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PROVIDING EXPERIMENTAL SUPPORT FOR MTERT AS AN ADULT NEURAL STEM CELL MARKER USING A TRIPLE TRANSGENIC MOUSE LINE

by

Caroline Dean Curtis

A Thesis Submitted in Partial Fulfillment

of the Requirements for a Degree with Honors

(Biology)

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ABSTRACT

Mouse telomerase reverse transcriptase (mTERT) is a gene that is expressed by cells that need to continually divide without the characteristic shortening of telomeres that accompanies DNA replication. Here we provide experimental evidence for mTERT as a novel and unique marker of adult neural stem cells (ANSCs). We use a triple transgenic mouse line that is designed so that mTERT-positive cells will glow green, via Green Fluorescent Protein (GFP), when the animal ingests doxycycline. This inducible model allows mTERT positive cells to be tracked and identified easily. Dissociated brain tissues were taken from these animals and sorted via a Fluorescence-Activated Cell Sorter into GFP-positive and GFP-negative cells. GFP+ cells were shown to exhibit similar gene expression patterns to that of ANSCs. For further support, the triple transgenic animals were exposed to known neurogenic stimuli, namely exercise and fasting. The brains of these animals were eventually removed, sliced, immuno-stained and imaged using a fluorescent microscope. Imaging allowed us to identify mTERT+ cells in the choroid plexus, but not in the hippocampus, a classic neurogenic niche.

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INTRODUCTION

Background & scope

For centuries now, science has continued to make massive strides in understanding the human body, but one organ is still somewhat a mystery to us. Ironically that organ is the one responsible for all scientific discoveries: the brain. The brain is responsible for the proper timing and functioning of all other organ systems in the body, as well as thoughts and movements. Many studies that look into the functions of the brain typically focus a function that is applicable to a specific disease pathology. In this study we endeavor to elucidate a reliable way to mark adult neural stem cells in order to further research the potential for healing the brain after degeneration, a venture that could allow for research that will affect this field for years to come.

Neurodegenerative diseases, such as Alzheimer's Disease, are characterized by the death of brain cells, without regeneration to replace them. Briefly, some hypothesize that the presence of tau tangles collapse the microtubules of neurons which cuts off their supply of nutrients causing cell death. Amyloid beta proteins have been hypothesized to play a role in Alzheimer's by creating hard insoluble plaques throughout the brain. Short of finding a way to rid the brain of these harmful proteins, one option for treatment could be replacing the damaged cells.

Traumatic brain injuries, or TBIs, cause approximately 2.5 million emergency room visits per year (CDC, 2016). Like Alzheimer's, TBIs can often cause brain damage via cell death which can lead to neurological deficits. TBIs can cause lasting damage and

even death. Common treatments for TBIs focus on alleviating the long term symptoms if any are present. This occurs through therapy, such as occupational or physical therapy, as healing the brain itself is not yet attainable.

Neural precursor cells

The simple answer to these problems of brain damage and degeneration seems to be to heal the brain itself. This could possibly be done through the study and attempt to manipulate adult neural stem cells (ANSCs). These cells could potentially hold the key to being able regenerate brain cells after damage or degeneration as they are able to differentiate into any somatic cell given the correct signals. Studying ANSCs in the brain however has proved very challenging. It was previously believed that adults did not have any ANSCs, although it has since been proven that neural stem cells exist as a population in the brain throughout the entire life (Merkle and Alvarez-Buylla, 2006). However, the ANSC population does markedly decrease with age (Luo et al., 2006, Olariu, Cleaver, and Cameron, 2007, Amrein et al., 2004).

ANSCs have been described as residing in two major niches in the brain in mammals. The first is the subgranular zone (SGZ) of the dentate gyrus (DG). Here cells will differentiate and migrate into the granular cell layer upon stimulation. Processes from these cells will then branch into the molecular layer of the DG. The second niche is the subventricular zone of the lateral ventricles. In mice ANSCs of the SVZ differentiate and migrate to the olfactory bulb to integrate into the neural circuitry there. In humans SVZ ANSCs differentiate and migrate to the striatum instead.

Neural precursor cells exist in six general categories that are dependent on location within the brain as well as morphology, behavior, and genetic expression (A summary of all of the genes discussed in this paper can be found in Supplementary Table 1 below). In the sub-granular zone (SGZ) of the dentate gyrus (DG), type I cells are the earliest form of precursor cells. These cells display expression of Hes5, Sox2, Brain Lipid Binding Protein (BLBP), Glial Fibrillary Acidic Protein (GFAP) (Giachino et al., 2010) and nestin (Zhao, Deng, and Gage, 2008). Type I cells are typically referred to as Neural Stem Cells (NSCs) and can be divided into three subcategories: radial quiescent, horizontal quiescent, and horizontal dividing (Giachino et al., 2010). These categories classify cells by morphology as well as activity, although it has been hypothesized that quiescent cells could move to a state of active division under appropriate conditions. Type II cells, known collectively as Intermediate Progenitor Cells, have been split into early Type IIA cells and later Type IIB cells depending on gene expression. Type IIA cells are characterized by the expression of BLBP, Sox2, and MASH1 with no Hes5 or GFAP expression. Type IIB cells are known to express BLBP, Sox2, DCX, Prox1, and NeuroD. Type II cells are able to self-renew as well as generate astrocytes and neurons (Zhao, Deng, and Gage, 2008) and are localized to the SGZ. Type III cells continue to express DCX and NeuroD, but lack the expression of other common Type II markers. These cells are known as migratory neuroblasts. SGZ ANSC's are organized with Type I cells being located nearest to the surrounding blood vessels with precursors located slightly further away. Once a cell has committed to the neuronal lineage and differentiated into a Type III cell it will migrate a short distance into the granular cell

layer where it will become integrated into the pre-existing neural circuitry as a granular neuron (Mandyam, 2013).

In the sub-ventricular zone (SVZ) a letter system is used to classify neural stem cells, although a correlation can be seen between the gene expression data of the two classification systems. Type B cells are most analogous to the type I cells above. Type B cells, which express GFAP, can be active or quiescent and are characterized as slowly proliferating astrocytes (Doetsch et al., 1999). These cells show a radial morphology and can give rise to oligodendrocytes, astrocytes, and type A cells (Chaker et al., 2016). Quiescent cells are discernible from active due to the expression of epidermal growth factor receptor (EGFR), which is present in the active stem cells but, absent in the quiescent one (Doetsch et al., 2014). Lineage tracing studies have determined that Type A cells are the cells that eventually create new neurons in the olfactory bulb through migration via the rostral migratory stream (Doetsch et al., 1999). Type C cells are similar to type II cells above in that they are described as rapidly dividing immature precursor cells (Doetsch et al., 1999). They express MASH1, can only divide up to three times, and are commonly described as transit-amplifying cells (Karl et al., 2012). Type A cells are migrating neuroblasts that express DCX and PSA-NCAM and can only divide once or twice. These cells will ultimately migrate down the rostral migratory stream (RMS) to the olfactory bulb (OB)

The ANSC's of the SVZ all work together in creating new cells and ensuring that they make it to the OB. Type A cells form chains which then migrate through tunnels created by the processes of Type B cells (Riquelme, Drapeau, and Doetsch, 2008). These Type A cells have been created by clumps of Type C cells that are present next to the

migratory chains (Riquelme, Drapeau, and Doetsch, 2008). These cells express Dlx, MASH1, and the LewisX antigen, but not GFAP (Aguirre et al., 2004). Once in the OB these cells migrate radially into distinct layers that make up the neural circuitry of the OB (Duan et al., 2008).

In this thesis cells will be referred to as ANSCs (Type I & Type B cells), transit amplifying cells (Type C & Type IIA cells), intermediate progenitor cells (Type IIB cells), and migratory neuroblasts (Type A & Type III cells).

In order to study ANSC's it certainly helps to be able to mark them easily and clearly. This has not been accomplished though, due to a lack of a specific and unique marker for ANSC's. Markers do exist, but they also mark other populations which introduces a need for double staining to be certain that you are looking at the correct cells, which will limit the ability to lineage trace these cells. Double staining is not only time consuming and tedious, it also reduces the possibility of cross staining for another cell population or variable. If there was a marker that only marked ANSC's, this would eliminate the tedious and confusing process of staining twice and allow for easier identification. We are proposing that this marker is Telomerase Reverse Transcriptase (TERT), or for the purpose of this experiment mouse Telomerase Reverse Transcriptase (mTERT).

The role of mTERT

TERT is a gene that is responsible for the lengthening of telomeres. In normal cells the telomeres at the end of our chromosomes shorten with each replication cycle, eventually leading to cell senescence. Cellular senescence occurs when the telomeres are

no longer present and the cell ceases to divide. In the average somatic cell telomeres are not regenerated during the life of the cell. mTERT+ cells however are different. These cells have their telomeres replaced so that they cannot reach cell senescence and can keep dividing. In order to prevent senescence in stem cells TERT is used to maintain the length of the telomeres.

mTERT has been shown to be present in slowly cycling intestinal stem cells (Montgomery et al., 2010). Intestinal stem cells are very common due to the transient nature of the epithelial lining of the gut. Most intestinal stems cells are rapidly cycling cells that maintain a healthy gut lining. However, researchers have hypothesized that a potential second population of slowly cycling cells must also be present in order to protect against genetic mutations as well as aid in repair of tissue damage. The Breault Lab, at Children's Hospital/Harvard Medical, endeavored to find and mark these cells using mTERT and were successful in their venture. ANSCs have been shown to have a slowly cycling population in B cells (Ramírez-Castillejo et al., 2006). The Breault Lab's research has allowed for the hypothesis that mTERT could mark slowly cycling Neural Stem Cells in addition to Intestinal Stem Cells. The Breault Lab has also allowed for the examination of this hypothesis by permitting our lab to use their triple transgenic mouse line, which allows for lineage tracing of mTERT cells to determine how they proliferate, migrate, differentiate, and integrate into the existing neural circuitry.

Research tools & plan

In this transgenic mouse line mTERT+ cells can be induced to express an indelible green fluorescent protein marker (further detail in the methods section). In order

to determine whether mTERT+ cells showed typical ANSC patterns, it is necessary to use common neural stimulation techniques and lineage tracing. Exercise, specifically running in mice, has been repeatedly shown to increase hippocampal neurogenesis (Van der Borght et al., 2007, Winocur et al., 2013, Luo et al., 2007). Novel objects added to the home cage, sometime referred to as Enriched Environment, have also been shown to increase neurogenesis (Vega-Rivera et al., 2016, Garthe, Roeder, and Kempermann, 2015). These two sources together will allow for a significant amount of neurogenesis in our experimental group. These effects can be strain specific, but thus far we have no reason to believe that our mice have any deficiencies in neurogenesis. Although the control group will experience neurogenesis due to the novel object (the locked running wheel) the experimental group should experience significantly more, which will allow for us to visualize the difference in the brains and their mTERT+ populations. In both groups this should hopefully allow for enough of a stimulus to increase neurogenesis.

Similar to exercise, fasting has been shown to promote hippocampal neurogenesis in adult mice (Hornsby et al., 2016, Park et al., 2013). Although the mechanism is currently not known, it has been hypothesized that diet restriction causes a mild stress response which then stimulates the production of stress resistant proteins which stimulate the creation of new neurons (Gillette-Guyonnet and Vellas, 2008). These proteins include neurotrophic factors, protein chaperones, and anti-apoptotic proteins.

Female mice were used in the running study because it has been repeatedly shown that females run significantly more than their male counterparts, regardless of strain (Koteja et al., 1999). With so few mice this increase in running activity was crucial to obtain a power significant enough to show true effects. Female mice are used in many

high impact exercise and neurogenesis papers as well (Herring et al., 2016, Klein et al., 2016). Thus far all of our cohorts have had male and female mice and no sex difference has been observed in neurogenesis.

Our aim is to determine whether mTERT is a novel and unique marker for ANSCs. We hypothesize that by stimulating neurogenesis we will see patterns that are typical of quiescent ANSCs exhibited by the mTERT+ cells. Support for this hypothesis will be sought using genetic expression studies via quantitative Polymerase Chain Reactions (qPCR), imaging to confirm the location of the mTERT+ cells using a fluorescent microscope, and co-staining with two known proliferation markers to identify precursor cells.

METHODS

Animals

All animals were housed according to Institutional Animal Care and Use Committee (IACUC) specification with a 12 & 12 light and dark cycles. The strains used were mTERT-GFP and mTERT-rtTA oTETCre Rosa-mTmG (mTERT mTmG will be used from this point on for clarity). mTERT GFP is an endogenous reporter that marks cells that are currently expressing mTERT with a green fluorescent protein. This protein is no longer expressed upon the cessation of mTERT expression.

mTERT mTmG is an inducible triple transgenic cre line. The cells of these mice are marked with a red membrane protein unless doxycycline is added to their water supply. Upon ingestion of doxycycline any newly formed cells that express mTERT will be indelibly marked with a green fluorescent membrane protein (GFP). These cells will express this GFP even if mTERT ceases to be expressed, which allows for the aforementioned lineage tracing studies. Below in figure X is the exact mechanism behind this triple transgenic line. In mTERT-expressing cells a reverse tetracycline controlled transactivator (rtTA) is transcribed. The rtTA is not active until bound by a tetracycline derivative (in this case doxycycline, or dox). Once activated the rtTA-dox complex binds to the promoter region on OTet and allows for the transcription of the cre gene, which will eventually form a cre protein. Cre excises anything between the two LoxP sites, in

our case a membrane tomato and a stop. This allows for the expression of membrane GFP in only mTERT-expressing cells.

Figure 1

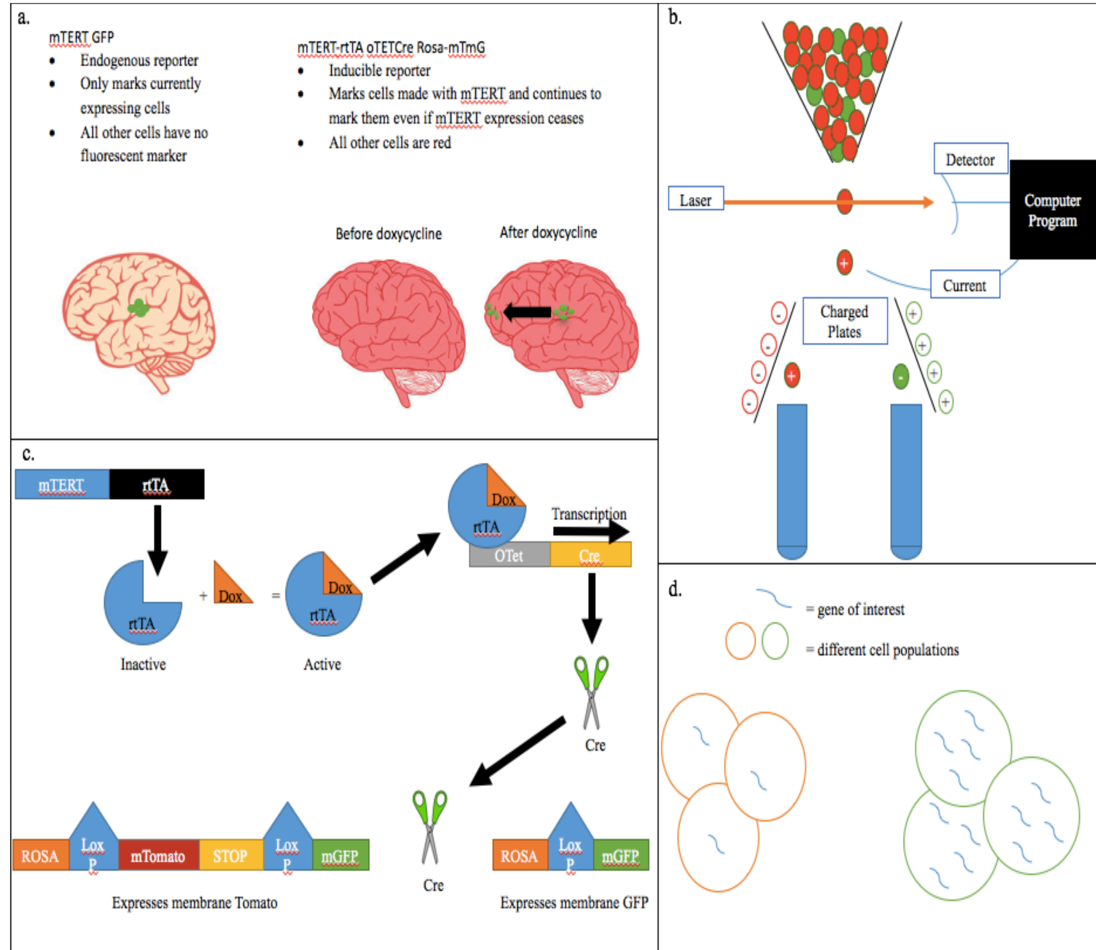

Figure 1a: This figure demonstrates the differences between the brains and cell markers for each strain. Figure 1b: This brief visual explanation of the mechanism behind FACS shows how cell color determines the charge a cell receives and therefore which vial the cell is sorted into. Figure 1c: The transgenes at work. This figure shows the mechanism behind the triple transgenic line mTERT-rtTA oTet-Cre ROSA-mTmG. Figure 1d: qPCR will amplify any gene of interest based on the primers used. Each cycle of replication doubles the number of copies of the gene. Exponential increases in expression will make differences more apparent.

Experimental cohorts

Exercise experiment

Four female mice were used per group. The “run” group had a running wheel in their cage that was free to spin. The “lock” group had a running wheel in their cage, but it was locked via a zip tie, so that it could not spin and no running could be accomplished. A three-week pulse of doxycycline was given before the addition of the wheels. Once the wheels were added the doxycycline was taken away for the chase period. The chase/wheel period lasted for eleven days. At this time, they were sacrificed and perfused. This experiment was originally run on male mTERT GFP mice, but due to nonspecific signal of unknown origin, another run was scheduled using female mTERT mTmG mice.

Fasting, feeding, & re-feeding experiment

This cohort was aged matched and used both sexes. This group was given a doxycycline pulse for three weeks and a water chase for five days. Two males and two females had continuous ad libitum access to food. Three males and two females were fasted for 24 hours before euthanization. Three males and two females were fasted for 24 hours and then given ad libitum access to food for 24 hours before euthanization. All groups were injected with 50mg/kg bromodeoxyuridine (BrDU) 24 hours before euthanization. These interventions were intended to show increased neurogenesis upon stimulation.

Tissue collection, storage & processing

Euthanization, dissection & storage

Mice were injected with a mixture of ketamine and xylazine prior to perfusion according to their body weight. Once the mouse was determined to be completely under anesthesia it was restrained. An incision was made into the animal and the heart was found. The perfusion needle was inserted into the left ventricle, the perfusion machine was started at 8.11mL/minute, and the right atria was clipped. The first fluid to perfuse was PBS buffer, and once this ran clear out of the body, Amresco was perfused to fix the tissue. Amresco is a fixative commonly used in the preservation of brains. Upon completion the mouse was removed from the machinery and the brain was dissected out. This was placed into a labeled cassette and put into a jar of Amresco in order to post fix. After 24 hours of Amresco the tissue was put through a sucrose gradient in order to protect it from the harmful effects of freezing. The gradient went from 5% to 15% to 30% sucrose solutions. From the sucrose gradient the tissue went into OCT (Optimal Cutting Temperature Compound), after being cut into 5 or 6 smaller sections using a brain block. Each piece was arranged in a plastic container with OCT poured over it and frozen via an ethanol and dry ice slurry. These tissues were then stored in a -20°C freezer.

Slicing

All slicing was done using a Leica CM 3050 S Cryostat set at -21°C with 7um slices. Tissue blocks were trimmed on a 10um slice until the tissue was clearly visible. At

this point the 7um slice setting was used to cut serial sections. Two sections were put on each slide with one slice occurring between them that was not used. Between each slide five slices were discarded. Once slicing was completed for the day tissues were dried in a 37°C oven for 12-18 hours. From here they were stored once again at -20°C.

Immunostaining

Slides were chosen based on location in the brain. Multiple slides of comparable location were taken from each animal. Slides were brought to room temperature over a period of thirty minutes. They were then fixed in ice cold acetone, followed by a rinse using Millipore Rinse Buffer (Cat #20845). Sudan black is used for twenty minutes to block for auto-fluorescence. After this tissues are again rinsed using the rinse buffer. Each slide has two slices on it. Each tissue has a hydrophobic barrier in the shape of an oval drawn around it using a pap pen (Dako Pen S2002). These barriers allow for drops of liquid to be pipetted directly onto the tissue without spreading. Millipore block (Cat #20773) is used for twenty minutes at thirty-seven degrees Celsius in order to block for non-specific staining. Tissues are then washed and a dilution of the primary antibody is added. The tissues with primary antibody are placed in a fridge overnight. The following morning tissues are washed again and a dilution of secondary antibody is added for ten minutes. Tissues are rinsed with wash buffer and then water. If the secondary antibody is not blue, DAPI can be used as a nuclear stain in order to more clearly visualize cells. For this to occur slides are put into a solution containing DAPI for five minutes and then well rinsed with water. A coverslip is then applied to each tissue using fluorescent mounting media (Millipore Cat #5013). Slides can be left flat to dry overnight and imaged the following day.

Table 1

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 1: All antibodies and stains used in immuno-staining

Imaging

Slides were imaged using a Nikon Eclipse E400 Fluorescence light microscope. Contrast was linear for all photos. Camera gain ranged from 100 to 800. Exposure ranged from 100ms to 800ms. All photos were captured using a 10X lens used in combination with a 10X objective for a total magnification of 100X. Immunostaining and imaging are used to determine specifically where cells reside in the brain.

Gene expression experiment

Fluorescence activated cell sorting

Four male and three female mTERT mTmG mice were given two weeks of doxycycline in their water, known as the "pulse", and then taken off doxycycline for 3 days, known as the "chase". At the end of the three days they were euthanized according to IACUC approved protocols and their brains were dissected. All areas caudal to the hypothalamus were removed and the majority of the cortex was removed. The brain was then finely sliced in 5mL of Pronase and Artificial Cerebrospinal Fluid (ACSF) (1mg/ml pronase in 15 ml ACSF). The brain pieces and reagents were then put into a 50ml tube in a shaking water bath at 37°C for 75 minutes. The sample was spun down and the pronase/ACSF was decanted and fresh Phosphate Buffered Saline (PBS) with 10% Fetal Bovine Serum (FBS). The tube was then placed back in the shaking water bath for 15 minutes. Fire polished pipettes were then used to triturate the samples in order to break up any remaining pieces of tissue. Three sizes were used for this with a 600um size being

used first. The other two sizes were each slightly smaller, but the exact sizes are unable to be determined. These were created by holding 600um tips over an ethanol lamp flame until the opening became slightly smaller. The samples were spun down again, decanted and suspended in PBS with 10% FBS again and transferred to FACS tubes. These were kept on ice and then transported to Jackson Laboratory via car.

Samples were sorted by Dr. Will Schott using a FACS Aria II machine. The sort results are shown below in the results section. Gating can be seen in supplemental figure 1. These cells were then transported back to UMaine on ice in lysis buffer.

Cells had cDNA extracted from them by Dr. Kristy Townsend. cDNA for this experiment did not require nanodropping to test for the amount of cDNA present because our results were expected to be largely a highly expressed or barely expressed response.

Quantitative polymerase chain reaction

For quantitative Polymerase Chain Reaction primers were chosen based on known expression in the brain. Our specific primers were ordered based on primers used in other papers and cross checked for specificity via a nucleotide BLAST. This takes the sequence given and searches for its use. If it is a specific primer it will come up as the gene it is used for. If it is not, it may come up as a variety of genes. Below, in Table 1, are all of the primers that were used and their sequences.

All qPCR reactions were done using a BioRad CFX96 C1000 Thermal Cycler and SYBR. Reaction volume was 20 ul, with 15 ul master mix and 5 ul cDNA in each well of a 96 well plate. Each reaction was run in duplicate and reactions were not used in any

calculations if the duplicates differed by more than 0.5 arbitrary units. The process behind qPCR is explained in Figure 1 above. qPCR data were used to show the differences in gene expression between GFP+ and GFP- cells.

Table 2

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

Table 2: All forward and reverse primers used for qPCR were obtained from scientific papers and then checked for specificity.

RESULTS

GFP+ cells exhibit gene expression patterns similar to ANSCs but not other neural precursor cell populations

Analysis of gene expression of GFP+ cells in Figure 3 revealed a pattern similar to that of the quiescent slowly proliferating neural stem cells, seen in Figure 2. GFAP, nestin, SOX2, and Hes5 have all been identified as significantly upregulated in GFP+ cells when compared to GFP- cells. Possibly even more exciting than the significantly different genes are the genes that were not significantly different. These genes include MASH1, DoubleCortin, NeuroD, EGFR, and TrkB; all markers of more activated/committed/differentiated neural precursors.

GFP+ cell frequency is comparable to that of ANSCs in the brain

FACS sorting yielded numerical results related to the relative occurrence of GFP+ cells compared to GFP- cells. These results show an average of less than one percent of cells are GFP+ in the brains we sorted. This means ~99.1% of all cells sorted are GFP-. These data, combined with the knowledge that our aim was sorting neurogenic regions points to GFP+ cells making up a very small population in the adult brain. This small population combined with our other evidence supports our hypothesis that mTERT+ are indeed ANSCs.

Figure 2

Quiescent Slowly Proliferating Neural Stem Cell	Active Neural Stem Cells	Transit Amplifying Cells	Intermediate Progenitors/ Immature Neuroblasts	Migratory Neuroblasts	Mature Neurons

GFAP
MASH1
CD24
Tuj1 (B3-tubulin)
Brain Lipid Binding Protein
PSA-NCAM
Sox2
mTERT?
EGFR
NeuN
Nestin
NeuroD
TrkB
CD133
DCX
Hes5
Prox1
LeX

Figure 2: This chart is a comprehensive compilation of genetic markers for each kind of neural precursor. (Daynac 2016; Doetsch et al., 2014; Giachino et al., 2010; Karl et al., 2012; Zhoa, Deng, and Gage, 2008)

Figure 3

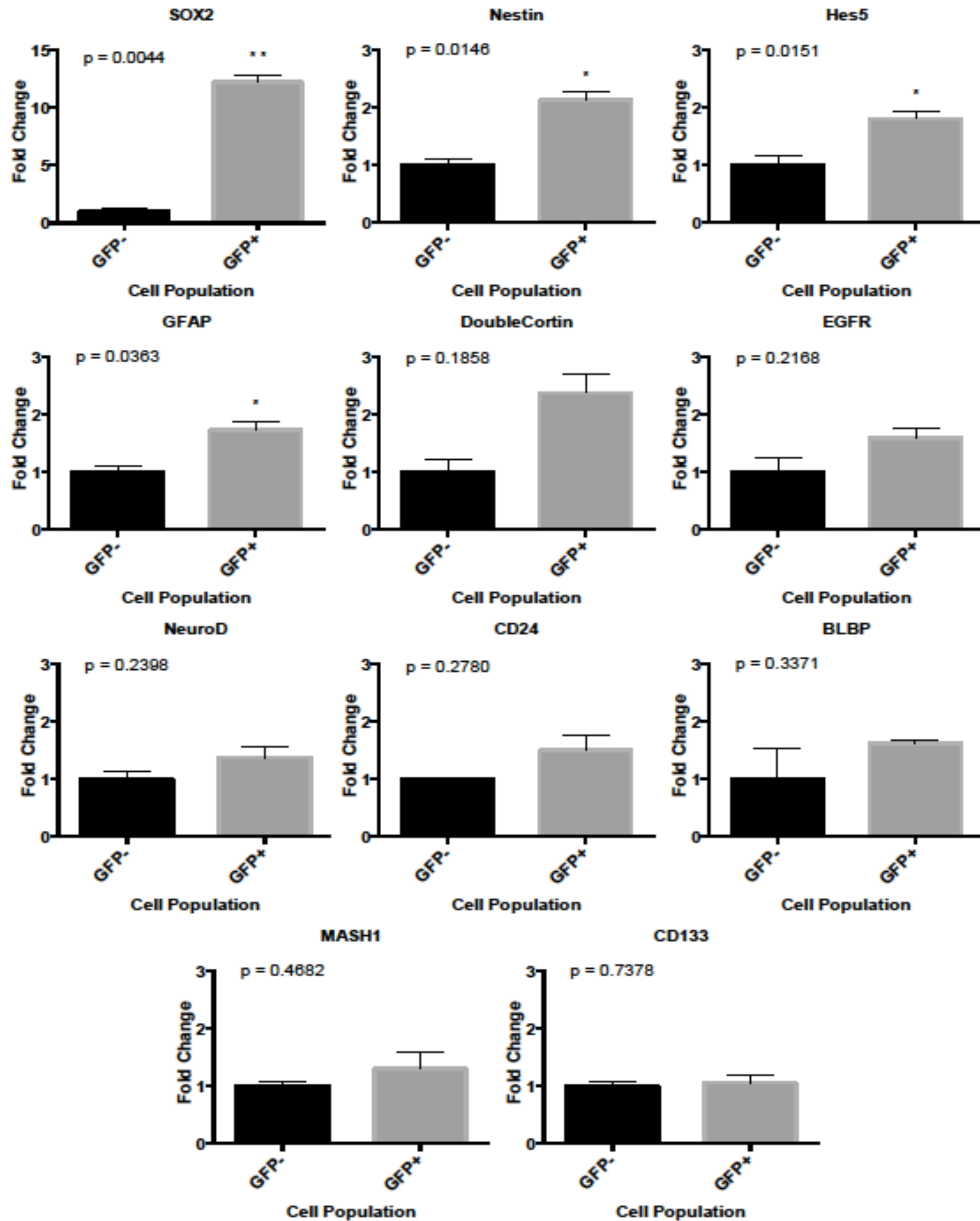

Figure 3: GFP+ cells and GFP- cells were assessed for gene expression. Five markers were significantly more expressed in GFP+ cells compared to GFP- cells: SOX2, Hes5, GFAP, Prox1 and Nestin. Seven tested do not display significant differences: DoubleCortin, EGFR, NeuroD, MASH1, CD24, BLBP, and TrkB. (Fold change ± SEM, significance determined using an unpaired student's t-test.

Figure 4

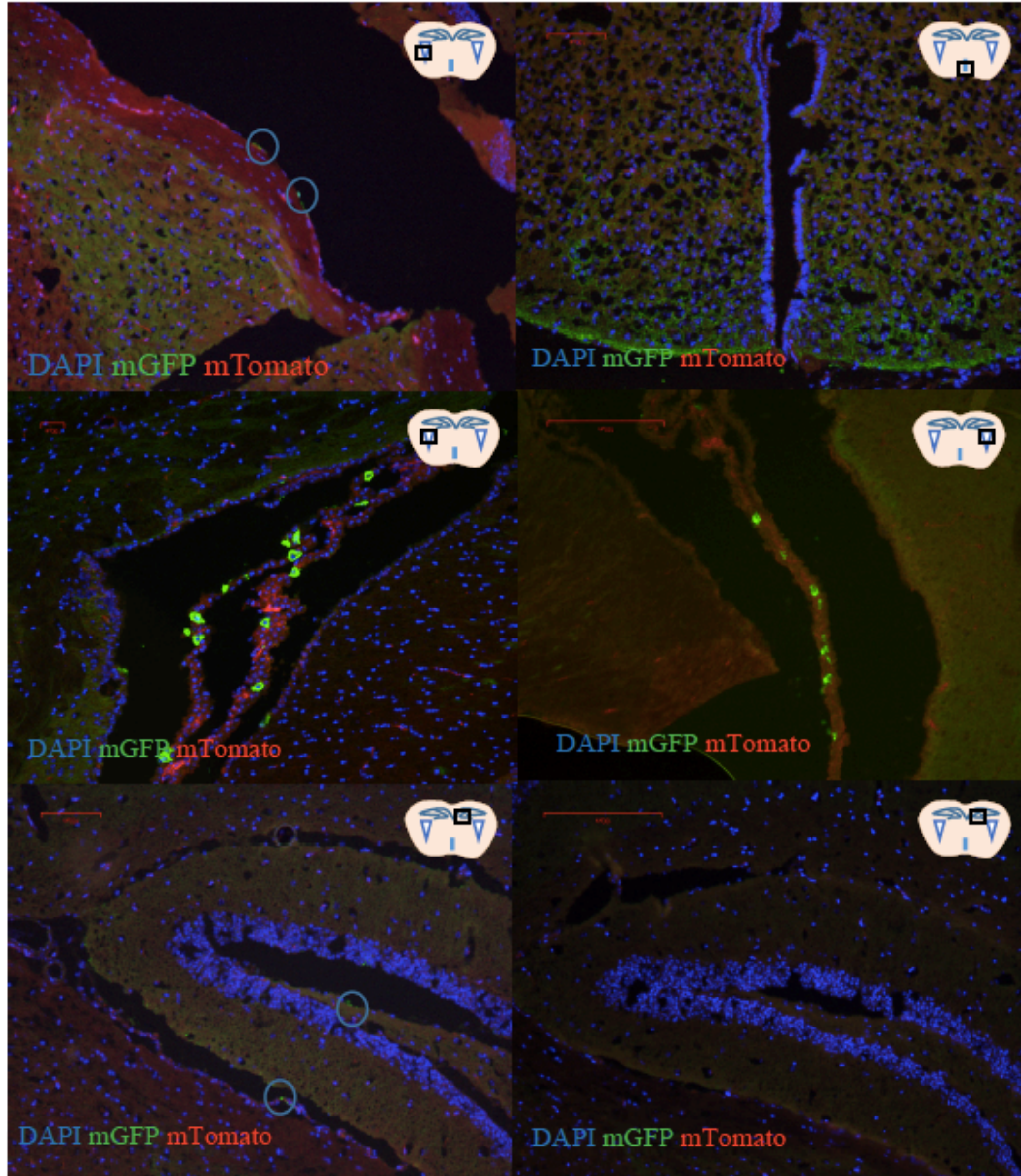

Figure 4: Brains from the exercise experiment and the diet restriction experiment both show a plethora of GFP+ cells in the choroid plexus in multiple brain sections and animals across all groups within the study. These images also show a small number of mTERT+ cells in the classic neurogenic niche of the SVZ and even fewer GFP+ cells in the DG.

GFP+ cells are heavily present in the choroid plexus but less present in classic neurogenic niches

GFP+ cells were immuno-stained for using either a conjugated GFP antibody or a primary GFP antibody coupled with a green fluorescent secondary. This, in combination with DAPI, a stain that binds to DNA, allowed for our initial discovery of mTERT in the choroid plexus by doctoral student Gabriel Jensen. Thorough investigation of the walls of the ventricles and the hippocampus of multiple animals across multiple cohorts revealed very few mTERT+ cells (figure 4).

GFP+ cells and classic proliferation markers

Testing for the presence of proliferation markers including BrDU and ki-67 was done on the restricted diet cohort and the exercise cohort respectively. Ki-67 is a protein that is only expressed in the nucleus of actively dividing cells (Sobecki et al., 2016). Due to the specific nature of its expression it can be used as an antigen when staining for precursor cells. Bromodeoxyuridine (BrDU) is a synthetic thymine analog that incorporates into the DNA of cells in the S phase of the cell cycle (Bio-Rad, 2017). Instead of adding thymine, cells that are exposed to BrDU will add BrDU in its place. This can then be used as an antigen for cell identification. Our immuno-staining revealed that Ki-67 is weakly present in the hypothalamus, which is typically used as a positive control, as well as some cortical areas. No ki-67 has been identified in mTERT+ cells as of publication (Figure 5). This is most likely due to an inaccurate antibody or to a lack of optimization. BrDU staining was not able to be optimized in time for publication, so no conclusions can be drawn using this marker.

Figure 5

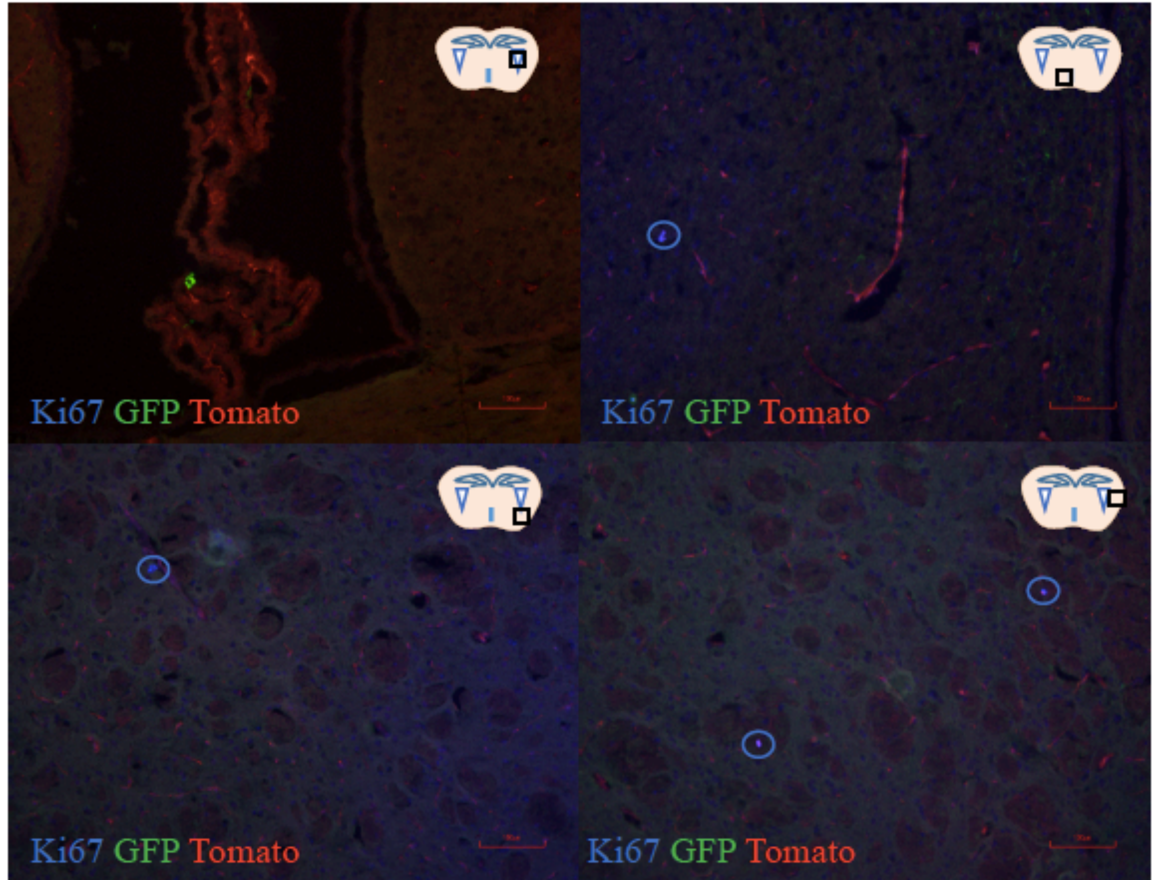

Figure 5: The blue circled spots above mark possible Ki-67+ cells. They are the approximate shape and size of a nucleus. No blue staining was visible in the choroid plexus of any animal that was tested.

DISCUSSION

The results above, in Figure 3, point to mTERT+ cells showing similar, if not the same, gene expression patterns when compared to the ANSC cell population of neural precursors. This expression pattern rules out the possibility of other precursors by the expression of GFAP and the lack of expression of EGFR. GFAP is only present in ANSCs, so the mTERT+ cells would need to start at the ANSC phase to express this. EGFR is only detected in activated cells, so our cells appear to be residing in a state before activation occurs.

The qPCR results on the FACS sorted cells show us a very general pattern of expression that points to mTERT+ cells being early neural precursors. An important part of this data set is that the GFAP expression is significantly different. If mTERT+ were not ANSCs they would exhibit typical precursor markers but not GFAP. This tells us that mTERT+ cells at least start as ANSCs. EGFR expression also provides a critical clue in the search for mTERT cell identity. EGFR is not significantly different between GFP+ and GFP- cells, although a trend is apparent. This finding led us to believe that mTERT+ cells are quiescent, as EGFR is a marker that is associated with activation.

The fact that so many genes typical of later stages of the neuronal lineage are not significant is also quite a relevant finding in that it points to an earlier stage of development. While these genes were not significantly different, a trend can be seen among many of them. The nature of our experiment allows for cells to stop expressing mTERT, differentiate, and migrate while still remaining marked with the GFP marker. This chase effect could allow some formerly mTERT+ cells to be in the GFP+

population. The trend seen in the non-significantly different genes could be caused by differentiation of mTERT+ cells. This would begin alter the gene expression data, which could be what we are seeing in our graphs in Figure 3.

GFAP is thought to only be found in glial cells of the Central Nervous System (CNS), which fits the classic description of ANSCs as “radial glial cells” (Cerilli and Wick, 2016). The upregulation of this gene in combination with the other three genes points to the GFP+ population being at least mostly stem cells as opposed to another kind of glial cell. SOX2 is an important transcription factor in neural tube development and has been shown to consistently mark early neural precursors (Lee et al., 2012). Nestin has also been shown to reliably mark early neural precursors (Cheng et al., 2015). It has been suggested that Hes5 keeps ANSCs in a proliferative state and prevents terminal differentiation, which would be necessary in the ANSC population regardless of activation or quiescence (Nieto-Estévez et al., 2016).

Taken together this supports our hypothesis that mTERT+ cells are indeed ANSCs and most likely quiescent cells. Two of these factors point to stem cells being held in a proliferative stage and not differentiating, which is supported by the fact that the mice that these cells were harvested from had no novel neurogenic stimuli introduced during this experiment. The mouse brains we used would have been in a state of neural stasis, so ANSCs would have had no reason to be actively differentiating beyond the basal neurogenic level.

Prox1 showed a very unexpected pattern of expression in our gene expression experiment. There are several possible explanations for this. Currently, the majority of

markers for each stage of a neuronal lineage, while widely accepted, are still not fully understood. It could be possible that Prox1 experiences a bimodal expression and is present in both qANSCs and immediate progenitors. It could also be possible that the primers used in this instance were not specific enough. Further testing will be conducted before any conclusion is reached.

It is important to restate that the markers shown in Figure 3 have been assembled after a thorough review of the literature. It must be noted, though, that the nomenclature for adult neural precursors is not consistent across this field. I have therefore organized a table comparing the nomenclature used in my references. This table can be found in Appendix B labeled as Supplementary Table 2. Another issue regarding these markers is that it is typically assumed that expression of these markers is either fully expressed or fully absent in each cells type. It could be that gene expression is more variable than this and certain genes may be slightly or moderately expressed as opposed to absolutely expressed or not.

If these cells are indeed quiescent, mTERT could provide an invaluable new tool in the field of neuroscience. Being able to confidently mark qANSCs with only one marker would cut down on time, cost, and labor in the study of quiescent neural stem cells. This would also allow for cells to be followed from quiescence in the presence of a variety of stimuli. If neurogenesis were to occur, we could expect to see increased mTERT expression due to the symmetric and asymmetric division that qANSCs would undergo upon stimulation. Increased mTERT expression could therefore allow us to quantify levels of neurogenesis in response to aging, neurogenic stimuli, and even injury.

Our preliminary immunostaining data, collected by Gabriel Jensen, showed an unexpected picture. mTERT+ cells were almost exclusively in the choroid plexus, but non-existent in the more studied neurogenic niches. However, upon closer inspection we were able to identify a small number of GFP+ cells in more classic neurogenic niches, such as the subventricular zone and the dentate gyrus. The low levels of GFP in these areas quickly led to the hypothesis that classically described niches could exclusively, or at least primarily, house more mature precursors.

We were able to distinguish a very strong GFP signal from the median eminence of the hypothalamus. This is the area of the brain where regulatory hormones are released. It acts to connect the hypothalamus to the pituitary gland. This area would indeed need to adapt to changes in energy status and may use neural stem cells to accomplish this. It was still surprising that the vast majority of GFP+ cells reside in the choroid plexus.

The choroid plexus (CP) is an epithelium that secretes cerebrospinal fluid (CSF) as well as some of the many signaling factors that can be present in CSF such as growth factors, cytokines, and neuropeptides (Krzyzanowska et al., 2015). The CP is present in all four ventricles of the brain and acts as a blood-CSF barrier (Lun, Monuki and Lehtinen, 2015). This crucial barrier allows for the rapid transport of water and some solutes from the circulatory system to the epithelial cells of the CP in order to make CSF (Lun, Monuki and Lehtinen, 2015).

Based on our immunostaining images we began to speculate that the choroid plexus may be the most primitive niche for ANSCs, which would then be able to migrate to the more well-known niches of the ventricular/sub-ventricular zone and the sub-

granular zone of the dentate gyrus (DG). Due to the ambiguous nature of previous markers it has been difficult to conclude that the neural precursors in more well-known niches are indeed the most primitive. If the CP was in fact the home of the most primitive cells it is possible that these cells could migrate to either of the other two niches or differentiate and then migrate to the other niches. Looking at this spatially it is also a possibility. The CP resides in ventricles which would provide plenty of access to the VZ/SVZ. The dentate gyrus is very close to the dorsal third ventricle which also houses CP (Paxinos and Franklin, 2013). This could allow for a small enough distance for the migration of stem cells into this region. Another key piece of supporting evidence is our timeline. It is estimated that it could take up to eight weeks for an ANSC to become a mature adult neuron (Duan et al., 2008). Our chase period for the running group was the longest at eleven days and the fasting and refeeding group only had a five-day chase. This could explain why we are seeing very few mTERT+ cells chasing to the more well-known niches.

It is also quite possible that the CP is only the most primitive niche for the VZ/SVZ and that the DG runs on a whole different system. Proximity and access is definitely more prominent in the VZ/SVZ. While both previously hypothesized niches show similar general patterns of behavior they also exhibit distinct differences related to function. Stem cells from the VZ/SVZ typically migrate to the olfactory bulb and integrate into the neural circuitry at that sight, while stems cells in the DG only migrate one cell layer up to become granule neurons.

In theory, the choroid plexus would be a well-suited environment for qANSCs. It provides ease of access to some of the most crucial brain regions in terms of basic

function and adaptability. Neural stem cells have frequently been described as requiring a nearby blood vessel, which the choroid plexus is directly in contact with. This contact with the blood vessel would allow the choroid plexus access to changing internal conditions which would allow for rapid activation of adaptive mechanisms, in the form of stem cells activation, if needed.

Proliferation marker staining did not appear to mark any GFP+ cells thus far. It may be possible that the neurogenic stimuli were not strong enough or present for a long enough time period to induce activation in the mTERT+ cells. It is also possible that our protocol is not fully correct. Unfortunately, BrDU was not able to be used to supplement the ki-67 data due to protocol optimization difficulties. In order to further our understanding using these markers they will be used in future experiments that involve stronger neurogenic stimuli and longer chase periods. Both techniques will need further optimization in order to draw any conclusions.

In order to further test our theory, we will be performing another FACS sort on mTERT-mTmG mice as well as mTERT-GFP mice. GFP mice will be used for further gene expression studies. This will allow full confidence in our gene expression data as mTERT-GFP mice only express green fluorescence in cells that are currently expressing. These cells will be exclusively mTERT+ and therefore our previous problems with impure populations will no longer be applicable. The mTERT-mTmG cells will be used for a neurosphere formation assay. This is a cell culture assay routinely used to prove neural stem cell lineage. Sorted cells will be added to a specific cell culture medium and allowed to grow. If they form floating spheres, we will be able to conclude that they originated from neural precursors.

Cells from mTERT-mTmG neurospheres will then be used for a Neural-Colony Forming Cell Assay (NCFC). This assay definitively distinguishes ANSCs from other neural precursors (Azari et al., 2011). Cells are sorted into a specific cell medium and allowed to grow for five to eight days. At the end of the growth period, colonies are measured to determine cell origin. Colonies that are above 2mm are formed from ANSCs and anything below 2mm originates from a neural precursor from a later stage. If we see colonies above 2mm we will be able to conclude that at least some of our GFP+ cells are ANSCs which means that, at the very least, mTERT+ cells begin as the most primitive ANSCs.

In this series of experiments, we were not able to conclusively determine differences due to the experimental treatments. Our immunostaining offered us a first look into the location of mTERT+ cells, but not an unbiased quantification method. In the future we will be using unbiased stereology in order to remedy this. Unbiased stereology will allow for us to fairly asses the number of mTERT+ cells in comparison with the total number of neurons in the brain.

In addition to the aforementioned experiments, we have begun to examine the brains of aged mice (19 months). In gathering preliminary immunostaining data, we have been able to identify GFP+ cells again in the choroid plexus as well as in the median eminence (see supplementary Figure 1). Further analyses of these brains will be conducted using unbiased stereology, a protocol that will allow us to quantify the number of cells in each brain in order to more accurately compare cell number between young and old mice. The conclusions from this experiment will allow us to determine if the number of mTERT+ cells decreases with age, as would be expected of ANSCs.

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APPENDICES

APPENDIX A

IACUC APPROVAL

UNIVERSITY OF MAINE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
581-1498

PLEASE DISPLAY ON OR NEAR ANIMAL CAGE

INVESTIGATOR: Townsend, Kristy
TITLE OF PROTOCOL: CNS Stem Cells and Adult Neural Plasticity Project
PROTOCOL NUMBER: A2014-11-02
APPROVAL PERIOD: 12/5/2014 - 12/4/2017

SPECIES	# APPROVED	LOCATION
Mice	134	Small Animal Research Facility

APPENDIX B

SUPPLEMENTARY MATERIAL

Supplementary Table 1

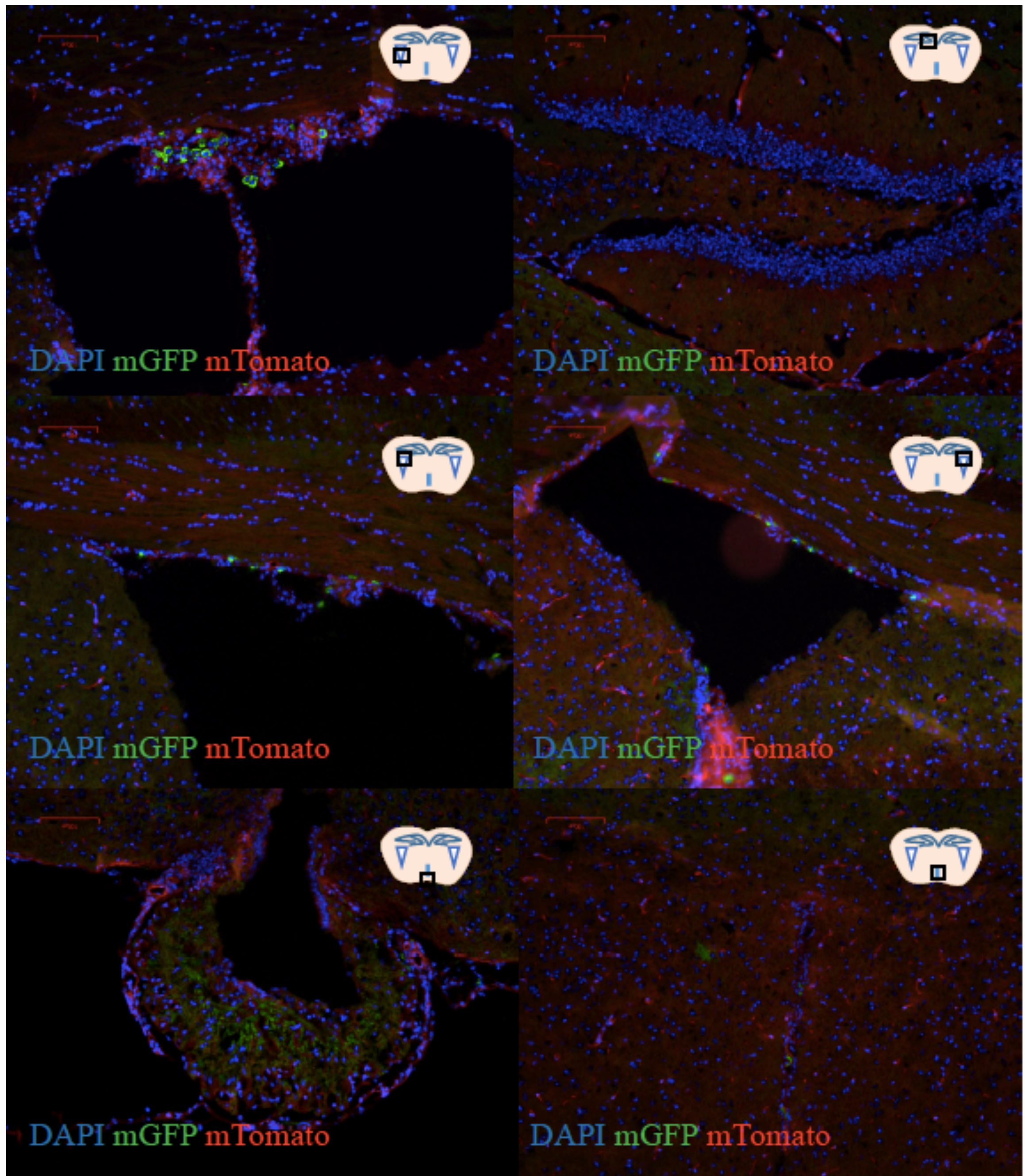
Abbreviation	Name	Function
CD133/PROM1	Prominin 1	Originally thought to mark ANSCs, proven to only accurately mark embryonic stem cells. In the adult brain CD133 marks a diffuse population of cells in the brain that are not correlated to ANSCs. Role in the organization of the apical domain of epithelial 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.
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)

Supplementary Table 1: This table is a succinct summary of the genes that are mentioned throughout this thesis (Holmberg Olausson et al., 2014, *GeneCards*, Cerilli and Wick, 2016, Lee et al., 2012, Quartu et al., 2008, Reference, 2017, Doetsch et al., 2014

Supplementary Figure 1



Sup. Figure 1: Images of the brain of an aged mouse (~19 months). These images are comparable to those shown in figure 4. (N=1).

Supplementary Table 2

<table>
<tr><th>Townsend Terms</th><th>Number System</th><th>Letter System</th><th>Glia-centric</th><th>Other Common Names</th><th>Other Common Names (continued)</th></tr>
<tr><td rowspan="2">Quiescent Adult Neural Stem Cell (qANSC)</td><td rowspan="2">Type I</td><td rowspan="2">Type B</td><td>Radial Quiescent Glial Cell</td><td rowspan="2">Label Retaining Cells</td><td rowspan="2">Slowly Proliferating Astrocytes</td></tr>
<tr><td>Horizontal Quiescent Glial Cell</td></tr>
<tr><td>Activated Adult Neural Stem Cell (aANSC)</td><td>Type I</td><td>Type B</td><td>Active Horizontal Glial Cell</td><td></td><td></td></tr>
<tr><td>Transit Amplifying Cells (TACs)</td><td>Type IIA</td><td>Type C</td><td></td><td>Transit Amplifying Progentors (TAPs)</td><td>Rapidly Dividing Immature Precursors</td></tr>
<tr><td>Immature Neuroblasts</td><td>Type IIB</td><td></td><td></td><td>Intermediate Progenitor Cells</td><td></td></tr>
<tr><td>Migratory Neuroblasts</td><td>Type III</td><td>Type A</td><td></td><td></td><td></td></tr>
<tr><td>Mature Neurons</td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Sup. Table 2: This Townsend Terms Table compares the different designations for adult neural precursor cells.

AUTHOR’S BIOGRAPHY

Caroline (Carrie) Dean Curtis is a biology major with a neuroscience minor. In her sophomore year, Carrie got involved in the Townsend lab as one of the first three research assistants to be hired. This allowed her to build the skill, confidence, and critical thinking necessary to complete this thesis.

Carrie performed in the marching and pep bands all four years of her undergraduate career and the concert band for two years. During this time, she played the trombone, sousaphone, tuba, ukulele, and the bass guitar. During her freshman year, Carrie became a brother of the honorary band service fraternity Kappa Kappa Psi. She hopes to continue to be able to pursue music as a hobby in the future.

Upon completion of the graduation requirements Carrie will be matriculated into the graduate program at the University of Maine and will begin work towards earning her Master’s degree in the Townsend Lab. Her work on the “mTERT” project will continue during this time.