Hippocampal neurogenesis in the SERT ALA56 mouse model to autism

by

Julieta Maria Di Mase

A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Science

In Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, FL

August 2019

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Kathleen Guthrie, Department of Biomedical Sciences, and has been approved by all members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

SUPERVISORY COMMITTEE:

Kathleen Guthrie, Ph.D.
Advisor

Kenneth Dawson-Scully, Ph.D.

Jianning Wei, Ph.D.

Randy D. Blakely, Ph.D.

Sarah Milton, Ph.D.
Interim Chair Department of Biological Sciences

Ata Sarajedini, Ph.D.
Dean, Charles E. Schmidt College of Science

Robert W. Stackman Jr., Ph.D.
Dean, Graduate College

August 1, 2019
Date

Acknowledgements

I want to thank my committee: Dr. Kathleen Guthrie for her patience, support, trust and mentorship; Dr. Ken Dawson-Scully for opening the door to the incredible world of research since day one, and his trust and understanding; Dr. Jianning Wei for showing me the essence of neurogenesis and support; and Dr. Randy Blakely for allowing me to use his high-in-demand mice, supporting my research, and trusting me to do this experiment.

I want to thank my family: My parents for always being supportive and taking me to school at the crack of dawn when I was unable to drive for half a year; my Godmother Lucy, for also driving me back and forth for months when I was “medicated” and not very nice (to put it nicely); my sister; and my encouraging boyfriend for putting up when I was insanely stressed and taking care of things.

To my lab mates for teaching me the ropes of research work. Olena, Stephanie and Melissa, for when I did not know what I was doing but were more than patient with my shenanigans; Katrina and Britnee for when I sort of knew what I was doing, but still needed a hand; and Aruna for those very last steps when I needed more than 24 hours in a day.

Last but definitely not least, I want to thank Geri for always being supportive and looking out for me, and Becky and Sharon for helping me sort my way in this journey called a “graduate degree”.

Thank you all.

In memory of my baby Milano 5-7-14 – 7-20-19.

Abstract

Author: Julieta Maria Di Mase

Title: Hippocampal Neurogenesis in the SERT Ala56 Mouse Model of Autism

Institution: Florida Atlantic University

Thesis Advisor: Dr. Kathleen Guthrie

Degree: Master of Science

Year: 2019

The causes of autism spectrum disorder (ASD) are not all known, but it is suspected that the serotonin transporter (SERT) plays an important role for some subjects with ASD. Mutations in the *SLC6A4* gene, that encodes SERT, including the Ala56 mutation (Gly56Ala), have been found in some autism patients. This mutation makes the transporter more active and reduces the probability of serotonergic neurotransmission in the brain, which is linked to behavioral changes that are associated with core domain deficits of ASD [1].

Depression also has been linked to decreases in the availability of serotonin (5-hydroxytryptamine; 5-HT) in the central nervous system (CNS), and is associated with reduced hippocampal neurogenesis. Selective serotonin reuptake inhibitors (SSRIs), drugs used to block SERTs, are used to treat depression and/or anxiety by inhibiting SERT to increase synaptic 5-HT levels.

In this in vivo study, I tested whether mutant SERT Ala56 knock-in (KI) mice exhibit a difference in the number of proliferating neural stem cells in the adult hippocampus, and/or in the number of surviving adult-born granule cells, compared to their normal, wildtype (WT) littermates. One group of animals was treated with bromodeoxyuridine (BrdU) and mice were euthanized 18-20 hours later, allowing only the hippocampal stem cells (SCs) and amplifying progenitor cells (APCs) to incorporate the BrdU during this time. For a second group of animals, BrdU was injected daily for 7 consecutive days, and mice were euthanized 5 weeks after the first injection to allow maturation and incorporation of new adult-born granule cells (GCs) in the hippocampus. Results of BrdU-labeled cell counts showed no significant differences in numbers of proliferating cells in the subgranular zone (SGZ), or in numbers of new adult-born GCs, between WT and KI mice, indicating that basal levels of adult hippocampal neurogenesis are not affected by the SERT mutation.

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General Background and Significance

Adult hippocampal neurogenesis in rodents:

Neurogenesis was initially thought to be restricted to the prenatal period of development, as initially proposed by Santiago Ramón y Cajal [1]. However, with the advancement of neuroscience, medicine, and experimental techniques, new research provided evidence that neurogenesis was possible in adulthood as well [2,3]. Early work by Joseph Altman first described neurogenesis in adult rats, and has since been demonstrated in numerous studies in both rodents and non-human primates [2,4–9]. In the adult brain, ongoing neurogenesis occurs in two regions: the subventricular zone (SVZ) that lines the anterior lateral ventricles and which produces γ-aminobutyric acid (GABA)ergic/dopaminergic neurons destined for the olfactory bulb, and the SGZ in the hippocampal dentate gyrus (DG, Figure 1), which generates local glutamatergic GCs [9]. The hippocampus is a limbic region located in the medial temporal lobe of the brain. Its main functions are related to aspects of learning and memory, and some aspects of mood control, hence the connection to SSRIs [5,10].

Factors such as exercise, exposure to enriched environments, hormones, growth factors, neurotransmitters and drugs can alter levels of neurogenesis in the rodent hippocampus [11–15]. Regarding human hippocampal neurogenesis,

many questions remain unanswered, and the topic continues to be debated regarding whether significant numbers of new neurons are generated in adult humans [5,6,16]. Some human studies have suggested that hundreds of new neurons are integrated into the hippocampal DG every day, while other studies argue that human neurogenesis is restricted to early development and the juvenile period [5]. A recent study by Arturo Alvarez-Buylla's group, showed that proliferating progenitor cells and numbers of young neurons declined sharply in the human brain by year 1 of age, and only a few cells were observed by 7 and 13 years of age. They also saw that in post-mortem brains of adult patients with epilepsy and healthy adults, new neurons in the DG were undetectable [5] .However, in a recent published paper, the evidence in support of adult hippocampal neurogenesis in humans has been reviewed in detail [4]. In 1998, Eriksson and his team studied the GCL and SGZ in the DG of the hippocampi from patients who had received infusions of BrdU for tumor-staging studies with no other treatment that could potentially affect cell generation, and analyzed the postmortem brains. They discovered that the adult human brain can undergo cell division in hippocampus, and that these new cells can differentiate into cells that were morphologically and phenotypically similar to neurons. Even though they could not prove that these newly generated cells were functional due to the nature of this experiment; this result indicated adult neurogenesis in the hippocampi in the same location as previous studies had seen in the DG of adult rodents and monkeys [4,17]. In addition, in a study by Maura Boldrini et al., SSRIs and tricyclic antidepressants (TCA) were tested in medicated patients with major

depressive disorder (MDD), with a non-treated MDD control group, to determine if the drugs increased neurogenesis in the human DG. Clinical data were obtained from psychological evaluations, and toxicological and neuropathological examination of all subjects at autopsy. They found that antidepressants increased dividing progenitor cell numbers in the anterior human DG, results that were consistent with findings in rodents [18]. The different results of these studies show that adult human hippocampal neurogenesis still needs to be further investigated. However, the data from the Boldrini study suggests that increases in levels of synaptic 5-HT may be linked to hippocampal neurogenesis in both humans and rodents treated with antidepressants/SSRIs.

The process of adult hippocampal neurogenesis has been studied in detail in rodents. As illustrated in Figure 2, within the hippocampus, radial glial-like type 1 cells located in the SGZ form the stem cell population [17,19]. Type 1 cells divide slowly to generate non-radial stem cells that are transit-amplifying Type 2a (intermediate progenitor cells with glial phenotype), and then Type 2b cells (intermediate progenitor cells with neural phenotype), which are fate-specified progenitors [11,20]. These two progenitor cell types are mitotic and Type 2b cells divide to produce post-mitotic neuroblasts that express the microtubule-associated cytoskeletal protein doublecortin (DCX) (Type 3 cells). Some neuroblasts undergo apoptosis at this stage, but if the cell survives it continues to differentiate and develop the morphology of an immature neuron with an apical dendrite, migrating upward in the granule cell layer, forming dendritic branches, and integrating as a mature neuron. New neuroblasts develop into immature

neurons over 2-3 weeks after they are born, and by 4 weeks they become fully functional granule cells (GC) in the granule cell layer (GCL) [17,19]. Only about half of all new GCs survive to 4 weeks and beyond, with all others eliminated by apoptosis. The GCs that survive, are regulated by the local environment, stimulated via synapses, and are acted on by other factors such as neurotropic factors, that help them mature and integrate into the GCL [10,20].

Serotonin:

Serotonin, named by Maurice Rapport in 1948, who first isolated the molecule in his laboratory [21], is a neurotransmitter and neuromodulator. It is derived from the amino acid tryptophan and is found in the central nervous system (CNS, made by tryptophan hydroxylase-2, TPH-2), the enteric nervous system of the gut, specifically in enterochromaffin cells (made by tryptophan hydroxylase-1; TPH-1), and is also present in blood platelets [22,23]. TPH-2 is the enzyme that catalyzes the first and rate limiting step in the biosynthesis of CNS 5-HT [52]. In the rodent CNS, serotonergic neurons are mainly located in the dorsal, median raphe nuclei (DR: cell groups B6-B9), in the midbrain and brainstem, with their axons innervating almost every region of the forebrain, the hippocampus being one target structure, along with virtually all the rest of the limbic system (Figure 3). The projections from the DR and median raphe nuclei (MR: B5 and B8) to the hippocampus are topographically organized, meaning they have different projection patterns (Figure 3). In rodents, the ventral hippocampus receives moderately dense projections from the caudal dorsal

raphe, and essentially none from the median raphe nucleus (MR) (Figure 3) [24]. The DG receives DR projections in the molecular layer, the hilus and in the SGZ. [3,23].

A pre-synaptic 5-HT neuron releases 5-HT at its axon terminus (Figure 4). The diffusion of the chemical across the synaptic cleft permits it to bind to 5-HT receptors on postsynaptic cells, leading to activation of signal transduction pathways that modulate neural activity. There are at least 14 subtypes of 5-HT receptors and most are G-protein coupled receptors (GPCRs) that activate second messenger signaling in postsynaptic neurons [23–25]. There are also 5-HT receptors that are located presynaptically [24]. The signaling the receptors produce depends on the receptor type and location. 5-HT influences the respiratory system, cardiovascular function, the gastrointestinal system, ejaculatory and bladder control, muscle movements, sleep, arousal, pain, sensory perception, emotions and cognition [23]. The 5-HT reuptake transporter (SERT) is a large transmembrane protein found in the presynaptic terminal that recycles 5-HT released into the synaptic cleft (Figure 5). The integral membrane transporter is encoded by the gene *SLC6A4* [26]. The gene has two main promoter-variant alleles, short (S) and long (L), which translates to lower and higher transcription rates of SERT that can lead to less or more SERT protein respectively [25,27].

5-HT “reuptake” helps terminate 5-HT neurotransmission and SERT function depends on the concentration gradient of potassium ions in the cytoplasm and the concentration of sodium and chloride ions in the extracellular space [28]. The binding of Na+ and Cl- allows SERT to bind 5-HT and change

conformation (flip) to bring the 5-HT inside the cell [29]. After releasing the 5-HT, binding to potassium ions changes SERT conformation and promotes reorientation of SERT to bind extracellular 5-HT again [25]. The transported 5-HT can be re-packaged in the presynaptic neuron in vesicles for further cycles of release and reuptake [26,30].

Serotonergic signaling pathologies:

Serotonin signaling deficiency in the brain has been linked to human disorders such as depression, anxiety and other mood disorders, as well as age-related cognitive decline [31–33]. SSRIs, like fluoxetine, known more commonly as Prozac, given to human patients and animal models of these disorders support correlations between antidepressant effects, and possible increases in the availability of synaptic 5-HT caused by reducing 5-HT reuptake by SERT [34,35]. This correlation led to the 5-HT hypothesis of depression that suggests that imbalances or deficiencies in 5-HT neurotransmission in the brain contribute to clinical depression [23]. The SSRIs block SERTs in the presynaptic neuron, allowing released 5-HT to be available in the synaptic cleft for more time. Post-synaptic 5-HT receptors can then bind the neurotransmitter and thus cause a response in the post-synaptic neuron. Overall, SSRIs are thought to boost 5-HT neurotransmission in the brain, and for some patients with major depressive disorder (MDD), SSRI treatment reduces symptoms of depression, although this does not work in all patients [12,31,36,37].

The neurogenic link to depression:

The neurogenic hypothesis proposed in the 1990s suggested that depression may partly be due to reductions in hippocampal neurogenesis due to abnormal 5-HT signaling [38]. Almost all 5-HT receptor subtypes are localized to neurons in the hippocampus [23,24,32], and neural stem cells in the SGZ express the type 5-HT_{1A} receptor [31,33]. Chronic administration of SSRIs to rodents has been shown to enhance proliferation of SGZ progenitor cells and increase granule cell maturation in rodents [37,39]. An increase in extracellular 5-HT is what is thought to cause the antidepressant effect, partly by promoting neurogenesis in the adult hippocampus [21,27,28,30,33,34]. A study by Dr. Randy Blakely's laboratory at Vanderbilt University School of Medicine in 2016, supports a role for SERT antagonism in the acute and chronic actions of the commonly used SSRIs, fluoxetine and citalopram. In these experiments, the SERT Met172 mouse model (in which interactions of some antidepressants with SERT have been eliminated by knock-in mutation) failed to show improved behavior on forced swim test, or increased SGZ cell proliferation after fluoxetine and citalopram treatment to increase 5-HT [41]. Experiments also have shown that stress-induced rodent models of depression have impaired adult neurogenesis, and that hippocampal volume is reduced in some human MDD patients [12,38]. Increasing neurogenesis by SSRI treatment can improve behavioral features of anxiety and depression in adult rodent models of depression [28,36]. Additionally, more proliferating neural

stem cells have been reported in the DG of mice lacking monoamine oxidase A or B (MaoA/MaoB), enzymes that catalyze the oxidative deamination of amines like 5-HT, dopamine, and norepinephrine [42–44]. However, although these data suggest that increased 5-HT signaling in the DG contributes to increased neurogenesis, this finding may not be due to higher levels of 5-HT alone since MaoA/B also degrades other monoamines, as mentioned above, which could be part of the effect seen [45]. How 5-HT modulates neurogenesis is still unclear as the exact physiologic mechanisms are not all known. Experiments have shown that basal levels of hippocampal neurogenesis are normal in adult mice that completely lack 5-HT due to deletion of TPH-2, (TPH-2 knock-out, TPH-2 KO) but increases in neurogenesis caused by wheel running are absent in these animals, showing that neurogenic responses to other signals are impaired without 5-HT [38]. Yet another study revealed that reducing 5-HT in the adult CNS promoted neurogenesis in the hippocampus and reduced depression-like behaviors in mice [46], showing that the relationship between 5-HT and adult hippocampal neurogenesis is not fully understood. Interactions between neurogenesis and neurotransmitter signaling continues to be researched for a better understanding of the mechanisms.

Human autism and the SERT Ala56 mutation:

Autism spectrum disorder (ASD) is a juvenile male-dominant disorder usually diagnosed by age five, and characterized by core symptoms that include

impairments in language acquisition, repetitive behaviors, hyperserotonemia (an increase of 5-HT in platelets), abnormal social interactions, communication problems and anxiety [26,47]. On April 26, 2018, the Centers for Disease Control and Prevention (CDC) released new data on the occurrence of autism in the United States that identified 1 in 59 children (1 in 37 boys and 1 in 151 girls) as having autism spectrum disorder (ASD) (https://www.autismspeaks.org/what-autism/prevalence). Although there is no cure for autism, some early therapeutic interventions, like speech therapy, relationship development intervention, and other therapies that teach play, communication, and self-care can help ameliorate the core symptoms of ASD [27,29,42,44,48].

Research with rodent models and human patients indicates that some types of autism are due partly to defects in the 5-HT neurotransmitter system, particularly the 5-HT_{1A} and 5-HT_{2A} serotonin receptors [74], and in SERT. In an experiment in 2010 by Gould et al. at the University of Texas Health Science Center at San Antonio, acute doses of fluoxetine and buspirone (a partial 5-HT_{1A} receptor agonist) were given to BTBR mice. This is a mouse model used for ASD research because it exhibits reduced social interactions, impaired play, low exploratory behavior, and anxiety. The two drug treatments increase 5-HT signaling and both improved social interaction behavior in the BTBR mice. The authors suggest that this finding may lead to the “identification of additional therapeutic targets for treating human autism” [10,49].

A genetic variant of the SERT gene, SERT Ala56, was identified in patients with autism in 2012 [25,48]. Its frequency in ASD patients is 1% in

individuals in the United States [25,27,42]. This mutation inserts an alanine (Ala) in the place of a glycine (Gly) at amino acid position 56 in the protein, which results in a change in the structure of the transporter. The structural change is thought to "lock the transporter in a high-affinity conformation" for 5-HT, that leads to less 5-HT being available in the synaptic cleft, [29,47]. Expression of the mutant gene in mice creates a hyperactive SERT, and an *in vivo* study showed a significant increase in the rate of 5-HT removal from synapses in the hippocampus of mutant mice compared to their wildtype littermates. *In vitro* studies found reductions in the firing of raphe nucleus neurons and a higher sensitivity of the SERT Ala56 transporter [25,41]. The mice also show enhanced 5-HT receptor sensitivity and hyperserotonemia, which is high levels of 5-HT in blood platelets, all traits associated with patients that carry the Ala56 mutation [25]. Behaviorally, the mice show impaired social interactions and vocalization and sensory aversion, as well as an unusual tendency to hang and vault down from the tops of wire cages repeatedly [26], a repetitive-compulsive behavior, like OCD in some humans. These behaviors parallel ASD-associated core deficits seen in most humans with ASD. Most recently, these mice have been shown to exhibit deficits in multisensory integration, which is how information from the different senses are integrated by the nervous system, a deficit seen in ASD patients [29]. 5-HT signaling is known to be important for sensory development and function, and altered sensory processing has been linked with ASD including disrupted visual and auditory processing [26,42].

The experiment

In this thesis, I used the transgenic mouse model for the autism SERT Ala56 mutation. My first aim was to test the hypothesis that the mutation would change the proliferation of stem cells in the SGZ. My second aim was to test the hypothesis that the mutation, and the resulting rapid clearance of 5-HT in hippocampus, changes the number of surviving new-born granule cells in the hippocampus. Ultimately, I determined if the mutation altered basal levels of adult hippocampal neurogenesis, and if so, whether the alteration was due to a decrease or increase in either SC proliferation or in the numbers of surviving adult-born GCs.

In future work, it would be of interest to perform experiments that may lead to novel interventions to treat autism. My ideas include, but are not limited to, using this mouse model to potentially rescue the pathology of the transporter, by using SSRIs to block the mutant SERT and allow more time for 5-HT to activate post-synaptic neurons. This could allow better activation of the signaling pathway that leads to normal brain development and function.

Experimental Objective: Test the hypothesis that mice expressing the SERT Ala56 mutation will show alterations in adult hippocampal neurogenesis when compared to normal mice. This was done in the following three experimental aims that addressed different stages in the development of adult-born granule cells:

Aim 1. Test the hypothesis that the SERT Ala56 mutation alters the levels of stem cell proliferation in the dentate gyrus of adult mutant mice.

Aim 2. Test the hypothesis that the SERT Ala56 mutation alters the survival of adult-born dentate granule cells.

Aim 3. Test for alterations in the maturation of new granule cells in the hippocampus of SERT Ala56 mice.

The project used bromodeoxyuridine (BrdU). BrdU is a thymidine analog that incorporates into dividing cells during the S phase of DNA synthesis [50], and it is a commonly used tool for cell proliferation and cell cycle studies. BrdU was used as for cell birth-dating *in vivo*, combined with immunolocalization of marker proteins expressed by granule cells at specific stages of development. Microscopic cell counts in the dentate gyrus were used to quantify and compare numbers of BrdU-labeled cells in adult mutant and normal mice.

Chapter One:

Impact of SERT Ala56 mutation on SC proliferation in the SGZ of the hippocampus

1.1 Introduction

Neurogenesis was once thought to be specifically an aspect of the embryonic CNS development, but studies have shown that neurons are born in the adult CNS [2]. These new neurons are normally integrated into the adult hippocampus and the olfactory bulb [20]. Adult-born hippocampal neurons derive from proliferating stem cells located in the SGZ of the DG. If they survive to 4 weeks, they will differentiate into functional dentate GCs that release glutamate onto target cells in the hilus and pyramidal neurons in CA3, and receive glutamatergic and GABAergic inputs that control their activity [51]. Impairments in hippocampal neurogenesis have been proposed to play a role in mood disorders associated with reduced 5-HT, based on the effect of SSRIs [18,32,37]. The present study was carried out to determine if a specific mutation in the SERT gene, which causes an autism disorder and is associated with reduced 5-HT signaling, affects SCs and intermediate progenitor cells in the SGZ, where effects of central 5-HT on adult hippocampal neurogenesis have been proposed [9,47]. A rare, ASD-associated variant of the *SLC6A4* gene, which codes for the SERT, leads to

elevated 5-HT re-uptake by the presynaptic 5-HT neuron, subsequently, reducing the availability of extracellular 5-HT in the synaptic cleft [40,42,43,47,50].

Genetic mouse models, like the SERT knockout (SERT KO) mouse, are useful models for studying 5-HT effects on hippocampal neurogenesis. In SERT KO mice, the mice exhibit a 50% reduction in raphe serotonergic cell number [59,68]. This loss of serotonergic neurons significantly reduces 5-HT input to areas like the brain stem, cortex, striatum and hippocampus [68]. However, several rodent studies have demonstrated that the basal proliferation of adult hippocampal SCs and intermediate progenitor cells increased in SERT KO mouse models. A study by Lanufumey et al. in 2007, showed that mice lacking SERT had higher levels of 5-HT in the synaptic cleft, increased synaptic area, and increased numbers of proliferating cells in the DG [87]. A study in 2013 by Marguerita Karabeg et al. also showed increased basal proliferation in SGZ SCs, as well as more GCs, in the DG of adult SERT KO mice [69]. Results of studies that have increased or decreased 5-HT *in vivo* using either genetic or drug manipulations have produced variable results on SGZ cell proliferation using different gene targets and methods. These differences could be attributed to the different effects 5-HT has through the variety of receptor types, and compensatory changes in these that may occur in hippocampal cells and neurons that project to the dentate [9,23,32,49,69,61]. Additional studies with SERT KO mouse models are discussed in chapter 2 of this thesis.

Studies in which lesions destroyed serotonergic neurons in the raphe nucleus found that hippocampal 5-HT levels and adult hippocampal

neurogenesis were reduced [60]. Raphe neuron grafts restored the normal number of proliferating precursor cells and immature neurons in the DG [60,71]. Reducing 5-HT *in vivo* has also been achieved through inhibition of 5-HT synthesis with para-chlorophenylalanine (PCPA), which inhibits TPH-2 and consequently, decreases CNS 5-HT. A study by Vaidja et al. in 2006 showed a decline in the proliferation and survival of adult hippocampal progenitors following PCPA treatment in rats [72]. Yet, another study showed that chronic PCPA treatment led to increased survival of progenitor cells (Type 2) in the SGZ of the DG of WT mice [61,70], showing differences when compared to other models of 5-HT deficiency, like TPH-2 KO mice. Again, these differences could be attributed to the different methods used to reduce 5-HT *in vivo*.

The aim of the first part of this experiment was to test the hypothesis that the SERT Ala56 mutation alters the levels of SC proliferation in the DG of adult mutant mice by reducing 5-HT in hippocampus. I quantified numbers of BrdU+ cells in dentate SGZ at 18-20 hrs after BrdU treatment in WT and KI mice. The results did not demonstrate any association between the mutation and changes in the number of cells incorporating BrdU, when compared to WT mice (Figure 8). Although the SERT Ala56 coding variant results in SERT gain-of-function (GoF), the decrease in 5-HT levels had no effect on basal levels of SC BrdU incorporation.

1.2 Methods

Animals:

This was a collaborative project between Dr. Kathleen Guthrie and Dr. Randy Blakely at FAU. Adult male SERT Ala56 mice and wild type (WT) littermates (9-12 weeks old) were obtained from Dr. Blakely's laboratory in Jupiter, Florida. Dr. Guthrie's laboratory did not perform breeding and genotyping of the SERT Ala56 animals, which was done by Dr. Blakely's personnel. However, in Dr. Guthrie's laboratory, I managed the breeding and genotyping of a different mouse model of an ASD-related syndrome (Angelman Syndrome; AS) to train in these methods. Dr. Blakely's PhD student, Meagan Quinlin, performed BrdU injections in the mice in Jupiter, while I carried out similar injections in the Angelman mice in the Guthrie lab. Dr. Guthrie performed Ala56 mouse perfusions in the Blakely lab in Jupiter. I performed Angelman mouse perfusions in Dr. Kathleen Guthrie's laboratory.

Mouse tail DNA collection and genotyping:

At 17-22 days of age, Angelman mice were anesthetized with isoflurane (20% solution in propylene glycol) in a small bell jar. About 0.5cm of the tail tip was cut and placed in 250ul of Direct PCR Lysis Reagent (Viagen Biotech, Inc), containing 2.5ul of proteinase K (20mg/ml), and tubes were placed in a heating block at 56ºC overnight to digest tissue. The temperature of the block was raised to 85.5ºC for one hour the next day to denature proteinase K. Tubes cooled at room temperature (RT) for 10 minutes and were centrifuged for 1 minute at 14,000 rpm to pellet the hair and other impurities to the bottom. The tubes were

stored frozen (-20oC) or the DNA solution used right away for PCR. For polymerase chain reaction (PCR) genotyping of AS mice, the primer DNA sequences were: P1 genomic forward, 5'-ACTTCTCAAGGTAAGCTGAGCTTGC-3'; P2 reverse, 5'-GCTCAAGGTTGTATGCCTTGGTGCT-3'; and P3 HPRT (mutant sequence) forward, 5'-TGCATCGCATTGTGTGAGTAGGTGTC-3'. PCR was performed using New England Biolabs 2X Master Mix containing Taq polymerase and 1-2ul of DNA solution, and cycling conditions were: 94ºC for 1 minute, 94ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 30 seconds (repeated 32 cycles), then 72ºC for 10 minutes, and storage at 4ºC. Final PCR samples were analyzed by electrophoresis using a 1% agarose gel that included lanes containing DNA molecular mass markers. At least 1 positive and 1 negative control DNA sample (from previous genotyping samples) and 1 blank PCR sample (water added instead of DNA) were included in each PCR set and gel. The gel was imaged with the Li-Cor Odyssey FC system to visualize DNA bands.

5-bromo-2'-deoxyuridine (BrdU) injections and brain collection:

All male mice were 9-12 weeks old when BrdU was injected interperitoneally (i.p.). For the short-term survival interval group (18-20hrs), a single injection of BrdU (150mg/kg weight) was given (N=6 WT mice, 7 mutant Ala56 knock-in mice). During the time mice were exposed to circulating BrdU, it was incorporated in the DNA of dividing cells during the synthesis (S) phase of the cell cycle, including the dentate radial-glial stem cells and transit amplifying

progenitors in the SGZ. Mice given a single BrdU injection were euthanized 18-20 hrs later to count dividing cells (in labeled S phase) in the SGZ. Mice were euthanized with 150mg/kg sodium pentobarbital (given i.p. in Euthasol) and were perfused through the heart with 0.9% buffered NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.35). Brains were dissected and post-fixed in 4% paraformaldehyde overnight in 4ºC, and then cryoprotected in 30% sucrose in 0.1M PB for 3 days at 4ºC. Fixed brains were embedded in 10% gelatin Type A and the brains/gelatin fixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose for 2-3 days. Brains were divided into anterior (olfactory bulbs to striatum) and posterior (caudal striatum to cerebellum) and snap frozen in isopentane (-45^{o}C) and stored at -80^{o}C.

Tissue processing:

Serial coronal sections (40 μm, 1 in 6) through the hippocampus were collected into 0.1M PB using a Leica cryostat (-21^{o}C). Free-floating immunohistochemistry was done using antibodies to BrdU (rat; 1:400, Accurate Chemical), or doublecortin (DCX; goat; 1:500; Santa Cruz Biotech). Sections rinsed in Tris-buffered saline (0.1M TBS solution) and endogenous peroxidase was quenched with 0.6% hydrogen peroxidase for 15 minutes. Sections were rinsed again in 0.1M TBS, then treated with 2N HCl acid at 37 ^{o}C followed by neutralizing for 10 minutes in 0.1M sodium borate (pH 8.5). Sections blocked in 5% normal goat serum with 0.3% Triton x-100 in 0.1M TBS for 1 hour. Tissue

was transferred to primary antibody (rat anti-BrdU; Accurate Scientific; 1:400) with 3% normal goat serum and 0.1% Triton X in 0.1M TBS. Sections incubated overnight at 4ºC on a rotator. Tissue was washed 0.1M TBS for 30 minutes. Secondary antibody was Vector Labs biotinylated goat anti-rat IgG in 0.1M TBS (1:200 dilution) with 2% normal serum added. Tissue incubated in the secondary antibody for 2 hours at room temperature (RT) with rotation, and was then washed in 0.1M TBS. Tissue was treated with avidin-biotin HRP complex (Vector Elite Kit) for 2 hours then washed in 0.1M TBS. The reaction product was developed with diaminobenzidine/H_2O_2 using Vector's IMPACT DAB Kit. Sections were rinsed in 0.1M TBS and were mounted in order (anterior to posterior) onto gelatin treated slides from 0.05M PB. Sections were stained with cresyl violet, dehydrated through alcohols, cover slipped with Permount and left to dry. Immunostaining for doublecortin was performed as above, but without HCl treatment, with 10% normal horse serum as blocker for 2 hrs., and with biotinylated anti-goat IgG (1:200; Vector Labs) as secondary antibody.

Quantification of labeled cells:

Counts of BrdU-labeled nuclei in the right and left hippocampus were made from every section in the series (1 in 6) with an OLYMPUS AX70 microscope using 100x objective magnification. Counts were conducted blind to genotype and were restricted to labeled nuclei in the SGZ in sections from mice that survived 18-20hrs after BrdU injection. Other labeled cells in the hilus or molecular layer were not included and were assumed to be glia. Counts were

performed at least twice for each subject. The mean total per mouse was averaged from these counts. Group means +/- SEM (standard error of the mean) were calculated from animal means. Comparisons of genotype results were analyzed with two-tailed, unpaired t-tests. The level of significance was considered to be $p<0.05$. Estimates of total numbers of BrdU+ SGZ cells were made by multiplying counts by six to take into account labeled cells in the intervening sections not included in the series.

1.3 Results

Numbers of cells in the SGZ that incorporated BrdU were similar in WT and KI mice:

To test whether SERT Ala56 knock-in mice had a difference in the number of mitotic cells (S phase) in the SGZ in comparison to their WT littermates, BrdU was injected i.p. and animals were perfused 18-20 hours later, allowing only the dividing stem cells and amplifying progenitor cells to incorporate the BrdU during this time. Coronal sections (40 um) through hippocampus were immunostained for BrdU (Figure 7). All sections in the series (1 in 6) through the hippocampus that contained the GCL were used for counting, beginning with the very first section in the series where the GCL was seen and ending in caudal of the hippocampus where no GCL was visible. Counts of BrdU+ cells in the SGZ were made in 6 WT mice and 7 KI animals (as shown in Table 1). Table 1 shows the cell counts, and also shows the age and weight of each mouse on the day of

BrdU injection for comparative purposes between the two genotypes. The mean number of BrdU+ labeled SGZ cells was 376 cells (+/-26 SEM) in WT mice, and was 367 cells (+/-23 SEM; $p>0.05$, unpaired two-tailed t test, $t_{(11)} =0.23$) in KI mice. The mean age of WT mice at BrdU injection day was 10.5 weeks and the mean body weight was 23.1 grams. The mean age of KI mice at BrdU injection day was 10.2 weeks and the mean body weight was 23.5 grams. These differences were not statistically different (Figure 8).

1.4 Discussion

New neurons are continually generated and functionally integrated into existing neuronal networks in the adult hippocampus and olfactory system. The hippocampus has been well studied in terms of neurogenesis. In the SGZ, SCs (radial-glial-like cells or Type 1 cells) give rise to proliferating progenitors that give rise to GCs. Neurotransmitters, including 5-HT, have been linked with the generation of adult-born neurons [53]. Research by Barry Jacobs laboratory demonstrated the stimulating effect of 5-HT on neurogenesis through 5-HT receptor signaling in adult neural progenitor cells in the DG [53]. They investigated the action of the 5-HT_{1A} receptor by administering 5-HT_{1A} receptor antagonists (NAN-190, p-MPPI and WAY-100635) to adult rats, and discovered a 30% reduction in proliferating cells in the DG [74]. A study by Daszuta et al. in 2004, showed that activation of the same receptor by the agonist 8-OH-DPAT, produced increases in the number proliferating cells [73]. These findings indicate

an important role of serotonergic signaling in regulating cell proliferation in the SGZ.

Mouse models with genetic manipulations targeting SERT, 5-HT receptors, and TPH-2 also have been useful for studying the role of 5-HT in hippocampal adult neurogenesis [38]. The effect that of TPH-2 KO, and VMAT2 KO (a vesicular serotonin packaging enzyme) produce in mice is to reduce 5-HT neurotransmission, making them somewhat comparable to the SERT Ala56 mutation, which also reduces of 5-HT neurotransmission. In both of these mice, basal cell proliferation in the SGZ were shown to be normal [9,34,38,47]. In the present study, changes in 5-HT availability in the hippocampus of Ala56 mice also failed to produce measurable changes in baseline proliferation of dentate progenitor cells. My results demonstrate there is no statistically significant difference in numbers of BrdU+ cells in the SGZ at 18-20 hrs post-treatment in WT and KI mice. When multiplied by 6 to estimate total labeled cells in all sections, the mean value for WT mice was 2258 labeled cells and mean for KI mice was 2201 labeled cells. This is similar to numbers reported by Klempin et al. in 2013 for WT mice and TPH-2 knock-out mice [54]. There was no evidence in my study to support the hypothesis that the SERT Ala56 mutation alters neurogenesis by changing SC proliferation in the hippocampal SGZ.

While the present study focused on the effect mutated SERT Ala56 has on proliferation of progenitors in the SGZ, neurotransmitter signaling effects on cell proliferation in the SGZ are not limited to serotonin. For example, ablation of entorhinal cortex (EC) neurons that project to the DG increases SGZ cell

proliferation, suggesting that glutaminergic input has some degree of importance in regulating hippocampal neurogenesis [62]. Metabotropic glutamate receptors (mGlu), a type of receptor that acts through a second messenger, have been detected in progenitor cells in the rodent hippocampus [30]. In studies of cultured dentated progenitor cells by Di Giorgi-Gerivini et al. (2005), treatment with a $mGlu_3$ receptor antagonist reduced proliferation. Treatment with a $mGlu_5$ antagonist also reduced cell proliferation *in vitro*, while genetic KO of $mGlu_5$ in mice reduced SGZ cell proliferation *in vivo* [55]. Di Giorgi-Gerivini's results indicate that $mGlu_5$ glutamate receptors act to regulate progenitor cell proliferation and survival in the SGZ. [55]. The different results seen in mGlu5 agonist and antagonist in these studies may be due to different receptor types that are expressed in the different cell types in the pool of SGZ progenitor cells (Type 1, Type 2a, Type 2b).

Acetylcholine also has been shown to affect proliferation of SCs in the DG. Long-term treatment with nicotine, an ionotropic (ligand-gated) acetylcholine receptor agonist, decreased cell proliferation in the SGZ [65]. Also, muscarinic acetylcholine receptors have been identified in radial-glial-like cells (Type 1) in the SGZ [66]. Ablation of medial septum cholinergic neurons that project to the DG, reduces cell proliferation in the SGZ, and impairs spatial memory [67,68]. A study by Van Kampen and Eckman in 2010, manipulated muscarinic M_1 receptors, the most abundant in the hippocampus, pharmacologically for 10 days using the muscarinic agonist, oxotremorine, which caused an increase in cell proliferation in the SGZ. They also observed a dose-dependent effect in cell proliferation in

response to the nonselective cholinergic agonist, carbachol. Lower doses showed a more selective muscarinic activation, while higher doses resulted in an increase in nicotinic receptor activation, and suppressed cell proliferation [56]. These results indicate that muscarinic receptors mediate the actions of endogenous acetylcholine in increasing adult neurogenesis, while nicotinic receptors have the opposite influence.

The DG also receives noradrenergic projections originating from the locus coeruleus, an area in the brainstem associated with physiological responses to stress and panic, and is the site for brain synthesis of norepinephrine (NE). A study in which reboxetine, a NE reuptake inhibitor, was administered to mice showed increased cell proliferation in the hippocampal SGZ, while ablation of noradrenergic neurons reduced cell proliferation [69]. These studies indicate different responses of progenitor cells in the SGZ to noradrenaline signaling depending on whether NE is more available (reboxetine) or severely reduced (neuron ablation). In addition, GABAergic interneurons in the DG release the modulator nitric oxide (NO), and this diffuses to neighboring cells. Under normal physiological conditions, NO has been shown to reduce SGZ cell proliferation and SC populations in 18 month old mice [2,51,71-73]. The data suggests that NO keeps proliferating SCs quiescent, and deficiency of NO accelerates depletion of the SCs.

In summary, my results in mice with reduced hippocampal 5-HT signaling due to SERT Ala56 mutation are consistent with results from TPH-2 KO mice. Previous studies demonstrated that basal proliferation of adult hippocampal

neural SCs is maintained at a normal rate in TPH-2 KO mice with severely reduced CNS 5-HT levels, suggesting that reductions changes in 5-HT signaling levels in the DG do not dramatically affect SC proliferation *in vivo* [11,54].

Chapter Two:

Impact of SERT Ala56 mutation on survival of adult-born GCs in the GCL of the hippocampus

2.1 Introduction

The finding of adult neurogenesis in the forebrain hippocampus has stimulated new research approaches for brain repair to find ways neurogenesis might be enhanced in different pathological conditions [13]. It is therefore of interest to study adult neurogenesis to understand how the process is regulated. Adult neurogenesis is closely regulated by the local dentate environment, or the "environmental niche" that includes the extracellular matrix and the various cell types, such as glial cells, endothelial cells (part of the vascular system), adult neural SCs, mature neurons and their inputs, and secreted molecular factors that affect cell proliferation and development [13,20,53,57]. Factors such as exercise and pharmacological treatments, including SSRIs, that are capable of affecting the niche microenvironment, are of great interest because they can change the survival rate of adult-born GCs. Interventions such as these that alter hippocampal circuit function can influence several aspects of adult neurogenesis in rodent disease models, suggesting potential ways to manipulate SCs for therapies [19,51,53].

Neuronal circuits that are thought to regulate distinct stages of adult neurogenesis in the hippocampus are complex and plastic. 5-HT is produced primarily by the dorsal raphe (DR), which contains about half of the mammalian nervous system's serotonergic neurons, is the major source of 5-HT axon projections in the brain, and is thought to function in regulating stress/anxiety and mood [12]. Chronic stress has been shown to negatively impact adult hippocampal neurogenesis, which can be positively regulated by antidepressant treatments in animal models, linking 5-HT neurotransmission to impairments in adult neurogenesis. This theory is supported by the work of several laboratories indicating decreased numbers of GCs and decreased GCL volume in the DG in untreated depressed patients, as well as increased hippocampal neurogenesis and GCL volume in depressed patients treated with antidepressant medication [11,18,54,56]. This links the serotonergic and neurogenic hypotheses of depression that may explain antidepressant effects.

The aim of the second part of this project was to test if the reductions in CNS 5-HT caused by the SERT Ala56 mutation affected the numbers of adult-born GCs that survive in the hippocampal DG in adult mutant mice. Following 7 days of BrdU treatment in WT and KI mice, the numbers of BrdU-immunoperoxidase+ cells in the hippocampal GCL were counted and compared 5 weeks later, when new GCs incorporating BrdU, would have matured and integrated. Alternate brain sections also were used to quantify BrdU+/NeuN+ double-labeled cells in the GCL using immunofluorescence. Staining for the

cytoskeletal protein doublecortin (DCX), expressed by immature GCs, was also performed to assess possible differences in this population.

2.2 Methods

5-bromo-2'-deoxyuridine (BrdU) injections and brain collection:

All male mice were 9-12 weeks old when BrdU treatment began. For the longer survival group interval (5 wks), mice were given single, daily injections of 50mg/kg of BrdU for 7 days (N=5 WT mice and 6 mutant mice). They were euthanized 5 weeks after the first injection to count numbers of surviving, mature adult-born granule cells. Mice were euthanized and perfused as described in Chapter 1. Brains were dissected, post-fixed, cryoprotected and frozen in the same manner.

Tissue processing:

Free-floating immunoperoxidase staining was performed for BrdU localization as described in Chapter 1. Immunoperoxidase staining for doublecortin (DCX) was performed as described for BrdU, using Santa Cruz goat antibody #sc-8066 at a dilution of 1:500, with HCl treatment omitted. For immunofluorescent staining, coronal brain sections through the hippocampus (40 µm, 1 in 6 series) were rinsed in Tris-buffered saline (0.1M TBS solution, then treated with 2N HCl acid at 37ºC, followed by neutralizing for 10 minutes in 0.1M sodium borate (pH 8.5). Tissue was then blocked in 10% normal goat serum with

0.3% Triton x-100 in 0.1M TBS for 2 hours, and was transferred to a cocktail of primary antibodies that included rat anti-BrdU (Accurate Scientific; 1:400) and rabbit antibody to NeuN (AbCam #EPR12763; 1:500), a nuclear protein expressed by mature neurons. Antibodies were diluted in 0.1M TBS with 5% normal goat serum added. Sections incubated overnight at 4ºC on a rotator. Tissue then washed 3 times (10 min each) in 0.1M TBS. Secondary antibodies used were AlexaFlour 488-labeled goat anti-rat (1:1000, Invitrogen/Fisher Scientific) and AlexaFlour 594-labeled goat anti-rabbit (1:1000). Secondary antibodies were diluted in 0.1M TBS and incubation was performed for 2 hrs at RT with rotation. Sections were then mounted in order (anterior to posterior) from 0.05 M PB to gelatin treated slides, and were air-dried in the dark. Slides were cover-slipped with Vectashield H-100 (Vector Laboratories, Inc.) and edges were sealed with nail polish.

Quantification of labeled cells:

BrdU cell quantification using immunoperoxidase labeling was done as described in Chapter 1, but the cells were counted in the GCL to quantify mature GCs. Counts of the immunofluorescent, labeled neurons were restricted to nuclei labeled for both BrdU and NeuN within the dentate GCL using a Zeiss 710 LSM confocal microscope at 40-63x objective magnification. Other, single-labeled BrdU+ cells that did not express NeuN, in the hilus or molecular layer, were not included and were assumed to be glia. The total number of new GCs per mouse was calculated from serial sections counts through hippocampus (1 in 6). Group

means +/- standard error of the mean (SEM) were calculated from animal means. Comparisons of genotype results were analyzed with two-tailed, unpaired t-tests for both BrdU+ immunoperoxidase data, and for counts of immunofluorescent, double-labeled, BrdU+/NeuN+ cells. The level of significance was considered to be $p<0.05$. Quantification of immature DCX+ neurons were not made since no qualitive difference were seen between the two genotypes.

2.3 Results

Survival of new adult-born granule cells was similar in WT and KI mice

To determine that the BrdU+ cells in the GCL were indeed neurons and not glial cells, immunostaining for the antigen neuronal nuclei, NeuN, allowed for the identification of mature BrdU+ neurons. NeuN is only produced by mature neurons, confirming that new labeled cells in the GCL are surviving, integrated neurons. Table 2 and Figures 10 and 11 show the results of labeled cell counts using immunoperoxidase and immunofluorescent techniques, as well as the total number of hippocampal serial sections sampled per animal. My results show that numbers of new mature GCs in the DG did not differ significantly with genotype (unpaired, two-tailed t-tests, $p>0.05$). The mean value of immunoperoxidase+ cell counts 5 weeks after BrdU treatment was 66 cells (+/- 10, SEM) per WT mouse, and in KI mice was 67 cells (+/- 6 SEM; $p>0.05$, unpaired two-tailed t test, $t_{(9)} =0.37$). Mean total counts of double-labeled cells obtained using immunofluorescence was 69 cells (+/- 4 SEM) for WT animals and 63 cells for SERT Ala56 mutants (+/- 6 SEM; $p>0.05$, unpaired two-tailed t test, $t_{(9)} =0.76$). The mean ages and body weights of mice of both genotypes is also shown.

DCX+ immature GCs were not quantified, but showed no notable differences between the WT and KI brain sections on visual inspection.

2.4 Discussion

The discovery of adult neurogenesis drove new advances in technology that uncovered unique properties and roles of these new adult-born neurons (GCs). However, it is still uncertain what advantages or special functions adult neurogenesis can provide. Also unclear is whether we can trigger new neurons to be generated, and if this would benefit patients that may have reduced adult neurogenesis due to imbalances in neurotransmitter systems that are associated with disorders like depression and autism.

Recent evidence of adult-born neurons' unique role in the rodent hippocampus has been described [3,13,58]. In a study by Alejandro F. Schinder and his team, adult-born GC functional outputs to CA3 were studied using the light-activated ion channel, channelrhodopsin, to determine the timing of critical periods in their developing connectivity, particularly feedback inhibition by GABAergic interneurons [59]. The electrophysiological analysis found that young, stimulated 4 week old adult-born neurons triggered less inhibitory feedback inhibition than mature GCs, suggesting an enhanced output by new GCs [59]. A study by Matteo Bergami's laboratory observed a critical period of anatomical response to environmental enrichment in adult-born GCs at 6 weeks of cell age, which correlated with twice as many mature, dendritic mushroom spines developing in the new neurons [60,71]. Together these studies show evidence of unique functions and responses in adult-born GCs within the hippocampal

network. Many additional studies have indicated that the primary role of adult-born hippocampal GCs is to add an additional level of plasticity in the existing circuitry through indirect and direct mechanisms, including their inhibition of mature GCs through activation of inhibitory hippocampal neurons [2,61,62]. Genetically reducing hippocampal 5-HT levels in rodent models like the TPH-2 KO mouse, where no 5-HT is synthesized, has no effect on basal hippocampal neurogenesis. However, the knockout blocks the increase in neurogenesis seen with wheel running, preventing the increase in new neurons that would normally occur [11,54]. This result showed 5-HT is necessary for the neurogenic response to physical activity. However, new GCs generated in response to running show a different, unique pattern of developing inputs and outputs, circuitry changes that do not occur in non-running mice [63]. Seemingly, this circuitry would not develop without 5-HT to trigger the increase in neurogenesis, and chronically reduced 5-HT could have permanent effects on normal hippocampal circuit function and plasticity.

Other manipulations of 5-HT levels cause a variety of changes in adult-born GC survival. The vesicular monoamine transporter 2 (Vmat2) is a protein that transports monoamines, like 5-HT, from the cell cytosol into the synaptic vesicles [38,61,70]. In Vmat2$^{sert\text{-}cre}$ conditional (C) KO mice, Vmat2 is deleted in serotonergic neurons (but not limited to only these neurons). The Vmat2 deletion in serotonergic neurons leads to significant 5-HT reduction in the brain but the survival of the serotonergic neurons is unaffected. Adult Vmat2$^{sert\text{-}cre}$ CKO mice show normal baseline proliferation of adult hippocampal progenitors in the SGZ

but surprisingly, enhanced survival of GCs [61,70]. In contrast, most studies with SSRIs, in which the effect is an increase in 5-HT by blocking its reuptake (and desensitization of 5-HT_{1A} pre-synaptic receptors), have shown that chronic treatment also leads to enhanced survival of new GCs [12,18,34,37,67]. The 5-HT_{1A} receptor is likely involved in regulation of hippocampal neurogenesis by the SSRI mechanism. Administration of the 5-HT_{1A}/5-HT_7 receptor agonist 8-OH DPAT increases neurogenesis in the SGZ [67]. In a study by René Hen's laboratory, the role of the 5-HT_{1A} receptor was confirmed in mice that are germline deficient in 5-HT_{1A} heteroreceptors in DG GCs. When given SSRIs no increase in GC neurogenesis was seen [18,31,33,34].

Dr. Randy Blakely's laboratory has shown that in the SERT Ala56 mice, 5-$HT_{2A/2C}$ and 5-HT_{1A} receptors show hypersensitivity to 5-HT, which may be a compensatory response to the lower levels of 5-HT stimulation caused by the mutation [29]. The hypersensitivity is due to increased 5-HT affinity caused by increased phosphorylation of the transporter protein by p38α mitogen activated protein kinase (MAPK). This group showed that genetic elimination or drug inhibition of p38α MAPK can slow 5-HT reuptake from the synapse [29]. These treatments also reduced the increased 5-$HT_{2A/2C}$ and 5-HT_{1A} receptor sensitivities and normalized despair-like behaviors the mice displayed when they were tested on the tail suspension test or forced swim test [27,64]. This finding could potentially lead to testing drugs that inhibit p38α MAPK to develop treatments that may reduce the symptoms of ASD in patients carrying the Ala56 mutation.

As mentioned previously, other signaling pathways that act to regulate GC birth and survival in hippocampus, are regulated by inputs from other CNS neuron populations that may be affected by reduced 5-HT due to the ALA56 mutation [51]. The SGZ/GCL receives multiple synaptic inputs from various brain regions. This includes dopaminergic fibers from the ventral tegmental area (VTA), which terminate mainly in the hilus, contact progenitor cells in the SGZ, and are part of the reward-system of the brain [3]. Acetylcholinergic input from the septum, glutamatergic inputs from entorhinal cortex (EC) and excitatory hippocampal neurons, and finally, the GABAergic connections from local interneurons all influence adult hippocampal neurogenesis [2,20,51,58,65]. GABAergic interneurons in the hippocampus, as well as GABAergic projection neurons in the septum, have been shown to regulate GC neurogenesis [3,65]. Local GABAergic input promotes GC maturation until glutamatergic input from EC projections make contacts with the neurons [2,10,13,53,55-57]. Serotonin may act on these input populations, as well as with other glutamatergic neurons within hippocampus, to affect GC neurogenesis. In an experiment by Dr. Francesc Artigas's laboratory in 2016, rats were treated with the antidepressant vortioxetine, a 5-HT_3 receptor antagonist, to test its effect on hippocampal pyramidal neuron activity and extracellular 5-HT concentration *in vivo* [67]. The results suggested that vortioxetine increases both glutaminergic and serotonergic neurotransmission by blocking 5-HT_3 receptors in GABAergic hippocampal interneurons [65,67].

In this study, I have evaluated the neurogenic effects of the SERT Ala56 mutation, which reduces synaptic 5-HT in adult rodent hippocampus. I found that

numbers of proliferating progenitor cells in the SGZ were not altered by the transporter mutation. After allowing enough time for the new neurons to develop, mature, and be incorporated in the GCL, my results also demonstrated that there was no significant difference in the numbers of new, surviving GCs between WT and KI mice. When BrdU+ cell counts were multiplied by 6 to estimate total numbers of labeled GCs in all hippocampal sections, the mean number for WT mice was 398, and for KI mice was 402. Estimations for the double-labeled BrdU+/NeuN+ cells were consistent with peroxidase-labeled cell counts. When multiplied by 6 total BrdU+/NeuN+ cells in WT mice averaged 414 and in KI mice averaged 375, numbers which are similar to numbers reported by Klempin et al. (2019). Therefore, there was no evidence to support the hypothesis that the SERT Ala56 mutation significantly changes basal levels of either adult-born dentate GC birth or survival in this mouse model.

Conclusion

The current prevalence of ASD in the United States is 1 in 59 children, more precisely, 1 in 37 boys and 1 in 151 girls. In addition to medical costs, intensive behavioral interventions for children with ASD cost $40,000 to $60,000 per child per year, according to the CDC in 2018. Therapies that ameliorate the symptoms of this disorder are scarce and new ones are desperately needed. The SERT Ala56 mutation has been linked to ASD, as well as hyperserotonemia, which is unique to this genetic disorder which appears to cause serotonergic system dysfunction [26,42]. Disturbances in the serotonergic system are related not only to the Ala56 mutation in ASD patients, but also to functional, anatomical and behavioral abnormalities that may occur in patients with psychiatric disorders such as OCD, anxiety, depression and mania [23] . Depression in particular is linked to 5-HT. Low levels of 5-HT are hypothesized to reduced hippocampal neurogenesis, and SSRIs increase neurogenesis, and improve depression in patients, presumedly by an increase neurogenesis in mouse models, and improve depression in treated patients, presumedly by increasing 5-HT neurotransmission. Serotonin has been stablished as an important factor that promotes neuronal development prenatally, however, some data on 5-HT and SSRIs effects on development of adult-born hippocampal neurons remains

contradictory, indicating the complexity of interactions between the 5-HT, neurogenesis and their combined role in neurological disorders [38].

While I did not find an effect of the Ala56 SERT mutation on basal levels of SGZ cell proliferation or survival in this mouse model, this does not rule out potential effects on other aspects of hippocampal neurogenesis, including effects on the plastic responses to wheel running, which is impaired in TPH-2 KO mice lacking 5-HT. It would be interesting in future research work with SERT Ala56 mice to test if using pharmacologic treatments, such as SSRIs, or environmental manipulations, such as wheel-running or cage enrichment, have different effects on hippocampal neurogenesis compared to normal animals [34,54]. Additional studies to determine if the connectivity of adult-born GCs in Ala56 mice is altered by the reduction in synaptic 5-HT could point to a role for 5-HT in their normal integration. There is evidence that 5-HT acts on 5-HT_{1A} receptors during early postnatal hippocampal development to regulate both neurogenesis and synaptic connectivity [1,21,32,68].

Appendices

Appendix A: Tables

TABLE 1. COUNTS OF IMMUNOPEROXIDASE-LABELED CELLS IN SGZ				
ANIMAL # AND GENOTYPE	**Age on day of injection (week and day)**	**Body weight (g) on injection day**	**SGZ cell count**	**Number of hippocampal sections**
4609 WT	10w4d	24	374	13
4638 WT	11w	26	409	12
4639 WT	11w4d	25	370	12
5004 WT	10w	22.1	238	12
5123 WT	10w	20	463	13
5134 WT	9w5d	21.6	404	11
4629 KI	10w	25	333	12
4631 KI	10w5d	26	285	14
4637 KI	11w	28	359	12
5006 KI	10w	25.5	424	11
5007 KI	10w	22.5	376	13
5135 KI	9w5d	18	294	12
5163 KI	10w	19.8	397	12

TABLE 2. COUNTS OF IMMUNOPEROXIDASE AND IMMUNOFLUORESCENT-LABELED CELLS IN THE GCL					
ANIMAL # AND GENOTYPE	**Age on day 7th of injection (week and day)**	**Body weight (g) on last injection day**	**Immunoperoxidase cell count of GCL**	**Immunofluorescent cell count of GCL**	**Number of hippocampal sections**
9103 WT	10w2d	20.4	111	62	14
9105 WT	10w4d	21.7	71	87	13
4343 WT	10w6d	20.2	46	65	13
4361 WT	11w1d	21.3	44	61	12
4354 WT	11w1d	20.9	60	70	13
9084 KI	10w6d	23.5	88	62	13
9086 KI	10w6d	20.4	50	32	12
9104 KI	10w2d	19.4	91	64	13
4341 KI	10w6d	19.8	67	77	12
4353 KI	11w1d	22.6	57	77	12
4342 KI	10w6d	21.6	49	63	14

Appendix B: Figures

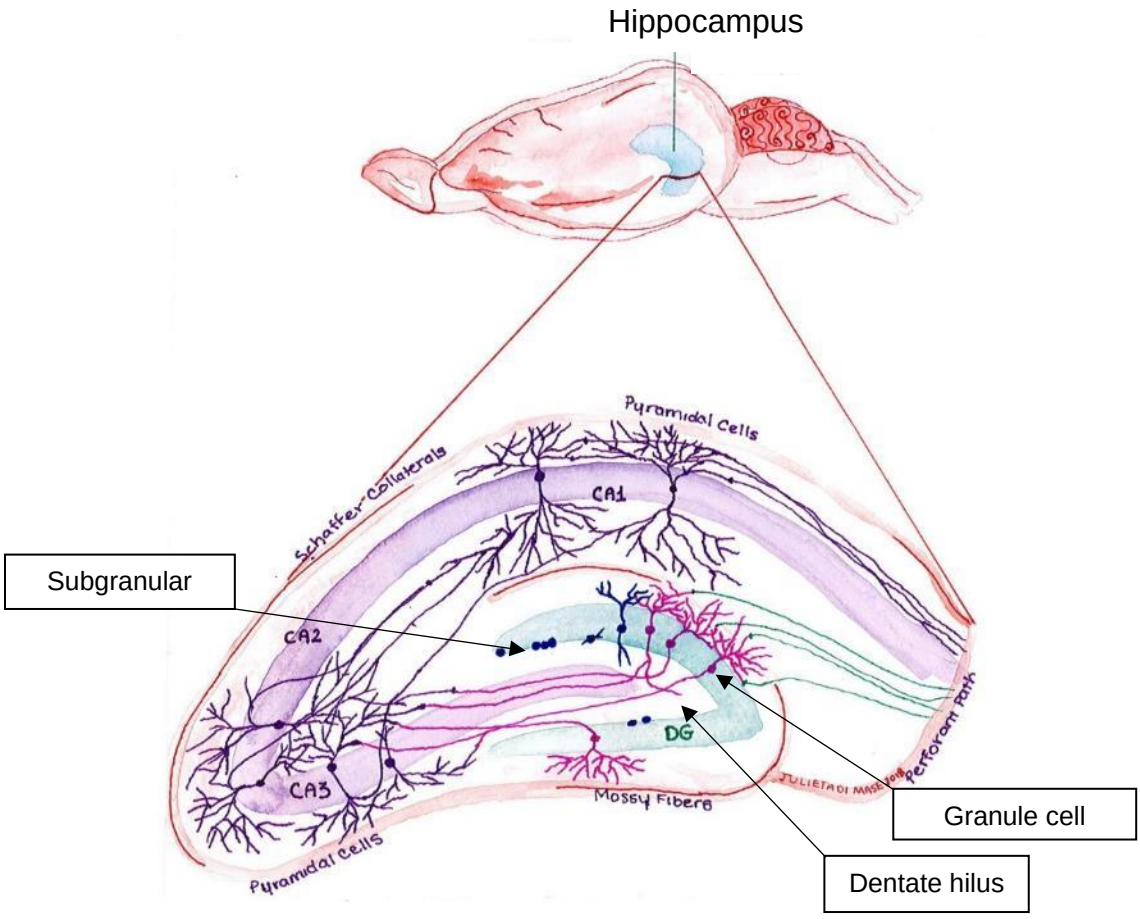

Figure 1. Illustration of a transverse section of the rodent hippocampus showing the location of the dentate gyrus (DG) granule cells.

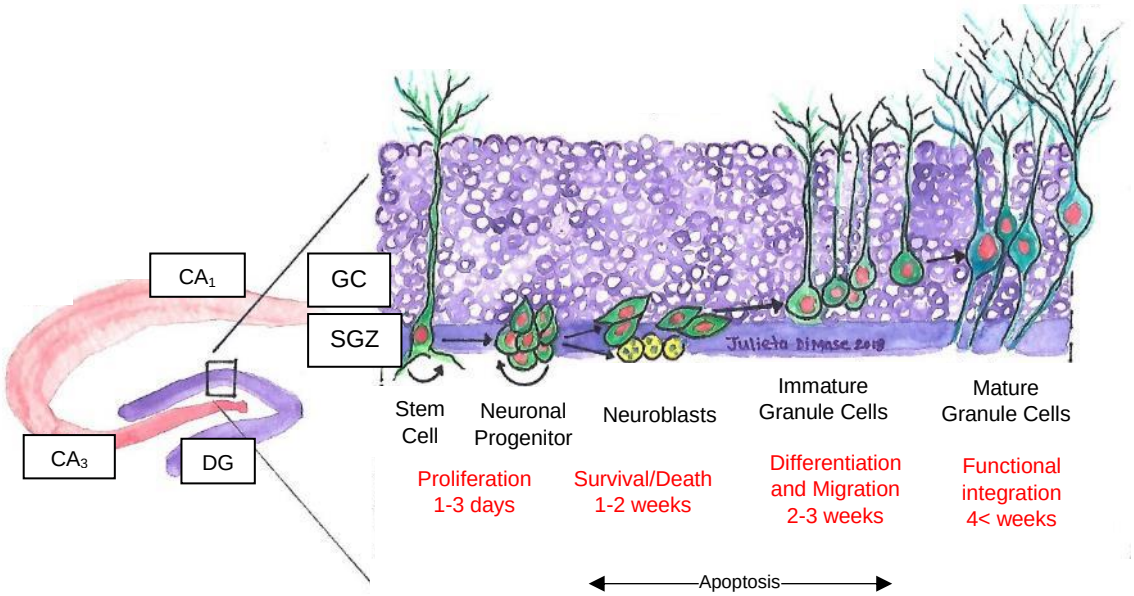

Figure 2. (Adapted from Koehl, 2015) Diagram of the hippocampal dentate gyrus (DG, left) illustrating the time course and stages of development of adult-born granule cells. CA1 and CA3, subfields of hippocampus: SGZ, subgranular zone: GC, granule cell layer.

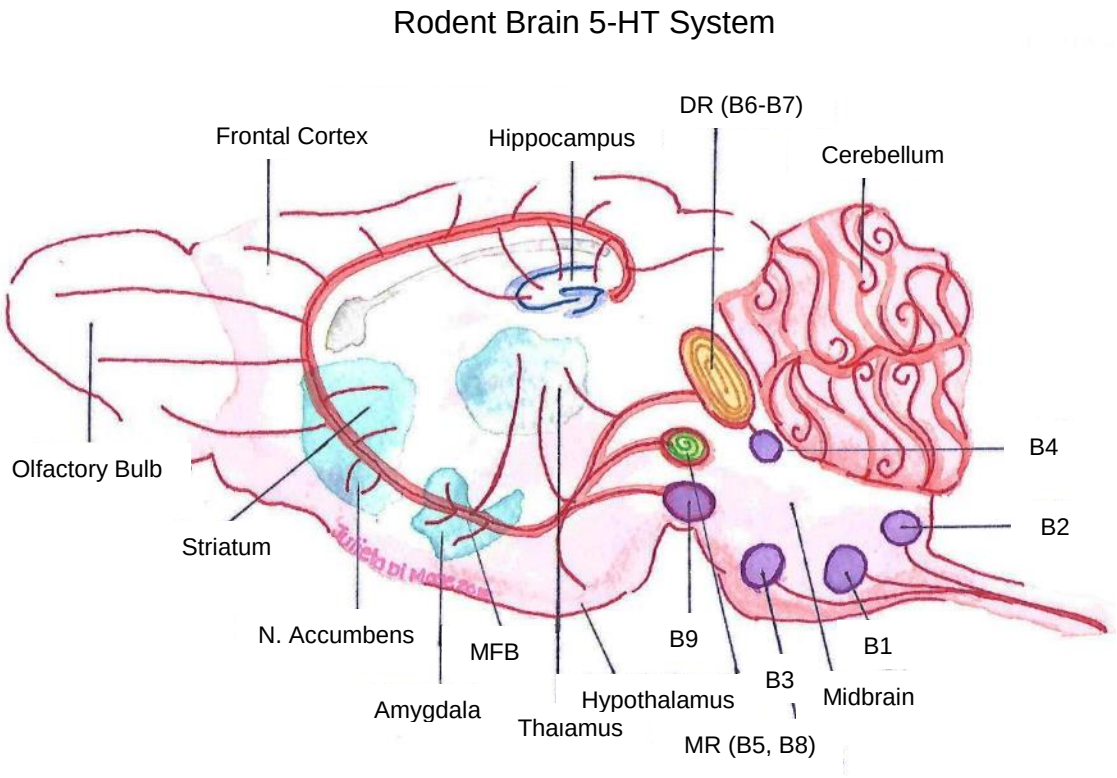

Figure 3. (Adapted from Lesch and Waider, 2012). Sagittal diagram of the rodent brain showing the serotonergic nuclei and their projections. The 5-HT neuronal clusters are divided into 9 raphe nuclei, B1-B9. The more caudal are B1-B3 in the medulla which project axons to the spinal cord. The more rostral nuclei are B6 and B7 and the median nuclei are B5 and B8, which project to different areas in the brain, including the hippocampus, with some overlap. The DR is the dorsal raphe nucleus; MFB is the medial forebrain bundle; MR is the median raphe nucleus [28]**.**

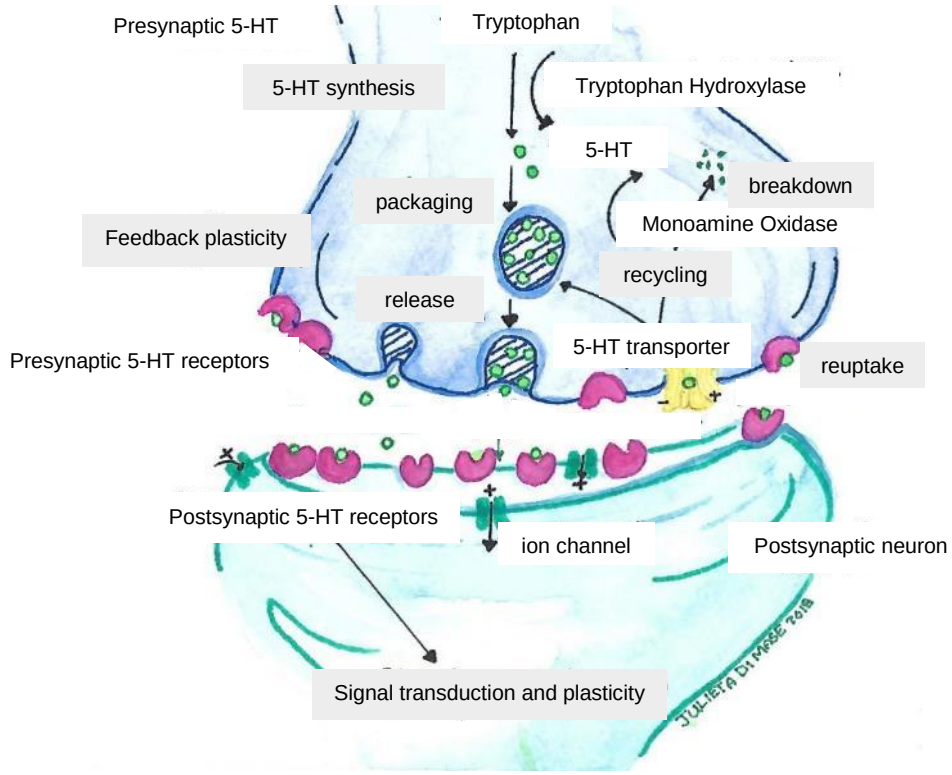

Figure 4. (Adapted from Rot et al., 2009) Diagram of the 5-HT synapse. 5-HT is packaged into presynaptic vesicles and released into the synaptic cleft when an action potential occurs. Once in the cleft, 5-HT can produce multiple actions. (1) 5-HT binds to 5-HT receptors on the postsynaptic neuron, and if enough binding occurs, an action potential follows the influx of positive ions into the postsynaptic cell. (2) 5-HT also binds to receptors in the presynaptic terminal, activating a negative feedback mechanism which will stop the release of 5-HT. (3) 5-HT is taken back into the presynaptic neuron by the SERT and it is recycled to be released again, or broken down by monoamine oxidase A (MAO) and excreted by the body [23]**.**

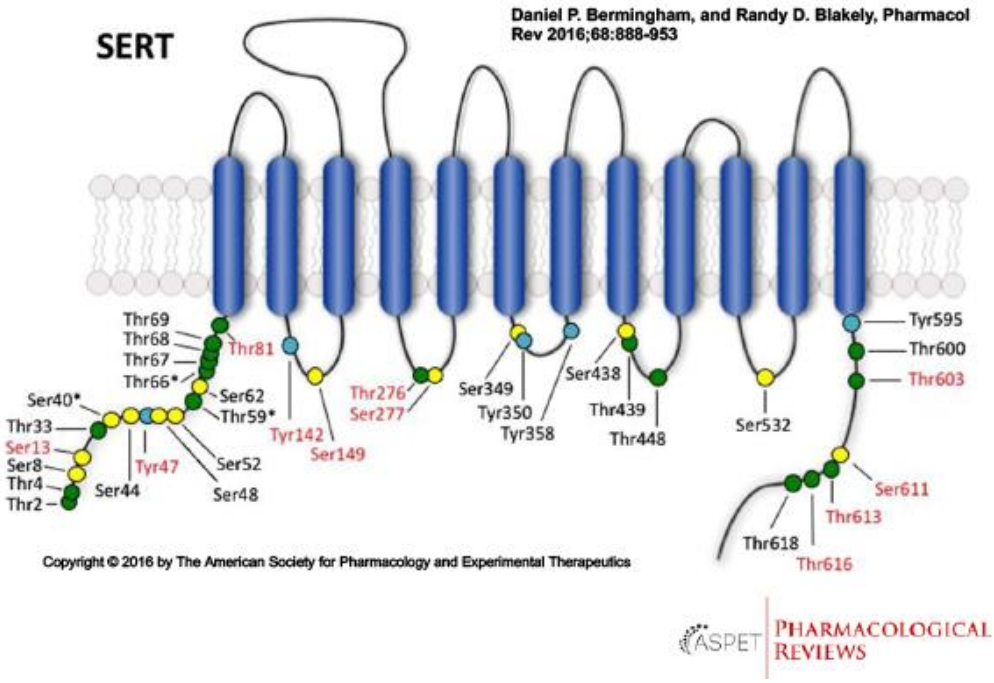

Figure 5. Model diagram of SERT (sodium-dependent 5-HT transporter), an essential membrane protein for the transport of 5-HT from the synaptic cleft into the pre-synaptic cell. The protein transverses the phospholipid bilayer of the cell membrane 12 times. Shown are threonines (green), tyrosines (blue) and serines (yellow) which are potential sites for phosphorylation of the transporter [43]**.**

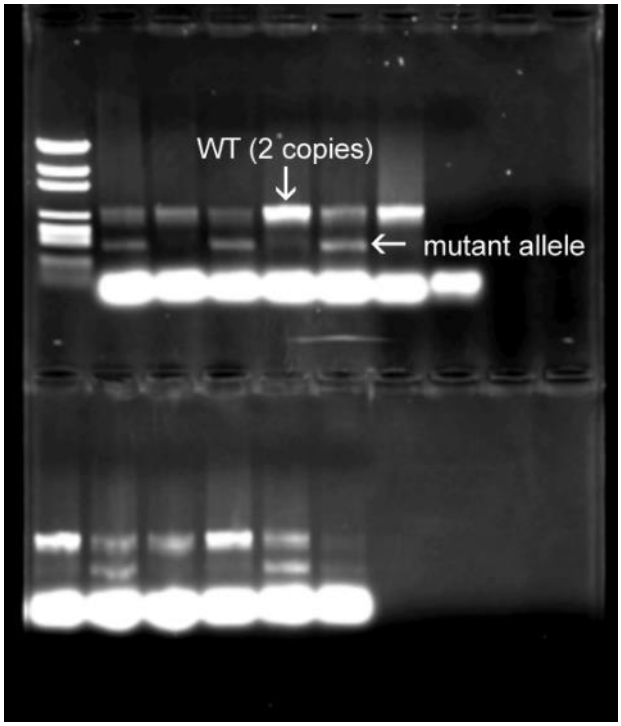

Figure 6. Electrophoresis of mouse DNA from PCR reaction samples for genotyping Angelman mice. The top left lane contains a molecular mass DNA ladder (Promega pGEM Benchtop Markers).

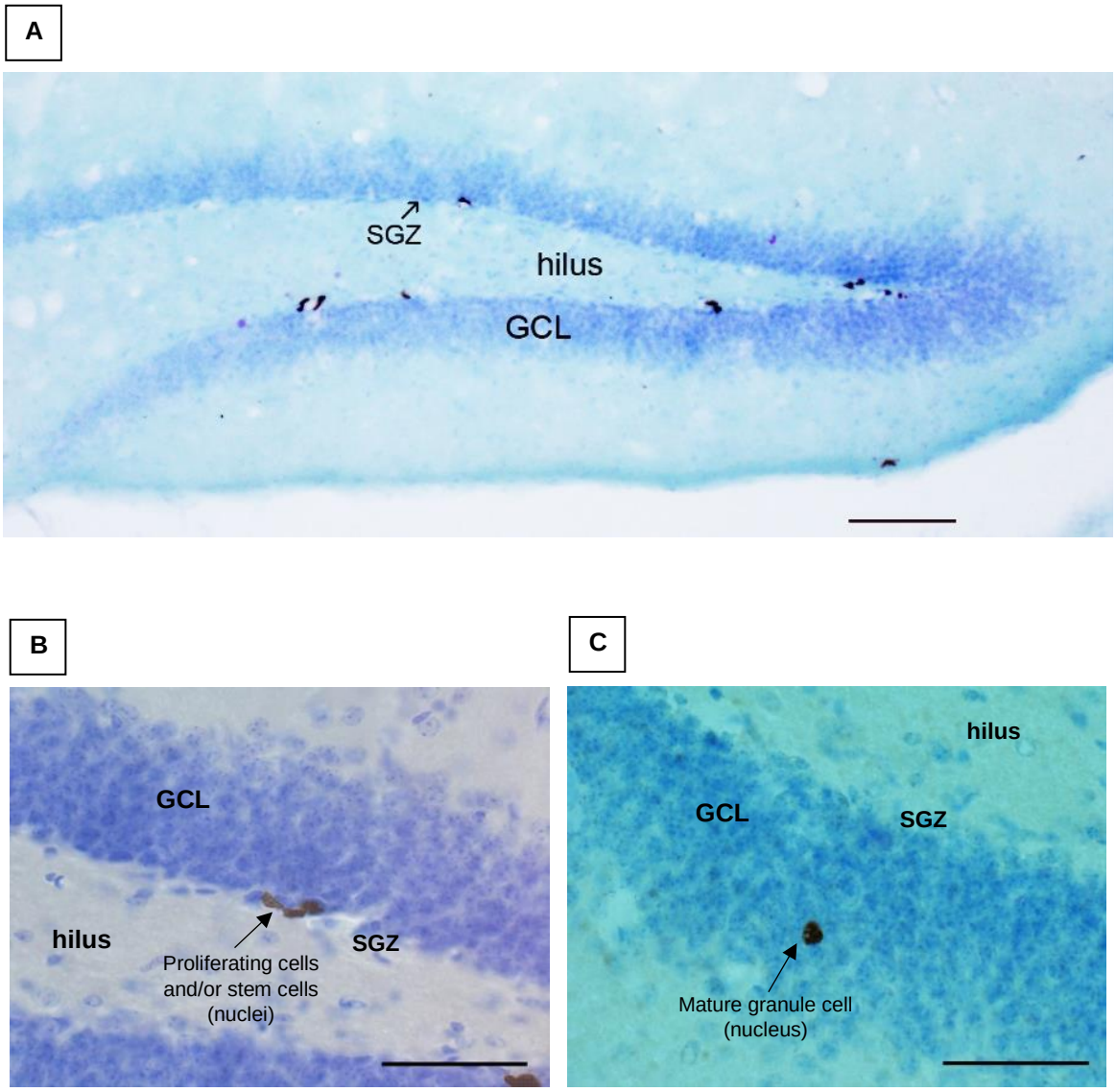

Figure 7. (A) Low magnification of the dentate gyrus (DG) showing BrdU+ cells in the subgranular zone (SGZ) 18-20 hrs after BrdU treatment. Tissue was stained with cresyl violet to make the GCL visible for better analysis. No BrdU+ cells are visible in the granule cell layer (GCL) at this time. Scale bar, 50um. **(B)** Higher magnification of BrdU+ cells (arrow) in the SGZ. Scale bar, 50um. **(C)** BrdU+, mature GC (arrow) in the GCL 5 weeks after BrdU treatment. Scale bar, 50um.

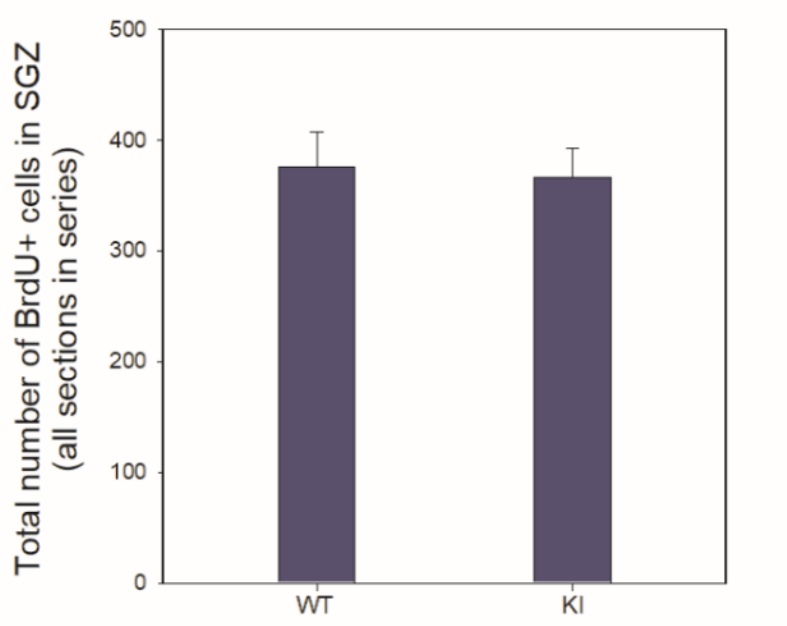

Figure 8. Bar graph comparing group mean numbers of proliferating BrdU+ cells counted in serial sections through the SGZ. Mice were perfused 18-20 hrs after BrdU treatment. There was no significant difference in numbers of labeled cells found between the WT (N=6) and KI (N=7) mice. Mean cell numbers were 376+/-26 standard error of the mean (SEM) in WT mice and 367+/-23 (SEM) in KI mice. ($p>0.05$, unpaired two-tailed t test, $t_{(11)} = 0.23$).

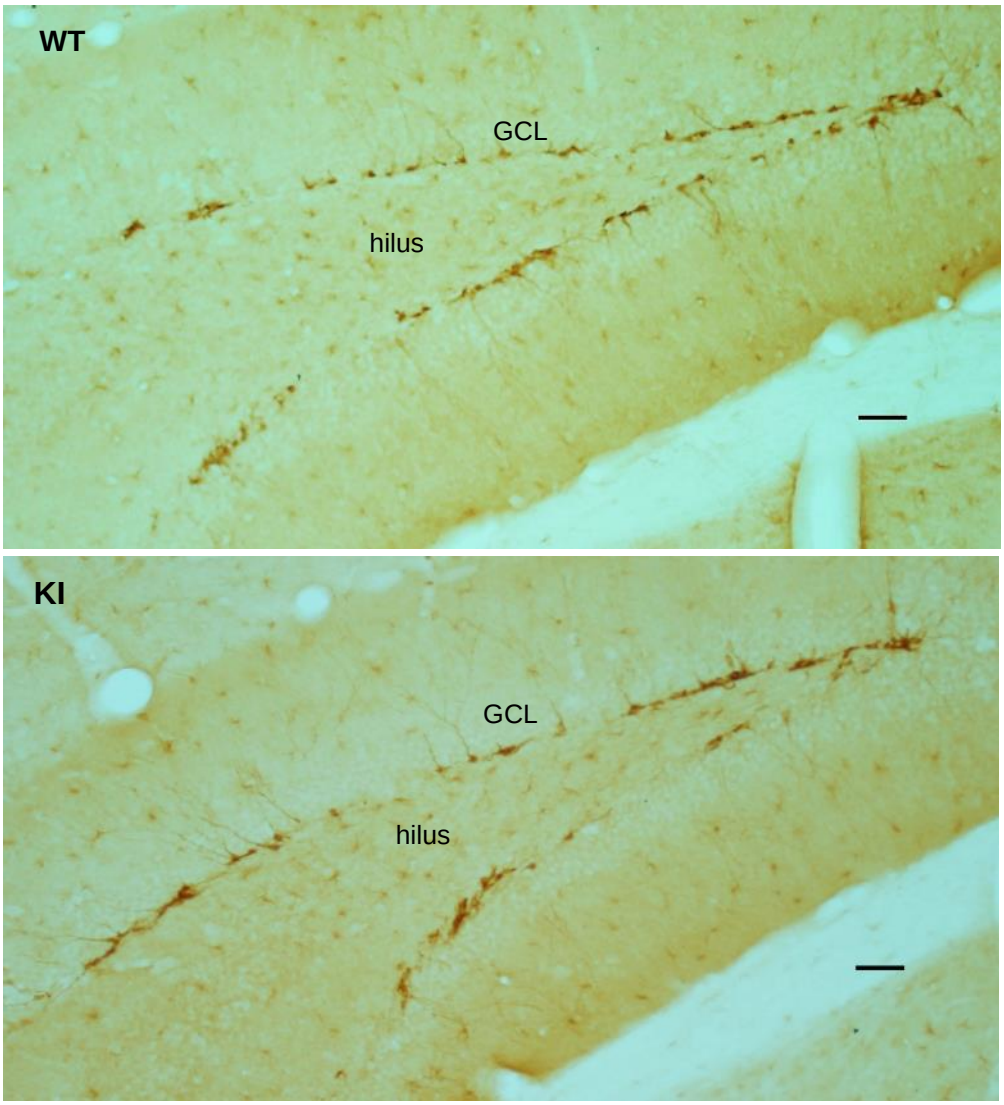

Figure 9. Low magnification image of the DG in a WT mouse (top) and a SERT Ala56 KI mouse (bottom) showing DCX+ immature GCs in 14-15 week old mice. Sections were selected to show labeling in the middle of the hippocampal section series (1 in 6). Scale bar, 50um.

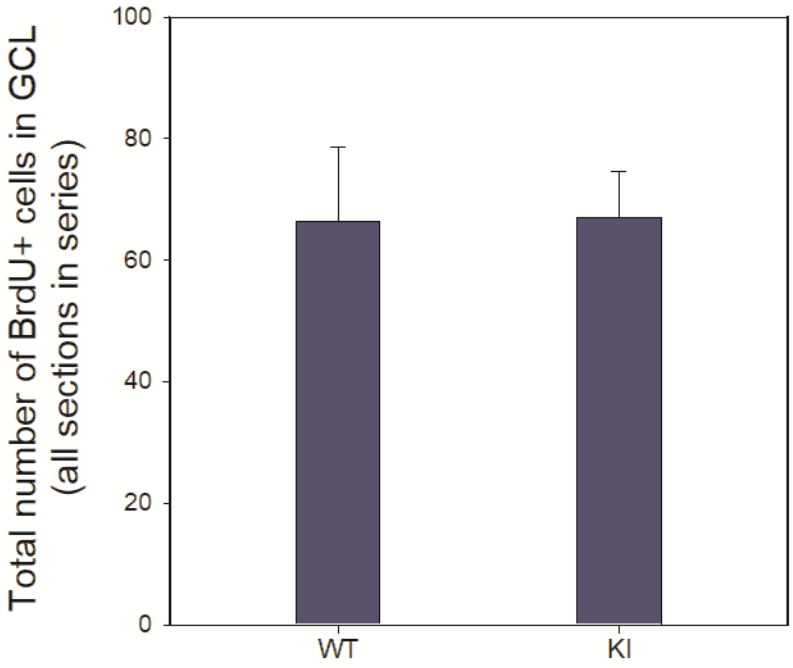

Figure 10. Bar graph of group mean counts of immunoperoxidase-stained BrdU+ cells counted in serial sections through the GCL at 5 weeks after BrdU treatment. There was no significant difference in numbers of BrdU+ cells found between the WT (N=5) and KI (N=6) mice. Mean values were 66+/-10 (SEM) for WT mice and 67+/-6 (SEM) for Ala56 KI mice. ($p>0.05$, unpaired two- tailed t test, $t_{(9)} =0.37$).

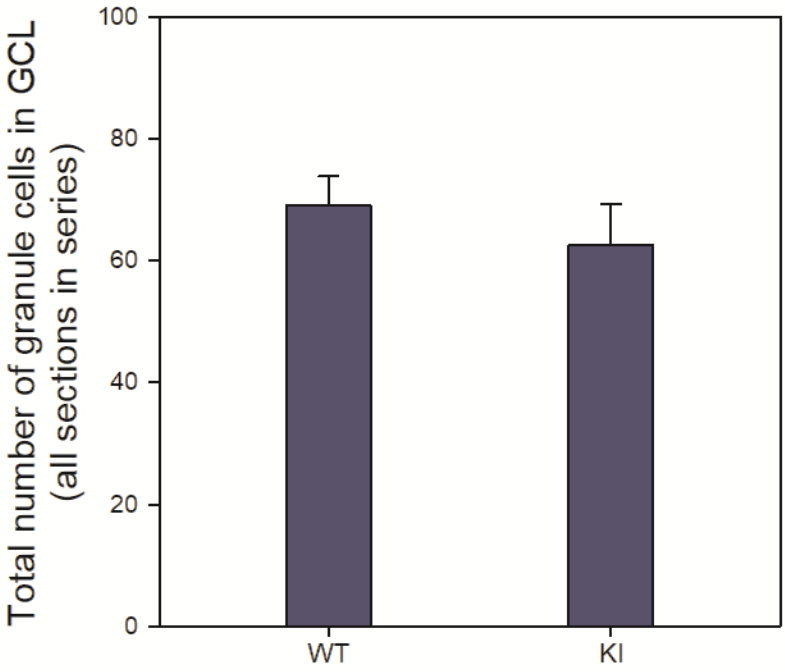

Figure 11. Bar graph of group mean counts of double-labeled mature GCs (BrdU+ and NeuN+ nuclei) within the GCL in sections from male WT mice and Ala56 mutants. There was no significant difference seen in numbers of mature GCs in WT (N=5) and KI (N=6) mice. Mean values were 69+/-4 (SEM) for WT mice and 63+/-6 (SEM) for Ala56 KI mice. ($p>0.05$, unpaired two-tailed t test, $t_{(9)} = 0.76$).

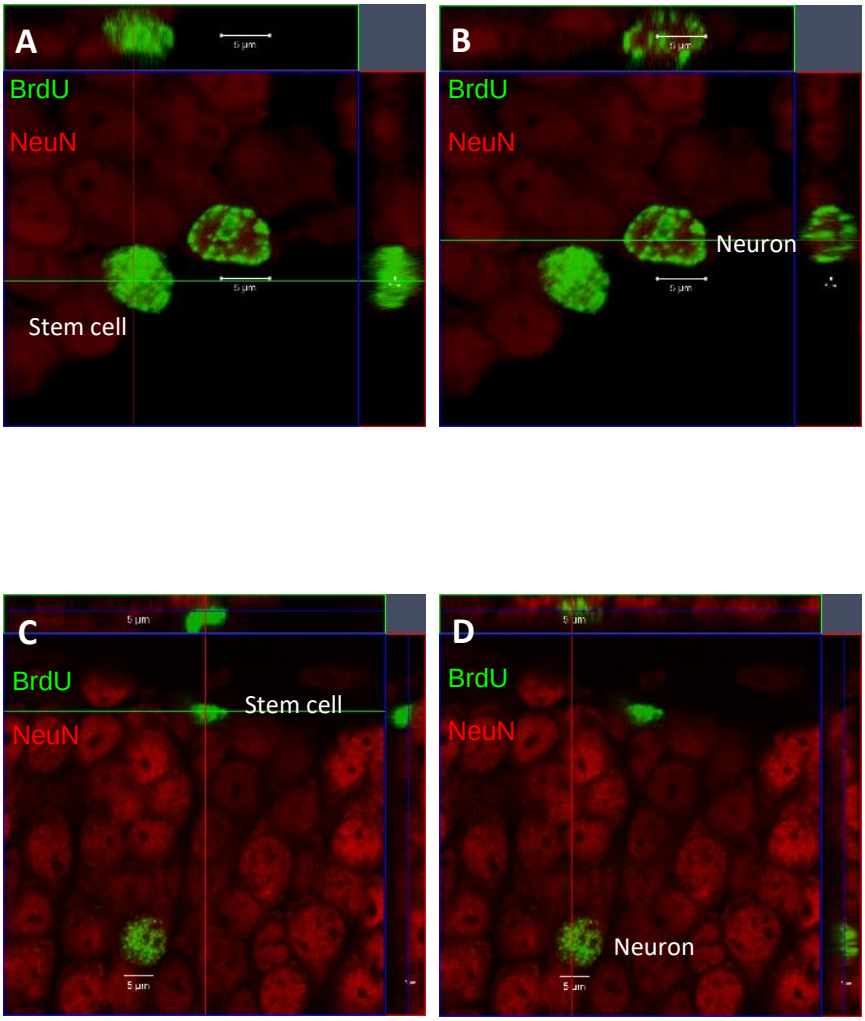

Figure 12. Orthogonal projections of confocal images of double-labeled neurons in the dentate GCL. (A-B) BrdU+ cells (green) in a WT mouse at 5 weeks after BrdU treatment at 63X objective magnification. The red label indicates NeuN expression. The double labeled cell (orthogonal projection shown in B) is an adult- born granule cell neuron. The single labeled cell (A, green only) is a probable stem cell or glial cell lacking NeuN expression. (C-D) Similar images showing double and single labeled cells in the GCL of a KI mouse. Two BrdU-labeled neurons, one without NeuN, in a KI mouse. Scale bar, 5um.

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