Winter 12-27-2018

Providing Experimental Evidence for Mouse Telomerase Reverse Transcriptase as a Novel and Unique Adult Neural Stem Cell Marker

Caroline Dean Curtis
University of Maine, cdbcurtis@gmail.com

PROVIDING EXPERIMENTAL EVIDENCE FOR MOUSE TELOMERASE REVERSE TRANSCRIPTASE AS A NOVEL AND UNIQUE ADULT NEURAL STEM CELL MARKER

by

Caroline Dean Curtis

B.S. University of Maine 2017

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Zoology)

The Graduate School

University of Maine

December 2018

Advisory Committee:

Kristy L. Townsend, Assistant Professor of Neurobiology

Alan M. Rosenwasser, Professor of Psychology

Seth Tyler, Professor of Zoology & Cooperating Professor of Marine Sciences

PROVIDING EXPERIMENTAL EVIDENCE FOR MOUSE TELOMERASE REVERSE TRANSCRIPTASE AS A NOVEL AND UNIQUE ADULT NEURAL STEM CELL MARKER

By Caroline Dean Curtis

Thesis Advisor: Dr. Kristy Townsend

An Abstract of the Thesis Presented in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Zoology)

December 2018

With the prevalence of neurodegenerative pathologies in our society today it is imperative that we begin to look at novel approaches to the underlying problem of dying neurons that are not replaced. Adult neural stem cells exist naturally and could potentially be manipulated into targeted repair of damaged brains, given substantial research. The first step in this process is to find a way to specifically mark the earliest subset of these cells, the quiescent adult neural stem cells. Here we provide evidence for the existence of a novel and unique qANSC marker in mouse telomerase reverse transcriptase (mTERT). mTERT has been shown to mark a slowly cycling quiescent stem cell population in the gut, and has been shown to be present in the brain. Here we will use two specific mouse models, including a lineage tracing model and a direct reporter model, in order to evaluate the location and behavior of mTERT+ cells within the brain. Through quantitative polymerase chain reaction we were able to determine that mTERT+ cell

populations express higher levels common neural stem cell markers than mTERT- cell populations. We were able to use fluorescent staining of brain slices in order to determine the niches for these cells as well as their co-expression patterns.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED

TBI	Traumatic brain injury
ANSC	Adult neural stem cell
qANSC	Quiescent adult neural stem cell
aANSC	Activated neural stem cell
TAC	Transit amplifying cell
NPC	Neural Precursor Cell
GFAP	Glial fibrillary acidic protein
EGFR	Epidermal growth factor receptor
bHLH	Basic helix loop helix
DCX	Doublecortin
FACS	Fluorescence activated cell sorting
SGZ	Sub-granular one
V-SVZ	Ventricular-sub-ventricular zone
RMS	Rostral migratory stream
OB	Olfactory bulb
ME	Median eminence
BBB	Blood brain barrier
CSF	Cerebrospinal fluid
HFD	High fat diet
BDNF	Brain derived neurotrophic factor
CNTF	Ciliary neurotrophic factor

ABBREVIATIONS CONTINUED

EdU	Ethynyldeoxyuridine
mTERT	Mouse telomerase reverse transcriptase
TERC	Telomerase RNA component
GFP	Green fluorescent protein
FACS	Fluorescence activated cell sorting
RNA	Ribonucleic acid
qPCR	Quantitative polymerase chain reaction
EGF	Epidermal growth factr
bFGF	Basic fibroblast growth factor
PSA-NCAM	Polysialylated neural cell adhesion molecule
ACSF	Artificial cerebrospinal fluid
IHC	Immunohistochemistry
PBS	Phosphate buffered saline
OCT	Optimal cutting temperature compound

CHAPTER 1

INTRODUCTION

Neurogenesis and its significance in current research

Neurogenesis research has proven to be highly relevant in the ongoing public health discussion in relation to neurodegeneration and neural injury. Neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, typically happen with aging and are the result of the loss of neurons without subsequent replacement or repair. While mechanisms of neuronal death and symptoms caused by neurodegeneration may vary, the core problem of neuronal loss is conserved between such diseases. In a similar fashion, neural injuries, such as traumatic brain injuries (TBIs), may also result in the death of neurons and subsequent deficits caused by their absence. TBIs are typically caused by mechanical force to the head, pressure due to swelling, or loss of blood caused by an injury, which is what leads to neuronal cell death. According to the CDC, approximately 1.7 million people experience some form of TBI per year, with adults over the age of 65 being particularly at risk. All of these alarming pathologies have the commonality of dead neurons without adequate compensation for the loss, and are at a disproportionate incidence in elderly populations.

This thesis will focus on adult neurogenesis, which will from here on be referred to simply as neurogenesis. It is important to note that developmental neurogenesis is another neurogenic process, although one that only happens during development. Developmental neurogenesis begins with the formation of the neural plate in the third week of gestation. Migrating cells arrange themselves in this plate formation based on signals from the embryonic node. This plate will then form ridges at either end that will

soon fuse to form the neural tube with the neural progenitor cells lining up along the center of this tube. Over the next several weeks the neural tube will undergo drastic morphological changes that will eventually lead to the formation of a small smooth brain. During the fetal period this brain will begin to develop the familiar pattern of gyri and sulci. Over the next several years the connections formed in this early brain will be built upon with further cell population growth, pruned, and refined as the human it belongs to interacts with their environment. This process continues through early childhood with the brain reaching approximately 90% of its adult size by age six in human (Stiles & Jernigan 2010).

Prior to the 1960's it was believed that after the cessation of developmental neurogenesis the brain was essentially static, not producing any new neurons. During the early 1960's Dr. Joseph Altman and his laboratory were able to prove that postnatal neurogenesis existed in both rats, and cats using thymidine-H^3 labeling. Thymidine labeling is a reliable way to mark cells that are preparing to divide as it is able to incorporate into newly synthesized DNA. In 1965 they were able to use thymidine labeling on young rats, adult rats, and rats with lesions to further support their hypothesis that adult neurogenesis would occur. The Altman lab was able to determine that neurogenesis in the dentate gyrus existed after the rat brain was fully developed although the number of thymidine labeled cells did steadily decline as animals aged. Lesions did not affect the rate of hippocampal neurogenesis in this study, although the lesions were made in the cortex, so it is possible that they affected the rate of neurogenesis in another niche.

In 1969, again using thymidine labeling, Dr. Altman's lab was able to show not only the existence of a persistent ANSC niche in the sub-ventricular zone, but also of the rostral migratory stream. In rats ranging in age from newborn to adult this niche was present and showed signs of proliferation. In addition, thymidine labeling allowed for the tracking of cells over up to 180 days post injection. From this a pattern began to emerge showing a high density of thymidine labeled cells in the SVZ up to 24 hours after injection, and these cells then beginning to migrate toward the olfactory bulb before reaching it 3-6 days after injection. This study supported the existence of a robust ANSC niche in the lateral ventricles of the brain.

Adult neurogenesis is the process in which neural precursor cells can create new cell types in order to provide for the needs of the brain. While several kinds of neural precursor cells exist, the most primitive of these are adult neural stem cells (ANSCs). These cells can either be in a quiescent, slowly proliferating state (qANSCs) or in an active, dividing state (aANSCs). ANSCs are multipotent with the potential to differentiate into neurons, glia, or oligodendrocytes, cell differentiation and commitment is regulated by specific signals in the brain. The classic canon pathway of the neuronal lineage is qANSCs become activated and then divide and differentiate into transit amplifying cells (TACs). TACs can then further divide and differentiate into neuroblasts which will migrate and differentiate into neurons, which will integrate into the existing neural circuitry. If this process could be understood mechanistically it is possible that these cells could be manipulated to target and repair specific areas, namely injured ones.

Markers used to identify neural precursors

Typically, neural precursors are characterized and distinguished from one another by a unique pattern of gene expression. qANSCs and aANSCs exhibit similar patterns of expression. The few known expression differences relate to qANSCs' lack of common activation markers. The markers that are shared between the two types of ANSCs are Nestin, glial fibrillary acidic protein (GFAP), Hes5, and Sox2 (sex determining region Y box 2). Nestin is a Type IV intermediate filament which has been found to aid in the survival, proliferation, and renewal of neural stem cells and has been hypothesized to aid in the migration of these cells (Suzuki et al., 2010). Recently the presence of nestin in qANSCs has been contested, however it is possible that it is still present in all ANSCs at different levels (Chaker, Codega, & Doetsch 2016). Nestin is also expressed in the vascular endothelial cells of other tissues around the body, such as the pancreas (Klein et al., 2003). GFAP is a Type III intermediate filament commonly found in astrocytes, is used for support and strength, and has been shown to mark ANSCs (Doetsch et al., 1997). Hes5 is a basic helix-loop-helix (bHLH) transcriptional repressor known for the regulation of cell differentiation in several tissues throughout the body and whose expression is downregulated upon differentiation (Basak & Taylor 2007).

Sox2 is responsible for maintaining ANSCs in an undifferentiated state in both developmental and adult neurogenesis (Graham et al., 2003). Repression of this gene results in premature exit from the cell cycle and differentiation. This is the most common ANSC marker and it has been used in several studies, however it marks both active and quiescent ANSCs so these studies are unable to differentiate between the two (Graham et al., 2003; Kazanis et al., 2010; Marqués-Torrejón et al., 2013).

Currently, the major differences between the expression patterns of qANSCs and aANSCs lie in the two activation markers which begin to be expressed by the aANSCs. These markers include, EGFR (epidermal growth factor receptor), a receptor tyrosine kinase which can lead to cell proliferation via signaling cascades (Doetsch et al., 2014) and ASCL1 (Achaete-Scute Complex Homolog) is a bHLH transcription factor which aids in commitment and differentiation of ANSCs. TACs continue to express ASCL1, Sox2, and EGFR but cease to express common ANSC markers. These cells undergo rapid proliferation and can divide up to three times (Alvarez-Buylla et al., 1999; Karl et al., 2012). These cells will differentiate to become neuroblasts.

Neuroblasts continue the expression of EGFR but also begin to express several new markers. NeuroD1 is a bHLH transcription factor and regulates the expression of the insulin gene. This transcription factor, is expressed in neuroblasts and has been shown to aid in the regulation of neurogenesis, morphogenesis of dendrites, retinal neuron formation, inner ear sensory neuron formation, endocrine islet cell formation, enteroendocrine cell formation, and hippocampal formation (Gao et al., 2009). Another common neuroblast marker is doublecortin, or DCX. DCX is a protein that directs migration in the developing brain by binding to microtubules and regulating their stability and organization (Gleeson et al., 1999). NeuN is a gene that begins its expression in neuroblasts and continues to mark mature neurons. It is an RNA binding protein involved in the regulation of pre-mRNA splicing. Tuj1, more commonly known as beta III tubulin is one of two protein families that form the microtubules found in mature neurons. It may also play a role in axonal migration and differentiation. In addition to Tuj1, TrkB can be

used to mark neurons, as it is a highly expressed receptor in neurons. A summary of all markers mentioned here may be found in Table 1.

Recently, single cell transcriptomics has been used to look for combinations of the aforementioned markers in a single cell. In brief, a specific area of an organ, such as a small part of the brain, is micro-dissected out and cells from this area are sorted via fluorescence activated cell sorting (FACS). The sorting is accomplished based on the presence or absence of a few predetermined markers chosen by the experimenter. These cells can then be taken individually and put through RNA-sequencing which allows for the detection of specific gene expression in each cell. This process has allowed us to learn that the neuronal lineage exists on a continuum with cell clusters along this continuum representing distinct progenitor subtypes (Dulken et al., 2017; Shin et al., 2015). In addition, differences between *in vivo* and *in vitro* populations have been shown using this method with *in vitro* neurosphere cells exhibiting an increase in inflammatory and cytokine signaling genes (Dulken et al., 2017). Hippocampal and ventricular progenitor populations also exhibit some differences in marker expression, although they are very similar for the most part (Dulken et al., 2017; Shin et al., 2015). The most pronounced difference between these two populations is the presence of Hopx gene expression in hippocampal, but not ventricular, stem cells (Shin et al., 2015).

Major neurogenic niches

Currently there are two well-studied neurogenic niches, the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the ventricular subventricular zone (V-SVZ) of the lateral ventricles. In the SGZ cells will undergo the classic pattern of differentiation and migrate up a single cell layer into the granular cell layer of the dentate

gyrus, where they become fully mature granule cell neurons (Mandyam, 2013). In the V-SVZ cells will undergo differentiation up until the point of becoming a neuroblast and then they migrate down the rostral migratory stream (RMS) to the olfactory bulb (OB) where they will integrate into the pre-existing neural circuitry (Duan et al., 2008). In humans, V-SVZ neurogenesis results the neuroblasts migrating to the striatum, as humans do not require the same level of OB turnover as rodents (Ernst et al., 2014). Striatal neurogenesis does occur in rodents as well, but not as commonly as OB neurogenesis (Song et al., 2017; Jeon et al., 2017; Kreiner et al., 2017).

Minor neurogenic niches

While the aforementioned niches are the most well-characterized, there exist other, smaller niches which have begun to be researched. These niches include the hypothalamus and the median eminence (ME), the cerebellum, and the choroid plexus of the lateral ventricles. The hypothalamus and ME work in concert with one another where neurogenesis is involved, but in a sexually dimorphic way. The ME is a circumventricular organ, meaning it is not within the blood brain barrier (BBB), whereas the hypothalamus is. This distinct difference allows for each to receive signals via different pathways. The ME receives signals from the blood and the hypothalamus is able to gather information from contact with cerebrospinal fluid (CSF) via tanycytes connected to the ventral third ventricle. Wrathall et al., 2012 was able to show in females, acute high fat diet (HFD) intervention increased cell proliferation in the hypothalamus and decrease cell proliferation in the ME, while long term HFD did the opposite in both regions. In males only the hypothalamus was shown to be affected, with no inverse correlation to the ME present.

The cerebellum is a vital area for balance and motor coordination. This area has been shown to contain nestin+sox2+S100B- cells which reside among Bergmann glia (Ahlfeld et al., 2017). Neurogenic stimulation in this area can be assessed by rotarod training.

The choroid plexus lines part of the ventricles in the brain and filters blood in order to produce CSF. This vital part of the blood-CSF barrier has also been known to create and harbor growth factors (Krzyzanowska et al., 2015). It is composed almost entirely by epithelial cells linked via tight junctions and this protective chain of cells houses a stromal space, which houses macrophages and dendritic cells, in addition to blood vessels. In addition, the CSF facing side of the epithelial cells host epiplexus cells, which associate with the microvilli of the epithelial cells (Kaur et al., 2016). This area provides an ideal environment for stem cells due to its proximity to both blood vessels and CSF.

Neurogenic interventions in adult neurogenesis

Several interventions have been shown to naturally stimulate or inhibit neurogenesis. These interventions are usually applied in order to affect neurogenesis in a specific niche. Voluntary exercise has been shown to stimulate hippocampal neurogenesis (Van der Zee et al., 2007; Tannock et al., 2013; Zhu et al., 2007). Environmental enrichment, such as adding toys or tunnels the home cage, may also be used to enhance hippocampal neurogenesis (Kempermann et al., 2015).

Both male and female mice experience an increase in V-SVZ neurogenesis after mating, with females experiencing a further increase in RMS neurogenesis under a paced mating paradigm (Corona et al., 2016). Exposure to the odors of a sexually experienced

male can stimulate SVZ neurogenesis in females with the highest new cell density in the ventral portion of the SVZ (Corona et al., 2016). Exposure to novel non-sexual odors, such as banana scent, can also cause an increase of new cells in the SVZ (Corona et al., 2016). In addition, feeding behaviors can affect neurogenesis. Mice fed a powdered diet experience a marked decrease olfactory neurogenesis due to the decrease in mastication (Utsugi et al., 2014). This was behaviorally assessed by whether animals would avoid bad smells in a Y-maze and cellularly assessed by the amount of BrDU+ cells in the OB. This phenotype could be reversed by the replacement of the soft diet with a hard chow diet for three months (Utsugi et al., 2014). The aforementioned methods are known to cause the appearance of new cells in the olfactory pathway, but the V-SVZ niche is not limited to this pathway.

Striatal neurogenesis can be increased by stimulating a small population (~0.5% of the cells in the striatum) of glutamatergic neurons within the striatum which then release glutamate which is known to enhance the proliferation and survival of neural progenitors (Song et al., 2017). Furthermore, striatal neurogenesis can be enhanced by riluzole, a drug that inhibits the release of glutamic acid and leads to an increase in serum levels of brain derived neurotrophic factor (BDNF) (Kreiner et al., 2017). Although low dose (5 mg/kg) riluzole had no effect on the behavioral deficits experienced by a Parkinsonian mouse model, there was an increase in the number of new cells in the SVZ. Behavioral recovery, or lack thereof, was assessed by rotarod trials which measure how long the mice are able to stay on an accelerating rotarod. Cellular recovery was assessed by the presence of ki67+ cells in the SVZ and the number of dendritic spines present on

the neurons of the striatum. No significant difference was seen between drug and control groups in terms of dendritic spine number.

The addition of exogenous factors into the mouse brain, another neurogenic stimuli, commonly involves neurotrophic factors. BDNF is a member of the epidermal growth factor family (EGF family). BDNF has been shown to promote the survival and differentiation of SVZ progenitor cell and increase the number of these cells in the RMS and OB (Pencea et al., 2001). BDNF has two receptors, TrkB and p75, with TrkB being a high affinity receptor and p75 being a low affinity receptor. BDNF and TrkB play a major role in energy balance as disruption of either of these can lead to changes in food consumption, activity level, and weight. A loss of either BDNF or TrkB has been shown to cause hyperphagia, obesity, and hyperactivity (Rothman et al., 2012). Injection of BDNF causes a decrease in the consumption of food and subsequently a decrease in weight (Rothman et al., 2012). This is likely linked to the high levels of expression of TrkB in the ventromedial hypothalamus, a lesion of which has also been shown to cause hyperphagia and weight gain. The relationship between BDNF and energy balance is reciprocal; changes in BDNF can cause diet changes just as diet changes may affect levels of BDNF. Alternate day fasting increased BDNF levels in mice, while food deprivation had an inhibitory effect (Rothman et al., 2012). This information taken together also lends support to the hypothalamus and median eminence as potential niches, as these areas are highly involved in energy balance and BDNF signaling.

Ciliary neurotrophic factor (CNTF), similarly to BDNF induces weight loss via a decrease in food consumption, however unlike BDNF this dietary change is accompanied by an increase in energy expenditure (Ahima et al., 2008). CNTF induces cell

proliferation specifically in the hypothalamus which results in cells that respond to leptin, the body's satiety signal. This increased response to leptin could be the mechanism which induces the dietary changes associated with CNTF. The proliferative capability of the hypothalamus is another key piece of evidence in support of the hypothalamus as a proliferative niche. Vascular endothelial growth factor (VEGF) has also been shown to increase cell proliferation via the Flk1 receptor, which is expressed in neural progenitor cells.

In order to assess whether proliferation occurs in areas of interest ethynyldeoxyuridine (EdU) is used. EdU is incorporated into newly synthesized DNA and thus is able to act as a marker of proliferation. Our lab was able to show EdU incorporation in mice, specifically in the SVZ and the hypothalamus.

Mouse Telomerase Reverse Transcriptase

In the Townsend Lab we endeavored to find a specific and unique marker for qANSCs. The lack of such a marker has made these cells difficult to study, as currently it is necessary to stain for at least two antigens and determine whether one is missing. This experimental design is difficult to accomplish with confidence as it is unknown whether a flaw in staining caused the absence of a signal or whether the signal just naturally absent. Our goal is to determine whether mouse telomerase reverse transcriptase (mTERT) could be a unique and specific marker for qANSCs.

Mouse telomerase reverse transcriptase (mTERT) expression allows for the telomeres at the end of DNA strands to be repaired. In typical cells these telomeres do not undergo lengthening and therefore are eventually depleted after numerous replication cycles. The loss of these telomeres results in apoptosis. In cells that must divide for

longer periods of time, such as stem cells, these telomeres can be replaced by mTERT in conjunction with the telomerase RNA component (TERC). TERC acts as a template from mTERT to reverse transcribe from in order to lengthen the telomeres, protecting the DNA and allowing cell survival.

mTERT was originally found to mark slowly cycling stem cells deep within the crypts of the intestines by Dr. David Breault's lab at Harvard Medical/Children's Hospital Boston (Breault et al., 2011). This slowly cycling population could become activated and differentiate and divide in order to replenish lost cell types during injury. Since then it has also been found to mark small populations of cells in the kidney, endometrium, and heart (Song et al., 2011; Deane et al., 2016; Richardson et al., 2012). Quiescent neural stem cells have previously been described as slowly cycling astrocytes, which is congruent with the original discovery's conclusion; mTERT cells are indeed slowly cycling.

Our lab is able to study this marker for specificity using two unique transgenic mouse lines. mTERT-GFP is a direct reporter line which expresses a green fluorescent protein (GFP) in any cell in which mTERT is expressed and ceases expression when mTERT ceases to be expressed. mTERT-rtTA::oTet-Cre::ROSA-mTmG is a triple transgenic lineage tracing line. This line uses a tetracycline controlled cre system in order to allow for the indelible marking of cells expressing mTERT. The hyphens in the strain name above indicate genes which are linked with one another. ROSA, a ubiquitously expressed gene in mice, precedes the transcription of mTmG, which contains a membrane tomato marker, then a stop signal, then a membranous GFP. In the absence of either mTERT or doxycycline only the membrane tomato protein is expressed in the cell.

Anytime mTERT is expressed in a cell an inactivated rtTA will be as well. Once doxycycline, a tetracycline derivative, is introduced into the mouse via their drinking water the rtTA will become active. The activated rtTA will then bind to the oTet promoter which will allow for the transcription of Cre. The membrane tomato protein and the stop signal of the mTmG, which are flanked by LoxP sites, will be targeted by the Cre protein. Each LoxP site will be cut down the center with the sequence between them being circularized. The remaining LoxP sites on the strand are then spliced back together so the ROSA and membrane GFP are adjacent. This results in a membrane GFP marker being present in lieu of a membrane tomato marker.

Due to the removal and subsequent circularization of the membrane tomato and stop signal the GFP marker will be indelible and the red signal will never return regardless of mTERT expression status. In this manner we are able to mark mTERT positive cells for a period of time in which doxycycline is administered called the pulse. Upon the removal of doxycycline from the drinking water during a period called the chase, these cells will remain marked and proceed with development. During the chase, interventions may be used in order to alter the behavior of these cells. For example, neurogenic stimuli can be used to increase neurogenesis which could induce differentiation and migration in GFP marked populations. This technique of labeling and following cells is referred to as lineage tracing.

CHAPTER 2

METHODS

Animals

All mice were kept on a 12-hour light dark cycle in a room temperature environment. mTERT:GFP mice were obtained from Dr. David Breault at Boston Children's Hospital/Harvard Medical and have C57BL/6J as a genetic background. mTERT:rtTA::oTet:Cre::ROSA:mTmG mice were also obtained from Dr. David Breault and have a mixed genetic background.

Gene expression study

Six-month old mTERT:rtTA::oTet:Cre::ROSA:mTmG mice were given a 2-week doxycycline pulse followed by a 2-day chase. At the conclusion of the chase animals were sacrificed via CO_2 anesthetization followed by cervical dislocation. Brains were then dissected out and all brain tissue posterior to the hypothalamus was removed and discarded. Remaining brain tissue was evenly and finely diced in a petri dish containing 1mg/mL pronase in artificial cerebrospinal fluid (ACSF). All of this was then transferred to loosely capped 50mL tubes containing 10mL of pronase/ACSF mixture. Samples were put into a shaking water bath set at 37°C for less than 75 minutes with vortexing every 10 minutes. Samples were spun down in a clinical centrifuge and ACSF/pronase was decanted and replaced with 10% FBS in ACSF, stopping the dissociation step (serum inhibits pronase). This was incubated at 37°C in a shaking water bath for 15 minutes. Each sample was then triturated through a 600μm, a 300μm, and a 150μm fire-polished

pipette respectively until no chunks remained in the sample. Each sample was moved to a sterile loosely capped FACS tube and transferred on ice to Jackson Labs for sorting.

Sorting was done by Will Schott of Jackson Labs. Dead cells were exluded from the sort via staining with DAPI, a common nuclear marker for dead cells. Each sample yielded a GFP+ and a GFP- results tube. GFP+ cells were sorted directly into lysis buffer, while GFP- cells were sorted into PBS. Upon completion of the sort, all samples were taken back to the University of Maine on ice where they were immediately placed into -80°C.

Both GFP+ and GFP- samples had RNA extracted using the Zymo Quick-RNA MiniPrep Kit (Zymo Research, R1051). RNA was reverse transcribed into cDNA which was then diluted and stored at -20°C. Gene Expression was measured by qPCR using SYBR Green as the fluorophore. All samples were run in replicate. Analysis was completed using GraphPad Prism. For primer forward and reverse sequences see Table 2.

Neurosphere assay

Mice were treated as previously described in the "Gene Expression Study" section of the methods, however instead of sorting into lysis buffer or PBS, all samples were sorted into NeuroCult Complete Proliferation Media. These samples were transported back the University of Maine on ice, where they were plated. Media was changed every seven days and plates were examined daily. Images were taken by Magda Blaszkiewicz.

Immunohistochemistry

Animals used for immunohistochemistry (IHC) were anesthetized using a ketamine/xylazine mixture at a concentration of 20mg/mL and 10mg/mL respectively. Animals were given a dose of 100μL/gram of body weight. After successful

anesthetization, confirmed via toe pinch, each animal was transcardially perfused first with PBS and then with Amresco HistoChoice Fixative (Sigma-Aldrich, H2904). Brains were then removed, put into individual cassettes, and fixed overnight in HistoChoice. After 16 hours brains were moved to a sucrose gradient with 10%, 20%, and finally 30% sucrose in PBS.

Each brain was flash frozen in OCT and sliced on a cryostat at a thickness of 7μm. Serial sectioning was achieved by adding two tissue sections to each slide for six continuous slides. Twelve brain slices were then discarded. This pattern was repeated until no tissue remained. All slides were then baked in an incubator at 37°C overnight and then stored in -20°C until staining.

Staining began by allowing slides to come to room temperature for 30 minutes, followed by fixing the slide in ice cold acetone for 15 minutes. Slides were washed in Millipore Rinse Buffer (Millipore, #20845) for 3-5 minutes. Membranes were made permeable Nuclear antigens were retrieved using 0.2% Triton X-100 in PBS for exactly 10 minutes. Cytoplasmic antigens were retrieved by placing slides in 0.3% Tween-20 in PBS for 30 minutes. Regardless of antigen localization slides were microwaved on low for 20 minutes total in DAKO Target Retrieval reagent (Cat #S1700) with a 3 minute break halfway through. Sudan Black was used to block autofluorescence by incubating slides in it for 20 minutes. After this incubation slides were rinsed using Millipore Rinse Buffer and hydrophobic barriers were drawn using a pap pen. Millipore block (Cat #20773) was added to each slide and slides were allowed to incubate at 37°C for 20 minutes. Following this incubation primary antibody was added and slides were left overnight at 4°C. The following day slides were rinsed in Millipore Rinse Buffer and

then allowed to incubate in the secondary antibody for 10 minutes in the dark. Slides were again rinsed in rinse buffer, followed by a water rinse. Slides were counterstained in 100ng/mL of DAPI for 5 minutes and then rinsed thoroughly in water. Fluorescent mounting media (Millipore Cat# 5013) and 1.5mm thickness cover slips were used to mount slides. For primary and secondary antibody concentrations see Table 3.

CHAPTER 3

RESULTS

Gene expression in the mTERT-GFP+ cell population

After FACS sorting mTERT-GFP+ populations and mTERT-GFP- populations from 6-month old mTERT:rtTA::oTet:Cre::ROSA:mTmG mice, RNA was extracted and reversed transcribed into cDNA so that gene expression could be assessed via quantitative polymerase chain reaction (qPCR).

In Figure 1 four out of the six tested ANSC markers show upregulation in the mTERT-GFP+ cell population when compared to the mTERT-GFP- cell population. It is important to keep in mind that due to our animal model this is a comparison of cells that expressed mTERT during our 2-week pulse to other brain cells of a non-specific population. In essence upregulation in the mTERT-GFP+ population indicates that the gene of interest is higher in these cells than in a collective average of other brain cell types.

Gfap, hes5, sox2, and *nestin* are all significantly more expressed in the mTERT-GFP+ population providing evidence for mTERT-GFP+ cells being some of the earliest neural precursors, ANSCs. *Blbp* does not show a significant difference between populations although there is a trend towards upregulation in the mTERT-GFP+ population. *Cd133* is not upregulated in the mTERT-GFP+ population and no trend is apparent. *Cd133* has been shown to be a reliable marker for developmental NSCs, it has been shown to mark a diffuse population in the adult brain that is not correlated to ANSCs (Olausson et al., 2014).

Several trends seem to appear in the later precursor markers as well. This could be due to the fact that the mTERT:rtTA::oTet:Cre::ROSA:mTmG line marks cells indelibly, so from the very beginning of the pulse any cell that is marked will remain marked despite any changes in gene expression that may occur. These trends are not significant, while there is significance in the difference between earlier precursor markers which indicates that these cells at least begin to be marked in the earlier precursor phases. *Prox1* is the only later precursor marker to be significantly different between mTERT-GFP+ and mTERT-GFP- populations.

Trkb is a BDNF receptor that is highly prevalent on neurons and shows no difference between populations, showing that the mTERT-GFP+ population is not composed of neurons.

mTERT-GFP+ cells form neurospheres

mTERT-GFP+ cells from a 4-month old mTERT:rtTA::oTet:Cre::ROSA:mTmG mouse formed neurospheres when cultured, although in very low numbers (Figure 1). Active and quiescent ANSCs exhibit strikingly different behavior in culture with ANSCs robustly giving rise to neurospheres while qANSCs only form neurospheres 0.82% of the time in the presence of bFGF and EGF (Codega et al., 2014). Our assay began by plating 800 mTERT-GFP+ cells, which according to Codega et al., 2014 means we could reasonably expect ~6 neurospheres if no cell death occurred, indicating that as long as our protocol is correct our low yield may be a sign of a qANSC population.

***In vivo* location of mTERT-GFP+ cells and their co-localization**

When looking at figures 2, 3, and supplemental figure 2 a pattern emerges of mTERT-GFP+ cells residing in the ventricles, specifically in the choroid plexus. In

supplemental figure 2C an mTERT-GFP+ cell is visible with a clear protrusion, S2D shows a potential model of how a qANSC would reside in a choroidal niche with a similar projection. mTERT-GFP+ cells were also regularly found in the subventricular zone, a classic ANSC niche. mTERT-GFP+ cells were found in the hypothalamus, albeit less regularly than in the lateral ventricles. Typically, in this niche mTERT-GFP+ cells could be found in the walls of the ventral 3rd ventricle or in the median eminence, both areas that have been previously shown to be neurogenic, specifically in response to diet. mTERT-GFP+ cells were distinctly absent from the SGZ of the dentate gyrus in all samples imaged, although sox2,nestin, and EGFR were present in this region.

Figures 4, 5, and supplemental figure 4 elucidate co-staining patterns of occasional overlap with early markers such as sox2, nestin, and EGFR although no overlap with neuroblasts marker DCX. Sox2 overlap with mTERT-GFP+ cells of mTERT:rtTA mice were observed in the choroid plexus as well as the wall of the ventral third ventricle. Nestin co-localization in mTERT:rtTA mice occurred in the choroid plexus as well. EGFR co-staining was detected in the hypothalamus in mTERT:rtTA mice and the SVZ in mTERT:GFP mice. DCX was present in both the SVZ and the olfactory and was able to display part of the rostral migratory stream (figure 4A).

Taken together, the above histological data highlights the need for further co-staining studies in both lines, although it does give us some idea of the possible identity of these cells. Many of these cells appear to exhibit the genetic expression and location patterns of ANSCs. They are not neuroblasts as evidenced by their lack of DCX as well as their locations within the brain, but this is the most definite we are able to be about the identity of these cells at this point.

CHAPTER 4

DISCUSSION

Hippocampal neurogenesis debate in the field of neuroscience

Adult hippocampal neurogenesis has recently become a hotly debated topic, due to the presence of papers that draw opposing conclusions. Here we will look at two central papers published one month apart and a variety of peripheral papers that support or discredit their claims. Each of these papers uses the hippocampi of deceased human patients in order to establish the neurogenic changes that occur with aging. Immunohistochemistry is employed in order to compare the co-expression of a variety of neural and neural precursor markers and their relative presence or absence at each age.

According to Sorrells et al., neurogenic activity wanes after the first year and is essentially non-existent in adult humans and macaques (Sorrells et al., 2018). Using DCX and PSA-NCAM to mark young neurons this group established a clear absence of neuronal production in adult subjects. This lab used cadaver brains ranging from 14 gestational weeks to 77 years of age as well as brain samples removed from epileptic patients during resection surgeries.

This is consistent with the decrease in self renewal and neurogenic capacity of ANSCs seen in aging (Trinchero et al., 2017). This could also be linked to the scarcity of adult born granule cells in middle-aged (5-8 months) mice (Trinchero et al., 2017). A sharp decrease in neurogenesis and ANSCs in particular could result in a correlated decrease in new granule cells as well. The newborn granule cells that were present in middle-aged mice had a significantly delayed development compared to their counterparts in young mice (Trinchero et al., 2017). However, this delay could be

mitigated through voluntary exercise which also resulted in a correlated increase of BDNF levels (Trinchero et al., 2017).

The opposing paper claims that although qANSC pools decrease in size in the aging human hippocampus immature neuron pools and neurogenesis remain stable (Boldrini et al., 2018). This lab employed the use of brains from human cadavers ranging in age from 14 to 79 years of age. As a control measure all subjects used in this research were ensured to be free of brain diseases, medications, cognitive impairments, recent significantly stressful life events, and global functioning impairment.

Although these papers will influence the interpretation of our research, the fact remains that our work is in mice, where the research is more decided about the existence of adult hippocampal neurogenesis. Thus given the stark lack of mTERT+ cells in the DG we have formed several possible hypotheses. The first of these is that ANSCs in the ventricular niches are a completely unique cell population from those in the hippocampal niches. Although their objectives would be the same their journeys would be vastly different with ventricular cells having to migrate to the OB and hippocampal cells only needing to move up one cell layer. This could result in different neuronal and progenitor markers being expressed.

Prox1 may exhibit divergent expression in the neural lineage

While *prox1* has been previously identified as a neuroblast marker, we see its upregulation in our mTERT-GFP+ cell population. Several hypotheses exist to explain this data. It is possible that *prox1* could mark earlier precursor populations in addition to being a neuroblast marker. *Prox1* has been shown to function in a tissue-dependent manner, acting as a tumor suppressor in liver cells (Nagai et al., 2003) and a proto-

oncogene in colorectal cells (Petrova et al., 2008). Neuroblasts do not divide, which could indicate that *prox1* acts as a tumor-suppressor gene in the brain. If this is the case it could follow that *prox1* expression is upregulated in qANSCs and then ceases expression in the actively dividing aANSCs only to exhibit expression once again after the cell has reached the neuroblast stage and ceased division.

Neurosphere formation of qANSCs

As mentioned previously qANSCs plated with EGF and bFGF will form neurospheres about 0.82% of the time. This offers a possible explanation for our low yield of neurospheres in our GFP+ population. In the GFP- population it is important to recall that these are simply cells that do not express GFP and therefore these cells could represent a wide variety of cell populations. In addition, if neural precursors were present in this population they may not have been able to form neurospheres due to the signals given off by the cells around them, as such a variety is not typically expected to produce neurosphere.

Stem Cell Markers

Figures 4, 5, and supplemental figure 4 bring about the idea of expression that tapers on and off instead of abruptly beginning and ending. Especially in the case of EGFR, we see a pattern of sparse co-expression with GFP+ cells, which could indicate cells that are transitioning between qANSCs and aANSCs, but that have not yet fully assumed their new identity, nor shed their old one. Unbiased stereology should be considered in order to quantify the number of mTERT-GFP+ cells that express EGFR in order to determine whether this is the true expression of GFP+ cells or a common transition phase into an activated state. We would also be able to use this method to

determine if the majority of these cells express sox2 or other ANSC markers or if only a small subpopulation express these markers. If there is a majority, it may point to mTERT being a reliable stem cell marker. However, if these cells do not reliably co-express markers such as sox2 and nestin, we may need to consider if they are perhaps an inducible stem cell or possibly a stem cell companion cell.

Luo et al., 2015 brought to light the possibility of a rare stem-capable ependymal cell population by using single-cell transcriptomics to find minor cell populations within the brain. mTERT-GFP+ cells could potentially fit into this category based on the locations we have observed them occupying in the brain. We have seen them in the choroid plexus, which is known to be made of ependymal cells. We have also observed them in the walls of the lateral and ventral third ventricles, both of which contain layers of ependymal cells. Further analysis and higher resolution imaging would be needed in order to determine if these cells truly reside in the SVZ or if they perhaps are present in the ependymal layer. The existence of these rare stem-capable ependymal cells has been further supported by Hendrickson et al., 2018. Although promising this theory does have its opponents who believe that this population does not exist (Shah et al., 2018; Muthusamy et al., 2018), although the rarity of these cells could result in difficulty finding them.

Conclusions

Based on the above data and discussion we can support the hypothesis that mTERT marks a subpopulation of neural precursor cells in the adult mouse brain. The population as a whole expresses several ANSC markers without significant upregulation of later precursor markers so we can further support the position that this population is an early

precursor, most likely an ANSC. Neurosphere data offers further backing that these cells are indeed ANSCs and possibly quiescent. Histological data allowed for the emergence of a pattern of expression of mTERT-GFP+ cells within the brain that closely follows the accepted expression pattern of ANSCs. Co-localization data leaves open the possibility of mTERT-GFP+ cells being ANSCs, either quiescent or activated. Taken together an image begins to emerge of the prospect of mTERT being able to accurately mark ANSCs, although further experiments will be needed to draw more concrete conclusions.

BIBLIOGRAPHY

Ahima, R. S., & Antwi, D. A. (2008). Brain Regulation of Appetite and Satiety. *Endocrinology and Metabolism Clinics of North America,37*(4), 811-823. doi:10.1016/j.ecl.2008.08.005

Ahlfeld, J., Filser, S., Schmidt, F., Wefers, A. K., Merk, D. J., Glaß, R., . . . Schüller, U. (2017). Neurogenesis from Sox2 expressing cells in the adult cerebellar cortex. *Scientific Reports,7*(1). doi:10.1038/s41598-017-06150-x

Alvarez-Buylla, A., García-Verdugo, J., Lim, D., Caillé, I. and Doetsch, F. (1999). Subventricular zone Astrocytes are neural stem cells in the adult mammalian brain. *Cell*, 97(6), pp.703-716.

Association®, A. (2013). *Latest Alzheimer's facts and figures*. [online] Available at: http://alz.org/facts/ [Accessed 3 Nov. 2016].

Basak, O., & Taylor, V. (2007). Identification of self-replicating multipotent progenitors in the embryonic nervous system by high Notch activity and Hes5 expression. *European Journal of Neuroscience,25*(4), 1006-1022. doi:10.1111/j.1460-9568.2007.05370.x

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., . . . Mann, J. J. (2018). Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell,22*(4). doi:10.1016/j.stem.2018.03.015

Breault, D., Algra, S., Fogli, L., Ambruzs, D., Baffour-Awuah, N., Henderson, D., Kranendonk, M., Farilla, L., Richmond, C., Carlone, D. and Montgomery, R. (2010). Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proceedings of the National Academy of Sciences*, 108(1), pp.179-184.

CDC (2016). *TBI: Get the facts*. [online] Available at: http://www.cdc.gov/traumaticbraininjury/get_the_facts.html [Accessed 3 Nov. 2016].

Chaker, Z., Codega, P., & Doetsch, F. (2016). A mosaic world: Puzzles revealed by adult neural stem cell heterogeneity. *Wiley Interdisciplinary Reviews: Developmental Biology,5*(6), 640-658. doi:10.1002/wdev.248

Chen, X., Liu, C., Tu, F., Huang, T., Xiang, B., Pan, J., Liu, M., Pang, Q. and Zhao, Y. (2016). Treadmill exercise promotes Neurogenesis in Ischemic rat brains via Caveolin-1/VEGF signaling pathways. *Neurochemical Research*.

Codega, P., Silva-Vargas, V., Paul, A., Maldonado-Soto, A., Deleo, A., Pastrana, E., & Doetsch, F. (2014). Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche. *Neuron,82*(3), 545-559. doi:10.1016/j.neuron.2014.02.039

Corona, R., Retana-Márquez, S., Portillo, W., & Paredes, R. G. (2016). Sexual Behavior Increases Cell Proliferation in the Rostral Migratory Stream and Promotes the Differentiation of the New Cells into Neurons in the Accessory Olfactory Bulb of Female Rats. *Frontiers in Neuroscience,10*. doi:10.3389/fnins.2016.00048

Damkier, H., Brown, P. and Praetorius, J. (2013). Cerebrospinal Fluid Secretion by the Choroid Plexus. *Physiological Reviews*, 93(4), pp.1847-1892.

Deane, J. A., Breault, D. T., & Gargett, C. E. (2014). Telomerase reverse transcriptase is an epithelial progenitor marker in the mouse endometrium. *Reproduction Abstracts*. doi:10.1530/repabs.1.p354

Doetsch, F., García-Verdugo, J. M., & Alvarez-Buylla, A. (1997). Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain. *The Journal of Neuroscience,17*(13), 5046-5061. doi:10.1523/jneurosci.17-13-05046.1997

Doetsch, F., Drapeau, E. and Riquelme, P. (2008). Brain micro-ecologies: Neural stem cell niches in the adult mammalian brain. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1489), pp.123-137.

Doetsch, F., Pastrana, E., DeLeo, A., Maldonado-Soto, A., Paul, A., Silva-Vargas, V. and Codega, P. (2014). Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche. *Neuron*, 82(3), pp.545-559.

Duan, X., Kang, E., Liu, C., Ming, G. and Song, H. (2008). Development of neural stem cell in the adult brain. *Current Opinion in Neurobiology*, 18(1), pp.108-115.

Dulken, B. W., Leeman, D. S., Boutet, S. C., Hebestreit, K., & Brunet, A. (2017). Single-Cell Transcriptomic Analysis Defines Heterogeneity and Transcriptional Dynamics in the Adult Neural Stem Cell Lineage. *Cell Reports,18*(3), 777-790. doi:10.1016/j.celrep.2016.12.060

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., . . . Frisén, J. (2014). Neurogenesis in the Striatum of the Adult Human Brain. *Cell,156*(5), 1072-1083. doi:10.1016/j.cell.2014.01.044

Fuss, J., Gass, P., Weber-Fahr, W., Lutz, B., Ende, G., Bindila, L., Auer, M. and Biedermann, S. (2016). Restricted vs. Unrestricted wheel running in mice: Effects on brain, behavior and endocannabinoids. *Hormones and Behavior*, 86, pp.45-54.

Gage, F., Deng, W. and Zhao, C. (2008). Mechanisms and functional implications of adult Neurogenesis. *Cell*, 132(4), pp.645-660.

Gao, Z., Ure, K., Ables, J. L., Lagace, D. C., Nave, K., Goebbels, S., . . . Hsieh, J. (2009). Neurod1 is essential for the survival and maturation of adult-born neurons. *Nature Neuroscience,12*(9), 1090-1092. doi:10.1038/nn.2385

Garland, Jr., T., Carter, P., Swallow, J. and Koteja, P. (1999). Energy cost of wheel running in house mice: Implications for Coadaptation of Locomotion and energy budgets. *Physiological and Biochemical Zoology*, 72(2), pp.238-249.

Giachino, C., Taylor, V., Kempermann, G., Haas, C., Götz, M., Fabel, K., Haussler, U., Knuckles, P., Basak, O. and Lugert, S. (2010). Quiescent and active Hippocampal neural stem cells with distinct Morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell*, 6(5), pp.445-456.

Gillette-Guyonnet, S. and Vellas, B. (2008). Caloric restriction and brain function. *Current Opinion in Clinical Nutrition and Metabolic Care*, 11(6), pp.686-692.

Gleeson, J. G., Lin, P. T., Flanagan, L. A., & Walsh, C. A. (1999). Doublecortin Is a Microtubule-Associated Protein and Is Expressed Widely by Migrating Neurons. *Neuron,23*(2), 257-271. doi:10.1016/s0896-6273(00)80778-3

Graham, V., Khudyakov, J., Ellis, P., & Pevny, L. (2003). SOX2 Functions to Maintain Neural Progenitor Identity. *Neuron,39*(5), 749-765. doi:10.1016/s0896-6273(03)00497-5

Holmberg Olausson, K., Maire, C., Haidar, S., Ling, J., Learner, E., Nistér, M. and Ligon, K. (2014). Prominin-1 (CD133) Defines Both Stem and Non-Stem Cell Populations in CNS Development and Gliomas. *PLoS ONE*, 9(9), p.e106694.

Hornsby, A., Redhead, Y., Rees, D., Ratcliff, M., Reichenbach, A., Wells, T., Francis, L., Amstalden, K., Andrews, Z. and Davies, J. (2016). Short-term calorie restriction enhances adult hippocampal neurogenesis and remote fear memory in a Ghsr-dependent manner. *Psychoneuroendocrinology*, 63, pp.198-207.

Kazanis, I., Lathia, J. D., Vadakkan, T. J., Raborn, E., Wan, R., Mughal, M. R., . . . Ffrench-Constant, C. (2010). Quiescence and Activation of Stem and Precursor Cell Populations in the Subependymal Zone of the Mammalian Brain Are Associated with Distinct Cellular and Extracellular Matrix Signals. *Journal of Neuroscience,30*(29), 9771-9781. doi:10.1523/jneurosci.0700-10.2010

Jeon, H., Ryu, S., Kim, D., Koo, S., Ha, K., & Kim, S. (2017). Acupuncture Stimulation at GB34 Restores MPTP-Induced Neurogenesis Impairment in the Subventricular Zone of Mice. *Evidence-Based Complementary and Alternative Medicine,2017*, 1-9. doi:10.1155/2017/3971675

Jessberger, S. (2016). Stem cell-mediated regeneration of the adult brain. *Transfusion Medicine and Hemotherapy*, 43(5), pp.321-326.

Karl, M., Shi, Y., Ye, P., Murai, K., Sun, G. and Li, S. (2012). Characterization of TLX expression in neural stem cells and Progenitor cells in adult brains. *PLoS ONE*, 7(8), p.e43324.

Kaur, C., Rathnasamy, G., & Ling, E. (2016). The Choroid Plexus in Healthy and Diseased Brain. *Journal of Neuropathology & Experimental Neurology,75*(3), 198-213. doi:10.1093/jnen/nlv030

Kempermann, G., Roeder, I. and Garthe, A. (2015). Mice in an enriched environment learn more flexibly because of adult hippocampal neurogenesis. *Hippocampus*, 26(2), pp.261-271.

Keyvani, K., Teuber-Hanselmann, S., Hermann, D., Wang, Y., Stang, A., Roggendorf, C., Karim, F., Yavuz, I., Krieter, D., Krüssel, S., Bolczek, B., Metzdorf, J., Münster, Y. and Herring, A. (2016). Late running is not too late against Alzheimer's pathology. *Neurobiology of Disease*, 94, pp.44-54.

Klein, T., Ling, Z., Heimberg, H., Madsen, O. D., Heller, R. S., & Serup, P. (2003). Nestin Is Expressed in Vascular Endothelial Cells in the Adult Human Pancreas. *Journal of Histochemistry & Cytochemistry,51*(6), 697-706. doi:10.1177/002215540305100601

Kreiner, G., Rafa-Zabłocka, K., Chmielarz, P., Bagińska, M., & Nalepa, I. (2017). Lack of riluzole efficacy in the progression of the neurodegenerative phenotype in a new conditional mouse model of striatal degeneration. *PeerJ,5*. doi:10.7717/peerj.3240

Krzyzanowska, A., García-Consuegra, I., Pascual, C., Antequera, D., Ferrer, I. and Carro, E. (2015). Expression of Regulatory Proteins in Choroid Plexus Changes in Early Stages of Alzheimer Disease. *Journal of Neuropathology & Experimental Neurology*, 74(4), pp.359-369.

Li, Z. (2013). CD133: A stem cell biomarker and beyond. *Experimental Hematology & Oncology*, 2(1), p.17.

Lipp, H., Bologova, N., Poletaeva, I., Slomianka, L. and Amrein, I. (2004). Marked species and age-dependent differences in cell proliferation and neurogenesis in the hippocampus of wild-living rodents. *Hippocampus*, 14(8), pp.1000-1010.

Lun, M., Monuki, E. and Lehtinen, M. (2015). Development and functions of the choroid plexus–cerebrospinal fluid system. *Nature Reviews Neuroscience*, 16(8), pp.445-457.

Mandyam, C. (2013). The Interplay between the Hippocampus and Amygdala in Regulating Aberrant Hippocampal Neurogenesis during Protracted Abstinence from Alcohol Dependence. *Frontiers in Psychiatry*, 4.

Manzanero, S., Erion, J. R., Santro, T., Steyn, F. J., Chen, C., Arumugam, T. V., & Stranahan, A. M. (2014). Intermittent Fasting Attenuates Increases in Neurogenesis after Ischemia and Reperfusion and Improves Recovery. *Journal of Cerebral Blood Flow & Metabolism,34*(5), 897-905. doi:10.1038/jcbfm.2014.36

Marqués-Torrejón, M., Porlan, E., Banito, A., Gómez-Ibarlucea, E., Lopez-Contreras, A., Fernández-Capetillo, Ó, . . . Fariñas, I. (2013). Cyclin-Dependent Kinase Inhibitor p21 Controls Adult Neural Stem Cell Expansion by Regulating Sox2 Gene Expression. *Cell Stem Cell,12*(1), 88-100. doi:10.1016/j.stem.2012.12.001

McDonald, M., Mobley, J., Reiserer, R., Bernardo, A., Harrison, F., Ross, J. and Rao, S. (2015). Differential proteomic and behavioral effects of long-term voluntary exercise in wild-type and APP-overexpressing transgenics. *Neurobiology of Disease*, 78, pp.45-55.

Nagai, H., Li, Y., Hatano, S., Toshihito, O., Yuge, M., Ito, E., . . . Kinoshita, T. (2003). Mutations and aberrant DNA methylation of thePROX1 gene in hematologic malignancies. *Genes, Chromosomes and Cancer,38*(1), 13-21. doi:10.1002/gcc.10248

Obermair, F., Fiorelli, R., Schroeter, A., Beyeler, S., Blatti, C., Zoerner, B. and Thallmair, M. (2010). A novel classification of quiescent and transit amplifying adult neural stem cells by surface and metabolic markers permits a defined simultaneous isolation. *Stem Cell Research*, 5(2), pp.131-143.

Park, J., Glass, Z., Sayed, K., Michurina, T., Lazutkin, A., Mineyeva, O., Velmeshev, D., Ward, W., Richardson, A. and Enikolopov, G. (2013). Calorie restriction alleviates the age-related decrease in neural progenitor cell division in the aging brain. *European Journal of Neuroscience*, 37(12), pp.1987-1993.

Paxinos, G. and Franklin, K. (2013). *Paxinos and Franklin's the mouse brain in stereotaxic coordinates*. 1st ed. Amsterdam [etc.]: Elsevier/Academic Press.

Pencea V, Bingaman KD, Wiegand SJ, et al. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *Journal of Neuro-Ophthalmology,22*(2), 135-136. doi:10.1097/00041327-200206000-00020

Petrova, T. V., Nykänen, A., Norrmén, C., Ivanov, K. I., Andersson, L. C., Haglund, C., . . . Alitalo, K. (2008). Transcription Factor PROX1 Induces Colon Cancer Progression by Promoting the Transition from Benign to Highly Dysplastic Phenotype. *Cancer Cell,13*(5), 407-419. doi:10.1016/j.ccr.2008.02.020

plc, A. (2017). *Fluorescence activated cell sorting of live cells*. [online] Available at: http://www.abcam.com/protocols/fluorescence-activated-cell-sorting-of-live-cells [Accessed 13 Jan. 2017].

Ramírez-Rodríguez, G., Estrada-Camarena, E., Oikawa-Sala, J., Gómez-Sánchez, A., Ortiz-López, L. and Vega-Rivera, N. (2016). The neurogenic effects of an enriched environment and its protection against the behavioral consequences of chronic mild stress persistent after enrichment cessation in six-month-old female Balb/C mice. *Behavioural Brain Research*, 301, pp.72-83.

Reference, G. (2017). *DCX gene*. [online] Available at: https://ghr.nlm.nih.gov/gene/DCX [Accessed 3 Jan. 2017].

Richardson, G. D., Breault, D., Horrocks, G., Cormack, S., Hole, N., & Owens, W. A. (2012). Telomerase expression in the mammalian heart. *The FASEB Journal,26*(12), 4832-4840. doi:10.1096/fj.12-208843

Rothman, S. M., Griffioen, K. J., Wan, R., & Mattson, M. P. (2012). Brain-derived neurotrophic factor as a regulator of systemic and brain energy metabolism and cardiovascular health. *Annals of the New York Academy of Sciences,1264*(1), 49-63. doi:10.1111/j.1749-6632.2012.06525.x

Shin, J., Berg, D., Zhu, Y., Shin, J., Song, J., Bonaguidi, M., . . . Song, H. (2015). Single-Cell RNA-Seq with Waterfall Reveals Molecular Cascades underlying Adult Neurogenesis. *Cell Stem Cell,17*(3), 360-372. doi:10.1016/j.stem.2015.07.013

Sobecki, M., Mrouj, K., Camasses, A., Parisis, N., Nicolas, E., Llères, D., Gerbe, F., Prieto, S., Krasinska, L., David, A., Eguren, M., Birling, M., Urbach, S., Hem, S., Déjardin, J., Malumbres, M., Jay, P., Dulic, V., Lafontaine, D., Feil, R. and Fisher, D. (2016). The cell proliferation antigen Ki-67 organises heterochromatin. *eLife*, 5.

Steiner, B., Winter, Y., Hellweg, R., Spranger, J., Wiedmer, P., Rivalan, M., Empl, L., Iggena, D., Jonas, W. and Klein, C. (2016). Exercise prevents high-fat diet-induced impairment of flexible memory expression in the water maze and modulates adult hippocampal neurogenesis in mice. *Neurobiology of Learning and Memory*, 131, pp.26-35.

Stiles, J., & Jernigan, T. L. (2010). The Basics of Brain Development. *Neuropsychology Review,20*(4), 327-348. doi:10.1007/s11065-010-9148-4

Song, J., Czerniak, S., Wang, T., Ying, W., Carlone, D. L., Breault, D. T., & Humphreys, B. D. (2011). Characterization and Fate of Telomerase-expressing Epithelia during Kidney Repair. *Journal of the American Society of Nephrology,22*(12), 2256-2265. doi:10.1681/asn.2011050447

Song, M., Yu, S. P., Mohamad, O., Cao, W., Wei, Z. Z., Gu, X., . . . Wei, L. (2017). Optogenetic stimulation of glutamatergic neuronal activity in the striatum enhances neurogenesis in the subventricular zone of normal and stroke mice. *Neurobiology of Disease,98*, 9-24. doi:10.1016/j.nbd.2016.11.005

Sorrells, S. F., Paredes, M. F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K. W., . . . Alvarez-Buylla, A. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature,555*(7696), 377-381. doi:10.1038/nature25975

Tannock, I., Huang, J., Wojtowicz, J. and Winocur, G. (2013). Physical exercise prevents suppression of hippocampal neurogenesis and reduces cognitive impairment in chemotherapy-treated rats. *Psychopharmacology*, 231(11), pp.2311-2320.

Torres-Alemán, I., Trejo, J., Soya, H., Yamamura, Y., Inoue, K., Tejeda, G., Llorens-Martín, M. and Nishijima, T. (2013). Cessation of voluntary wheel running increases anxiety-like behavior and impairs adult hippocampal neurogenesis in mice. *Behavioural Brain Research*, 245, pp.34-41.

Trinchero, M. F., Buttner, K. A., Cuevas, J. N., Temprana, S. G., Fontanet, P. A., Monzón-Salinas, M. C., . . . Schinder, A. F. (2017). High Plasticity of New Granule Cells in the Aging Hippocampus. *Cell Reports,21*(5), 1129-1139. doi:10.1016/j.celrep.2017.09.064

Utsugi, C., Miyazono, S., Osada, K., Matsuda, M., & Kashiwayanagi, M. (2014). Impaired mastication reduced newly generated neurons at the accessory olfactory bulb and pheromonal responses in mice. *Archives of Oral Biology,59*(12), 1272-1278. doi:10.1016/j.archoralbio.2014.07.018

Van der Zee, E., Eggen, B., Bos, T., Havekes, R. and Van der Borght, K. (2007). Exercise improves memory acquisition and retrieval in the y-maze task: Relationship with hippocampal neurogenesis. *Behavioral Neuroscience*, 121(2), pp.324-334.

Vicario-Abejón, C., Morales, A., Pickel, J., Li, L., Oueslati-Morales, C. and Nieto-Estévez, V. (2016). Brain insulin-like growth factor-i directs the transition from stem cells to mature Neurons during Postnatal/adult Hippocampal Neurogenesis. *STEM CELLS*, 34(8), pp.2194-2209.

Wick, M. and Cerilli, L. (2016). *Glial fibrillary acidic protein - ScienceDirect topics*. [online] Available at: http://topics.sciencedirect.com/topics/page/Glial_fibrillary_acidic_protein [Accessed 1 Dec. 2016].

Wrathall, J., Chung, J., Wu, J. and Lee, H. (2012). SOX2 expression is upregulated in adult spinal cord after contusion injury in both oligodendrocyte lineage and ependymal cells. *Journal of Neuroscience Research*, 91(2), pp.196-210.

Yang, Q., Tang, F., Chen, J., Song, X., Shen, C., Wang, L., Yu, P. and Cheng, W. (2015). Sonic Hedgehog signaling Mediates Resveratrol to increase proliferation of neural stem cells after oxygen-glucose deprivation/Reoxygenation injury in Vitro. *Cellular Physiology and Biochemistry*, 35(5), pp.2019-2032.

Yang, Y., Chen, Y., Liu, H., Wang, J., Ma, X. and Ma, C. (2017). Physical exercise induces hippocampal neurogenesis and prevents cognitive decline. *Behavioural Brain Research*, 317, pp.332-339.

Zhu, D., Han, X., Zhang, Z., Wang, W., Zhu, X., Zhou, Q., Jiang, J. and Luo, C. (2007). Voluntary exercise-induced neurogenesis in the postischemic dentate gyrus is associated with spatial memory recovery from stroke. *Journal of Neuroscience Research*, 85(8), pp.1637-1646.

APPENDIX A: TABLES

Table 1: Gene Summaries

Abbreviation	Name	Function
CD133/PROM1	Prominin 1	Originally thought to mark ANSCs, proven to only accurately mark embryonic stem cells. In the adult brain CD133 marks a diffuse population of cells in the brain that are not correlated to ANSCs. Role in the organization of the apical domain of epithelial cells.
EGFR	Epidermal Growth Factor Receptor	Expressed in active ANSCs, but not quiescent. Ultimately leads to cell proliferation via signaling cascades. Receptor tyrosine kinase that dimerizes and auto-phosphorylates upon ligand binding. This activates pathways that promote cell survival, neuronal functions, and metabolism.
GFAP	Glial Fibrillary Acidic Protein	Class III intermediate filaments needed for support and strength. Only found in astrocytes.
Hes5	Class B Basic Helix-Loop-Helix Protein 38	Basic Helix-Loop-Helix transcription repressor downstream of Notch. Important regulation of cell differentiation. Expression is downregulated upon differentiation.
Nestin	Nestin	Type IV intermediate filament found to promote survival, renewal, and proliferation in neural progenitor cells. May play a role in the trafficking and distribution of intermediate filaments in progenitor cell division.
SOX2	Sex Determining Region Y Box 2	Keeps NSCs undifferentiated. Regulates several genes associated with embryonic development.
DCX	Double Cortin	Codes for a cytoplasmic protein that binds microtubules. Directs migration in the developing brain by regulating the organization and stability of microtubules.
BLBP	Brain Lipid Binding Protein	Small highly conserved protein that binds fatty acids. Involved in the establishment of radial glial fibers in the developing brain which are necessary for the migration of immature neurons.

Continuation of Table 1: Gene Summaries

MASH1/ASCL1	Achaete-Scute Complex Homolog	Codes for a Basic Helix-Loop-Helix (BHLH) transcription factor. Plays a role in commitment and differentiation in neuronal lineages. Essential for the generation of olfactory and autonomic neurons.
NeuroD	Neuronal Differentiation 1	Codes for a Basic Helix-Loop-Helix (BHLH) transcription factor. Regulates the expression of the insulin gene. Involved in the regulation of neurogenesis, morphogenesis of dendrites, retinal neuron formation, inner ear sensory neuron formation, endocrine islet cell formation, eneteroendocrine cell formation, and hippocampal formation. Promotes differentiation into granule cells in the DG and islet cells in the pancreas.
PSA-NCAM	Polysialated Neural Cell Adhesion Molecule	Common in developing nervous systems and areas of neurogenesis. Allows for the migration of precursor cells and synaptogenesis.
Prox1	Prospero Homeobox 1	Transcription factor involved in progenitor cell regulation and cell fate determination. Heavily involved in embryonic development and neurogenesis.
TrkB	Neurotrophic Receptor Tyrosine Kinase 2	"TRKB is the high-affinity receptor for BDNF and mediates BDNF signaling, regulating several aspects of neural plasticity including long term potentiation, neurogenesis and memory" (de Miranda et al., 2015)

Table 2: Antibodies Used

Antibody	Species	Type	Concentration	Color	Catalog Number
Anti-GFP	Rabbit	Primary	1:1000	N/A	Ab6556
Anti-Ki67	Rabbit	Primary	1:1000	N/A	Ab15580
Anti-BrDU	Sheep	Primary	10ug/mL	N/A	Ab1893
Alexa Fluor 488	Goat anti-rabbit	Secondary	1:1000	Green	A11070
Alexa Fluor 350	Goat anti-rabbit	Secondary	1:1000	Blue	A11046
Alexa Fluor 350	Donkey anti-sheep	Secondary	1:1000	Blue	A21097
Anti-GFP	Goat	Conjugated	1:1000	Green	Ab6662

Table 3: qPCR Primers

Primer	Forward Sequence	Reverse Sequence
Cyclophilin	CAAATGCTGGACCAAACACAA	AAGACCACATGCTTGCCAT
Hes5	GCACCAGCCCAACTCCAA	GGCGAAGGCTTTGCTGTGT
BLBP	TAAGTCTGTGGTTCGGTTGG	CCCAAAGGTAAGAGTCACGAC
EGFR	GCATCATGGGAGAGAACAACA	CTGCCATTGAACGTACCCAGA
GFAP	CGGAGACGCATCACCTCTG	AGGGAGTGGAGGAGTCATTCG
Nestin	AGGACCAGGTGCTTGAGAGA	TGGCACAGGTGTCTCAAGGGTAG
SOX2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Prox1	CGCAGAAGGACTCTCTTTGTC	GATTGGGTGATAGCCCTTCAT
CD24	GTTGCTGCTTCTGGCACTG	GGTAGCGTTACTTGGATTTGG
DoubleCortin	CATTTTGACGAACGAGACAAAGC	TGGAAGTCCATTCATCCGTGA
NeuroD	ATGACCAAATCATACAGCGAGAG	TCTGCCTCGTGTTCCTCGT
MASH1	CCACGGTCTTTGCTTCTGTTT	TGGGGATGGCAGTTGTAAGA

APPENDIX B: FIGURES

Figure 1: mTERT-GFP+ cells express ANSC markers and form neurospheres in culture

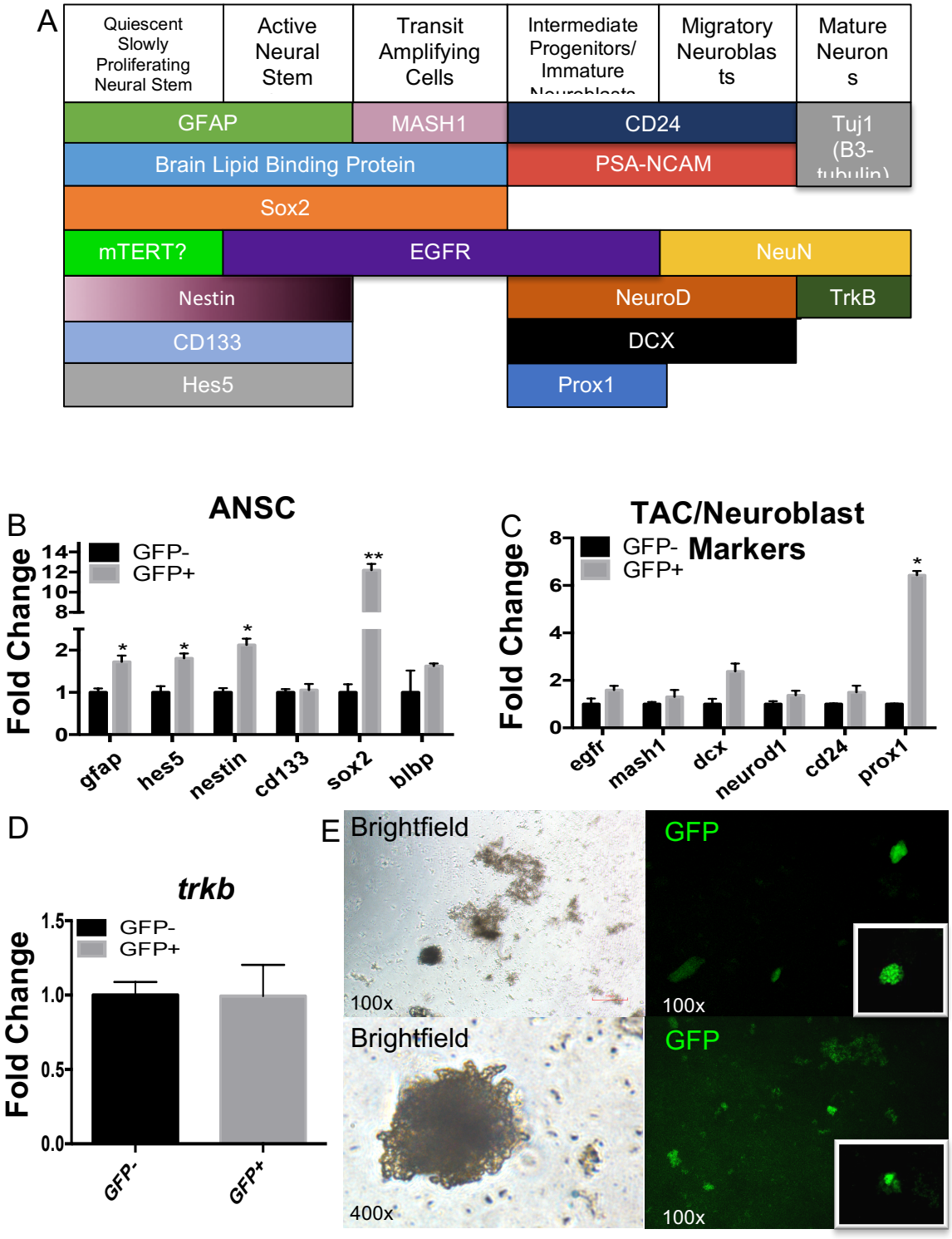
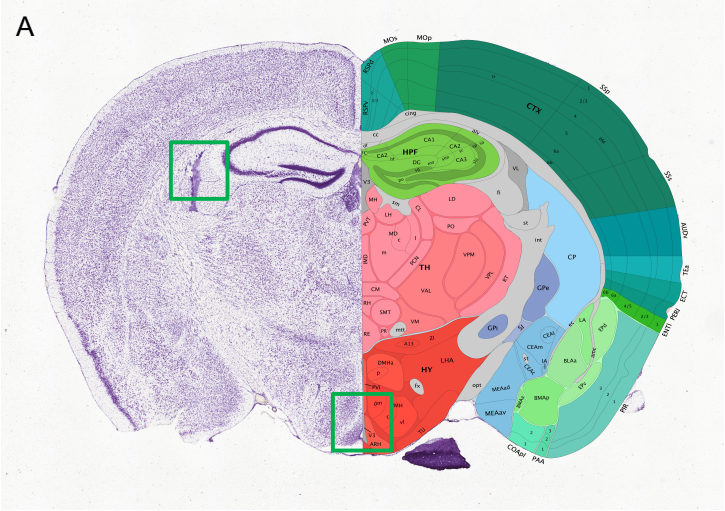
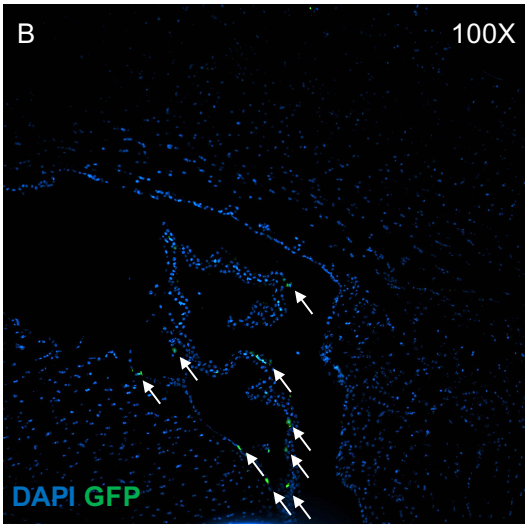


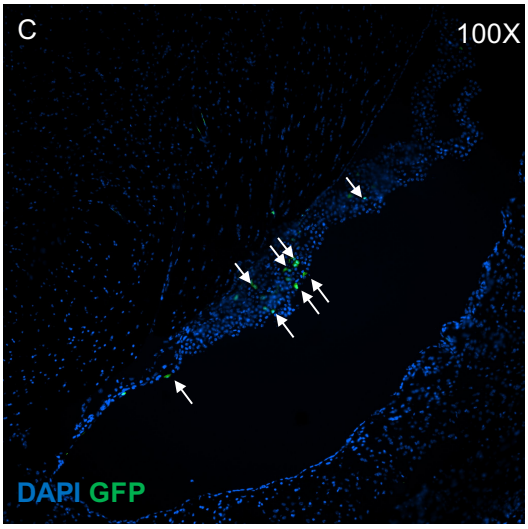
Figure 1: mTERT-GFP+ cells express ANSC markers and form neurospheres in culture

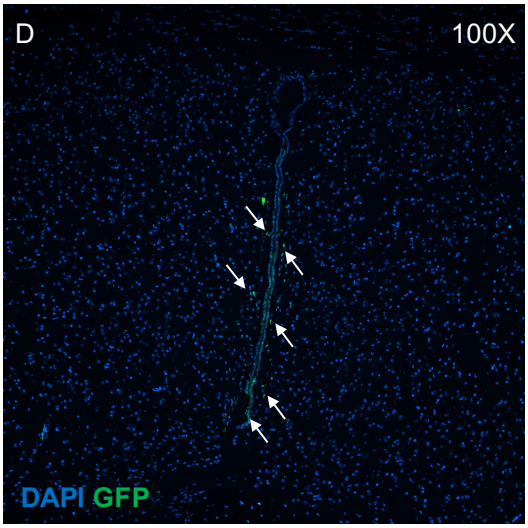
Precursors along the neuronal lineage may be differentiated by patterns of gene expression (A). qPCR shows that after a 2-week pulse and 2-day chase, mTERT-GFP+ cells from six 6-month old mTERT:rtTA::oTet:Cre::Rosa:mTmG mice express a significant upregulation in 4 out of 6 well-studied ANSC markers, namely, GFAP, nestin, Hes5, and SOX2 (B). qPCR analysis of later precursor markers and mature neurons reveal that mTERT-GFP+ cells only express upregulation in 1 out of 7 of these genes (C). Notably the marker for activation, EGFR, is not significantly upregulated in the mTERT-GFP+ population. TrkB, a receptor commonly found on neurons does not show any significant difference between populations (D) (n=4). mTERT-GFP+ cells from a 4-month old mTERT:rtTA::oTet:Cre::Rosa:mTmG mouse behave like ANSCs in culture by forming neurospheres (E). (n=1)

Figure 2: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, and the ventral third ventricle.









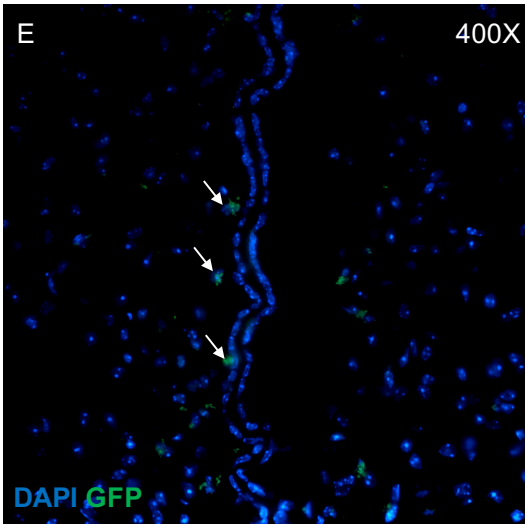


Figure 2: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, and the ventral third ventricle.

Immunostaining with an anti-GFP primary antibody (abcam, ab6556) and AlexaFluor 488 (Life Technology, A11070) in 6 (4 males, 2 females) 1 year old mTERT:GFP mice show GFP+ cells (marked by arrows) in the choroid plexus & the SVZ (A,B), the ventral third ventricle (C,D). mTERT-GFP+ cells were not found in the SGZ (n=6). Representative images are used here (A-D), but further images may be found in supplemental figure 2.

Figure 3: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the hypothalamus, and the olfactory bulb

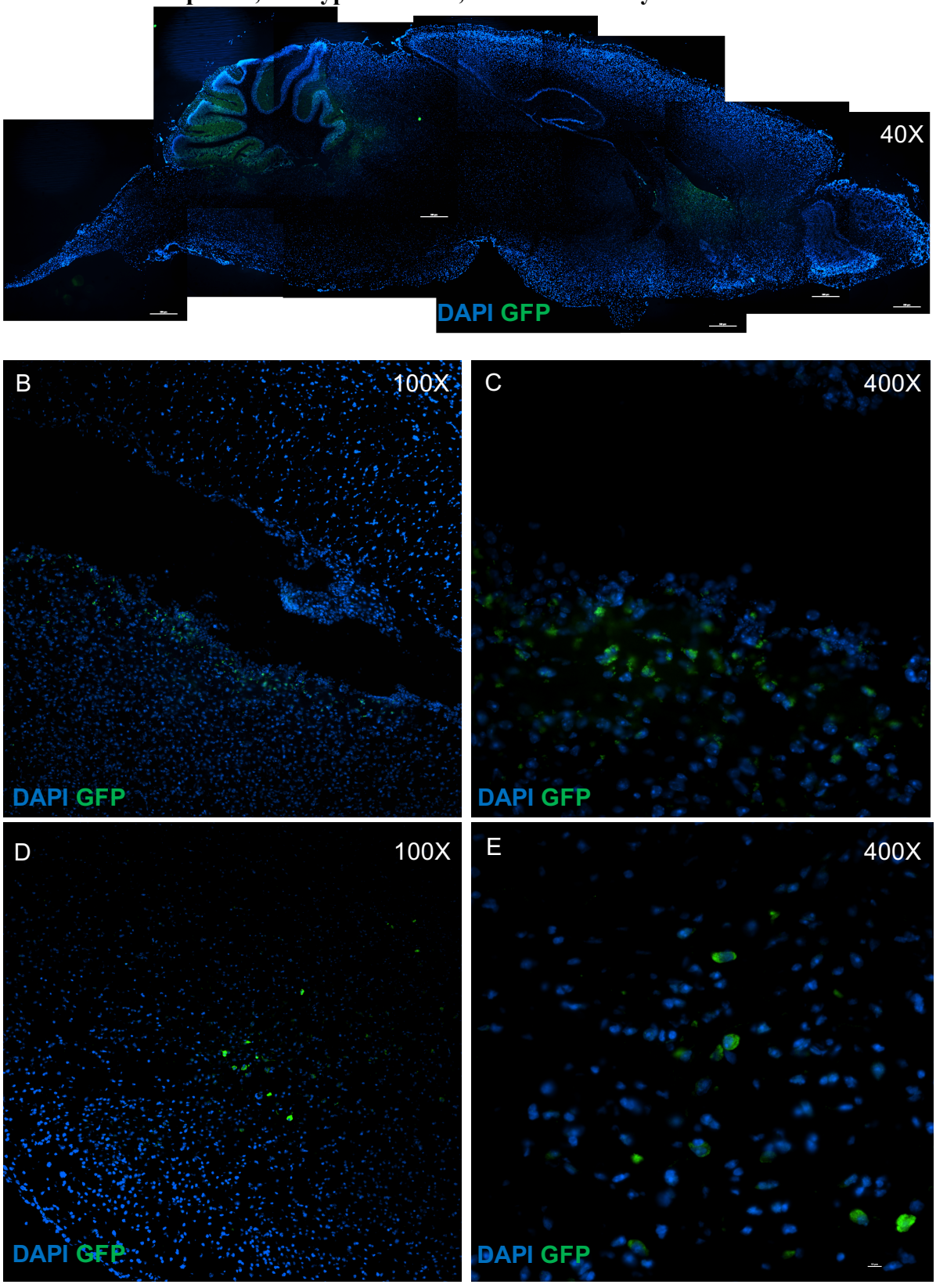


Figure 3: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the hypothalamus, and the olfactory bulb

Immunostaining with an anti-GFP primary antibody (abcam, ab6556) and AlexaFluor 488 (Life Technology, A11070) in 6 male 18 month old mTERT:GFP mice show mTERT-GFP+ cells (marked by arrows) in the choroid plexus & the SVZ (B,C), and the hypothalamus (D,E). mTERT-GFP+ cells were not found in the SGZ. 40X sagittal view for orientation (A) (n=6). Representative images are used here (B-D), but further images may be found in supplemental figure 3.

Figure 4: GFP+ cells do not co-express with neuroblasts marker doublecortin

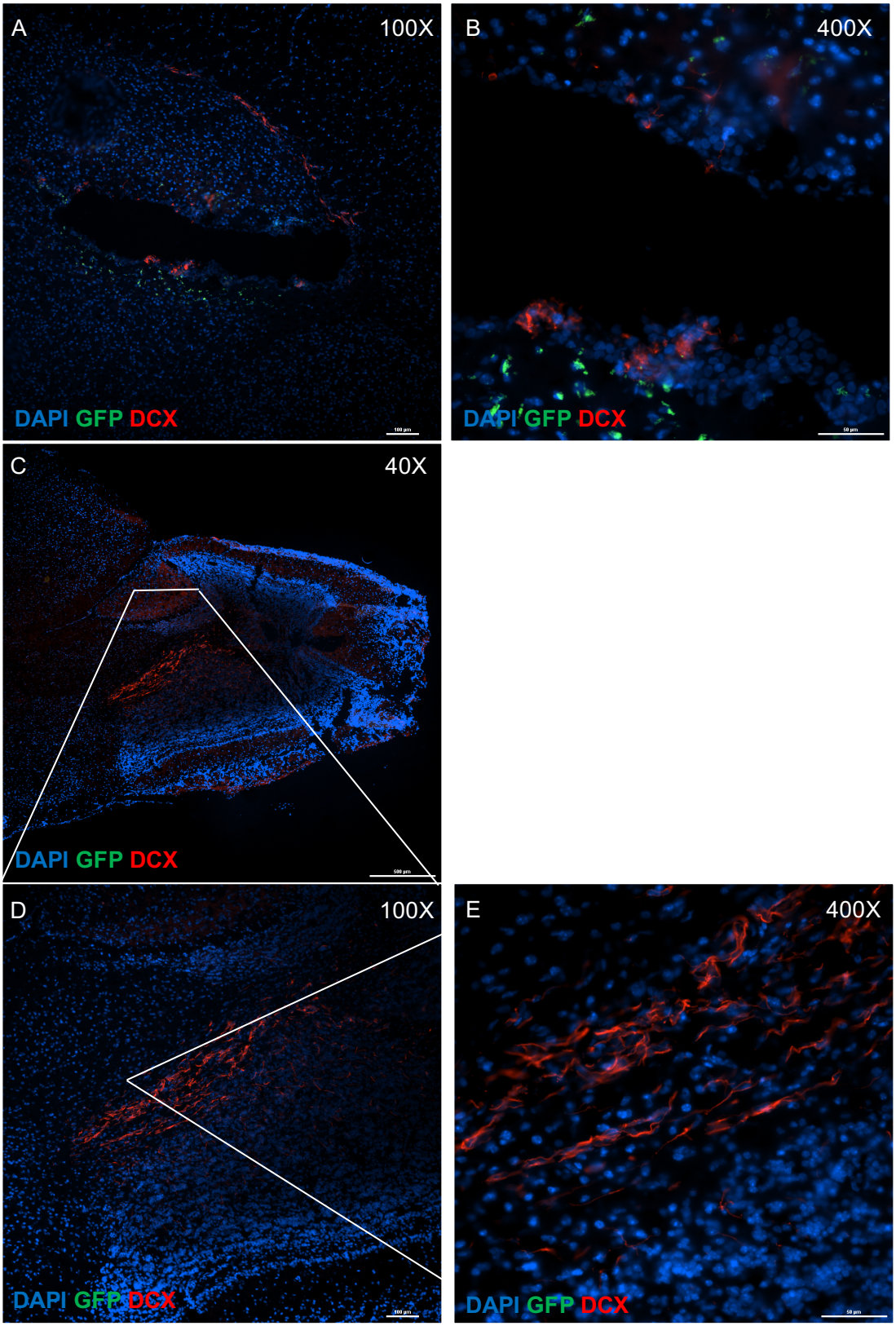


Figure 4: GFP+ cells do not co-express with neuroblasts marker doublecortin
Using fluorescence microscopy in conjunction with immunostaining, in 6 male 18 month old mTERT:GFP mice, mTERT-GFP+ cells were not found to co-express with the neuroblast marker doublecortin (abcam, ab18723) in the lateral ventricle (A,B) or the olfactory bulb (C-E). AlexaFluor 594 was used as a secondary (Life Technology, A11012) (n=6). Representative images are used here (A-E), but further images may be found in supplemental figure 4.

Figure 5: GFP+ cells sometimes co-express with activation marker EGFR

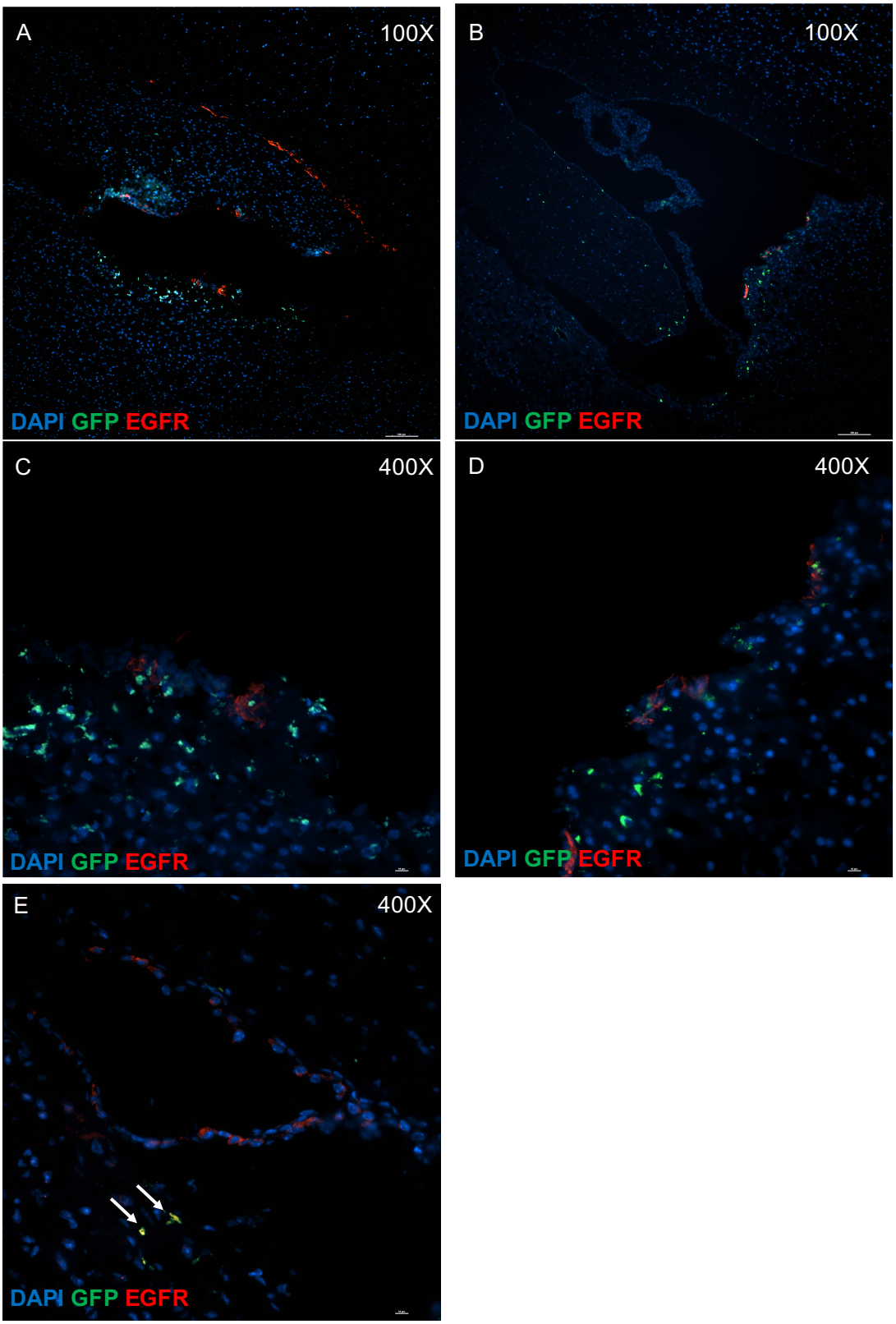
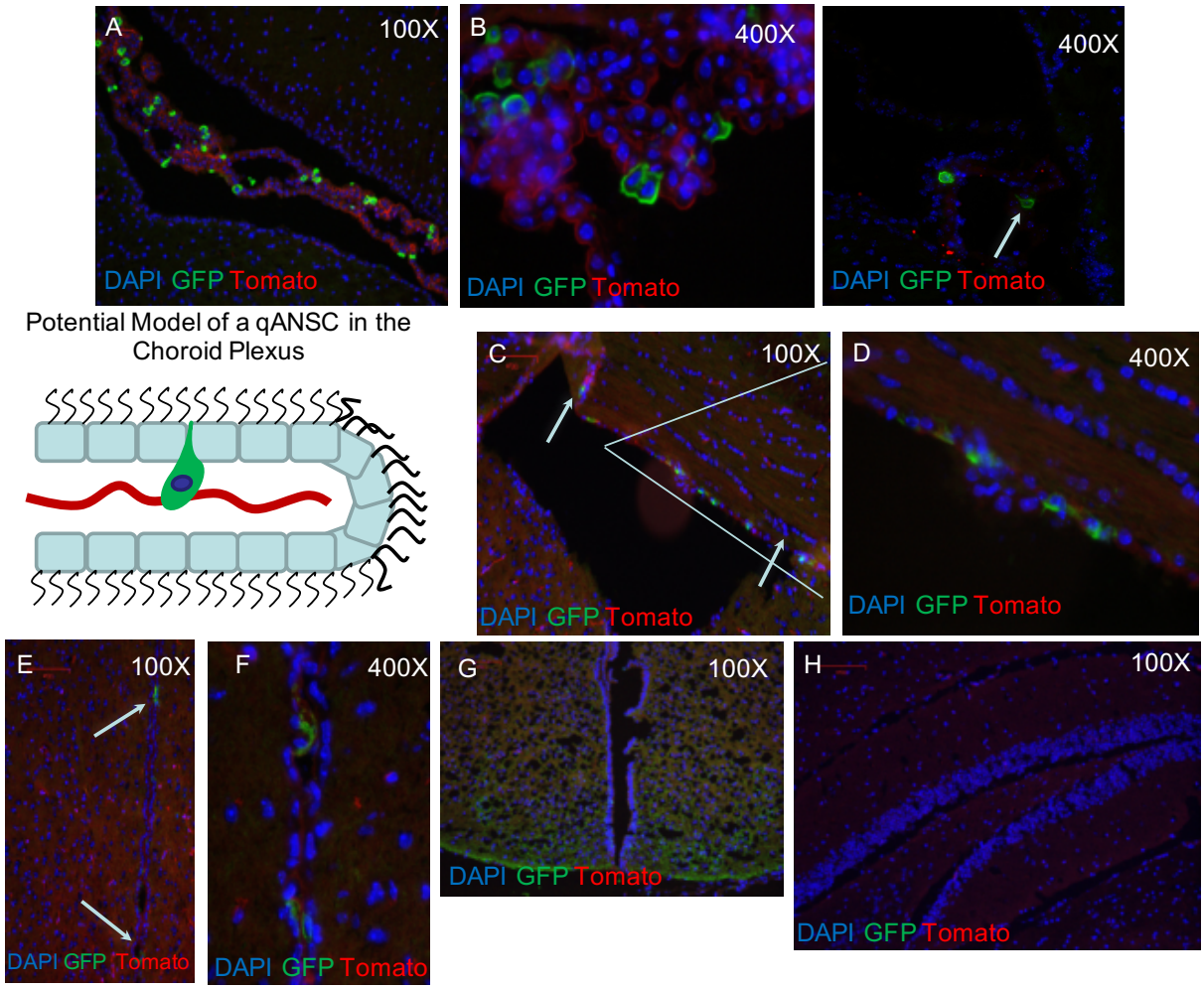


Figure 5: GFP+ cells sometimes co-express with activation marker EGFR
Using fluorescence microscopy in conjunction with immunostaining, in 6 male 18 month old mTERT:GFP mice, mTERT-GFP+ cells were seen to rarely co-express with the activation marker EGFR (abcam, ab 32562) in the lateral ventricle. Images A-D show no co-expression, while image E shows mTERT-GFP+ and EGFR co-expressed (marked by arrows). AlexaFluor 594 was used as a secondary (Life Technology, A11012) (n=6). Representative images are used here (A-E), but further images may be found in supplemental figure 5.

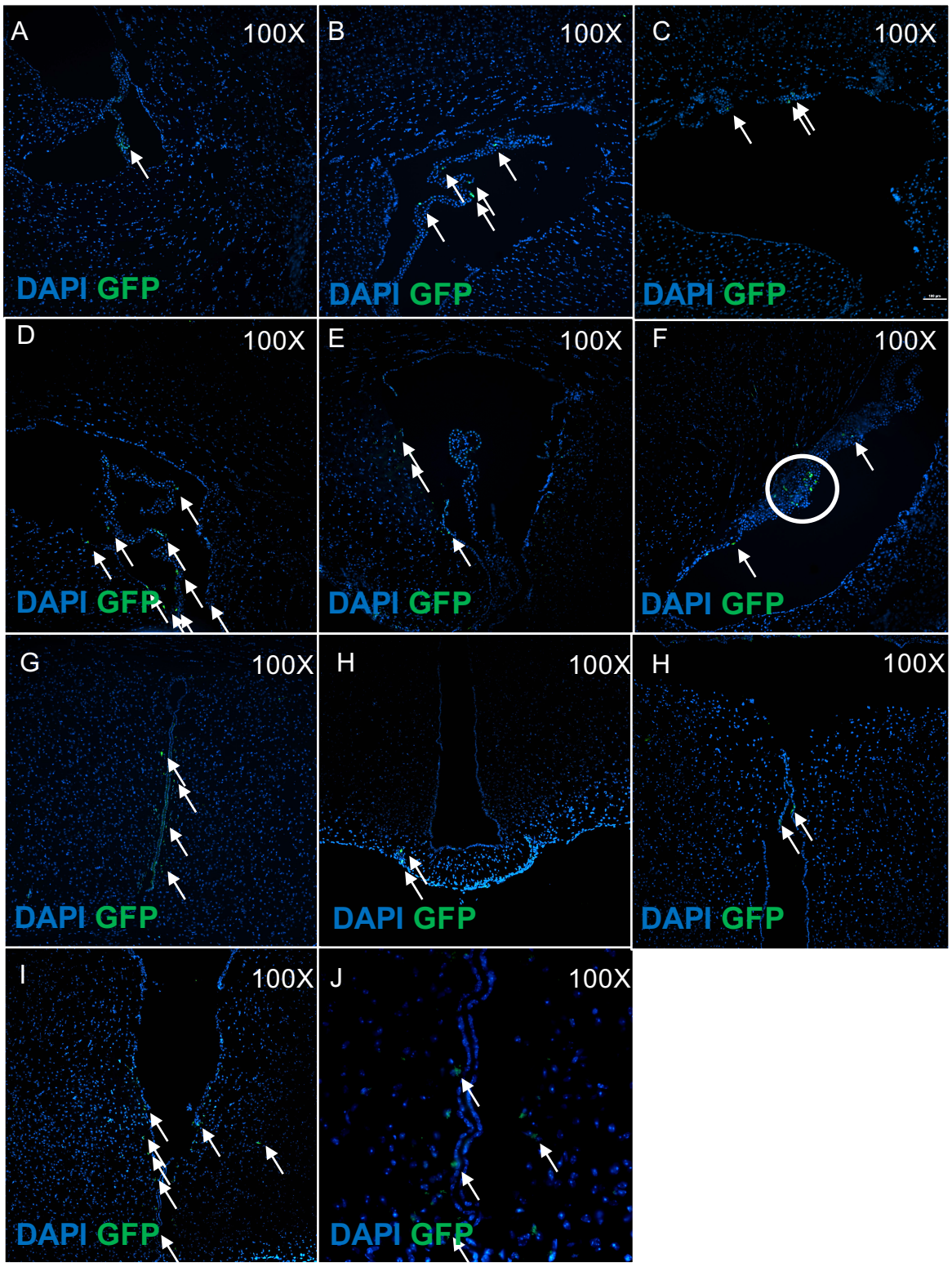
Supplemental Figures

Supplementary Figure 1: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the ventral third ventricle, and the median eminence



Supplementary Figure 1: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the ventral third ventricle, and the median eminence
Immunostaining with an anti-GFP conjugated antibody (abcam, ab6662) in a 20 month old mTERT:rtTA::oTet:Cre::ROSA:mTmG mouse show mTERT-GFP+ cells in the choroid plexus & the SVZ (A, B, & C), the ventral third ventricle, and the median eminence (E, F, G). mTERT-GFP+ cells were not found in the SGZ (H). Included is a diagram of the possible arrangement of qANSCs in the choroid plexus niche (D). (n=1)

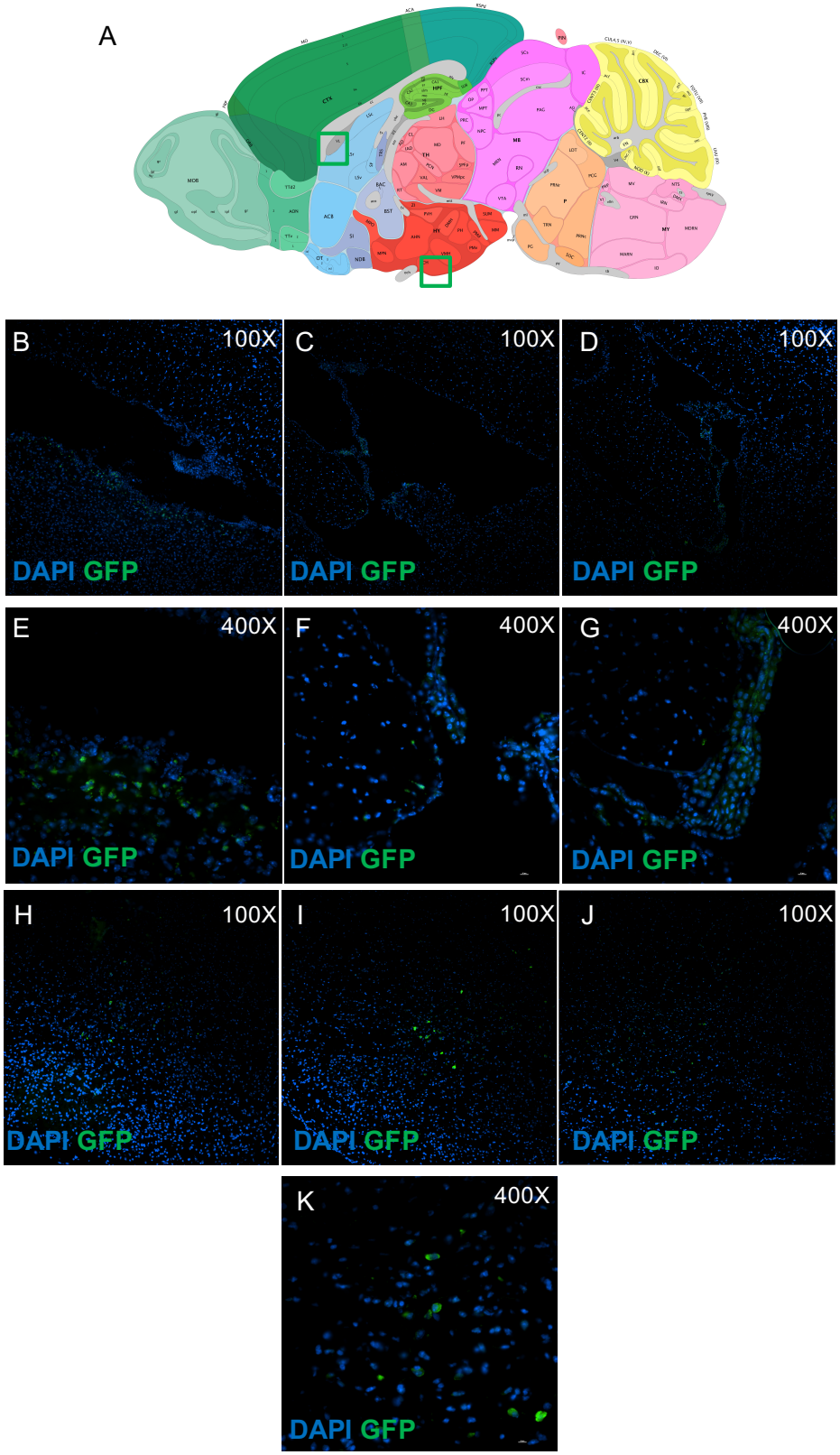
Supplementary Figure 2: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, and the ventral third ventricle



Supplementary Figure 2: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, and the ventral third ventricle

Immunostaining with an anti-GFP primary antibody (abcam, ab6556) and AlexaFluor 488 (Life Technology, A11070) in 6 (4 males, 2 females) 1 year old mTERT:GFP mice show GFP+ cells in the choroid plexus & the SVZ (A-F), the ventral third ventricle (G-J). Representative images can be found in Figure 2. (n=6)

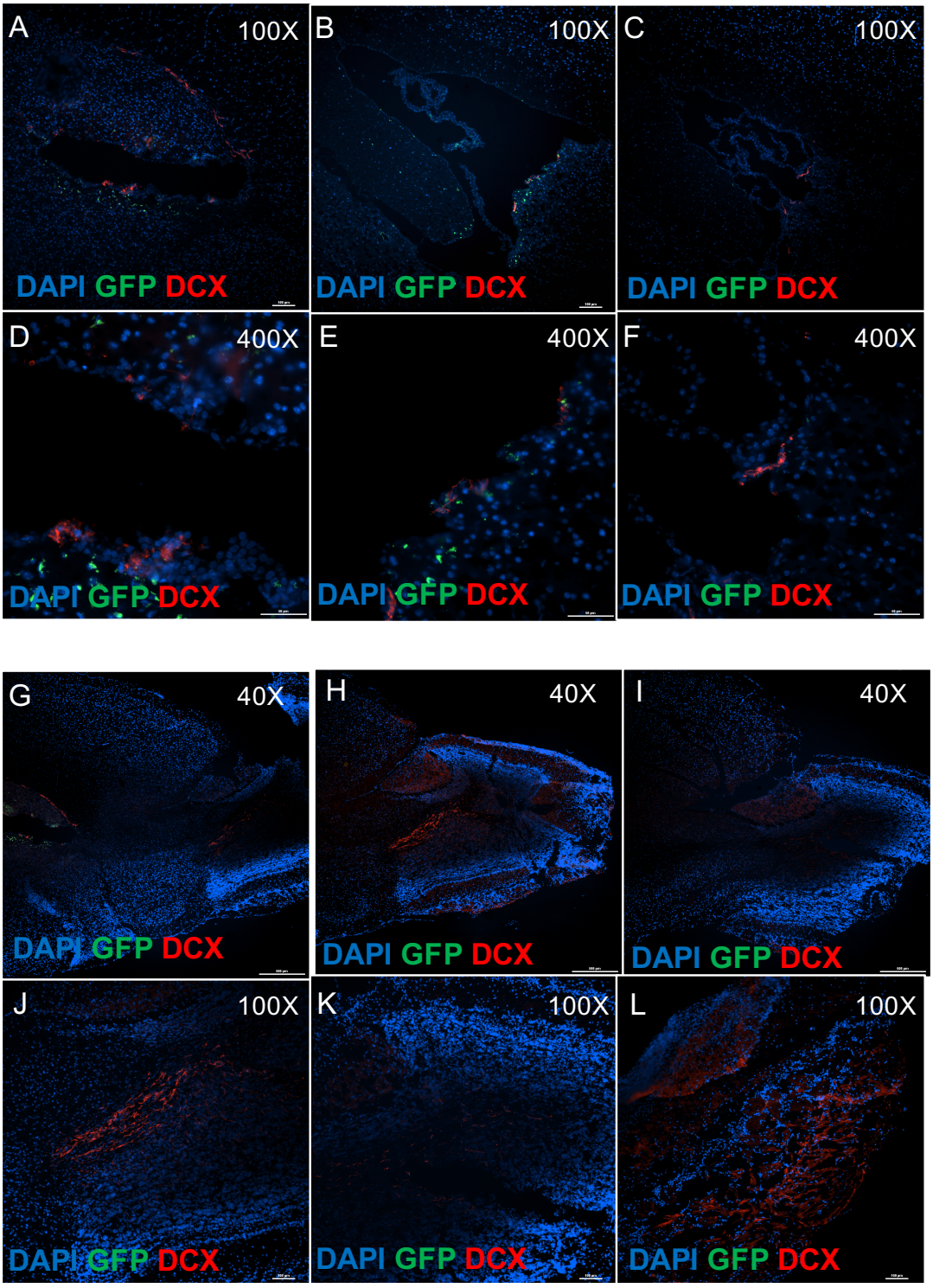
Supplemental Figure 3: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the hypothalamus, and the olfactory bulb



Supplemental Figure 3: GFP+ cells only reside in ventricular niches, including the SVZ, choroid plexus, the hypothalamus, and the olfactory bulb

Immunostaining with an anti-GFP primary antibody (abcam, ab6556) and AlexaFluor 488 (Life Technology, A11070) in 6 male 18 month old mTERT:GFP mice show mTERT-GFP+ cells in the choroid plexus & the SVZ (B-G), and the hypothalamus (H-K). mTERT-GFP+ cells were not found in the SGZ (L-N). 40X sagittal view from the Allen Brain Atlas for orientation (A). (n=6)

Supplemental Figure 4: GFP+ cells do not co-express with neuroblasts marker doublecortin



Supplemental Figure 4: GFP+ cells do not co-express with neuroblasts marker doublecortin

Using fluorescence microscopy in conjunction with immunostaining, in 6 male 18 month old mTERT:GFP mice, mTERT-GFP+ cells were not found to co-express with the neuroblast marker doublecortin (abcam, ab18723) in the lateral ventricle (A-F) or the olfactory bulb (G-L). AlexaFluor 594 was used as a secondary (Life Technology, A11012). (n=6)

Supplemental Figure 5: GFP+ cells sometimes co-express with activation marker EGFR

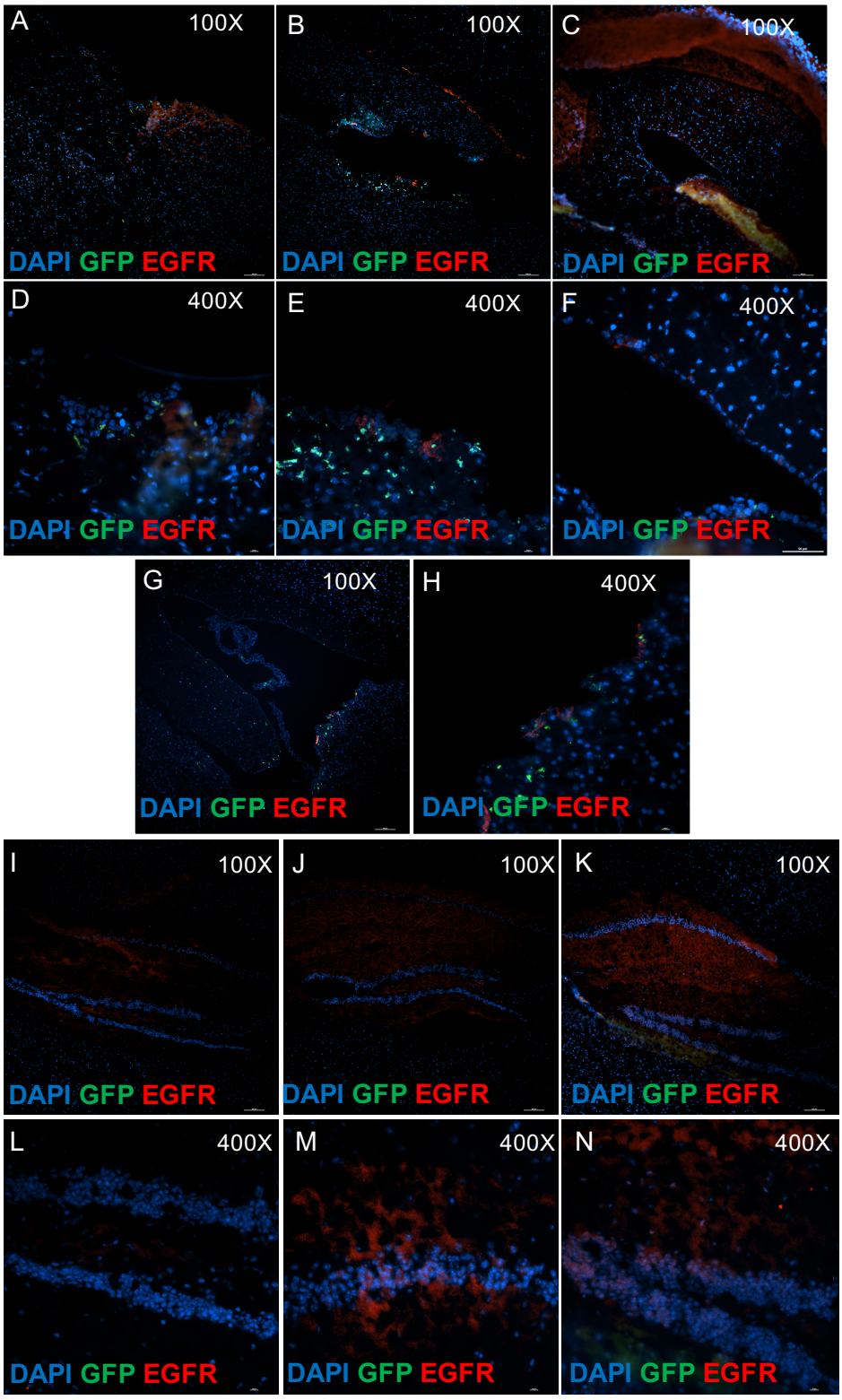
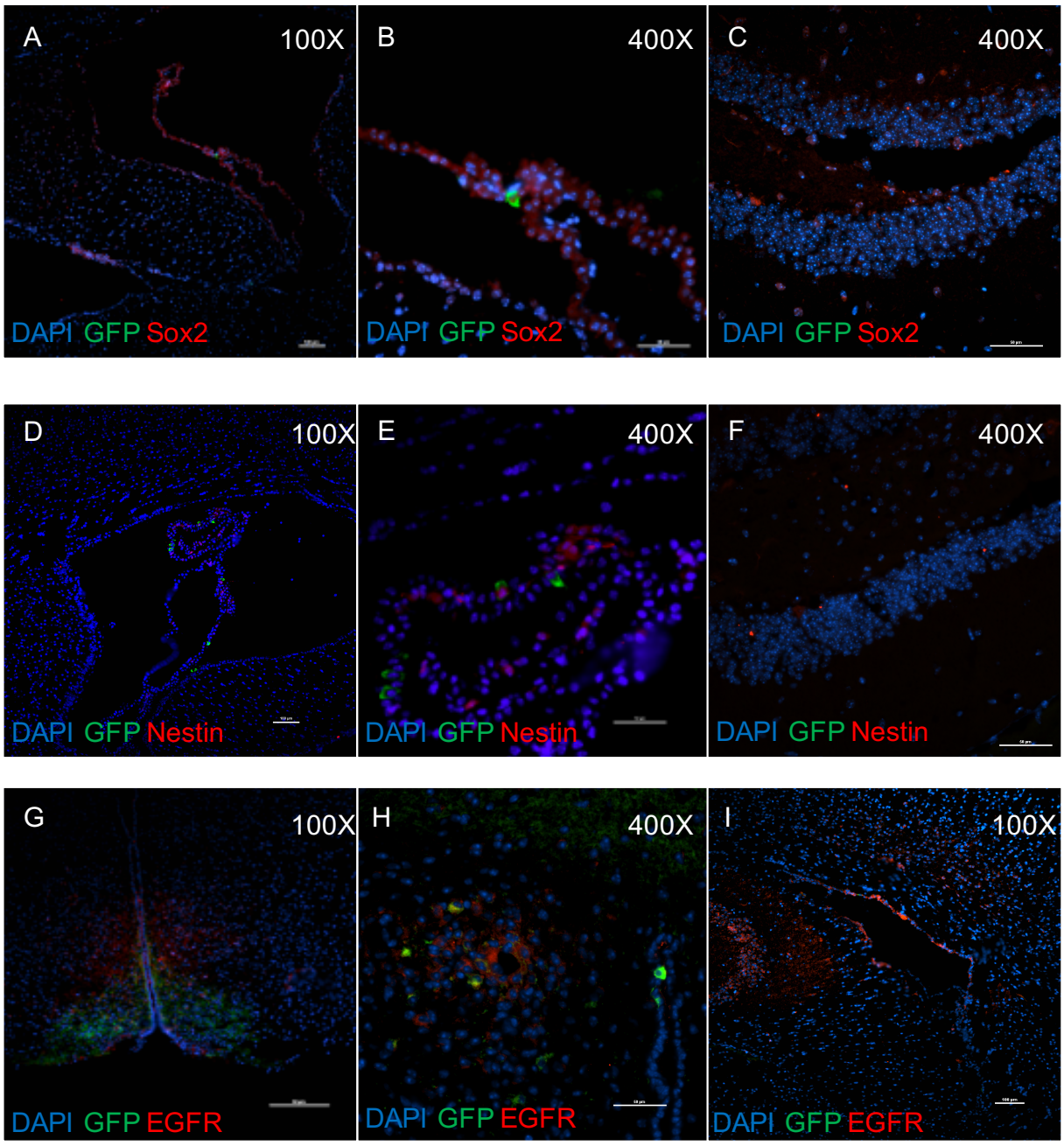


Figure 5: GFP+ cells sometimes co-express with activation marker EGFR
Using fluorescence microscopy in conjunction with immunostaining, in 6 male 18 month old mTERT:GFP mice, mTERT-GFP+ cells were seen to occasionally co-express with the activation marker EGFR (abcam, ab 32562) in the lateral ventricle (A-H) or the olfactory bulb (I-N). AlexaFluor 594 was used as a secondary (Life Technology, A11012). (n=6)

Supplementary Figure 6: GFP+ cells sometimes co-express with well-known ANSC markers, but not with the neuroblast marker doublecortin



Supplementary Figure 6: GFP+ cells sometimes co-express with well-known ANSC markers, but not with the neuroblast marker doublecortin

Using fluorescence microscopy in conjunction with immunostaining, in 7-month old mTERT:rtTA::oTet:Cre::ROSA:mTmG mice, GFP+ cells were seen to co-express with ANSC markers nestin (abcam, ab 11306) (A,B) and sox2 (Millipore, AB5603) (D,E), and activation marker EGFR (abcam, ab 32562) (G,H). AlexaFluor 594 was used as a secondary (Life Technology, A11012). These markers also appeared in their respective niches within the brain (C, F, I). (n=2)

AUTHOR BIOGRAPHY

Caroline Dean Curtis was born June 15th, 1995, in Camp Lejeune, NC into a military family. She grew up between North Carolina, Virginia, Arizona, and Maine. Caroline has three siblings, a brother and two sisters that she has remains close with. She attended the University of Maine as an undergraduate from 2013 to 2017, at which point she transitioned into a Master's as part of the 4+1 program. Caroline intends to move back to NC and pursue a career in industry. She is a candidate for the Master of Science degree in Zoology from the University of Maine in December 2018.