 3 mutations in APP and 2 mutations in PSEN1 (Eimer & Vassar, 2013; Oblak et al., 2021). These mice are known for their aggressive pathology as plaques begin to form as early as 2 months of age, which is closely followed by synaptic loss and cognitive defects at 4 months of age. Unlike other APP or APP/PSEN1 mutations, these mice

also display neuronal loss, however the lack of tau pathology present in the brains of these mice can be considered as a drawback (Eimer & Vassar, 2013; Oblak et al., 2021).

Among these various lines, the triple transgenic mouse model of AD (3xTG) is one of the only mouse models that develop both amyloid and tau pathology, as well as cognitive deficits and neuronal loss that closely mirror that of AD patients (Oddo et al., 2003; Roda et al., 2020; Sterniczuk et al., 2010). These mice contain the 3 mutations that are most commonly associated with familial AD – APP Swedish, microtubule-associated protein tau (MAPT) P301L and PSEN1 M146V (Oddo et al., 2003; Roda et al., 2020; Sterniczuk et al., 2010). At 4 months of age, they begin to display some cognitive impairments and amyloid beta accumulation intracellularly (Billings et al., 2005; Oddo et al., 2003; Roda et al., 2020). This is followed by more severe learning and memory deficits and the formation of amyloid beta deposits at 6 months of age (Billings et al., 2005; Oddo et al., 2003; Roda et al., 2020). Tau pathology develops at 12 months of age, well after the formation of amyloid beta plaques, as is the case for AD patients (Billings et al., 2005; Oddo et al., 2003; Roda et al., 2020). Since these mice allow for the simultaneous study of the roles of both amyloid and tau pathology in AD and share many similarities in the development of the disease in humans, the 3xTG mouse model is among the most widely used in AD research.

While these mice models are indeed essential tools in the study of AD, there are some careful considerations to be made when applying the findings to AD in humans. Namely, the mutations used to generate these models are based on familial AD, which could limit the translatability to the more common form of AD – sporadic AD. Regardless, many advances in our understanding of AD pathology have been accomplished through the use of these animal models.

1.5 Neurogenesis

An exciting topic within AD research is neurogenesis, the process of generating new neurons from neural stem cells (NSCs). This process was previously believed to be limited to the embryonic and postnatal development of the central nervous system and some recent findings continue to support the idea that the role of neurogenesis is negligible after early childhood (Cipriani et al., 2018; Hagihara et al., 2019; Koketsu et al., 2003; Kumar et al., 2019; Nano & Bhaduri, 2022; Snyder, 2019; Sorrells et al., 2018, 2021). However, this is hotly debated by emerging evidence which suggests that neurogenesis indeed persists throughout adulthood and plays a key role in neuroplasticity and maintaining cognitive function throughout the lifespan (Akers et al., 2014; Alam et al., 2018; Anacker et al., 2018; Boldrini et al., 2018; Borrett et al., 2022; Cipriani et al., 2018; Dennis et al., 2016; Hanspal & Gillotin, 2022; Kumar et al., 2019; Lacefield et al., 2012; Terranova et al., 2019; Toni et al., 2008; Zhou et al., 2022).

Adult neurogenesis primarily occurs in two specific regions of the brain – the subventricular zone (SVZ) in the lateral ventricles and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus. Neurons generated from the SVZ migrate via the rostral migratory stream (RMS) and contribute to the olfactory neural circuitry (Alvarez-Buylla & García-Verdugo, 2002; Lim & Alvarez-Buylla, 2016) and neurons derived from NSCs from the SGZ are believed to functionally integrate into the hippocampal network and enhance cognitive processes such as learning and memory (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). Neurogenesis can occur in other regions within the central nervous system (Cameron & Dayer, 2008; Oyarce et al., 2014), although the SVZ and SGZ continue to be the most characterized and defined niches.

1.6 Adult hippocampal neurogenesis

Adult hippocampal neurogenesis is characterized by a series of stages (Figure S1), the first begins with NSCs or Type 1 radial glia-like (RGL) cells which express markers such as glial fibrillary acidic protein (GFAP), Nestin and Sry-box2 (Sox2) (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). These Type 1 NSCs are typically quiescent and undergo self-renewal to maintain the NSC pool and give rise to type 2 neuronal progenitor cells (NPCs) (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). These type 2 NPCs then undergo proliferation before generating type 3 neuroblasts (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). Although distinct with respect to morphology, a subset of these type 2 NPCs – referred to as type 2a cells– share several markers with type 1 NSCs but can be distinguished through the expression of Ascl1, before beginning to express markers such as Tbr2 that are specific to the type of neuronal cell they will ultimately give rise to, at which point they are referred to as Type 2b cells (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). Type 2b cells also express markers such as doublecortin (Dcx) which continues to be expressed as the cells become Type 3 neuroblasts. Type 3 neuroblasts then exit the cell cycle, marking the birth of new neurons which express postmitotic neuronal markers such as NeuN and begin to grow axons and dendrites (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015). Maturation is completed when the new cells express Prox1 and calbindin and functionally integrate into the hippocampal network (Abbott & Nigussie, 2020; Kempermann et al., 2004, 2015; Kozareva et al., 2019; Ma et al., 2009; J. Zhang & Jiao, 2015).

Studies involving rodents suggest that adult hippocampal neurogenesis plays a functional role in important cognitive processes like learning, memory, attention and mood regulation (Alam et al., 2018; Anacker et al., 2018; Drapeau et al., 2003; Hagemann et al., 2013; Lacefield et al., 2012; Sahay et al., 2011; Saxe et al., 2006; Toni et al., 2008). Immunohistochemistry analysis of the above markers have indicated that the neurogenic process continues throughout the lifespan of rodents, which – when coupled with behavioural assays – suggest involvement in maintaining cognitive function. An ablation of new-born adult hippocampal neurons in mice resulted in poor Morris Water Maze results, suggesting impairments in spatial memory (Drapeau et al., 2003). Furthermore, elimination of Type I NSCs through either irradiation or genetic manipulation in mice resulted in decreased synaptic plasticity and impaired contextual fear conditioning (Saxe et al., 2006; Winocur et al., 2006). Conversely, enhancing hippocampal neurogenesis in mice through the use of exercise and external stimulation improved performance in pattern separation and the spatial memory (Sahay et al., 2011).

Additionally, studies found that cranial radiation therapy used for treatment in brain cancer patients resulted in impairments in cognitive function with processes such as a memory or attention, a side-effect that is believed to have been caused by attenuated hippocampal neurogenesis (Greene-Schloesser et al., 2013), supporting a functional role of neurogenesis in humans throughout the lifespan. This was further corroborated by two recent studies completed by independent groups, where neurogenic markers were analyzed in the brains of healthy human subjects at various ages (Moreno-Jiménez et al., 2019; Tobin et al., 2019). One study measured the amount of Nestin+Sox2+ NPCs and Dcx+ neuroblasts/immature and detected considerable amounts in subjects between the ages of 79-99 (Tobin et al., 2019). The subsequent study that measured the amount of Dcx+ cells in neurologically healthy human subjects agreed with these

findings as Dcx+ expression could be identified even within the brains of 80-year-old subjects (Moreno-Jiménez et al., 2019). This study also included a test of different tissue processing and immunostaining techniques which revealed that some methodological practices can affect the visualization of markers enough to alter the outcomes of the study (Moreno-Jiménez et al., 2019). Other approaches such as carbon-14 dating (Spalding et al., 2013) and, more recently, single-cell and single-nucleus transcriptomics (Borrett et al., 2022; Zhou et al., 2022) also provide evidence of the persistence of adult hippocampal neurogenesis.

In spite of this evidence supporting a functional role of adult hippocampal neurogenesis in continued cognition throughout the lifespan, adult hippocampal neurogenesis is reported to decline in an aged or neurodegenerative setting, which has been proposed to contribute to the associated cognitive impairments (Criado-Marrero et al., 2020; Hamilton et al., 2010, 2015; Hamilton & Fernandes, 2018; Moreno-Jiménez et al., 2019; Rodríguez et al., 2008a; Tobin et al., 2019; Vivar, 2015). Gaining a better understanding of the mechanisms underlying these deficits can provide insight on how neurogenesis can be targeted in these settings to restore cognitive function.

1.7 AD and neurogenesis

Targeting adult neurogenesis has been proposed as a potential therapeutic intervention to regenerate the neurons being lost and restore cognitive function in these patients (Briley et al., 2016; Shohayeb et al., 2018). However, it has been reported that NSCs in AD patients and mouse models exhibit defects that impair their progression through the neurogenic process (Criado-Marrero et al., 2020; Hamilton et al., 2010, 2015; Hamilton & Fernandes, 2018; Moreno-Jiménez

et al., 2019; Rodríguez et al., 2008a; Tobin et al., 2019; Vivar, 2015). Reduced amounts of BrdU+ cells (proliferative cells) were identified in 12-month-old APP mutant mice, and the use of the hormone ghrelin was capable of increasing the BrdU+ cells (C. Kim et al., 2017). Similar findings were reported in a study using 9-month-old APP/PSEN1 mice where decreased amounts of BrdU and Dcx positive cells were observed in the hippocampus of the mice (Faure et al., 2011). In the Tg2576 mouse model, showed defective survival of BrdU positive cells at 3 months of age – a defect that predated the onset of amyloid pathology (Krezymon et al., 2013). A subsequent study with 5x-FAD mice found that inhibiting adult hippocampal neurogenesis worsened cognition and neuronal loss in these mice (Moon et al., 2014). Conversely, inducing adult hippocampal neurogenesis resulted in improved cognitive abilities in the mice (Moon et al., 2014).

Several studies involving 3xTG mice have demonstrated that these mice also display defects in neurogenesis (Hamilton et al., 2010, 2015; Hamilton & Fernandes, 2018; Rodríguez et al., 2008a; Wirths, 2017). One such study found that there were decreased amounts of proliferating cells, Sox2 (Type 1 NSCs/Type 2a NPCs) cells and Dcx (Type 3 neuroblasts, immature neurons) cells in both neurogenic niches in the brains of 3xTG mice compared to wildtype at both 11 and 18 months of age (which represent middle and old age respectively) (Hamilton et al., 2010). Additionally, there was a corresponding decrease in the amounts of new neurons functionally added to the dentate gyrus and olfactory bulb neural circuits(Hamilton et al., 2010). These findings suggested that defects in 3xTG neurogenesis predate the formation of tangles and plaques (Hamilton et al., 2010). A subsequent study revealed decreased levels of Ki-67+ (proliferative) cells in both the SVZ and SGZ occurring as early as 2 months of age (Hamilton et al., 2015). These early defects correlated with the accumulation of lipid droplets in the SVZ of

these mice at 2 months of age; interestingly, increased lipid droplets could also be found in the SVZ of AD patient brains (Hamilton et al., 2015). Treatment with oleic acid resulted in impaired proliferation of these NSPCs in vitro, suggesting that aberrant lipid metabolism may underly the early defects of 3xTG neurogenesis (Hamilton et al., 2015).

Visualization and analysis of neurogenic markers in adult human brains revealed that hippocampal neurogenesis is impaired in AD patient brains compared to neurologically healthy and similarly aged counterparts (Moreno-Jiménez et al., 2019; Tobin et al., 2019). Nestin+Sox2+ NPCs could be found up until the 10^{th} decade of life, including in the brains of patients with mild cognitive impairment, however there were reduced amounts in these patients. A further study using the Braak characterization of neurohistopathology to establish the staging of AD determined that a loss of Dcx positive cells correlated to the progression of later stages of AD (Moreno-Jiménez et al., 2019).

While some cellular and molecular mechanisms behind these defects have been identified, further characterization can provide key targets in realizing the therapeutic potential of neurogenesis in the context of AD.

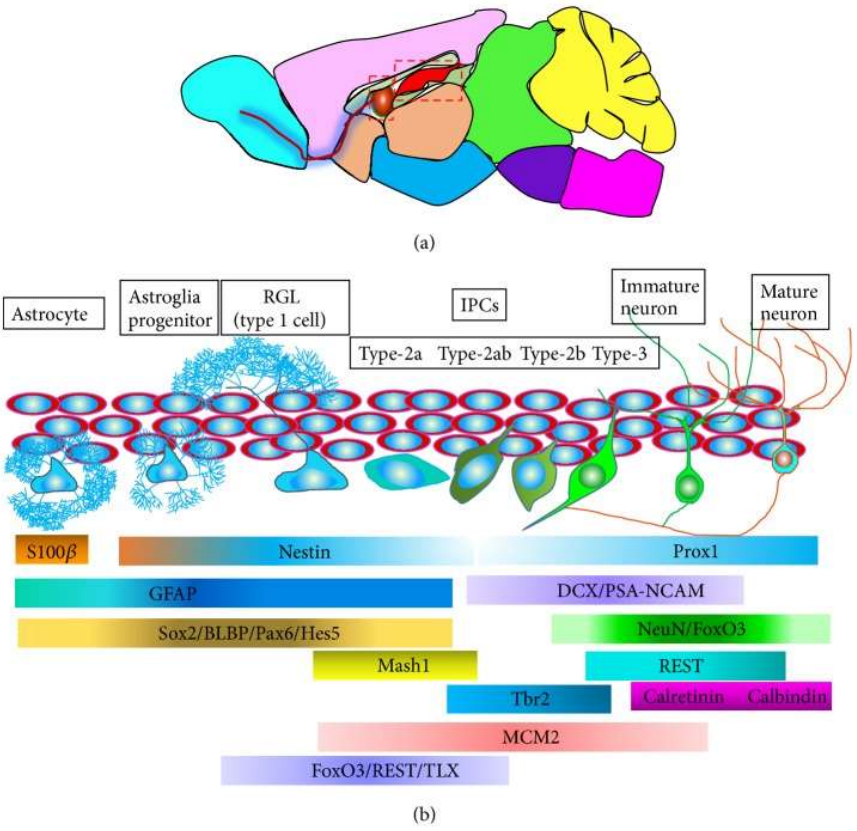

Figure S1: The molecular markers for different developmental stages of adult hippocampal neurogenesis. Figure from Zhang *et. al* (J. Zhang & Jiao, 2015)

1.8 Rationale underlying hypothesis and research objectives

To confirm the findings of studies on neurogenesis in 3xTG mice these findings (Hamilton et al., 2015, 2015; Hamilton & Fernandes, 2018; Rodríguez et al., 2008a; Wirths, 2017), a preliminary characterization of the expression of Sox2 (Type 1 NSCs and 2a NPCs) and Dcx (Type 2b NPCs, Type 3 neuroblasts and immature neurons) in the DG of 3-month-old wildtype and 3xTG mice was done in our lab (Figure S2). A smaller granule cell layer, less Dcx positive cells and less Sox2 positive cells in the 3xTG mice were observed, indicating that defects are occurring in the neural precursor populations at the 3-month-old timepoint, prior to the

manifestation of cognitive impairments, which is consistent with the previously mentioned study [69].

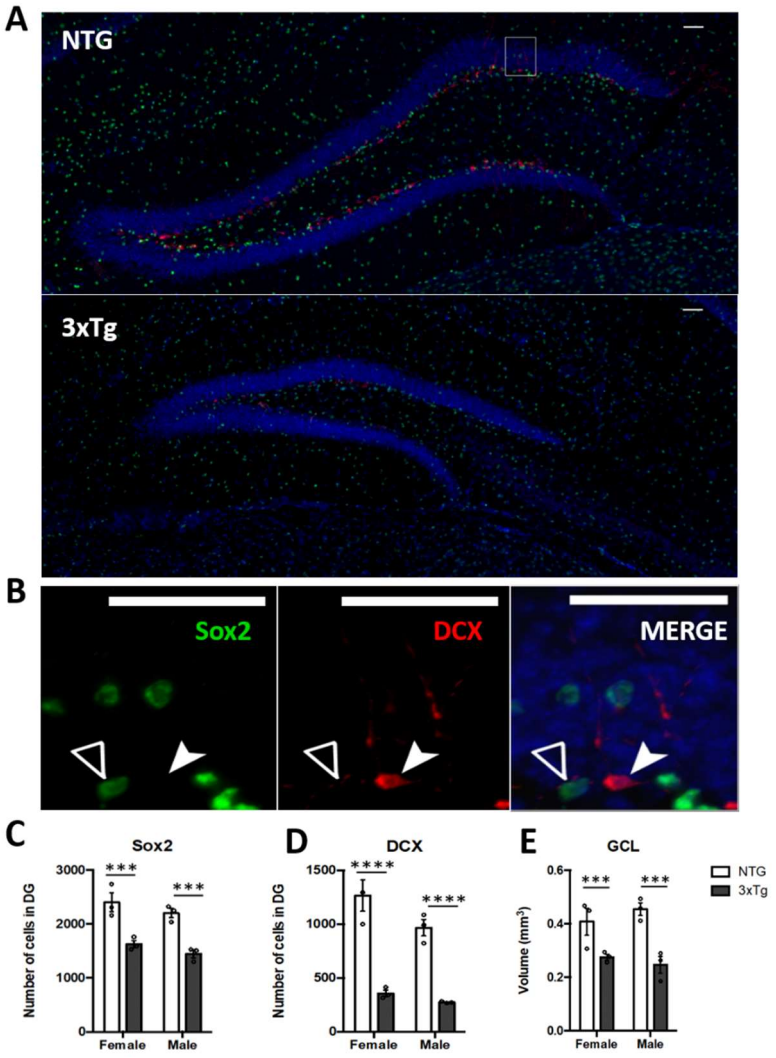

Figure S2: Preliminary characterization reveals deficiencies in 3xTG neural precursor cells at 3 months of age. The dentate gyrus (DG) of 3-month-old wildtype (NTG; n=3 male and n=3 female) and 3xTG Nestin-CreER^{T2}-RosaYFP (n=3 male and n= 3 female) reporter mice was sectioned and stained for either (A) Sox2 (green) and Dcx (red). (B) Arrowheads denote examples of a Sox2 or Dcx positive cell. The granule cell layer (GCL) and counts of positive cells in the subgranular zone (SGZ) were measured blindly and the averages were subsequently compiled for analysis. All quantification, analysis, tissue preparation, imaging and figure preparation was completed by R.H. and M.M. Scale bar indicates 50 μm, a two-way ANOVA test was used for statistical analysis, *p<0.05, **p<0.01, ***p<0.001.

The mechanisms underlying this loss of NSPCs is not well understood, and so we (Y. Liu et al., 2022) conducted a single cell RNA sequencing (scRNA-seq) experiment comparing the gene expression of cells in the SGZ of the brains of 3-month-old wildtype and 3xTG mice from our Nestin-CreER^{T2}-RosaYFP reporter line (Figure S3) to better understand the loss of these cells. Following treatment with tamoxifen, the Cre-recombinase induced expression of YFP, but only in Nestin expressing cells as the transgene under the control of the Nestin promoter. Since Nestin is expressed in Type 1 NSCs and Type 2a NPCs (J. Zhang & Jiao, 2015), YFP expression would begin in these cells and persist as they progress through the different stages of neurogenesis (Cicero et al., 2009; Zhu et al., 2012). Using established markers, individual cell populations could be mapped and identified (Figure S3A) and comparing the proportions of wildtype and 3xTG cells in each population revealed defects occurring in specific cell populations (Figure S3B). Of particular interest was the increased proportion of 3xTG quiescent NSCs (qNSC), but decreased proportions of activated NSCs (pNSC), TAPS and neurons relative to the wildtype. Additionally, many dysregulated genes and pathways were identified through our sequencing results.

These results suggest that there are cellular and molecular defects in neurogenesis within the 3xTG mouse model occurring at a timepoint that predates the onset of neurohistopathological markers and clinical symptoms. Better characterization of these defects can not only shed light on the mechanisms by which 3xTG neurogenesis is impaired but also introduce novel therapeutic strategies whereby neurogenesis can be used to restore cognitive function in AD patients.

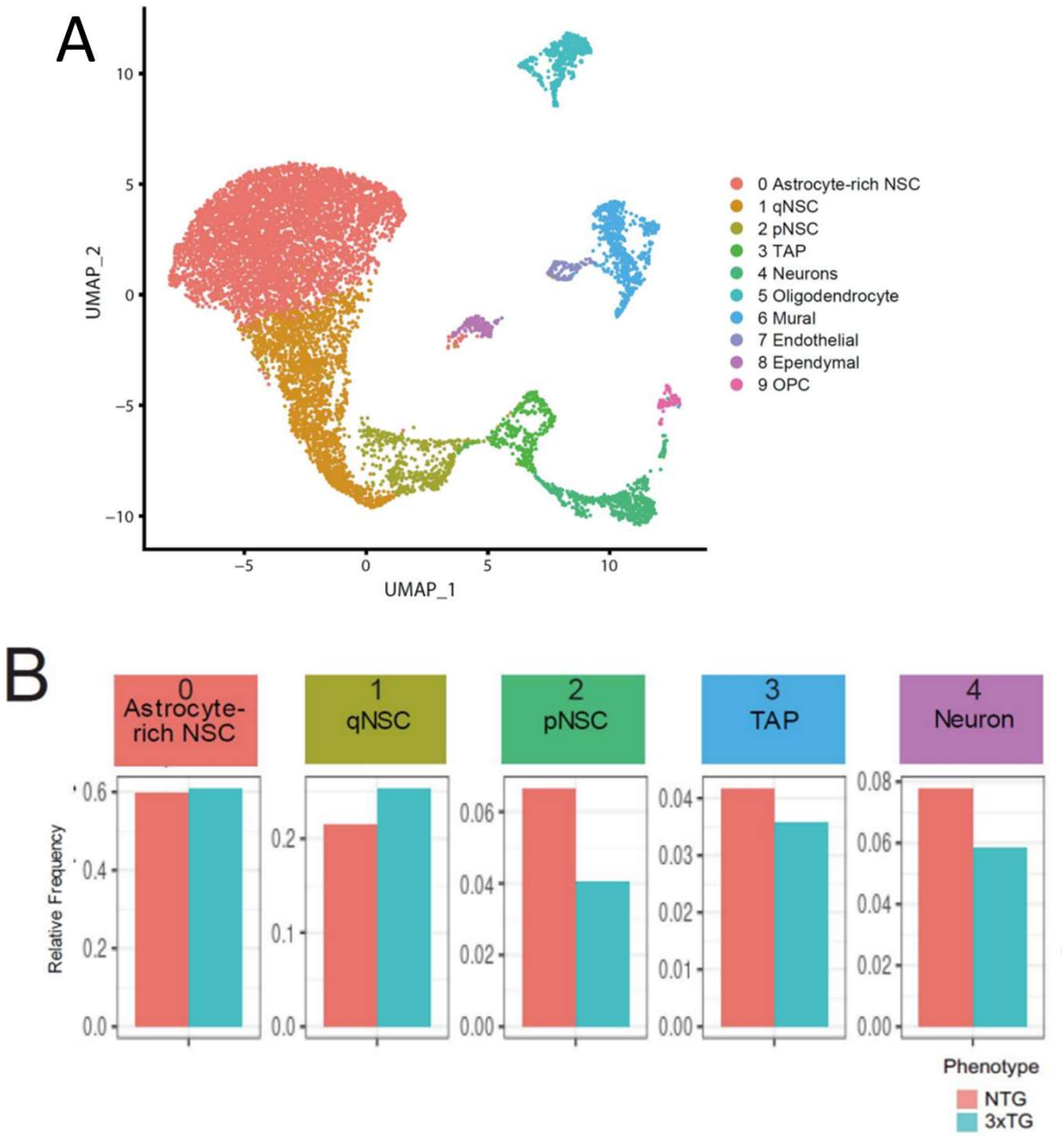

Figure S3: Defects in specific cell populations in 3xTG neural precursor cells identified through scRNA sequencing. Cells from the dentate gyrus (DG) of 3-month-old wildtype and 3xTG Nestin-CreER^{T2}-RosaYFP reporter mice were isolated using FACS before being sent for RNA sequencing at the single cell level (Y.L. and B.F.). (A) Mapping of the individual cell populations were determined using clustering and populations were identified based on the expression of established markers. (B) Comparing proportions of wildtype and 3xTG cells reveals defects in specific cell populations. All data analysis and figure preparation were completed by Y.L. and B.F. (Y. Liu et al., 2022)

HYPOTHESIS AND RESEARCH OBJECTIVES

Hypothesis: Characterization of cellular and molecular defects in 3xTG NSPC function will help to reveal novel therapeutic strategies to restore defects in AD neurogenesis.

Aim #1: To characterize the cellular and molecular defects in neurogenesis and the expression of dysregulated targets in the neurogenic niches in the brains of the 3xTG mouse model of AD.

Aim #2: To characterize the cellular and molecular defects that are intrinsic to 3xTG NSPC functions.

2. MATERIALS AND METHODS

2.1 Mice colony maintenance

All mice were housed at the University of Ottawa Animal Care and Veterinary Services and cared for according to CCAC guidelines. The 3xTG mice used were of the following breeding: B6;129-Tg(APPSwe,tauP301L)1Lfa*Psen1*^{tm1Mpm}/2J (stock no.: 32830-JAX, the Jackson Laboratory). These mice contained mutations in Amyloid Precursor Protein swedish (APP-Swe), Microtubule Associated Protein Tau (MAPT) P301L, and Presilinin-1 (PSEN1) M146V (Oddo et al., 2003). 3xTG mice were routinely genotyped according to the JAX genotyping protocol (genotyping primers can be found in Table S1). The wildtype, or nontransgenic (NTG) counterpart mice used were of standard B6129SF2/J (stock no.: 101045, the Jackson Laboratory) breeding. These two colonies were used for all *in vitro* experiments (Figures 5-7) and immunohistochemistry analysis of molecular targets (Figure 4). 3xTG and NTG mice crossbred onto an inducible Nestin-CreERT2-RosaYFP background (Cicero et al., 2009; Zhu et al., 2012) that were gifted by Dr. Suzanne J Baker at the University of Tennessee Health Science Center allowed for conditional YFP expression in all Nestin-expressing cells. This was used for lineage tracing during the immunohistochemistry analysis of the neurogenic populations (Figures 1-3). In these mice, an estrogen-sensitive Cre-Recombinase protein is contained within Nestin-expressing cells. Cre recombination was induced by administration of tamoxifen (TAM; an estrogen analog) which would result in the cleavage of loxP sites to omit the stop codon precluding YFP expression. As a result, YFP would be expressed in Nestin-positive cells following TAM administration. All mice were euthanized at three months of age with euthanyl

(Sodium Pentobarbital) solution injected intraperitoneally at 120mg/kg in accordance with Animal Care and Veterinary Services of the University of Ottawa protocol #ME-1772.

2.2 TAM treatment and EdU labeling

TAM was prepared by dissolving in 100% ethanol and corn oil to prepare a final concentration of 50 mg/mL. A dose of 200 mg/kg of body weight of mice at two months of age for five consecutive days was administered via oral gavage over the course of 5 successive days (Khacho et al., 2016). TAM treatments took place one month prior to animal sacrifice – at 2 months of age – as this is the reported amount of time it takes for the neurogenic process to be completed in healthy settings, therefore allowing for the lineage tracing of Type I NSCs as they proceed through neurogenesis (Khacho et al., 2016). EdU (5-ethynyl-2'-deoxyuridine) was used to label proliferative cells *in vivo* (Figures 1 and 2). To do so, 10μL/g of body weight of a 5 mg/mL EdU solution (Clickbase, BCK647-IV-IM-M) was administered to the mice via intraperitoneal injection 2 hours before the euthanization of the mice. EdU was subsequently stained according to the instructions provided by the manufacturer.

2.3 Tissue fixation and cryosectioning

To fix the brain tissue, the chest of the mice was opened to expose the heart and a perfusion needle was placed in the left ventricle. The right atrium was snipped, and 0.9% cold saline solution was dispensed through the needle and subsequently followed by administration of cold 4% paraformaldehyde (PFA) solution. The brains were then harvested and placed in 4% PFA solution and rocked at 4°C for 24 hours before then being deposited in 20% sucrose in 1x PBS

and 0.3% sodium azide solution for at least 48 hours. The brains were later cut in half to separate the two hemispheres – one of which was returned into the sucrose solution to remain stored at 4°C. The other hemisphere was placed in -40°C isopentane solution (Thermo Fisher Scientific) for 1 minute before being removed, covered with aluminum foil and stored in dry ice before being cryosectioned. The Leica CM 1850 cryostat was used to section the brain tissue into 30um thick, free-floating serial coronal sections of the SGZ. The temperature of the cryostat was set to -22°C and Tissue-Tek OCT Compound (Sakura Finetek) was used to mount the brain onto the block. The cryosections were serially collected from the beginning to the end of the DG and placed into nine wells of a 24-well plate with each well containing 0.1% sodium azide in 1x PBS solution. The sections were stored at 4°C until ready for immunohistochemistry analysis.

2.4 Immunohistochemistry

Prior to staining, the sections were washed four times in 1x PBS for 5 minutes on the rocker. The sections were then placed in 1mL of PBS-TritonX-100-Tween20 containing primary antibodies (see Table S2 for details of antibodies used) and incubated overnight at 4°C. Sections were subsequently washed in 1xPBS for 3 consecutive times, each lasting 5 minutes and on the rocker. They were then incubated in secondary antibody solutions and DAPI (1:10000, Sigma-Aldrich, D9542-10MG) for 2 hours at room temperature before being washed once again with 1xPBS for 5 minutes on the rocker, three successive times. Finally, the sections were mounted in Immunomount solution (Genetex) onto Superfrost Plus Microscope Slides (Fisherbrand, Thermo Fisher Scientific). Mounted slides were stored at 4°C in the dark until ready for imaging.

2.5 Imaging and quantification of labelled cells

Prepared slides were imaged under the 20x objective of either the GE DeltaVision Elite-Olympus IX-71 microscope or the Zeiss Axioscan.Z1 Slide Scanner. Images were processed through Zen blue or ImageJ to split scenes or generate projections of the 5 z-stacks collected, respectively. Positive cells were quantified as 1-2 cell layer along the SGZ for all markers, with the exception of Prox1, where Prox1-positive cells were counted strictly on the border of the SGZ. This is due to the fact that the entire GZ – which is composed of Prox1+ mature neurons – was stained, thereby making it difficult to establish a 1-2 cell layer. All quantification was completed through the use of the ImageJ software and the sum of all sections was multiplied by 9 to generate an estimation of the total number of positive cells per SGZ (Khacho et al., 2016).

2.6 SVZ Tissue Culture

SVZ tissue culture was performed as described before (Julian et al., 2013; Vanderluit et al., 2004). Brains were harvested from euthanized mice and in ice-cold artificial cerebral spinal fluid (ACSF;pH 7.4 solution of 26 mM NaHCO3, 124 mM NaCl, 5 mM KCl, 2mM CaCl2*2H2O, 1.3 mM MgCl2*6H20, 1X Pen/Strep dissolved in sterile water and sterile filtered). The brains were sectioned coronally with 1 mm spacing into slushy ACSF using the Adult Mouse Brain Slicer Matrix (Zivic, BSMAS005-1) and the SVZ was dissected from these coronal sections with use of the Leica MZ6 stereo microscope. Dissected SVZ tissue was subsequently stored in 500 µL of cold ACSF in an Eppendorf tube until dissections were completed for all samples. Afterwards, the ACSF solution was removed and replaced by 500 µL of warm papain digestion medium (20U/mL Papain Suspension (Worthington, PAP3126) and 1.2 mM EDTA (Invitrogen, 15675-

038) resuspended in DMEM/F12 (Invitrogen Life Technologies, 11320033)). The SVZ tissue was dissociated mechanically using a homogenizer pestle (Thermo Fisher Scientific) until bigger chunks no longer remained. The tissue in digestion media was then incubated for 10 minutes at 37°C while being rotated orbitally. Following this, 500 µL of resuspension media (0.5 mg/mL Dnase1 (Roche, 11284932001) and 10% fetal bovine serum resuspended in DMEM/F12) was added to the sample and triturated 5-10 times with a P1000 micropipette. 750µL of the solution was transferred into a clean 15mL falcon tube. The remaining 250 µL was resuspended in another 1mL of resuspension media and subsequently transferred to the falcon tube. Afterwards, 1.9 mL of resuspension media was added to the falcon tube to achieve a final volume of 3.9 mL before adding 1.1 mL of 90% Percoll in PBS. The falcon tube was then inverted 5 times to mix the solutions and the cell suspension was then spun at 500x*g* for 12.5 minutes at 4°C. The supernatant was removed and the cell pellet was washed twice which consisted of mixing by inversion with 10 mL of DMEM:F12 and centrifugation at 500x*g* for 5 minutes. After removing the supernatant, the cell pellet was resuspended in 500 µL of growth media that consisted of DMEM:F12, PenStrep, 20 ng/mL EGF (Sigma Aldrich, cat. no.: E1257), 20 ng/uL FGF2 (Sigma Aldrich, cat. no.: F0291-25UG), B27 with vitamin A (BD) and 2 µg/mL of heparin (Sigma Aldrich, cat. no.: H3147). The above protocol is adapted for tissue culturing from the methodology described to isolate NSPCs in preparation for direct FACS isolation by Iqbal *et. al.* (M. A. Iqbal et al., 2022) and based on neurosphere culture techniques reported in prior literature (Julian et al., 2013; Vanderluit et al., 2004). The cells were counted and seeded at clonal density of 2 cells/µL in ultra-low attachment 10mm dishes (Thermo Scientific). The cells were grown for one week, undisturbed, in a 37°C incubator, before either being replated for neurosphere quantification or RNA extraction.

2.7 Neurosphere quantification

Following a week of unperturbed growth of the originally isolated NSPCs, the formed neurospheres were resuspended and plated in 6 well plates (BioLite, low attachment). Neurospheres were triturated until a single cell solution which was then seeded at 500 cells/mL in the 24-well plates and left unperturbed for another week. The resulting neurospheres were then quantified using a threshold whereby spheres with a diameter smaller than 50 μm were excluded – as done in prior studies (Julian et al., 2013; Soares et al., 2021). The Axiovert S100 microscope was used to visualize and quantify the neurospheres. The Q-Imaging QICAM microscope camera was used to capture live and static images of neurospheres which could be opened in the Eclipse software program to measure the diameter of the neurospheres when establishing the threshold. This method of neurosphere quantification was optimized across different culturing methods and validated through the use of Rotenone – a vetted assay that results in an inhibition of neurosphere formation (Khacho et al., 2016) (Figure S4).

2.8 RNA extraction and qPCR

Neurospheres were collected into a 15mL falcon tube and spun at 500 xg for 5 mins. The supernatant was removed, and the pellet was resuspended in 500 μL of TriZol reagent (ThermoFisher Scientific, Invitrogen, cat. no.: 15596026 and 15596018). After resuspension with either a P1000 tip (or a needle for particularly large cell pellets), the solution was transferred into RNAse and DNAse free tubes. RNA extraction was performed according to the manufacturer's protocol. After removing potential genomic DNA contaminants with the RNase-free DNase set (QIAGEN, cat. no.: 79254), the RNA samples were then converted into cDNA

through ThermoFisher Scientific SuperScript IV VILO Master Mix (ThermoFisher Scientific, cat. no.: 11756050) as per the manufacturer's protocols. Samples were prepared through 1:10 cDNA dilutions in 10mM Tris-HCl buffer, and the standards prepared through a 1:5 serial dilution. Further details on the primers used can be found in Table S1. The samples were loaded in Fisher Scientific qPCR 96 well plates and run in a BioRad CFX96 qPCR machine. All analysis was done via the accompanying BioRad CFX Maestro analysis software and Excel. Each gene was examined on 3-4 biological replicates. The expression of all genes was calculated using ΔΔCT method (TD Schmittgen, 2008) normalized to the expression of a housekeeping gene Rps26 (40S ribosomal subunit protein S26). The sequence of primers used can be found in Table S1.

2.9 Galactose media

Following a week of unperturbed growth of the originally isolated NSPCs, the formed neurospheres were resuspended and cultured in freshly prepared galactose media. This media was composed of sterile water, HEPES, D-galactose (Sigma-Aldrich, G0750-10G), PenStrep and the same growth factors as the above media (FGF2, EGF, Heparin and B27 without vitamin A). The cells were subsequently plated in 24-well plates for 7 days and quantified as described above.

2.10 Statistical analyses

All statistical analyses were completed through excel. Unpaired and two-tailed student *t-test* was used to determined significance for each experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3. RESULTS

3.1 *In vivo* characterization of NSPC and newborn neuron populations in the DG of 3xTG brains

A preliminary characterization conducted by our lab indicated that defects in neurogenesis are occurring in 3xTG mice at 3 months of age, as there were less Sox2 positive NSPCs and less Dcx positive neuroblasts and immature neurons (Figure S2)(McNicoll et al., in review). Furthermore, a scRNA-seq experiment revealed deficiencies in specific cell populations (Figure S3) (Y. Liu et al., 2022). Characterizing these impairments at the cellular level is key to gaining a deeper understanding of how neurogenesis is impaired in the 3xTG mice.

To further investigate these cellular defects, we performed an immunohistochemistry analysis using a Nestin-CreER^{T2}-RosaYFP reporter line to track NSPCs as they progressed through the different stages of neurogenesis. The Nestin-CreER^{T2}-RosaYFP reporter line is a tamoxifen-inducible line where the expression of YFP is induced in Nestin-expressing cells following treatment of tamoxifen (Cicero et al., 2009; Zhu et al., 2012). This reporter line was cross-bred with the 3xTG and wildtype lines to facilitate study of NSPC populations and capturing the progression of Nestin-expressing cells through the distinct stages of neurogenesis ((Cicero et al., 2009; Oddo et al., 2003; Zhu et al., 2012). The mice were treated with tamoxifen at 2 months of age and brains were harvested and processed one month later. This would result in all early stage NSPCs expressing Nestin (a marker of Type I NSCs) to begin expressing YFP, and this induction of YFP expression 4 weeks prior to the harvest of the brains allowed for the progression from NSC, NPC, TAP and finally to neurons (Figure S4). In healthy controls, it typically takes one month for this entire process of neurogenesis to be completed (Khacho et al., 2016); therefore, by our timepoint of interest at 3 months of age, we would be able to assess how

the Nestin-positive NSCs were progressing through the neurogenic process in the 3xTG compared to the wildtype mice.

First, we set out to determine if there are any defects occurring prior to the differentiation of 3xTG NSCs. Sox2 – a transcription factor involved in the homeostasis of NSCs – has been shown to mark Type I NSCs that retain the ability to self-renew into identical Type I NSCs and differentiate into other neural cell types, as well as proliferating IPCs before its eventual downregulation as the cells commit to differentiation and become postmitotic (Favaro et al., 2009; J. Zhang & Jiao, 2015). Therefore, immunohistochemistry analysis of this cell population can indicate impairments in either the self-renewal, survival and/or proliferation of Type I NSCs and IPCs populations of the 3xTG mice which precede their differentiation.

Serial coronal cut sections of these brains taken from 3-month-old wildtype and 3xTG mice of our reporter line were stained with Sox2 and YFP (which marks the original Nestin+ population when beginning the tamoxifen treatments). Sox2 presented as a clear and bright nuclear signal and was counted along a 2-cell layer underneath the granular zone that was marked by DAPI (J. Zhang & Jiao, 2015). YFP staining was quantified similarly, however the staining was cytoplasmic and therefore often compared with DAPI to distinguish between the cells. Quantification revealed significantly decreased amounts of Sox2+ and YFP+ cells in the 3xTG mice compared to the wildtype mice (Figure 1). There were approximately one third less Sox2+ cells and nearly half as much YFP+ in the 3xTG mice. This suggests that not only are there fewer Type I self-renewing NSCs and/or IPCs in the 3xTG mice, but there is also an overall reduction in the total amount of cells belonging to the neurogenic lineage. Interestingly, we did not detect a difference in the amount of double positive Sox+YFP+ within the YFP+ population, suggesting that while there is a deficiency in the amount of Sox2+ cells, the

proportion within the neurogenic lineage is not affected. While this result alone is not enough to rule out defects in self-renewal, survival or proliferation of the 3xTG Type I NSCs and IPCs, it can indicate that defects causing reduced 3xTG neurogenesis are occurring after this stage.

To identify whether defects were occurring as the NSPCs undergo differentiation, we stained for Dcx – a microtubule-associated protein that is expressed in dividing NPCs until their differentiation into immature neurons, at which point it begins to be downregulated (Klempin et al., 2011; J. Zhang & Jiao, 2015). As a result, Dcx can be used as a marker of differentiating NPCs and neuroblasts, and so our immunohistochemistry analysis of it can reveal potential defects in differentiation, among other things. Due to the fact that Dcx is a microtubule-associated protein, it is expressed as a cytoplasmic stain along the SGZ layer. As observed in our Sox2+ counts, we continued to observe a decrease in YFP+ cells, again confirming an overall reduction of the neurogenic lineage within the 3xTG brains (Figure 2). We also observed a statistical decrease by nearly half of Dcx+ positive cells in 3xTG mice compared to wildtype mice (Figure 2). Additionally, the proportion of double positive Dcx+YFP+ cells within the YFP+ population is decreased in the 3xTG brains, which contrasts the finding with the Sox2 population which was not proportionally affected. This suggests that the 3xTG NPCs are faced with a differentiation defect that impairs their ability to progress through neurogenesis.

Next, to assess potential defects in proliferation, we used EdU – a thymidine analog that integrates into the DNA of cells in the S phase of cell division and fluoresces at 647 nm – which is often used to label proliferative cells (Le Bras, 2021). It was injected into the mice 2.5 hours before tissue collection where actively proliferating cells would incorporate it and later present with a nuclear stain along the SGZ. As such, we were also able to identify decreased amounts of EdU+ or proliferative cells across the total population in 3xTG mice, but also within the Sox2+

and Dcx+ cell populations (Figure 1 and 2). These results suggest that there are impairments in the proliferative capabilities of 3xTG NSPCs.

Taken together, these results indicate that 3xTG neurogenesis is compromised at 3 months of age. Not only are there less 3xTG NSPCs to begin with, but these NSPCs are faced with challenges that hinder their proliferation and differentiation – which could ultimately impair their ability to progress through neurogenesis.

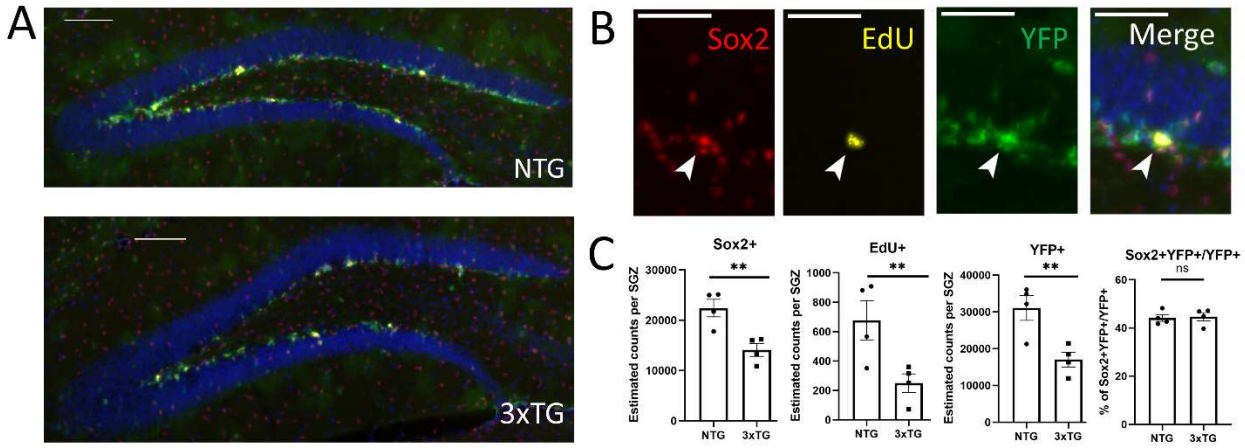

Figure 1: Immunohistochemistry analysis reveals loss of NSPC pool however the Sox2+ population is not proportionally affected. (A) The dentate gyrus (DG) of 3-month-old wildtype (NTG; n=4) and 3xTG Nestin-CreER^{T2}-RosaYFP (n=4) reporter mice was sectioned and stained for Sox2 (red), EdU (yellow) and YFP (green). (B) Arrowheads denote examples of cells positive for all three markers in each respective staining. (C) The DG surface area and counts of Sox2+, EdU+ and YFP+ cells in the subgranular zone (SGZ) were measured blindly and the sum for each sample was multiplied by 9 to determine counts per SGZ. The amount of Sox2+ cells was also analyzed proportionately within the YFP+ population (Sox2 and YFP+/YFP+). Tamoxifen treatments, perfusions and imaging was done by R.H. and M.M. sectioned and stained all sections. Y.L. randomized the sections and all quantification and analysis for the sections stained was completed by A.A. Data represented as mean ± SEM, n = 4. Scale bar indicates 100 μm, unpaired two-tailed Student's t-test was used to determine significance, *$p < 0.05$, ns = not significant, NTG = nontransgenic or wildtype.

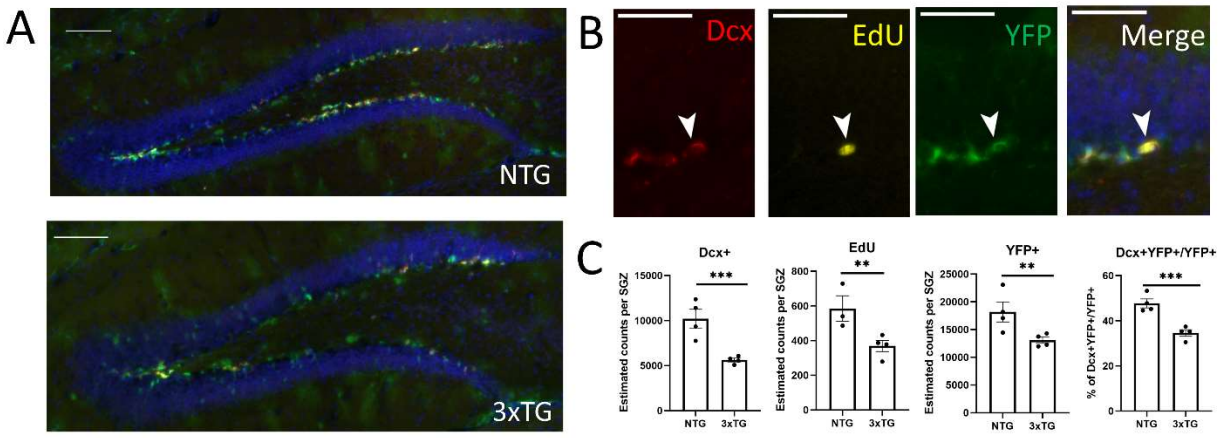

Figure 2: Immunohistochemistry analysis reveals defects in the DCX positive NSPC population occurring in the subgranular zone of three-month-old 3xTG mice. (A) The dentate gyrus (DG) of 3 month old wildtype (NTG; n=4) and 3xTG Nestin-CreER^{T2}-RosaYFP (n=4) reporter mice was sectioned and stained for Dcx (red), EdU (yellow) and YFP (green). (B) Arrowheads denote examples of cells positive for all three markers in each respective staining. (C) The DG surface area and counts of Dcx+, EdU+ and YFP+ cells in the subgranular zone (SGZ) were measured blindly and the sum for each sample was multiplied by 9 to determine counts per SGZ. The amount of Dcx+ cells was also analyzed proportionately within the YFP+ population (Dcx and YFP+/YFP+). Tamoxifen treatments, perfusions and imaging was done by R.H. and M.M. sectioned and stained all sections. Y.L. randomized the sections and all quantification and analysis was completed by J.C. Data represented as mean ± SEM. Scale bar indicates 100 μm, unpaired and two-tailed Student's t-test was used to determine significance, *p < 0.05, ns = not significant, NTG = nontransgenic or wildtype.

3.2 Further immunohistochemistry analysis of distinct cell-specific markers to identify defects in specific neurogenic populations

While these results validated our preliminary findings (Figures S2 and S3) and provided some clarification on the cellular defects of 3xTG neurogenesis, we were unable to pinpoint where exactly this loss was occurring. Sox2 and Dcx expression span several stages of neurogenesis (Figure S1), making it challenging to identify where exactly this loss is occurring. However, using other markers that are more specific to the distinct cell populations can allow for better characterization of these cellular defects. To accomplish this, we assessed the amount of

GFAP, Ascl1, Tbr2 and Prox1 positive cells (Figure 3) (see Figure S1 for more details on expression of markers). These markers were stained and analyzed alongside the YFP marker in order to determine the proportion of cells that were able to progress to the distinct stages.

GFAP, a type III intermediate filament that is expressed in astrocytes and Type I NSCs (Imura et al., 2003; J. Zhang & Jiao, 2015), presented as a cytoplasmic stain that could be seen throughout the brain sample. It was therefore quantified within a 2-cell layer from the border of the granular cell layer as that is where it is specific to Type I NSCs. Interestingly, a difference could not be detected between both the total amount of GFAP+ cells and proportional analysis within the YFP population (GFAP and YFP+/YFP+), despite a statistically significant, steep decrease in the amount of YFP+ cells (Figure 3B).

Prox1 – a protein that regulates granule cell maturation and is used to identify mature neurons – stained the entire GCL, making the quantification of this marker alone particularly challenging despite counting along the border of the GCL (Figure 3C). As a result, no difference could be detected in the total amount of Prox1+ cells. However, the quantification within the YFP+ population proved to be a more effective counting strategy and we observed a significant reduction in the proportion of 3xTG Prox1+ cells was identified (Figure 3D).

Ascl1, sometimes referred to as Mash1, is a transcription factor that is expressed in Type 2a IPCs during their exit from the cell cycle and has been shown to play a key role in regulating the activation of quiescent NSCs (Bottes et al., 2021; E. J. Kim et al., 2011; Sueda et al., 2019; J. Zhang & Jiao, 2015). Without Ascl1, NSCs remain quiescent and no Type 2 IPCs are formed (Sueda et al., 2019). Additionally, progeny of Ascl1+ cells within the DG result in the neuronal lineage exclusively, suggesting a key role in the activation of NSCs during the neurogenic process (E. J. Kim et al., 2011). Quantification of Ascl1, which presented as a bright nuclear

stain that appeared exclusively in the SGZ, revealed a trend of decrease in the total amount of Ascl1+ cells and indications that it could also be proportionally affected as there was also a trend of decreased Ascl1+YFP+/YFP+ in the 3xTG mice (Figure 3F). However, these trends were not found to be statistically significant, despite the total amount of YFP+ cells again being significantly decreased.

Tbr2, another transcription factor that regulates the commitment of Type 2ab to Type 2b IPCs to the neuronal lineage, was also characterized due to the reported essential role that Tbr2+ cells have in the progression throughout the neurogenic lineage (Hodge et al., 2008; J. Zhang & Jiao, 2015). Similarly, Tbr2 also presented as a nuclear stain within the SGZ and was found to be decreased in the 3xTG brains. The total amount of Tbr2+ cells was found to be statistically significantly reduced by approximately half, and despite a trend of decreased proportion within the YFP+ population being observed, it was not significant (Figure 3H).

Taken together, these results suggest that not only is there the loss of the overall NSPC pool in 3xTG mice, but these NSPCs exhibit defects in their progression through neurogenesis, resulting in decreased amounts of neuroblasts, immature neurons and mature neurons.

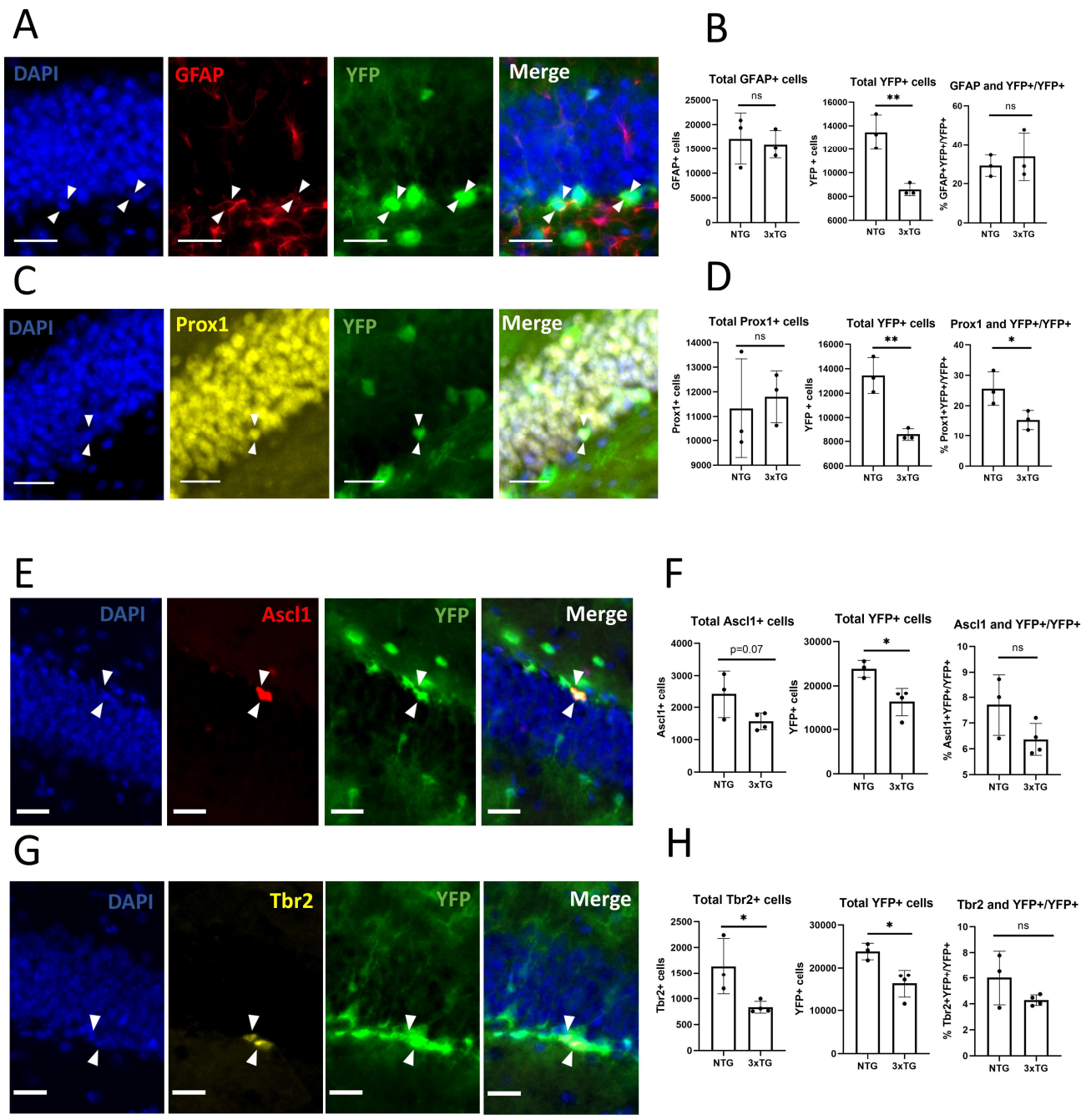

Figure 3: Further immunohistochemistry characterization of Nestin-positive and Nestin-derived cells reveals loss within the Prox1 population. The dentate gyrus (DG) of 3-month-old wildtype (NTG; n=4) and 3xTG Nestin-CreERT2-RosaYFP (n=3) reporter mice was sectioned and stained for the following markers: (A) GFAP (red) and YFP (green), (C) Prox1 (yellow) and YFP (green), (E) Ascl1 (red) and YFP (green) and (F) Tbr2 (yellow) and YFP (green). Arrowheads denote examples of cells positive for both markers in each respective staining. (B, D, F, H) The amount of positive cells of each marker per subgranular zone (SGZ) was quantified and the amount of double positive cells was normalized to the total number of YFP positive

cells. Tamoxifen treatments and perfusions was done by R.H. and sectioning by M.M. A.A. and S.P. stained either A and C or E and G, respectively. All quantification and analysis completed by A.A. Data represented as mean ± SEM. Scale bar indicates 100 μm, Student's t-test was used to determine significance, *p < 0.05, ns = not significant, NTG = nontransgenic or wildtype.

3.3 Immunohistochemistry analysis reveals dysregulation of molecular targets implicating 3xTG neurogenesis

Having characterized cellular defects in the brains of 3xTG mice, we next sought to identify the molecular basis of these deficits. Further analysis of our sequencing data revealed dysregulation of specific molecular targets that could potentially underly the identified cellular defects in 3xTG neurogenesis. These targets were involved in a number of cellular functions necessary for NSPC proliferation, differentiation and survival. In particular, two targets – lysophosphatidic acid receptor 1 (LPAR1) and homeodomain-only protein homeobox (Hopx) – stood out based on, not only their cellular functions but also their dysregulation within our single cell RNA sequencing study (Y. Liu et al., 2022).

LPAR1 is a GPCR which has previously been reported as a functional marker of NPCs and was found to regulate hippocampal precursor cell proliferation through the AKT/MAPK pathway (Matas-Rico et al., 2008; Walker et al., 2016). Studies involving knocking out the LPAR1 pathway have been directly implicated in impaired DG neurogenesis and decreased proliferation of NPCs (Matas-Rico et al., 2008; Walker et al., 2016). In our scRNA-seq data, we observed an upregulation of Lpar1 primarily in the primed NSC population (primed for activation) in the NTG sample, however this was completely absent in the 3xTG sample (Y. Liu et al., 2022). Additionally, through analysis of RNA dynamics, we observed repressed Lpar1 induction in the 3xTG primed NSC population which aligned with an abnormal return to

quiescence, implicating it in the impaired progression through neurogenesis (Liu et al., 2022). Given LPAR1's roles in NPC proliferation and DG neurogenesis along with our single cell findings, it was selected for further study.

Hopx is a homeobox protein that is expressed in Type 1 radial glial like cells in the DG where it has been shown to be involved in regulating hippocampal neurogenesis through the Notch signalling pathway (Li et al., 2015). When Hopx is knocked out in animals, the amount of Sox2-positive and quiescent NSCs are decreased, along with decreased Notch signalling, whereas BrdU+ and Dcx+ cells are increased, suggesting Hopx uses the Notch pathway to maintain quiescence of NSCs (Li et al., 2015). According to our sequencing data, Hopx expression is decreased in the quiescent NSC population, which also presented with dysregulation of the Notch1 pathway (Liu et al., 2022) – and given its cellular functions, especially in the context of neurogenesis, it is also an interesting target for further characterization.

Attempts to validate the dysregulation of these targets through qPCR analysis of RNA taken from NSPCs isolated via FACS of NSPCs from wildtype mice were unsuccessful, which is likely due to the challenges of attempting to validate single cell RNA-seq targets with a heterogenous pool of distinct cell populations. As a result, we attempted to characterize the *in vivo* expression of these targets via immunohistochemistry analysis in 3xTG and NTG brains. Sox2 was used as a co-stain for both as it is reportedly co-expressed with both Hopx and LPAR1 (Li et al., 2015; Walker et al., 2016; J. Zhang & Jiao, 2015) and marks the Type 1/2a population that Hopx and LPAR1 are seemingly impaired in. Additionally, this could serve as validation of the previous defect in the Sox2 population as observed in Figure 1.

The total amount of Hopx+, which was stained as a bright nuclear stain, was found to be statistically decreased by nearly half in 3xTG mice compared to the wildtype (Figure 4C). Similar to Figure 1, we continue to see a significant decrease in the amount of Sox2+ and YFP+ cells (Figure 4B, D). Interestingly, we did not find a proportional decrease of the Hopx+ cells within the YFP+ population (Figure 4E), thereby confirming our previous findings that the type I quiescent NSC population is not proportionally affected.

Lpar1 – which is a cell surface receptor – could be found strictly along the SGZ and appeared as a stain of the outer border of the cell. Similar to Hopx, we observed a statistically significant decrease of the total amount of Lpar1 positive progenitor cells (Figure 4H), along with a decrease in the amount of Sox2 positive cells (Figure 4G) and double positive cells (Figure I), in the DG of 3xTG mice. Additionally, to demonstrate that this reduction in Lpar1 positive progenitors is not solely due to the reduced pool of NSPCs previously identified (Figure 1-2), we identified a decrease in Lpar1 positive progenitors within the Sox2 population (Figure 4J). This reduction suggests that there is a loss of the activated NSC population, which LPAR1 function may underly.

Taken together, this not only validated our sequencing findings that Hopx+ and Lpar+ progenitors, which generally label the quiescent and primed NSC populations respectively, are decreased in the DG of 3xTG brains, but suggests that they may be interesting targets involved with these cellular defects.

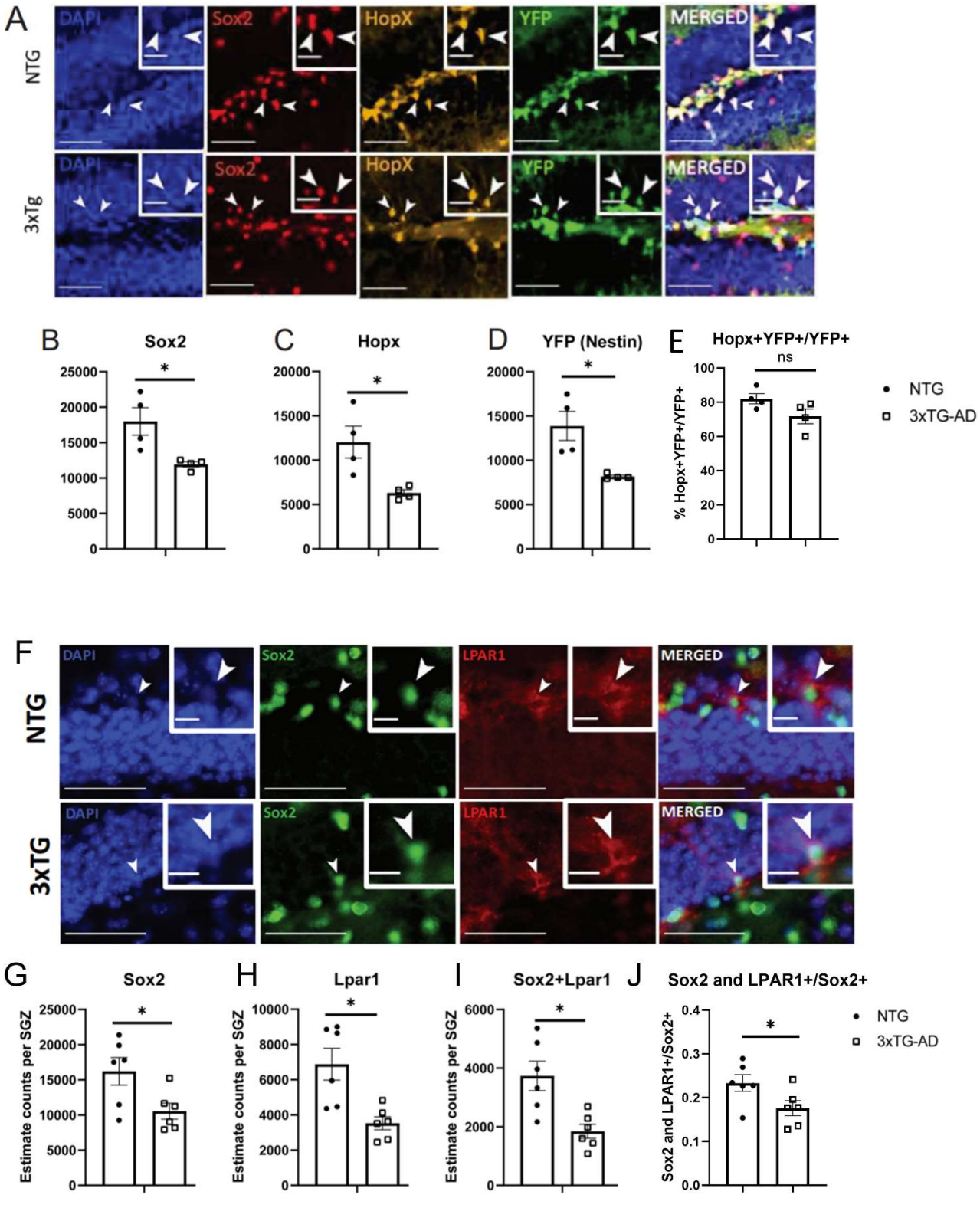
A
NTG
3xTg
DAPI
Sox2
HopX
YFP
MERGED
B
Sox2
C
Hopx
D
YFP (Nestin)
E
Hopx+YFP+/YFP+
ns
% Hopx+YFP+/YFP+
NTG
3xTG-AD
F
NTG
3xTG
DAPI
Sox2
LPAR1
MERGED
G
Sox2
H
Lpar1
I
Sox2+Lpar1
J
Sox2 and LPAR1+/Sox2+
Estimate counts per SGZ
Sox2 and LPAR1+/Sox2+
NTG
3xTG-AD

Figure 4: Immunohistochemistry analysis indicates decreased number of LPAR1 and Hopx positive progenitor cells in the DG of 3xTG mice. The dentate gyrus (DG) of 3-month-old wildtype (NTG; n=4) and 3xTG Nestin-CreERT2-RosaYFP (n=4) reporter mice was sectioned and stained for Sox2 (red), Hopx (yellow) and YFP (green) positive cells (A) and later quantified (B-D). The DG of 3-month-old wildtype (NTG, n=6) and 3xTG mice (n=6) was sectioned and stained for Sox2 (green) and LPAR1(red) positive cells (E) and later quantified (F-H). Arrowheads denote cells that are triple positive for Sox2, Hopx and YFP (A) or Sox and LPAR1 (E). Sectioning, staining and imaging of the brain slices was performed by M.M. Image quantification was performed blindly and later analyzed by M.M. for Hopx data and A.A. for Lpar1 data. Data represented as mean ± SEM. Student's t-test was used to determine statistical significance, *p<0.05, **p<0.01, ns = not significant. Scale bar in (A) and (E) indicates 100 μm.

3.4 Neurosphere assays reveal an *in vitro* defect in 3xTG NSPCs

Having validated the key cellular defects and molecular targets identified through our sequencing results *in vivo*, we next aimed to characterize the intrinsic, cell-autonomous defects of 3xTG NSPCs. To what extent are the observed *in vivo* cellular and molecular impairments of 3xTG neurogenesis due to extrinsic, niche factors? Could they potentially be the manifestation of some inherent defects of 3xTG NSPC? To answer these questions, we first needed to establish an *in vitro* system which would allow us to characterize the NSPCs cellular and molecular functions outside of the influence of extrinsic factors.

To do so, we employed the neurosphere assay which has previously been used as a tool to study intrinsic NSPC biology in a controlled environment (Gil-Perotín et al., 2013; Marshall et al., 2007; Soares et al., 2021). In this assay, NSPCs are isolated from the either the SGZ or SVZ of the brains and then cultured in serum-free stem cell media containing mitogenic growth factors like EGF and FGF and left to grow unperturbed for one week. This resulted in the formation of spherical structures called neurospheres – that are a heterogenous composition of stem, progenitor and differentiated cells. Each neurosphere arises from a single NSPC that has self-renewal and proliferative capabilities; as a result, the number of neurospheres formed is

indicative of NSPC pool expansion and has also been used to measure self-renewal (Dadwal et al., 2015; Fatt et al., 2015; Gil-Perotín et al., 2013; Marshall et al., 2007; Soares et al., 2021). The size that neurospheres can grow to has been used as an estimator of NSPC proliferation rate, however many have cautioned against this assumption as size measurements can be skewed due to the tendency of neurospheres to aggregate and fuse (Gil-Perotín et al., 2013; Marshall et al., 2007; Soares et al., 2021). Although SGZ culture has been done in the past, there is generally a smaller cell yield which have a low propensity for growth, making it challenging for experimental use (Soares et al., 2021), therefore SVZ culture was the preferred method to go.

There are some challenges associated with using the neurosphere assay – namely, how sensitive it is to methodological techniques which can lead to variable and unreliable results (Pastrana et al., 2011). Due to this, a great deal of effort was put in to optimize tissue culture protocols and rotenone treatment was used to validate the effectiveness of the assay in our hands. Rotenone, a drug that inhibits the electron transport chain, has been established in some prior studies as having a toxic effect on the growth of neurospheres (Khacho et al., 2016). As a result, we grew neurospheres with or without the presence of rotenone and observed a dose-dependent decrease in neurosphere formation (Figure S5), thereby giving us confidence that our assay would be capable of capturing some differences between cultured wildtype and 3xTG NSPCs, if any.

NSPCs were isolated from dissected SVZ tissue of wildtype and 3xTG mice and grown for 1 week in serum-free, stem cell media enriched in growth factors until they formed neurospheres (termed passage 0 or P0). These neurospheres were collected, dissociated, and passaged again for 1 week (referred to as passage 1 or P1), before the subsequently formed neurospheres were later counted. We opted to quantify neurospheres from P1 as the cells are

more uniformed compared to P0 and have optimal capacity for growth compared to both P0 and later passages.

This quantification revealed a decrease in the amount of neurospheres formed from 3xTG NSPCs by approximately half when compared to those from wildtype samples (Figure 5A and C). This suggests that the expansion of the NSPC pool is impaired in 3xTG mice and may also indicate some deficiencies in self-renewal capabilities. We did not qualitatively observed any major differences of neurosphere size (Figure 5C).

Alzheimer's disease reportedly has a differential effect on the basis of sex – with females being more likely to develop AD, particularly early-onset AD (Ferretti et al., 2018). This prompted us to compare the growth of neurospheres between the two sexes in order to assess any sex-based defects. However, there was no statistically significant difference observed between sexes with respect to the amount of neurospheres formed (Figure 5B) suggesting that there are no sex-based defects we could characterize.

In sum, since 3xTG NSPCs yielded fewer neurospheres, we were able to identify a sustained reduction of the expansion of the 3xTG NSPCs pool in 3xTG brains, potentially due to impaired self-renewal abilities.

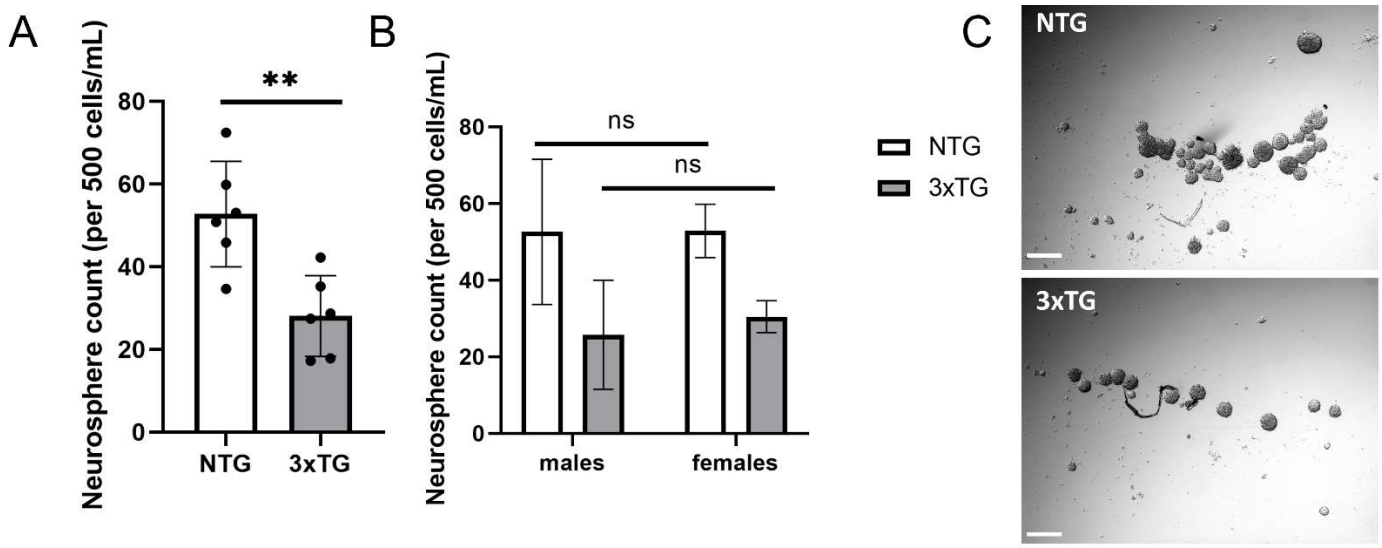

Figure 5: Decreased neurosphere formation from NSPCs cultured from the SVZ of 3xTG mice. NSPCs were isolated from the SVZ of wildtype (NTG; n=6) and 3xTG (n=6) mice brains and cultured for one week in stem cell media to grow neurospheres. These neurospheres were then dissociated and replated for one week before the subsequently formed neurospheres were quantified with respect to genotype (A, n=6) and sex (n=3) (B) based on the number of spheres formed from 500 cells/mL. Representative images of NTG and 3xTG neurospheres is shown (C). Data represented as mean ± SEM. Unpaired, two-tailed Student's t-test (A) and two-way ANOVA (B) was used to determine statistical significance, *p<0.05, **p<0.01, ns = not significant. Scale bar in (C) and indicates 100 μm.

3.5 qPCR analysis reveals molecular defects in cultured 3xTG NSPCs

To better understand the results from our *in vitro* assay and further interpret its biological relevance, we next sought to characterize the molecular phenotype of cultured 3xTG NSPCs. This would allow for the discovery of dysregulated pathways that can explain the cellular phenotype we observe and will permit for better comparison of our *in vitro* system to the defects we have characterized *in vivo.*

Through our neurosphere assay, we observed fewer neurospheres forming from 3xTG NSPCs compared to wildtype NSPCs – indicating impaired expansion of the NSPC pool (Figure 5). Our sequencing results revealed many potential molecular pathways that could explain these

results. Particularly, several mitochondrial and lipid metabolic pathways were affected, where mitochondrial genes along with lipid metabolism was found to be upregulated in 3xTG (Y. Liu et al., 2022). These pathways play key roles in supplying the energy demands of NSPC functions during the progression through neurogenesis. Since this upregulation was found to be occurring particularly in the transition between NSCs primed for activation and TAP (Y. Liu et al., 2022), we reasoned that it could be an attempt to compensate for an energetic insufficiency. Additionally, bulk RNA sequencing analysis revealed dysregulation of redox genes (McNicoll et al., in revision), which, when combined with an upregulation of mitochondrial metabolism, can lead to oxidative stress conditions. These molecular defects – dysregulated metabolism and oxidative stress – could account for impairments of the NSPC expansion that we observed through our neurosphere assay.

Based on our findings, we selected 4 upregulated molecular targets for further characterization: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), Peroxisome proliferator-activated receptor gamma (PPARγ), carnitine palmitoyltransferase 1 (Cpt1α) and Superoxide Dismutase 3 (SOD3). PGC1α – a master regulator of mitochondrial pathways and biogenesis – whose expression is induced in response to exercise and has been shown to enrich mitochondrial performance in many high energy demand tissues such as muscles or brain (Halling & Pilegaard, 2020; Handschin & Spiegelman, 2008; Jodeiri Farshbaf et al., 2016; Villapol, 2018). PGC1α is a transcription factor which goes on to regulate the expression of other genes through binding with and activating other transcription factors like the PPAR and NRF families, to upregulate mitochondrial OXPHOS, mitochondrial biogenesis genes and metabolism of lipids, carbohydrates and proteins (Halling & Pilegaard, 2020; Handschin & Spiegelman, 2008; Jodeiri Farshbaf et al., 2016; Nikolić et al., 2011;

Villapol, 2018). Through PPARγ, PGC1α goes on to upregulate lipid and carbohydrate metabolism as well as upregulating mitochondrial genes (Corona & Duchen, 2016). CPT1α, an enzyme required for fatty acid oxidation (Knobloch et al., 2017) and SOD3, an enzyme that attempts to reduce oxidation in response to oxidative stress conditions (Wert et al., 2018), were also selected.

Characterization of these genes via qPCR revealed an overexpression in PGC1α, PPARΓ, Cpt1α and SOD3 in the 3xTG mice (Figure 7). PGC1α was upregulated in the 3xTG mice along with PPARγ relative to the housekeeping gene RPS26, suggesting induction of lipid metabolic pathways and increased expression of mitochondrial biogenesis and OXPHOS genes. The increased expression of Cpt1α also supports increased lipid metabolism within 3xTG NSPCs – specifically, of fatty acid oxidation. Finally, SOD3 upregulation indicates increased antioxidation within 3xTG NSPCs, which can point towards oxidative stress conditions. These results suggest that there is a dysregulation of some metabolic and oxidative stress pathways at the molecular level within 3xTG NSPCs, which can explain the cellular defect observed through the neurosphere assay.

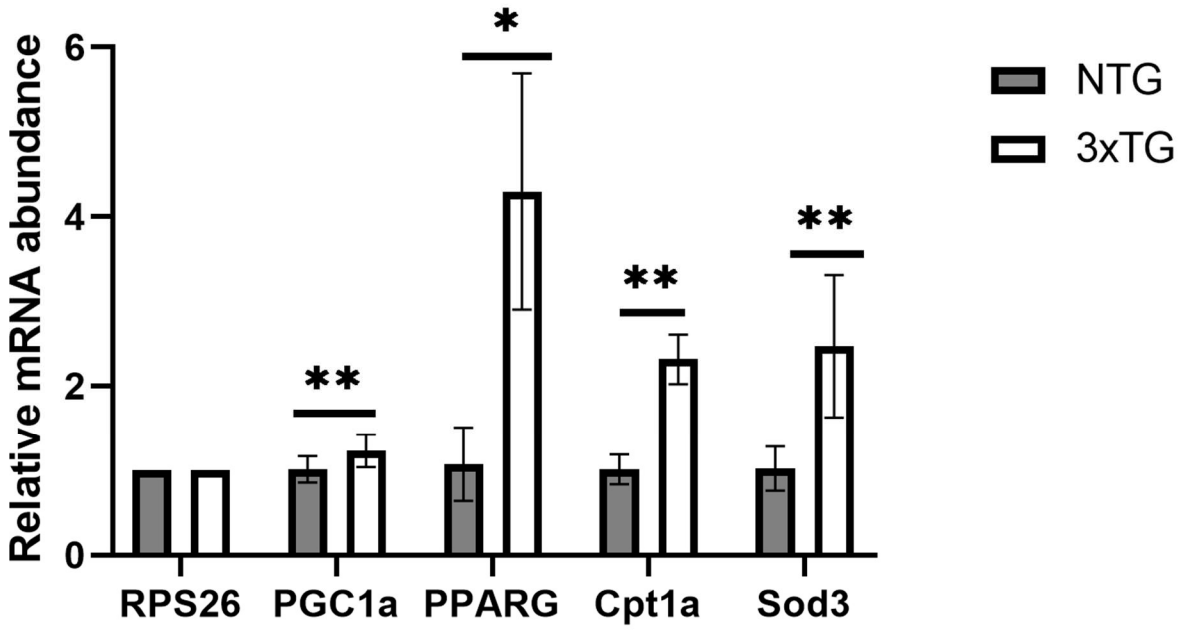

Figure 6: qPCR analysis reveals dysregulation of molecular pathways in 3xTG NSPCs. NSPCs were isolated from the SVZ of wildtype (NTG; n=4) and 3xTG (n=4) mice brains and cultured for one week in stem cell media to grow neurospheres. These neurospheres were then dissociated and replated for one week in stem cell media before the subsequently formed neurospheres were collected and treated with TriZol to extract RNA which was then converted into cDNA. Expression level of key genes –PGC1α, PPARΓ, Cpt1α and Sod3 –relative to the expression of housekeeping gene, Rps26 were analyzed via qPCR, ± SEM. Unpaired, two-tailed student's t-test was used to determine statistical significance, *p<0.05, **p<0.01, ns = not significant. A.A contributed to the *in vitro* culture while Y.L. completed the qPCR analysis.

3.5 Improved neurosphere formation of 3xTG NSPCs observed when culturing in low glucose media

The results from our molecular characterization – both through sequencing (Y. Liu et al., 2022) and our in vitro analysis – demonstrate dysregulation in lipid and mitochondrial metabolism as well as increase in antioxidant genes. An upregulation of mitochondrial metabolism in 3xTG NSPCs was particularly striking as mitochondrial respiration is required for

the production of ATP to supply the energy required for NSPCs to proliferate and differentiate (Khacho et al., 2016, 2019). As a result, the switch from glycolysis to OXPHOS respiration has been used to distinguish between NSC and NPC – where quiescent NSCs depend more on glycolysis and NPCs rely more on OXPHOS to supply their needs (Khacho et al., 2016, 2019). This upregulation can be seen as an attempt to meet energy requirements, especially considering increased lipid metabolism, where lipids are being used as an alternative energy source.

However, an aberrant increase in mitochondrial respiration can result in oxidative stress conditions which can interfere with NSPCs function (Khacho et al., 2019; Markesbery, 1999; Uttara et al., 2009). The upregulation of SOD3 and other redox genes could be a compensatory response to oxidative stress condition – also pointing towards the presence of oxidative stress in 3xTG NSPCs which could have impaired their growth into neurospheres (Singh & Bhat, 2012; Wert et al., 2018). Galactose is often used to model an aged setting by removing glucose – the substrate for glycolysis – thereby requiring the NSPCs to rely on mitochondrial respiration as their main energy source and inducing oxidative stress (Aguer et al., 2011). Therefore, we reasoned that, by assessing the ability of wildtype and 3xTG NSPCs to grow into neurospheres in this low glucose and oxidative stress environment, we could then identify if 3xTG NSPCs were indeed adapted to oxidative stress conditions.

Interestingly, we found that the trend in normal TC conditions (Figure 5) was reversed when culturing the NSPCs in 5 mM galactose conditions – where there were larger and more neurospheres from the 3xTG samples compared to the wildtype samples (Figure 7). Despite seeing reduced neurosphere formation in galactose compared to normal conditions (Figure 6), there were more than double as much neurospheres grown from the 3xTG NSPCs compared to the wildtype NSPCs (Figure 7A). Additionally, there was a nearly 10-fold reduction in the

wildtype neurosphere formation in galactose conditions compared to the growth in normal conditions (Figure 6A), whereas the 3xTG neurosphere growth declined by only 1.5-fold. This suggests that, while both wildtype and 3xTG NSPCs had impaired growth in oxidative stress conditions, the 3xTG neurospheres were able to respond better to these environmental challenges. We also qualitatively observed a marked decrease in the sizes of the neurospheres (Figure 7B) formed compared to when grown in normal tissue culture conditions (Figure 5C). However the 3xTG neurospheres did appear slightly bigger than the wildtype neurospheres in the galactose conditions (Figure 7B), once again suggesting that 3xTG neurospheres were better at adapting to these oxidative stress conditions.

In sum, 3xTG NSPCs were better situated in a glycolysis-deprived and oxidative stress environment. This may be the result of the upregulated metabolic and redox pathways in 3xTG NSPCs seen in both our sequencing data and our qPCR analysis. Together, these results suggest that 3xTG NSPCs may be under constant oxidative stress, which explains their adaptation to those conditions. Taken together, we were able to characterize some intrinsic cellular defects of 3xTG neurogenesis and were successful in our attempts to establish an *in vitro* system with a meaningful biological readout.

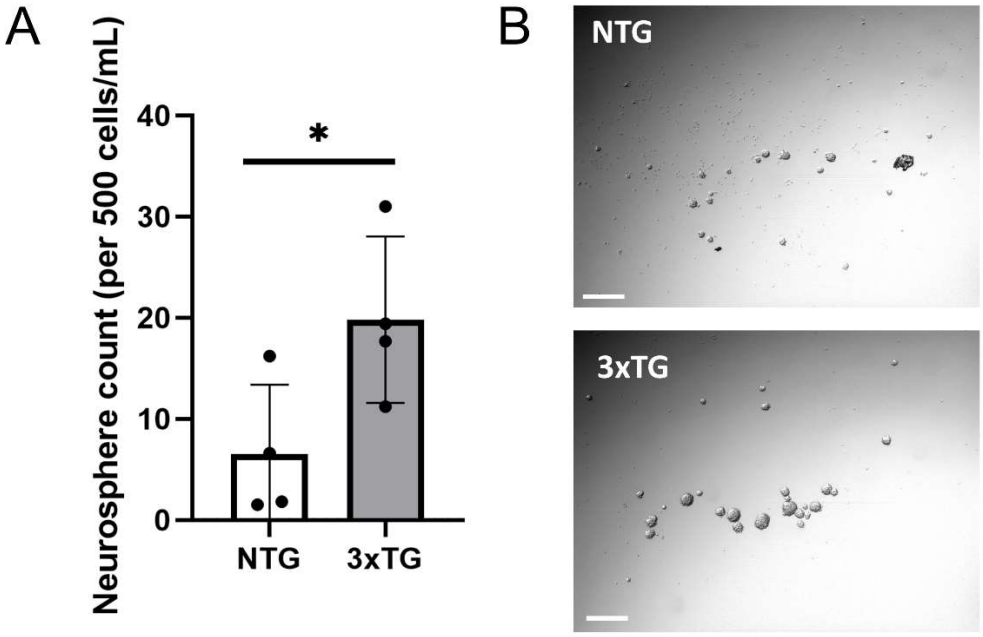

Figure 7: Increased 3xTG neurosphere formation relative to wildtype when cultured in galactose-based media. NSPCs were isolated from the SVZ of wildtype (NTG; n=4) and 3xTG (n=4) mice brains and cultured for one week in stem cell media to grow neurospheres. These neurospheres were then dissociated and replated for one week in galactose-based media before the subsequently formed neurospheres were quantified based on the number of spheres formed from 500 cells/mL (A). Representative images of NTG and 3xTG neurospheres is shown (B). Unpaired, two-tailed Student's t-test was used to determine statistical significance, *$p<0.05$, **$p<0.01$, ns = not significant. Scale bar in (B) and indicates 100 μm.

4. DISCUSSION

4.1 Summary of results

Previous studies provided evidence that not only is adult hippocampal neurogenesis functionally relevant throughout the human lifespan, but it is also impaired in AD patients (Briley et al., 2016; Criado-Marrero et al., 2020; Hamilton & Fernandes, 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019; Vivar, 2015). This, combined with experiments involving AD animal models (Criado-Marrero et al., 2020; Hamilton et al., 2010, 2015; Hamilton & Fernandes, 2018; Rodríguez et al., 2008a), supports the notion that impaired neurogenesis may contribute to the pathogenesis of AD. Additionally, identifying the underlying causes of these defects of neurogenesis in an AD setting can allow for the development of novel therapeutic strategies to restore neurogenesis and, as a result, cognitive function in AD patients.

Defects of neurogenesis have previously been identified in the 3xTG mouse model (Criado-Marrero et al., 2020; Hamilton et al., 2010, 2015; Hamilton & Fernandes, 2018; Rodríguez et al., 2008a) – which was used throughout this study – and subsequent experiments by our group have contributed to these findings (Y. Liu et al., 2022, McNicoll et al. in revision). The purpose of this thesis was to further characterize this loss and identify cellular and molecular deficits that impair 3xTG neurogenesis. Through immunohistochemistry analyses of markers of neurogenic cellular populations and molecular markers, as well as the use of the *in vitro* neurosphere assay, we were successful in characterizing cellular and molecular defects in 3xTG neurogenesis. The findings of this study can be summarized as follows:

1. Immunohistochemistry analysis reveals that there are less Sox2+, Dcx+, EdU+ and YFP+ (or Nestin+) cells in the SGZ of 3xTG mice, suggesting that there are fewer NSPCS in 3xTG brains;
2. However, while there is a similar proportion of Sox2+ cells within the YFP+ population in 3xTG brains, there is a significant reduction of the proportion of Dcx+ cells;
3. Further characterization with markers that are more specific to the distinct NSPC populations within the YFP+ population indicate that the Prox1/mature neuron population is also proportionally affected;
4. Analysis of molecular markers via immunohistochemistry reveal that there are fewer LPAR1+ and Hopx+ progenitor cells within the SGZ of 3xTG mice;
5. Cultured NSPCs collected from the SVZ of 3xTG mice form fewer neurospheres when compared to the wildtype counterpart;
6. A qPCR analysis reveals an upregulation of PGC1α, PPARγ, Cpt1α and Sod3 in 3xTG cultured NSPCs;
7. When cultured in galactose media – which triggers a switch to OXPHOS and simulates an oxidative stress and aged environment – NSPCs from 3xTG mice formed more and larger neurospheres relative to the NSPCs from wildtype mice

Taken together, these results suggest that indeed there are cellular and molecular defects in 3xTG neurogenesis occurring, particularly in functions related to the progression

through the neurogenic process, and that some of the defects are cell-autonomous and inherent to 3xTG NSPC biology. Over the course of this study, we were able to characterize these defects in more detail which have contributed to our understanding of neurogenesis and its processes in an AD setting while also introducing novel therapeutic targets for future study. This understanding is paramount in advancing the field and realizing the true therapeutic potential of stimulating neurogenesis in AD.

4.2 *In vivo* characterization of defects in differentiation and proliferation within 3xTG neurogenic populations

Previous immunohistochemistry analyses involving 3xTG mice, reveal loss of Sox2, Dcx and EdU cells in the neurogenic niches of 3xTG brains (Figure S2) (McNicoll et al., in revision). We subsequently followed this up with a more in-depth characterization using our Nestin CreYFP reporter line whereby YFP expression would be induced in Nestin positive cells following tamoxifen administration. YFP expression was induced starting at 2 months of age and brains were harvested one month later, giving sufficient time for the Nestin positive cells to progress through neurogenesis (Figure S4). With the use of neurogenic markers, we could trace the lineage of the original Nestin positive cells and compare the progression of the progeny.

We first assessed the amount of Sox2, Dcx, EdU and YFP cells – which were all lower in the 3xTG SGZ (Figure 1 and 2). EdU, a nucleotide that is incorporated to the replicated DNA, is used to label actively proliferating cells (Le Bras, 2021). A lower amount of EdU positive cells indicates a deficiency in proliferation within the 3xTG mice. A decrease in the total amount of YFP+ cells indicates that there is a smaller pool of NSPCs in 3xTG brains compared to wildtype.

We quantified the amount of Sox2+ cells, a marker that is expressed in self-renewing early stage NSCs and proliferating TAP (Favaro et al., 2009; J. Zhang & Jiao, 2015), to assess whether this loss was occurring prior to the differentiation process. Similarly, Dcx+ cells, a marker that stains neuroblasts and immature neurons(Klempin et al., 2011; J. Zhang & Jiao, 2015), were measured to determine defects in the ability of 3xTG NSPCs to differentiate. We observed a decrease in the total amount of both of these progenitors, but given that there was an overall decrease in the amount of YFP+ cells, it was possible that this decrease was simply the result of the 3xTG mice having a smaller pool of NSPCs to begin with. To verify this, we counted the amount of double positive cells (Sox2+YFP+ or Dcx+YFP+) and divided it over the total amount of YFP+, which would allow us to compare the relative proportions within the neurogenic (YFP) population. While we continued to see a decrease of the proportion of Dcx+ cells in the 3xTG mice, the Sox2+ population was not proportionally affected. These findings suggests that, while there are less 3xTG NSPCs to begin with, fewer of the 3xTG NSPCs are able to progress through neurogenesis, potentially due to defects in proliferation and differentiation.

Similar results were also found in our single cell RNA-seq analyses where the proportion of quiescent NSCs appear to be slightly increased in the 3xTG sample whereas there were fewer of the NSCs primed for activation and less TAP and neurons (Figure S3)(Y. Liu et al., 2022). This proportional increase in quiescent NSCs and decrease of downstream cell types was interpreted as a defect in NSC activation in 3xTG mice (Liu et al., 2022). While the immunohistochemistry analysis lend support to these results, the identification of defects in proliferation and differentiation provides an idea of what this activation defect could comprise of. This is also in accordance with past findings that reported defects in the 3xTG neurogenesis in the SGZ (Hamilton et al., 2010, 2015; Rodríguez et al., 2008a). While we did not detect a

proportional difference in the Sox2+ population within the DG, it is important to note that we did not assess self-renewal or survival functions within these populations, therefore we cannot fully reject the idea that there are defects within this population as well. A more effective way of studying this would be through lineage tracing of NSCs with 3xTG mice which can be used to confirm this.

4.3 Further *in vivo* characterization reveals loss within specific cell populations

As the expression of Sox2 and Dcx span several stages, it is difficult to pinpoint exactly which population this loss is occurring, precluding further interpretation of our immunohistochemistry analysis finding. Our scRNA-seq analyses seems to point towards an activation defect (Figure S3)(Liu et al., 2022), so potential issues involving activation could result in a loss of NSCs primed for activation, TAP and mature neurons. Alternatively, this could be the result of a survival issue – perhaps the newborn neurons promptly die before functionally incorporating into the hippocampal network. Performing a more in-depth characterization of the neurogenic population, we completed a more specific characterization using 4 markers – GFAP (Type I NSCs), Ascl1 (Type 2a IPCs), Tbr2(Type 2b/c IPC) and Prox1(mature neurons) – within the YFP population (Figure 3). Due to the specificity of which stage these markers are expressed in, their analysis would allow for better distinction of the distinct NSPC populations within the YFP pool and allow us to identify in which population the loss was occurring.

While we continued to see a significant loss of YFP+ cells, only Tbr2+ cells were significantly decreased in 3xTG mice. The amount of Ascl1+ cells had a trend of being lower in 3xTG mice, but this trend was narrowly deemed insignificant (with a p-value of 0.07). No

statistical significance was observed in the amount of GFAP+ and Prox1+ cells. Since we continue to see a decline in the YFP+ population signifying a decrease in the entire NSPC pool in 3xTG brains, we expected to see significantly less amounts of all cell types. Large error bars within the wildtype sample could contribute to the lack of significance with Ascl1 and GFAP, so an extra biological replicate could be useful to solidify the trend for Ascl1 counts. Additionally, since Prox1 stained the entire granular cell layer, it was challenging to establish an effective counting strategy when establishing a threshold, making any analysis of the total amount of Prox1 positive cells unreliable. Therefore, analysis of this marker in the context of YFP would be far more informative.

However, when completing the analysis within the YFP population (Prox1+YFP+/YFP+), we observe a significant decrease of the Prox1 population in 3xTG mice. In other words, fewer of the Nestin positive cells were successful in making it to the final stages of neurogenesis. No other significant decrease was observed with respect to the relative proportion of GFAP+, Ascl1+ and Tbr2+ cells – however Ascl1 and Tbr2 did present with a trend towards with a decrease in 3xTG brains. Compared to our scRNA-seq analyses (Liu et al., 2022) and prior immunohistochemistry (Figures 1 and 2), it is plausible that there is a similar proportion of Type I NSCs (GFAP+), however we did anticipate a decline in our Type 2a IPC (Ascl1+) and Type 2ab/2b IPC (Tbr2+) populations within the 3xTG and wildtype neurogenic pools – especially due to their expression in the primed for activation NSC and TAP population, which we observed in our scRNA-seq analyses (Figure S3). Our failure to detect any significant change in these populations could be the result of large error bars which could be improved with an additional biological replicate.

Collectively, these results suggest that a loss within the Prox1 population is occurring in 3xTG mice. It is difficult, however, to assess where this defect begins and whether it is upstream to where this loss occurring. Perhaps there is a defect occurring in one of the early stages that persists throughout before ultimately contributing to the loss in mature neurons. Inconclusive findings make it challenging to determine if this is the case in the IPC populations – so perhaps the addition of another biological replicate to eliminate discrepancy and consolidate that trend would help. Alternatively, we could explore the use of other markers of the IPC population to confirm this. Additionally, it would be interesting to see if there is a loss occurring in one of the other stages, such as the NeuN+, immature neuron population. Coupling this analysis with the characterization of molecular pathways can shed light on what exactly is contributing to these defects.

4.4 Molecular dysregulation of LPAR1 and Hopx characterized *in vivo*

To make more sense of our findings and add context to our *in vivo* cellular defects of 3xTG neurogenesis, we turned to our sequencing data. Through our sequencing experiments, we were able to map out the distinct cell populations and analyze the expression of different genes to compare differences between the 3xTG and wildtype mice. Among the several dysregulated genes, we remarked that lysophoshphatidic acid receptor 1 (LPAR1) and homeodomain-only protein homeobox (Hopx) were both downregulated in the 3xTG mice.

Lpar1 is a GPCR that is capable of regulating the AKT/MAPT pathway. As such, it is involved in mediating proliferation and impairments in neurogenesis (Matas-Rico et al., 2008; Walker et al., 2016). Hopx is a protein involved with the regulation of the Notch1 pathway – a

pathway that is key in hippocampal neurogenesis through regulating NSC maintenance (Li et al., 2015). Lpar1 knockout mice and Hopx knockout mice both contain impairments in neurogenesis, where in the former there was defects in NSPC proliferation (Matas-Rico et al., 2008; Walker et al., 2016) and in the latter there was deficiency in the quiescent stem cell populations (Li et al., 2015). Our scRNA-seq findings revealed decreased Lpar1 expression in the 3xTG sample at a time when it is upregulated in the wildtype sample, corresponding to an activation defect occurring within the 3xTG NSCs primed for activation (Y. Liu et al., 2022). Additionally, Hopx and members of the Notch1 pathway were found to be dysregulated in the primed NSC population (Liu et al., 2022). We subsequently selected both of these molecular markers for further validation, due to their potential roles in neurogenesis and compatibility with the cellular defects we observed.

Following immunohistochemistry analysis of these molecular markers, we observed a decrease in the number of both LPAR1 positive and Hopx positive progenitors in the SGZ of 3xTG mice (Figure 4). Additionally, we did not observe a decline of Hopx+ progenitors within the YFP+ population, which effectively confirmed our previous findings where Sox2+ was not found to be proportionally affected. Similarly, analysis of Lpar1+ progenitors within the Sox2+ population also presented a statistically significant decline, confirming that the decrease was not due simply to a loss in the NSPC pool. It is important to consider that Hopx and Lpar1 label different Sox2+ populations – with Hopx labelling more the quiescent and primed NSC populations and Lpar1 labelling more the primed NSCs and TAP populations. The proportional decrease of Lpar1 can therefore also be interpreted as a deficiency in the primed NSC and TAP populations, which coincides with our sequencing findings (Figure S3).

While these findings suggests that there are impairments in the Hopx and LPAR1 progenitors that may result in the downstream cellular defects we previously identified (Figures 1-3), further characterization of these two pathways and their targets *in vivo* can allow us to decipher the mechanisms underlying these defects. Additionally, a more thorough investigation of other targets identified in our sequencing data can allow us to identify the dysregulation of the entire molecular network contributing to the deficits and explore further molecular targets. From there, we can begin to construct the pathway defects which can reveal the best ways to target the cellular defects and yield novel, druggable targets.

4.5 Intrinsic cellular defects in 3xTG neurogenesis as assessed by the neurosphere assay

Many of the cellular and molecular defects characterized through immunohistochemistry are the manifestation of complex interactions of the stem cell niche environment which have been shown to regulate NSPC functions. Supporting cells and the ligands they secrete can facilitate cellular processes such as proliferation or differentiation, thereby affecting stem and progenitor cell fates (Abbott & Nigussie, 2020; Kempermann et al., 2004; Kozareva et al., 2019). It is very possible that the cellular and molecular defects identified in the 3xTG mice through our immunohistochemistry analysis could be due to differences in niche factors and supporting cell types that create an environmental that negatively affects 3xTG neurogenesis. So next, we asked – could any of the observed defects be inherent to 3xTG NSPC functions?

To explore this, we employed the neurosphere assay – a cell culture technique where isolated NSPCs from one of the neurogenic regions of the brain can be isolated and subsequently grown into spherical aggregates known as neurospheres (Soares et al., 2021). Each neurosphere

is a heterogenous collection of NSPCs that is formed by a single NSPC that can self-renewal and proliferate (Pastrana et al., 2011; Soares et al., 2021). The quantification can be related to expansion of the NSPC and self-renewal capacity while the size used as an indicator of proliferation (Dadwal et al., 2015; Fatt et al., 2015; Pastrana et al., 2011; Soares et al., 2021). Despite the fact that neurospheres are essentially heterogenous cell populations, which is composed of NSCs, NPCs and differentiated cells, the neurosphere assay is a good way to study intrinsic NSPC biology due to the controlled *in vitro* system being one that promotes proliferation and growth (Pastrana et al., 2011; Soares et al., 2021). As a result, defects observed in the neurosphere assay can be direct measures of intrinsic cellular defects.

NSPCs taken from 3xTG brains formed fewer neurospheres, suggesting defects in NSPC pool expansion. While no major difference with respect to size was detected qualitatively (Figure 6A), however a systematic approach at quantifying size could prove otherwise. Since this defect was measured at the second passage (Passage 1 or sometimes referred to as secondary neurospheres) after the first neurospheres (passage 0 or primary neurospheres) were dissociated and replated, suggesting that the fewer neurospheres grown at this passage are the result of a sustained defect in NSPC expansion. Neurospheres were quantified at passage 1 due to technical considerations, such as more uniform growth, however it might be interesting to see if this defect can be observed beginning at P0. Measuring size quantitatively or secondary neurosphere formation – which is used to characterize self-renewal, where a single neurosphere is dissociated and replated in a well and the resulting neurospheres are quantified to assess self-renewal abilities – can reveal potential impairments in proliferation and self-renewal that underly this defect in 3xTG NSPC pool expansion.

We also assessed the amount of neurosphere formation on the basis of sex – as AD has been reported to have a sex-based defect (Ferretti et al., 2018) – however we were not able to detect any significant differences. This is in accordance with prior studies in our lab, where immunohistochemistry analysis of neurogenic markers did not find any sex-based defects in 3xTG neurogenesis at 3 months of age (McNicoll et al., in revision) and previous findings report differences in 3xTG neurogenesis based on sex present at later time points (Rodríguez et al., 2008).

In sum, we were able to identify and characterize an *in vitro* phenotype that presents an intrinsic 3xTG NSPC defect in NSPC expansion. Further characterization through this neurosphere assay measuring different cellular functions – such as self-renewal assays, differentiation assays, etc. – can reveal further inherent cellular defects.

4.6 Cell-autonomous dysregulation of molecular pathways

Another way to gain a better understanding of these cellular defects is through exploring the molecular basis of these defects. To do so, we opted to screen some of our dysregulated targets identified through our sequencing that could explain the results we were observing in our neurosphere assay. Our sequencing data revealed upregulation of mitochondrial and lipid metabolism pathways, as well as impaired redox genes, in 3xTG genes (Y. Liu et al., 2022, McNicoll et al., in revision). Metabolism plays a key role in the functions of NSPCs during its progression through neurogenesis (Khacho et al., 2019; Knobloch & Jessberger, 2017), so impairments in these genes can add context to our cell culture experiments.

Among the many dysregulated genes, PGC1a – a master regulator of mitochondrial biogenesis, and fatty oxidation genes (Nikolić et al., 2011) – and 2 of its binding partners, PPARγ and CPT1α were assessed. The expression SOD3, which is among a family of antioxidant enzymes that help protect tissue against oxidative stress (Singh & Bhat, 2012; Wert et al., 2018), was also analyzed. qPCR analysis of cultured NSPCs revealed that all these genes were significantly increased in 3xTG NSPCs (Figure 7), and we reasoned that this could have contributed to the reduced formation of 3xTG neurospheres for a variety of reasons.

NSCs rely mostly on glycolysis and their transition towards differentiation is mediated by a switch to mitochondrial respiration (Khacho et al., 2019). As a result, the dependence on glycolysis decreases as NSCs progress to NPC, before ultimately relying predominantly on neurons (Khacho et al., 2019).A premature induction of mitochondrial metabolism can interfere with NSC fate decisions and result in the depletion of the NSPC pool (Khacho et al., 2019). Alternatively, this increased expression could be a compensatory mechanism to make up for a mitochondrial defect inherent to 3xTG NSPCs. Furthermore, an increase in mitochondrial metabolism can lead to increases of ROS and establish oxidative stress conditions, and an upregulation of SOD3 support this idea.

Additionally, Cpt1α has been found to be highly expressed in quiescent NSCs and is subsequently downregulated in proliferating NSPCs (Knobloch et al., 2017). Its increased expression can mean that there is a defect in the proliferation of NPCs which can contribute to impaired growth of neurospheres. Alternatively, increased Cpt1α expression could suggest that the NSPCs are relying on lipid metabolism to supply energy needs which may indicate an inability to meet energy demands to supply the needs of NSPCs as they undergo processes like proliferation or cell division. The increased expression of Cpt1a is in accordance with previous

reports of the accumulation of lipids within the ventricular walls of both 3xTG mice and brain tissue from AD patients due to dysregulated lipid metabolism (Hamilton et al., 2015).

Metabolism plays a profound role in neurogenesis (Khacho et al., 2019; Knobloch & Jessberger, 2017), which makes the dysregulation of these metabolic pathways all the more intriguing. Taken together, our molecular findings provide valuable insight into the potential causes of the cell-autonomous deficits of 3xTG NSPC function identified through our neurosphere assay.

4.7 3xTG neurosphere growth in galactose-media suggests the presence of oxidative stress within 3xTG NSPCs

Given our sequencing and molecular *in vitro* characterization indicate that 3xTG NSPCs may be better adapted to oxidative stress conditions. Galactose media has been used to simulate oxidative stress as removing glucose – the key substrate of glycolysis – will require the NSPCs to use oxidative respiration rather than glucose to supply the energy demands (Aguer et al., 2011; Khacho et al., 2016). This overreliance on mitochondrial respiration can result in the accumulation of oxidative by-products, thereby inducing oxidative stress. As a result, culturing in galactose is an effective model of aging and oxidative stress environments – and therefore allow for the measurement of the ability of the wildtype and 3xTG NSPCs to grow in a stressed, aged setting.

Strikingly, when we instead grow the neurospheres in galactose media, we observed better neurosphere growth – both more and larger neurospheres – in the 3xTG NSPCs compared to the wildtype NSPCs (Figure 7). Despite there being fewer neurospheres compared to normal

tissue conditions for both wildtype and 3xTG samples, the decline was much greater in the wildtype samples. This could mean that the 3xTG NSPCS are better adapted to these oxidative stress conditions. These adaptive features that allow better growth in stressed conditions however may be impeding their growth in normal conditions. These results suggest that the 3xTG NSPCs express different genes that make them better equipped to function in an oxidative stress environment. Its possible that this differential gene expression, particularly that of redox genes like SOD3, is an adaptative response to the oxidative stress environment of 3xTG brains which persists throughout cultures. Alternatively, this molecular dysregulation – like overexpression of mitochondrial metabolism genes – could be a defect inherent to 3xTG NSPC function.

Another consideration is that, perhaps 3xTG NSPCs are not only better at managing oxidative stress conditions, but they possess a propensity for non-glycolytic metabolism, which was supported by lipid droplets occurring in 3xTG neurogenic niches (Hamilton et al., 2015; Hamilton & Fernandes, 2018). Culturing in galactose media is another way to assess the ability for NSPCs to thrive without glycolysis and the findings here show that 3xTG NSPCs were able to do so much better. This could be the result of the increase in lipid metabolism by genes like Cpt1α, which permitted the use of an alternative energy source as glycolysis – the primary energy source of NSCs which switches as they differentiate to neuron. Alternatively, this metabolic switch to OXPHOS could have triggered the NSPCs in the wildtype samples to differentiate into neurons (Khacho et al., 2019), which may explain the poor neurosphere formation. In this case, the increase in 3xTG neurosphere formation relative to wildtype samples may be indicative of a defect in attaining that OXPHOS switch effectively (due to mitochondrial defects) or in differentiation. This would certainly be in line with our previous findings throughout this thesis and in our previous studies (Liu et al., 2022, McNicoll et al., in revision).

There are many different interpretations of these findings; using other methods to measure oxidative stress conditions in 3xTG NSPCs as well as exploring other molecular pathways can help clarify what exactly is occurring to achieve this intriguing result.

4.8 Future directions

The ultimate objective of this study was to identify and characterize the cellular and molecular defects of 3xTG neurogenesis in order to devise novel approaches to restore neurogenesis in AD. While we were successful in characterizing these defects both within the 3xTG brain and those inherent to 3xTG NSPCs, there are several questions that arise and avenues this work can lead to in order for us to better understand these defects and learn how to best target them therapeutically.

Firstly, it would be interesting to add an extra biological replicate to complete the *in vivo* characterization of the cell-specific YFP population (Figure 3). While not necessary, some of the trends observed that were not statistically significant could be solidified with an extra replicate. Additionally, we could quantify other markers of adult hippocampal neurogenesis that could help us identify characterize populations we not included in our original analysis. This would allow us to have a more complete trajectory of the neurogenic process and identify defects occurring along the process.

Additionally, our neurosphere assays could be followed up with more experiments such as secondary neurosphere assays, where the amount of neurospheres formed from a single neurosphere is quantified. This can be used as a measure of self-renewal capabilities and would allow for the identification of any defects in self-renewal within 3xTG NSPCs. Additionally, we

could do differentiation and cell survival assays and identify any defects with respect to these two functions. We could also do some experiments to measure mitochondrial function or ROS content of 3xTG neurospheres in order to gain a better understanding of what is happening when these cells grow in galactose medium.

Using some of the molecular targets we have already identified, such as PGC1α, we can use drugs inhibiting or enhancing the specific target which can be screened through our *in vitro* neurosphere assay to assess. Eventually, we could move towards enhancing or attenuating the molecular targets *in vivo* and assess their impact on 3xTG neurogenesis in the brain. Any enhancements in neurogenesis can be followed up with behavioural assays in order to assess if the effectiveness at restoring cognitive function in AD mice. Characterizing more molecular targets will allow us to piece together a molecular mechanism by which these defects are occurring. This would be particularly intriguing with respect to our neurosphere cultures in galactose media – where characterization of other molecular pathways, like glycolysis, can permit for better conclusions to be drawn. Our sequencing data has a plethora of candidates which could be selected for subsequent *in vivo* and *in vitro* characterization.

CONCLUSION

In conclusion, **we were successful in our attempts to identify cellular and molecular defects of neurogenesis in the brains of 3xTG mice and in developing and characterizing an in vitro model of intrinsic, cell-autonomous defects of 3xTG NSPC molecular and cellular functions**. These findings agree with the several studies that have demonstrated how neurogenesis is affected in an AD setting. Here, we show how those defects include a loss of the overall NSPC pool and losses in the neuroblast, immature neuron and mature neuron populations. The use of an *in vitro* system to characterize intrinsic defects can be an effective tool in screening the effect of targeting potential molecular markers. Findings from this study can provide insight on novel approaches to rescue neurogenesis in AD and restoring the cognitive defects caused by the disease.

APPENDIX 1: Supplementary Figures

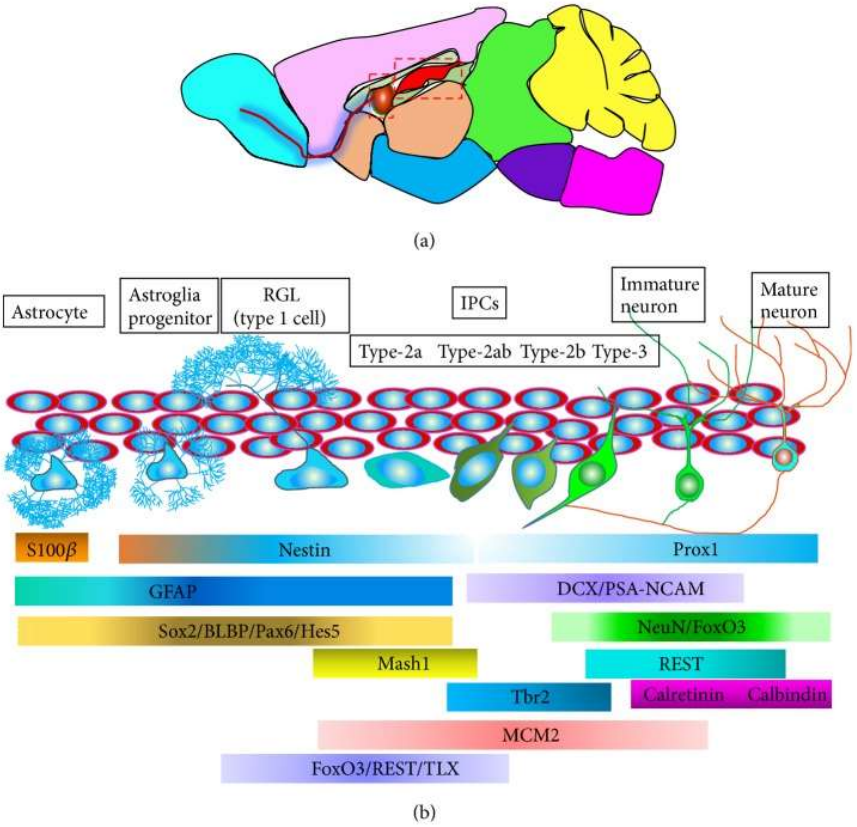

Figure S1: The molecular markers for different developmental stages of adult hippocampal neurogenesis. Figure from Zhang *et. al* (J. Zhang & Jiao, 2015)

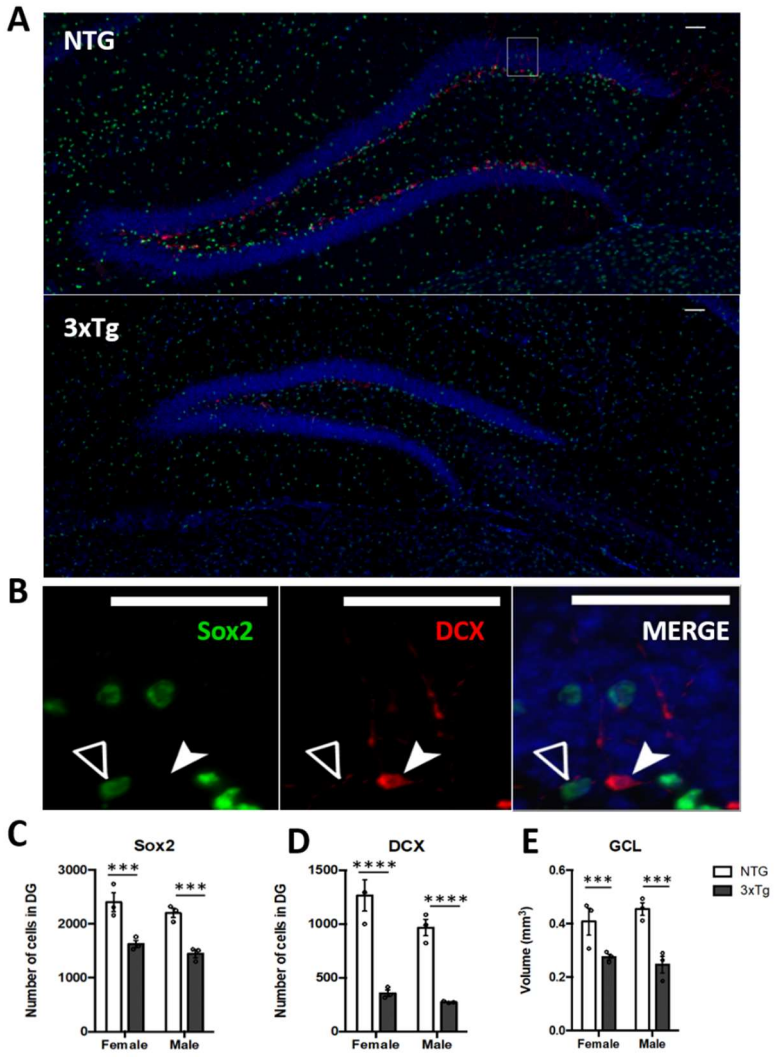

Figure S2: Preliminary characterization reveals deficiencies in 3xTG neural precursor cells at 3 month of age. The dentate gyrus (DG) of 3 month old wildtype (NTG; n=3 male and n=3 female) and 3xTG Nestin-CreER^{T2}-RosaYFP (n=3 male and n= 3 female) reporter mice was sectioned and stained for either (A) Sox2 (green) and Dcx (red). (B) Arrowheads denote examples of a Sox2 or Dcx positive cell. The granule cell layer (GCL) and counts of positive cells in the subgranular zone (SGZ) were measured blindly and the averages were subsequently compiled for analysis. All quantification, analysis, tissue preparation, imaging and figure preparation was completed by R.H. and M.M. Scale bar indicates 50 μm, a two-way ANOVA test was used for statistical analysis, *p<0.05, **p<0.01, ***p<0.001.

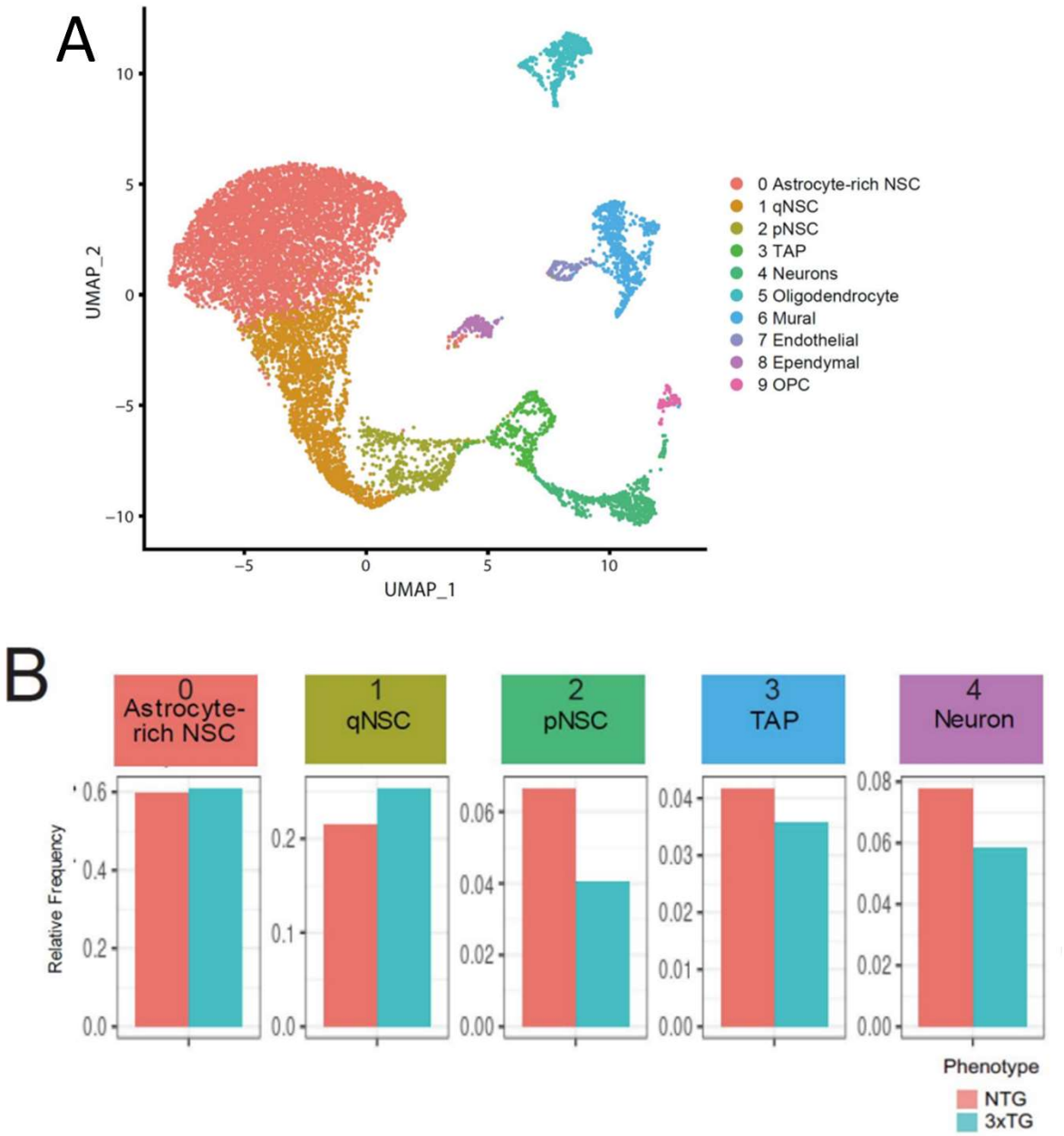

Figure S3: Defects in specific cell populations in 3xTG neural precursor cells identified through scRNA sequencing. Cells from the dentate gyrus (DG) of 3-month-old wildtype and 3xTG Nestin-CreER^{T2}-RosaYFP reporter mice were isolated using FACS before being sent for RNA sequencing at the single cell level (Y.L. and B.F.). (A) Mapping of the individual cell populations were determined using clustering and populations were identified based on the expression of established markers. (B) Comparing proportions of wildtype and 3xTG cells reveals defects in specific cell populations. All data analysis and figure preparation were completed by Y.L. and B.F. (Y. Liu et al., 2022)

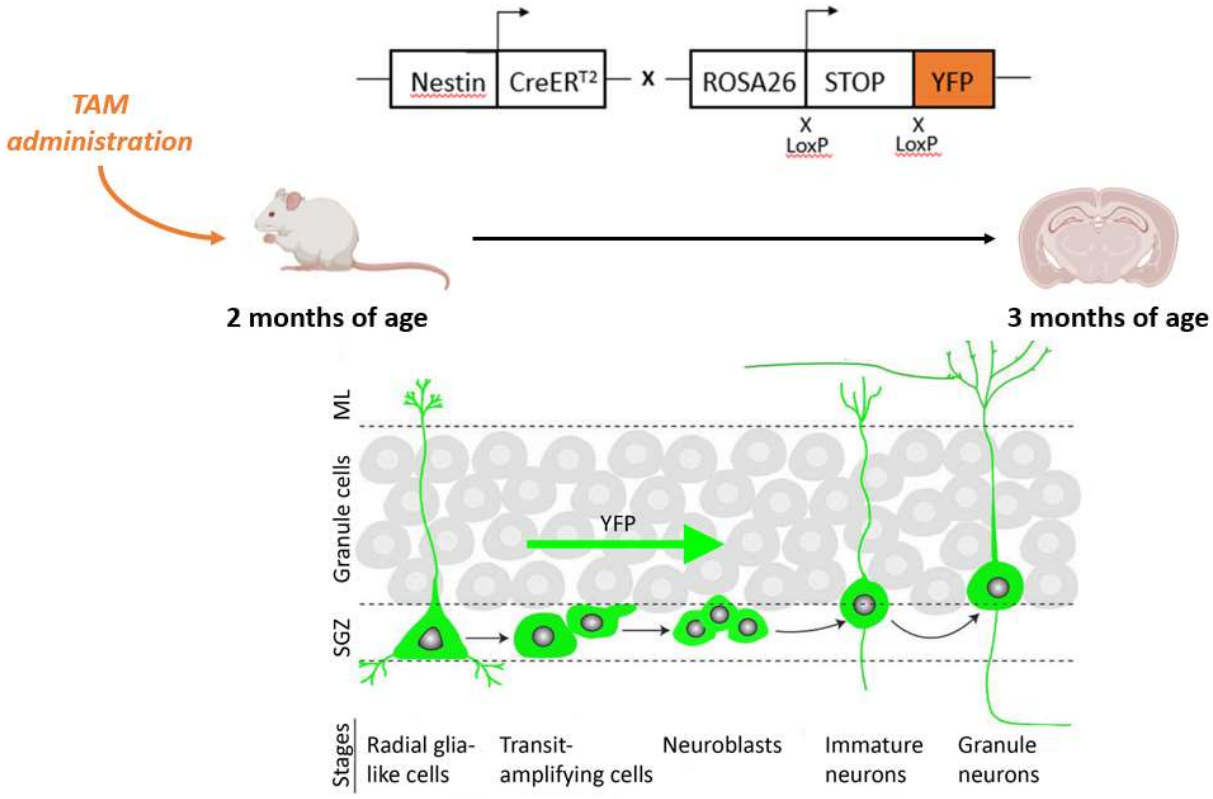

Figure S4: Timeline of Tamoxifen-administration to induce YFP expression for lineage tracing expression. Adapted from a figure created by R.H.

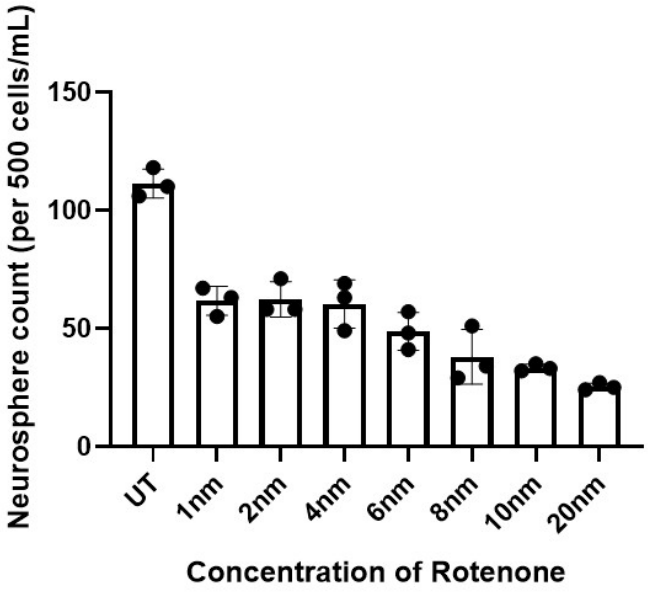

Figure S5: Decreased neurosphere growth with increasing concentration of Rotenone, validating our cell culture techniques. NSPCs were isolated from the SVZ of wildtype

(n=3) mice brains and cultured for one week in stem cell media to grow neurospheres. These neurospheres were then dissociated and replated for one week in the presence of rotenone before the subsequently formed neurospheres were quantified based on the number of spheres formed from 500 cells/mL.

APPENDIX 2: Supplementary Tables

Table S1: Primers used for genotyping and qPCR analysis. All primers were designed by Y.L.

Gene	Primer sequence (5' to 3')
Cre	*Transgene Forward:* GAACCTGATGGACATGTTCAGG *Transgene Reverse:* AGTGCGTTCGAACGCTAGAGCCTGT *Internal Positive Control forward:* TTACGTCCATCGTGGACAGC *Internal Positive Control Reverse:* TGGGCTGGGTGTTAGCCTTA
ROSA26YFP	*Mutant Reverse:* GCG AAG AGT TTG TCC TCA ACC *Common:* AAAGTCGCTCTGAGTTGTTAT *Reverse:* GGAGCGGGAGAAATGGATATG
Tg(APPSwe;tauP301L)1Lfa	*TF-Forward:* AGGACTGACCACTCGACCAG *TR-Reverse:* CGGGGGTCTAGTTCTGCAT
PSEN1$^{(tm1Mpm)}$	*IPC Forward:* CTAGGCCACAGAATGAAAGATCT *IPC reverse:* GTAGGTGGAAATTCTAGCATCATC
Rps26	*Forward:* GCCATCCATAGCAAGGTTGT *Reverse:* GCCTCTTTACATGGGCTTTG
PGC1α	*Forward:* GGTTCCCCATTTGAGAACAA *Reverse:* GGAGGAGTTGTGGGAGGAGT
PPARΓ	*Forward:* AAGAGCTGACCCAATGGTTG *Reverse:* TGAGGCCTGTTGTAGAGCTG
Cpt1α	*Forward:* GCTGCACTCCTGGAAGAAGA *Reverse:* ATAAGCCAGCTGGAGGGACT
SOD3	*Forward:* AGAGCCTGACAGGTGCAGAG *Reverse:* ACATGGTGACAGAGCCACAG

Table S2: Antibodies used for immunohistochemistry analysis.

Antibody target	Manufacturer	Catalogue no.	Host Species	Dilution
Sox2	Neuromics	GT15098	Goat	1:1000
Dcx	EMD Millipore	AB2253	Guinea Pig	1:1000
GFP	Abcam	Ab13970	Chicken	1:1000
GFAP	Chemicon	MAB3402	Mouse	1:1000
Ascl1				1:200
Tbr2	eBioscience	14-4875-82	Mouse	1:500
Prox1				
Hopx	Atlas Antibodies	HPA030180	Rabbit	1:2500
LPAR1	Cayman Chemicals	10005280	Rabbit	1:200

APPENDIX 3: Permission to reprint figures (as necessary)

Figure S1 permission to reprint

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