Hippocampal Neurogenesis in Rats: Gender and Strain Differences

By

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A thesis submitted to the faculty of Radford University

in partial fulfillment of the requirements for the degree of

Master of Arts in the Department of Psychology.

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October 2016

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Abstract

There is a comprehensive body of research indicating that neurogenesis continues throughout adulthood. This process by which new neurons are born and integrated into existing brain circuitry takes place in four stages: cell proliferation, differentiation, maturation, and survival. One of the primary regions where neurogenesis occurs is the hippocampus, which plays an extensive role in learning and memory. Though investigators have analyzed how various experimental interventions might affect neurogenesis, the research has almost exclusively been conducted in male Sprague-Dawley rats. However, various gender and strain differences are known to exist. For instance, females are more likely to consume addictive substances that inhibit neurogenesis while androgens in males have been shown to enhance cell proliferation. Additionally, Long-Evans rats have generally outperformed Sprague-Dawley rats in several hippocampal-dependent learning tasks. To date there has not been a comprehensive study to examine whether significant baseline gender and/or strain differences in hippocampal neurogenesis exist. To that end, adult (~6.5 months old) male and female Sprague-Dawley and Long-Evans rats were perfused without exposure to experimental manipulations. Brains were collected, sliced, and stained for Ki67 immunoreactivity, a common indicator of cell proliferation. Cells expressing Ki67 were counted and compared. It was predicted that Long-Evans rats and male rats would show significantly higher levels of neurogenesis than their respective counterparts. The results of this study indicate that there are no fundamental differences in neurogenesis between Long-Evans and Sprague-Dawley rats, or between male and female rats. Furthermore, there was not a significant interaction between the two factors.

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Acknowledgements

I would like to extend my sincerest gratitude for the mentorship I received from Dr. Dayna Hayes, without whom I would not have been able to complete a fraction of this project. Her advice and expertise has not only made this entire process possible, but she has gone beyond and above to assist me in making my academic career at Radford University a successful one.

I would also like to extend a special thank you to Dr. Ann Elliott and Dr. Pam Jackson for agreeing to sit on my committee and having the never-ending patience to work with me despite my over-extended schedule.

Finally, I would like to thank my professors over the past two years for their support and my family and friends for their endless love, support, and encouragement.

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Chapter 1: Overview of Literature

Although previously thought to occur only during development, more recent research indicates that neurogenesis continues throughout the lifespan. One of the primary regions where this birth of new neurons occurs is the hippocampus, which plays an extensive role in learning and memory (van Praag et al., 2002). In an effort to further understand this relationship, investigators have examined how various experimental interventions and tasks designed to target the hippocampus might affect adult hippocampal neurogenesis in rats. Unfortunately, this research has not been consistently conducted across genders or strains. Despite this general interest in changes in neurogenesis following various experimental interventions, there is a dearth of research comparing basal levels of neurogenesis. Differences in the basal levels of neurogenesis between strains and genders in rats could change the way results from experimental manipulations are interpreted, compared, and applied. For the purposes of the current proposed study, the primary focus will be on the basal levels of neurogenesis in male and female Sprague-Dawley and Long-Evans rats.

Neurogenesis in Rats

Neurogenesis is the process by which new neurons are formed and incorporated into the existing brain circuitry. There are four stages of neurogenesis: cell proliferation, the division of cells; differentiation, the commitment to a neuronal phenotype; maturation, the survival through a critical period of apoptotic cell death; and survival, maturation of surviving cells into functional neurons (Figure 1; Christie et al., 2011). Evidence has indicated that this process continues in the dentate gyrus, a critical sub-region of the adult hippocampus involved in the formation of new memories, the ventricular zone, and the olfactory system (Leuner, Gould, & Shors, 2002; Gage et al., 1995). In fact, adult neurogenesis has been conclusively demonstrated

to occur specifically in the subventricular zone around the anterior lateral ventricles and the innermost part of the granule cell layer of the dentate gyrus, known as the subgranular zone (Fontana, Nácher, Soriano, & Antonio del Río, 2006). In the rodent brain, pyramidal neurons in the entorhinal cortex and hippocampus are known to be generated prenatally. Granule cells in the dentate gyrus, however, are mainly born postnatally (Fontana et al., 2006). Forty years of research has demonstrated that neurons born in adulthood are assimilated into the granule cell layer, achieve biochemical and morphological characteristics of neurons, develop synapses and dendrites, extend axons into the CA3 region of the hippocampus, and generate action potentials (Figure 2; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002).

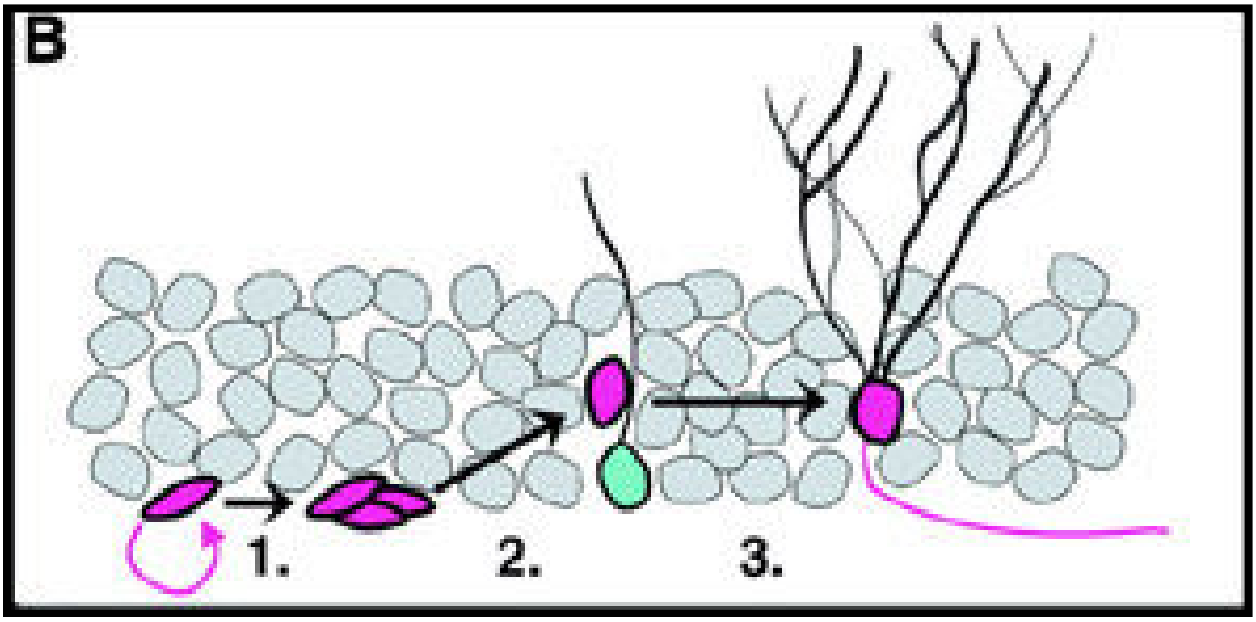

Figure 1: The Four Stages of Neurogenesis

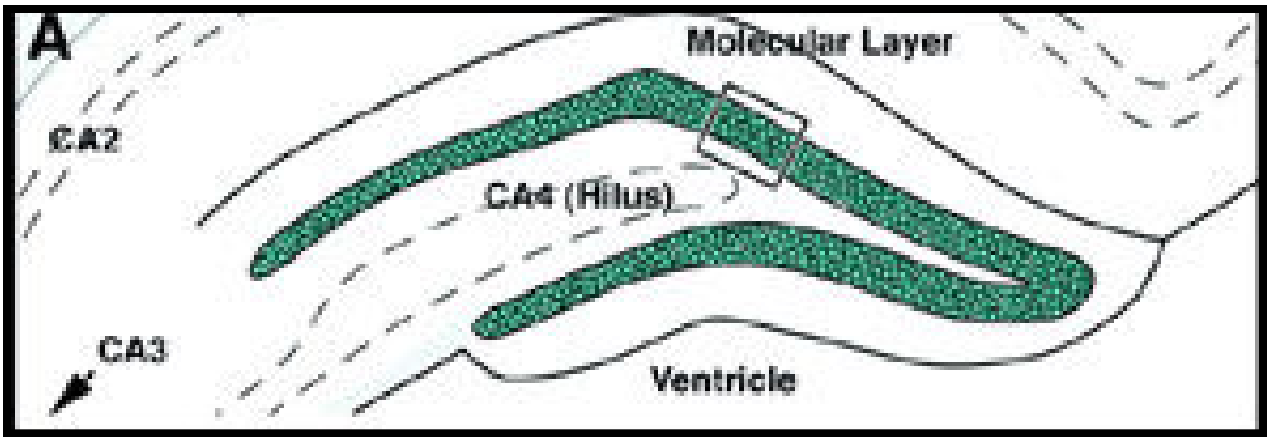

Figure 2: The Granular Cell Layer

It has been demonstrated that approximately 9,000 new cells are produced every day in the adult rat hippocampus, and most of these differentiate into new neurons (Shors et al., 2002). The precise role that new neurons play in learning, however, has yet to be discovered. Some researchers have suggested that new neurons increase learning simply by providing more cells to be integrated into existing functioning (Leuner et al., 2002). Others argue that certain unique properties of adult born neurons, such as a lower induction threshold for long-term potentiation, which is the strengthening of a synapse over time due to use, and insensitivity to gamma-Aminobutyric acid (GABA), a neurotransmitter mainly responsible for the inhibition of synaptic activity in the brain, may qualify new neurons for functions that mature cells are unfit to take on. By requiring less use for long-term potentiation and being insensitive to the effects of GABA, adult born neurons are particularly useful for processing the new associations formed while learning (Leuner et al., 2002). Furthermore, it is possible that the new neurons may be related to the temporary storage of information since it is believed that the hippocampus plays a time-limited role in memory storage. However, evidence that these new neurons survive longer than the time needed for time-sensitive memory storage suggests that they are being integrated into other hippocampal processes as well (Leuner et al., 2002). Moreover, an increase in neurogenesis is found in hippocampus-dependent learning but not hippocampus-independent learning,

therefore an animal must be engaged in a task for which the hippocampus is essential in order for learning to have an enhancing effect on neurogenesis in the region (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Ultimately, neurogenesis has been shown to be differentially affected by various experimental manipulations and conditions, including age, stress, exercise, learning, seizures, and environmental enrichment (Snyder, Kee, & Wojtowicz, 2001; Kempermann, Kuhn, & Gage, 1997; Abrous, Koehl, & Le Moal, 2005).

Detecting neurogenesis. There are four phases in the cycle of a cell during which the new cell can be detected: gap 1 (G_1), which represents growth; gap 2 (G_2) which represents preparation for cell division; synthesis (S), which represents DNA replication; and mitosis (M), which represents cell division (Christie et al., 2011). The cell cycle has been estimated to be about 24 hours in three-month-old rats. Evidence that cells are still in the cell cycle three days after initial divisions indicates that the progeny continue to divide during the first week after birth. The majority of newborn cells in the dentate gyrus are at least partially differentiated within three and seven days of birth. About 60% of the newborn cells die within one week of their generation when they do not terminally differentiate. The neurons generated in the dentate gyrus integrate into the preexisting structure approximately four to eight weeks after generation and reach a mature morphology four months after birth (Abrous et al., 2005).

One of the main methods of detecting neurogenesis in brain tissue is the use of 5-Bromo-2-deosyuridine (BrdU). The popularity of this method is unsurprising due to its specificity to dividing cells. BrdU is also particularly useful in tracing cell lineage and survival because of its long-term retention in divided cells and passage to daughter cells (Kee, Sivalingam, Boonstra, & Wojtowicz, 2002). While BrdU is a principal marker for new cells in brain tissue, it does have several potential drawbacks. First, it primarily detects cells that are in the synthesis phase of the

cell cycle. This only represents one fourth of the newly generated cells within the brain. It has also been suggested that BrdU may not only be labelling newly generated cells, but also cells undergoing DNA repair. Moreover, BrdU requires the denaturing of DNA and an extra injection while the animal is still alive, and the timing of such injections can be difficult (Christie et al., 2011). Furthermore, evidence shows that the blood brain barrier may prevent BrdU from penetrating brain tissue, particularly in older animals (Kee et al., 2002). BrdU has been shown to harm cells at high doses as well (Christie et al., 2011). Some data even indicate the possibility that BrdU produces mutated cells (Kee et al., 2002).

Ki67, on the other hand, is an endogenous marker that does not have any known adverse effects on living cells (Kee et al, 2002). Unlike BrdU, however, Ki67 can detect a cell in all four active phases of the cell cycle (Figure 3; Christie et al., 2011). Because Ki67 is present in all proliferating cells, it has become known as an excellent marker for determining the growth fraction of a given cell population. The functional role of Ki67 during cell proliferation remains unknown, but Ki67 expression and cell proliferation have been found to be closely linked to one another (Scholzen & Gerdes, 2000). While it is possible that Ki67 cannot detect cells in early G1, it has still been shown to detect 50% more cells than the BrdU method. It also eliminates the need for the extra handling that is required for BrdU injections (Kee et al., 2002).

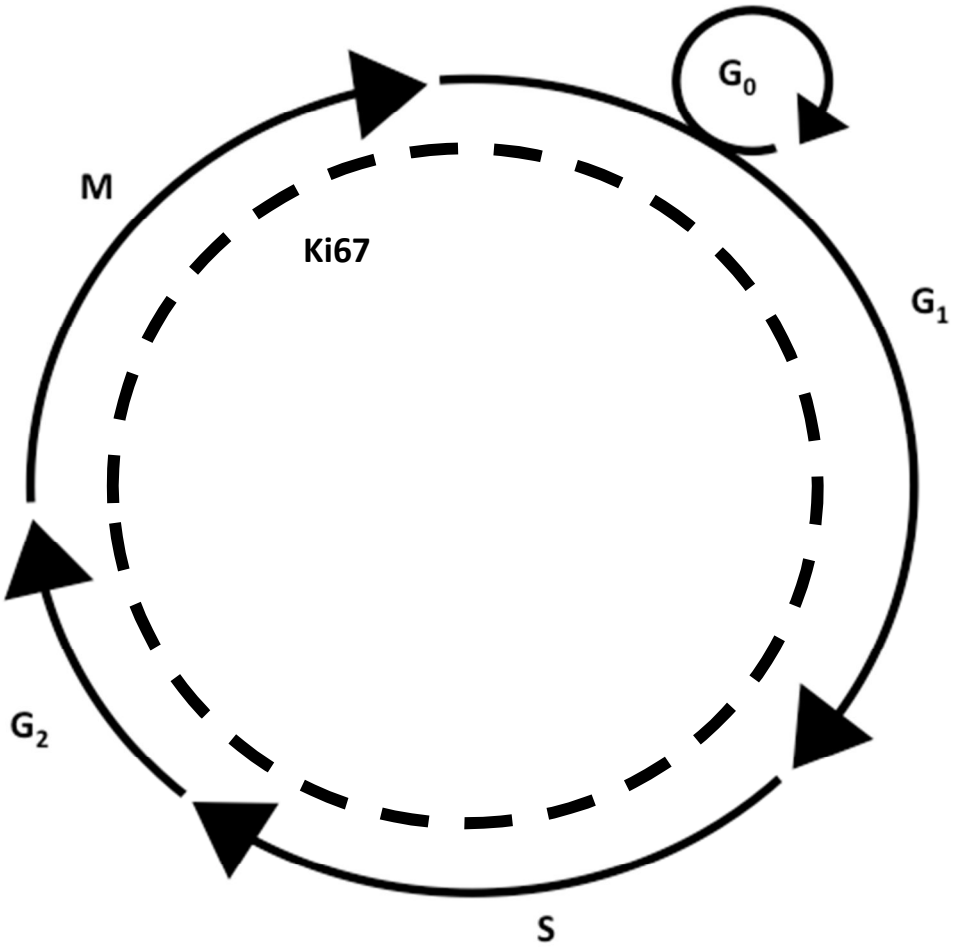

Figure 3: Ki67 Detection

Neurogenesis across the Lifespan

The first granule cells in the rat dentate gyrus appear at embryonic days 14-20, which is equivalent to the second trimester in humans. Between 15 and 20% of all granule cells are generated prenatally, with the remaining majority of cells forming between birth and postnatal day 18. However, the remaining 5-10% of cells are generated throughout adulthood (Amrein et al., 2011). Adulthood in rats begins at sexual maturity, which is typically around two to three months of age, and can be divided into three stages: adult (from sexual maturity until about ten months), middle-aged (about ten months to 20 months), and senescent rats (20 months and older; Abrous et al., 2005). Middle-aged rats have been shown to experience a drastic decline in

neurogenesis by 12 months of age. Over a two-month follow-up period after BrdU injections, researchers found a maximum of around 10,000 BrdU-positive cells in the dentate gyrus juvenile rats but only around 1,000 BrdU-positive cells in the dentate gyrus of middle-aged rats. Despite this reduction in proliferation, only a small decrease in neuronal differentiation was found, and the development of the remaining cells was found to proceed normally (McDonald & Wojtowicz, 2005).

As a rat ages the hippocampus undergoes several expected degenerative changes. These include neuronal cell loss and reduced synaptic density (Kuhn, Dickinson-Anson, & Gage, 1996). However, not all hippocampal changes are of a degenerative nature. Elevated postsynaptic efficacy of granule cell synapses, increased dendritic extent, and increased N-methyl-D-aspartate (NMDA) receptor function, a glutamate receptor important for controlling synaptic plasticity and memory functioning, are all indications of a compensatory increase in hippocampal functioning (Kuhn et al., 1996; Li & Tsien, 2009). Regardless of these compensatory increases in functioning, neurogenesis is reduced to low levels in middle-aged to senescent rats, with stability existing between 12- and 24-month-old animals (Amrein et al., 2011). Proliferation in the dentate gyrus has been shown to decline steadily throughout the aging process rather than an age-related decrease in granule cell volume (Kuhn et al., 1996). An examination of cell proliferation across the lifespan of Fischer 344/Brown-Norway male hybrid rats, for example, showed a statistically significant downward trend of cell proliferation in the dentate gyrus with age (Church et al., 2014). Some debate as to whether this reduced level of neurogenesis is due to a decrease in the number of progenitor cells or an increase in quiescence (i.e., the state of the cell when it is not dividing) of the progenitor cells (Amrein et al., 2011).

Research indicates that neurogenesis levels can be restored as the rat ages. Middle-aged retired breeders exposed to sexual experience have been shown to exhibit levels of cell proliferation higher than naïve controls and comparable to those found in young male rats. Such continuous exposure to sexual experience was also found to restore object recognition in middle-aged rats to the same levels as young adult levels. However, this improvement was not present in animals that spent the same amount of time away from a sexually receptive female as with one (Glasper & Gould, 2013). Learning continues to increase neurogenesis in aged rats, and with that the density of granule cells in the dentate gyrus increases during adulthood and is constant during the aging process (Leuner et al., 2002; Kuhn et al., 1996). Moreover, higher levels of newly generated cells as compared to cell death suggests the possibility that neurogenesis may be accompanied by continuous growth in the granule cell populations. Because of this system of continuous growth and death, it is possible that an animal may have a different set of neurons at the onset of adulthood as compared to the later stages in its life (Biebl, Cooper, Winkler, & Kuhn, 2000).

Behavioral Strain Differences

Memory tasks. Performance differences on certain learning tasks have been connected to differences in neurogenesis. In associative learning tasks, rats with the greatest behavioral performances also possessed more newborn cells at levels maintained two months after acquisition (Abrous et al., 2005). Initial studies of neurogenesis have shown that the effects of enrichment on hippocampal neurogenesis are different based on the genetic difference of various strains of mice (Abrous et al., 2005). Moreover, strain comparison studies in mice have shown that the strains with the fewest number of new neurons were the strains that performed the worst in learning the Morris water maze task. However, this correlation does not indicate whether or

not the different strains had different basal levels of neurogenesis prior to experimental exposure (Leuner et al., 2002). A similar comparison among rats has also shown that Long-Evans rats appear to perform better on the water maze task than Sprague-Dawley rats (Andrews, 1996). Treatment with methylazoxymethanol acetate, an antimitotic agent that decreases the number of newly generated neurons in the hippocampus, has been shown to have no effect on spatial memory acquisition in the water maze task, suggesting that neurogenesis may not play as big a role in hippocampal-dependent learning as previously thought (Shors et al., 2002). Moreover, some research suggests animals that require more trials to learn a task also retain more of the new neurons in the dentate gyrus (Dalla, Papachristos, Whetstone, & Shors, 2009). Other work focusing on the performance of Long-Evans rats in the water maze has found that the rats that prefer a place strategy over a cue strategy have lower levels of cell proliferation suggesting that lower levels of proliferation are associated with a more efficient hippocampus (Epp & Galea, 2009).

In a water maze comparison between male Wistar and Sprague-Dawley rats, the two strains performed equally well in the hidden platform version of the task. However, when the platform was made visible, Sprague-Dawley rats swam greater distances before finding the platform than did the Wistar rats. Upon completion of the task, brains were examined for differences in neurogenesis and no significant differences were found between control animals and experimental animals. Baseline levels between the two were also assessed and, while there were no significant differences in hippocampal cell proliferation as indicated by Ki67 positive cells, there were significantly more BrdU-positive cells in the Wistar strain than in the Sprague-Dawley strain. Overall, the Sprague-Dawley rats had 42% fewer positive BrdU cells. Because both strains had demonstrated similar baseline levels of cell proliferation, this could potentially

indicate that a higher number of newly formed cells are dying in Sprague-Dawley rats than in Wister rats (Van der Borght, Wallinga, Luiten, Eggen, & Van der Zee, 2005).

Recent research seems to suggest that Long-Evans rats have higher levels of neurogenesis than Sprague-Dawley rats. Using doublecortin (DCX) as a marker of neurogenesis, Epp, Scott, and Galea (2011) were able to show that, although both strains performed similarly in the Morris Water Maze task, only Sprague-Dawley rats showed an increase in neurogenesis in response to spatial training. However, their results also showed that when neither was exposed to the spatial training task, Long-Evans rats had a greater percentage of DCX-labeled cells, possibly indicating higher basal levels of neurogenesis. Moreover, when compared to Fischer-Norway, Dark-Agouti, Fischer 344, Wistar, Sprague-Dawley, and wild rats, Long-Evans rats performed significantly better on the water maze task than did the other included laboratory strains, and at a similar level as the wild rats (Harker & Whishaw, 2002). This result falls in line with previous research using Ki67 as a marker of neurogenesis, which also indicated that when compared to wild rats, Long-Evans rats and Brown Norway rats, Sprague-Dawley rats have significantly lower levels of hippocampal neurogenesis, while Long-Evans rats demonstrate levels comparable to wild rats (Figure 4; Epp, Barker, & Galea, 2009).

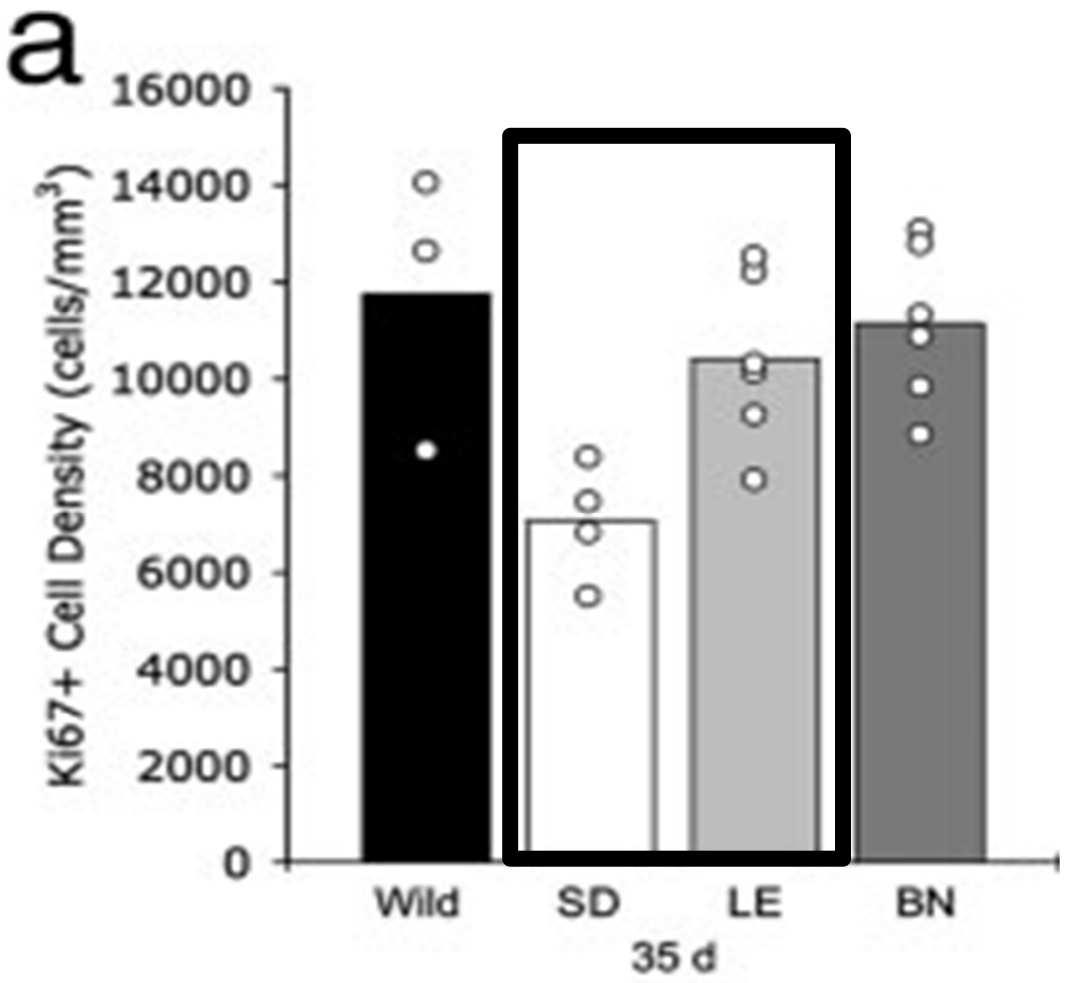

Figure 4: Strain Differences in Neurogenesis

In autoshaping tasks, pigmented strains learn to press the lever more quickly than do albino strains. Differences in handling, however, led to improvements in the performance of albino strains but not in pigmented strains (Andrews, 1996). Other studies suggest that while pigmented strains, such as Long-Evans, are more successful in autoshaping tasks and two-object discrimination tests, albino strains, such as Sprague-Dawley, are more successful in a two-island water maze task. However, wild Long-Evans have also been found to be superior to inbred Sprague-Dawley rats in the water maze task, suggesting adverse effects of inbreeding and albinism on cognitive functioning (Gökçek-Saraç, Wesierska, & Jakubowska-Doğru, 2015). Studies of open-field activity have also reported strain differences between Sprague-Dawley and

Long-Evans rats in response to stress. Specifically, in a behavioral model of depression influenced by stress, Sprague-Dawley females exhibited depression-like behavior but Long-Evans rats did not (Faraday, 2002).

A comparison of the performance on the radial arm maze of commonly used strains of rats found that, compared to Wistar, Long-Evans, and outcrossed Wistar/Sprague-Dawley rats, Sprague-Dawley rats had a higher overall number of errors, choices, and sessions until criterion accuracy was reached, therefore demonstrating a significantly slower rate of learning in Sprague-Dawley rats compared to all other strains. Sprague-Dawley rats also demonstrated a significantly higher number of working memory errors and reference memory errors compared to all other strains. There were no significant differences found in the performances of the other three strains included, suggesting that traits associated with albinism alone are not enough to significantly impair task performance (Gökçek-Saraç et al., 2015).

Homeostatic challenges. Homeostasis is the maintenance of the body's natural physiological states and has been shown to be affected by many different types of challenging factors, such as stress. These challenges have been shown to have an effect on neurogenesis in various different ways. Stress has been found to reduce neurogenesis and impair performance on hippocampal-dependent learning tasks. In some prenatal stress studies, learning abilities have been found to be negatively affected into adulthood after the application of early life stress (Leuner et al., 2002). Furthermore, male Sprague-Dawley rats reared in social isolation demonstrated learning and memory impairment which then improved drastically once regrouped. These behavioral differences were also accompanied by a decrease in neurogenesis, followed by an increase in neurogenesis after social regrouping (Lu et al., 2003). Strain comparisons have also shown that stress increases the acoustic startle response of Sprague-Dawley rats but not of

Long-Evans rats (Faraday, 2002). When exposed to environmental enrichment, Sprague-Dawley rats appear to recover from restraint induced stress faster than Long-Evans rats. When examining corticosterone levels, enriched Sprague-Dawley rats showed levels at 30% below baseline 120 minutes after restraint exposure while enriched Long-Evans rats demonstrated levels at about 50% above baseline (Konkle, Kentner, Baker, Stewart, & Bielajew, 2010).

Social stress has also been demonstrated to have an effect on neurogenesis. When exposed to social competition, dominant male Sprague-Dawley rats were found to have levels of cell proliferation measuring about 35-50% higher than the subordinate animals (Hoshaw, Evans, Mueller, Valentino, & Lucki, 2006). Reductions in hippocampal neurogenesis have been observed in animals raised in social isolation as compared to those raised in group settings (Abrous et al., 2005). Furthermore, social instability stress in adolescence leads to a deficit in performance on hippocampal-dependent tests in adulthood suggesting that stress disrupts the development of the hippocampus. In the same study, researchers found an initial increase in proliferation based on Ki67 immunoreactivity in the adolescents subjected to stress when compared to control animals. This difference did not last through adulthood (McCormick et al., 2012). When rats were prenatally stressed, however, cell proliferation was reduced from adolescence to old age at a more accelerated rate when compared to the typical age-related decline of cell proliferation. This was also linked to an alteration in learning and memory performance such that the typically seen learning-induced increase in cell proliferation was not observed in prenatally stressed rats (Abrous et al., 2005). Forced swim tests of stress have been found to have differing effects on typical behaviors based on strain. Overall, Sprague-Dawley rats have been found to be more active than Long-Evans rats when swimming. Long-Evans rats,

however, exhibited more frequent grooming behaviors than Sprague-Dawley rats (Konkle, et al., 2010).

A number of drugs of abuse, including alcohol, nicotine, and opiates, have also been linked to decreased neurogenesis in rats (Leuner et al., 2002). Some differences may also occur in the response of albino and pigmented strains to various drug manipulations; however, albinism alone does not appear to affect response to particular drugs (Andrews, 1996). Separate studies have confirmed that a four-day chronic binge exposure to alcohol decreases cell proliferation in both Sprague-Dawley and Long-Evans animals (Nixon & Crews, 2002; Leasure & Nixon, 2010).

Species-typical behavior. Several types of natural behavior patterns have been found to influence neurogenesis. These include experiencing maternal care, aggressive behaviors, and social interactions among others (Amrein et al., 2009). One area of particular focus to some researchers is locomotor activity. Research has shown that albino rat strains such as Sprague-Dawley typically show lower levels of locomotor activity than pigmented rat strains such as Long-Evans. This is made particularly evident when comparing Sprague-Dawley and Long-Evans performance in an open-field environment. While Long-Evans rats spent more time in the open-field actively walking, squatting, and rearing, Sprague-Dawley rats spent more time passively sniffing and body grooming. Moreover, Long-Evans rats displayed an average of 20.6 behavioral transitions per minute while Sprague-Dawley rats only displayed an average of 16.3 behavioral transitions per minute. Thus, Long-Evans rats showed a higher transition rate than Sprague-Dawley rats overall (van Lier, Drinkenburg, & Coenen, 2003).

Strain differences appear in the normal aging process of the rat as well. Some strains show more physical deterioration with age than others. Moreover, certain strains are more prone to ailments such as cataracts, cancers, arthritis, and other physical changes (Andrews, 1996). It

has been suggested that certain physical attributes associated with specific strains may have an effect on data obtained in behavioral tasks. For example, albino rats such as Sprague-Dawley rats may perform vastly differently in visual discrimination tasks than pigmented animals such as Long-Evans due to poor eyesight (Gökçek-Saraç, Wesierska, & Jakibowska-Doğru, 2015). In a comparison of several strains, visual acuity in pigmented strains (e.g. Fischer-Norway, Long-Evans, wild rats, and Dark Agouti) was superior to that in albino strains (e.g. Fischer 344, Wistar, and Sprague-Dawley; Harker & Whishaw, 2002). Albino strains are known to also show significant deterioration in visual acuity with age, and it is possible that bright light causes more damage to the albino rat's retina than to the pigmented rat's retina (Andrews, 1996).

Movement comparisons between pigmented and albino strains have shown that albino rats carry more weight on their rear limbs than pigmented rats. Differences have been found when comparing the way Long-Evans and Sprague-Dawley rats reach for a food pellet. Extensor and flexor movements in Sprague-Dawley rats were found to be reduced when compared to those of Long-Evans rats. Sprague-Dawley rats also appeared to hold their paws more laterally than Long-Evans rats, whose digits extend along the midline, when raising their paws from the floor. When advancing the paw toward the food pellet, the Long-Evans rats extend it above and beyond the food pellet whereas the Sprague-Dawley rats place the paw on the food in a sideways movement. The Long-Evans rats were also found to grasp the food fully within the paw while the Sprague-Dawley rats grasped it more distally. The Sprague-Dawley rats also incompletely rotated the paw when bringing the food to the mouth. Digits on the Long-Evans paws were also more fully extended than those on the Sprague-Dawley paws. Finally, Long-Evans grasp duration and time taken to bring the food to the mouth were shorter than the Sprague-Dawley rats (Whishaw, Gorny, Foroud, & Kleim, 2003). These types of species-specific differences

between Long-Evans and Sprague-Dawley rats may ultimately lead to differences in performance on the aforementioned hippocampal-dependent learning tasks, such as the water maze.

Known Gender Differences

Memory tasks. When comparing gender, researchers have found an overall advantage of male rats over female rats in working and reference memory, suggesting that males will likely display higher levels of neurogenesis than females. Specifically, in standard water maze and radial maze protocols, male rats have consistently outperformed female rats. These advantages have existed across different ages, arguing against the idea that sex effects in learning are due to differences in the rate of hippocampal development. Additional strain comparisons have shown an overall advantage to Sprague-Dawley rats. Sprague-Dawley males were found to have a significantly larger advantage over Sprague-Dawley females than Long-Evans males over Long-Evans females (Jonasson, 2005). Further gender analyses with the water maze have found that females swim longer distances and require more time than males to locate the hidden platform. Furthermore, females in proestrous spent significantly more time in the target quadrant during a spatial task than did non-proestrous females, and proestrous females demonstrated a trend to spend more time in the target quadrant than did males. However, this trend was not significant. When examined for differences in neurogenesis, spatial training increased neurogenesis in the hippocampus of the males but not the females, despite findings that better spatial performance was associated with higher levels of new cell activation in females but not in males (Chow, Epp, Lieblich, Barha, & Galea, 2013).

In contrast to previous research, a comparison of the performance of Long-Evans males and females in the water maze found no sex differences, raising the possibility that observed sex

differences may be due to differences in hippocampal development rather than differences in the mature hippocampus (Bucci, Chiba, & Gallagher, 1995). A similar study completed with male and female Sprague-Dawley rats found that sex-related effects in the water maze were limited to the initial response to new task requirements. Once the task was acquired, no sex or estrous cycle-related differences were found in a comparison of performance (Schmidt, Jacobson, & Markus, 2009). While a greater number of studies have found that males outperform females on the water maze task, these differences are reduced when pre-training protocols are added. Pre-training protocols not only provide the animals with the opportunity to learn the task before testing, but also help to reduce anxiety as demonstrated by thigmotactic (i.e. searching for contact with a solid object) behavior. This is especially beneficial for females because they show a greater tendency than males to display thigmotaxis during water maze testing. Age has also been found to be a factor in water maze performance, with adult males appearing to perform worse than females and no apparent differences being found between younger male and female rats (Simpson & Kelly, 2012).

Some studies have suggested that male and female rats use different strategies to locate the platform and that, when available, females will use odor trails from the previous rat to successfully find the platform. This is likely due to the fact that females appear to be more sensitive to odor than are males (Andrews, 1996). It has been suggested that ovarian hormones play a role in strategy use. A study of female Long-Evans rats in a cue-competition version of the water maze task found that overall, females preferred a cue-based strategy, but proestrous rats more often chose a place strategy when compared to non-proestrous rats. Interestingly, the rats that utilized a place strategy took longer to reach the platform and demonstrated significantly

more proliferating cells in the dentate gyrus. Males, on the other hand, have been found to choose place strategy and cue-based strategy in a 1:1 ratio (Rummel, Epp, & Galea, 2010).

In autoshaping tasks, male rats have demonstrated a faster ability to autoshape than female rats overall. However, females appear to extinguish behavior more quickly than males when reinforcement has been removed. Females also show a quicker ability to reverse a task than males. This may suggest a sex difference in perseverative tendencies in which males are more likely to perseverate on a particular behavior than are females (Andews, 1996). Males have also displayed significant improvements in performance over females in the radial arm maze. In the novel object recognition task, however, females have displayed better memory for novel objects compared to males. Females have shown significant discrimination between novel and familiar objects for up to a three hour inter trial interval while males have only demonstrated significant discrimination for up to one hour. Females have also out-performed males in operant conditioning tasks by learning to escape sooner than males do in avoidance tasks, but males tend to outperform females in response to fear conditioning by displaying more conditioned freezing behavior than females. The higher levels of fear-response freezing behaviors found in males was also found to be accompanied by significantly greater hippocampal-dependent excitatory postsynaptic potential and long-term potentiation, indicating greater hippocampal plasticity (Simpson & Kelly, 2012).

When training animals with trace eyeblink conditioning in order to determine gender differences in trace memory retention and neurogenesis, researchers found that female rats learned to time the conditioned response faster than male rats did and that more females than males reached the criterion level of responding during training (Dalla et al., 2009). This has been replicated in several studies, indicating that females are better at anticipating the unconditioned

stimulus and therefore emit a more well-timed eyeblink response (Simpson & Kelly, 2012). As a result of the training, twice as many new cells survived in the female than in the male hippocampus when compared to animals that were not trained. However, no difference existed between trained males and trained females. The results therefore appear to suggest that fewer new cells are surviving in naïve females than in males (Dalla et al., 2009).

Homeostatic challenges. Stress has also been shown to have differing effects on neurogenesis in males and females. After 12 days of restraint stress exposure, male Wistar rats had significantly less cell proliferation than did female Wister rats. Female rats also showed a reduction in cell survival after exposure to chronic stress whereas males did not. Interestingly, no differences in basal levels of cell proliferation were found between males and females, and males showed lower basal levels of cell survival than females (Hillerer, Neumann, Couillard-Despres, Aigner, & Slattery, 2013). Similarly, exposure to the odor of a predator has been linked to a decrease in cell proliferation in male but not female rats. This difference was found at two hours, 24 hours, and one week after exposure. However, this effect was transient and no longer present at three weeks after exposure (Abrous et al., 2005). In female rats, stress has been shown to significantly increase cell proliferation in the hippocampus regardless of reproductive state (Pawluski, van den Hove, Rayen, Prickaerts, & Steinbusch, 2011).

Chronic stress exposure has also been found to differentially affect individually housed males and females. In a study using Wistar rats, isolated males showed a decrease in BrdU-labelling in response to chronic stress while group housed stressed males did not. Isolated females, on the other hand, showed an increase in BrdU labelling after chronic stress exposure, and higher number of BrdU-positive cells than socially housed stressed females. Furthermore, it was found that the decrease in BrdU-positive cells in males was likely a result of a decrease in

cell proliferation, whereas the increase in BrdU-positive cells in females was more likely the result of changes in cell survival, not increased cell proliferation (Westenbroek, Den Boer, Veenhuis, & Ter Horst, 2004).

Sex differences in dendritic spine density as a response to stress have been reported as well. Stressed males were found to have a greater density of spines when compared to unstressed males and females. Changes were also found in the female hippocampus, however these changes were dependent on the stage of estrus the female was in at the time. Females that were stressed during estrus and perfused during diestrus 1 had a greater dendritic spine density than did unstressed females also perfused during diestrus 1. Females that were stressed during diestrus 2 and perfused during proestrus had a reduced density of spines than did unstressed females also perfused during proestrus. However, unstressed females in proestrus had a greater dendritic spine density than did unstressed females in diestrus 1 and unstressed males (Shors, Chua, & Falduto, 2001).

It is well known that female and male rats vary greatly in their responses to a wide range of drug treatments. This difference does not necessarily reflect a difference in cognitive ability, considering that baseline levels of anxiety and hormone levels can both interact with an administered drug (Andrews, 1996). Overall, female rats have a greater sensitivity to the rewarding effects of drugs of abuse compared to males. Female rats more readily acquire cocaine self-administration than do males and display a higher response to the progressive ration schedule, indicating greater incentive motivation in females than in males. Self-administration for cocaine was found to be highest during estrous. Female rats also more readily achieve acquisition criteria for methamphetamine self-administration and have an enhanced behavioral response to amphetamine administration than do males. Furthermore, female rats have been

found to more readily self-administer opioid drugs (such as morphine and heroin), nicotine, and alcohol in free choice paradigms. Females also display an increase in alcohol consumption over five weeks while males do not significantly increase intake over time (Simpson & Kelly, 2012). In a four-day chronic binge model, exposure to alcohol has been found to decrease cell proliferation in both females and males (Leasure & Nixon, 2010; Nixon & Crews, 2002).

Species-typical behavior. Research comparing gender differences has been more difficult to complete due to the hormonal changes females experience during the estrous cycle. Despite this, ovarian hormones have been found to affect the number of hippocampal synapses, the strength of hippocampal long-term potentiation, and to modulate hippocampal-dependent learning. Therefore, ovarian hormones have an important role in the regulation of hippocampal neurogenesis (Hillerer et al., 2013). Gender differences in both associative learning and neurogenesis have been found to be enhanced when estrogen levels are high (Dalla et al., 2009). Such differences are not always present, however, and research suggests that while estradiol exposure initially enhances cell proliferation, it eventually suppresses neurogenesis in the dentate gyrus (Abrous et al., 2005; Ormerod, Lee, & Galea, 2002). These dose-dependent estradiol-induced changes in cell proliferation could potentially influence hippocampus-dependent behavior and suggest that there may be an optimal level of neuron production and survival for optimal performance (Ormerod et al., 2002). Research has shown that a relative stability of hippocampal place cell firing characteristics exists across the estrous cycle, with the exception of mean firing rate, which is lowest during proestrus. Place cells are sensitive to location, speed, direction, and turning angle of the animal and are therefore pertinent to hippocampal learning and memory tasks. Stability of these cells across the estrus cycle suggests that there are likely

other factors to consider when sex differences are observed in behavioral tasks aside from ovarian hormones (Tropp, Figueiredo, & Markus, 2005).

When ovariectomised, researchers have found that exposure to various oestrogens (17β-E_2, 17α-E_2, E_1, and oestradiol benzoate) at different doses can lead to an increase in cell proliferation in the dentate gyrus of female rats, and that very low doses of such oestrogens can be as effective at increasing neurogenesis as high doses (Barha, Lieblich, & Galea, 2009). In contrast, castration of male rats was found to have no significant effect on hippocampal cell proliferation, but instead caused a decrease in cell survival. Furthermore, high levels of testosterone were found to increase hippocampal neurogenesis, and treatment with metabolite dihydrotestosterone, but not estradiol, was found to increase hippocampal neurogenesis. Put together, these findings indicate that androgens positively affect new cell survival in the dentate gyrus (Spritzer & Galea, 2007). These findings have been corroborated by Zhang, Konkle, Zup, and McCarthy. When females were treated with androgens, the number of BrdU positive cells found in the dentate gyrus was at levels comparable to those seen in males. However, treatment of males and females with both estrogens and androgens did not exhibit an enhanced effect of the hormones, suggesting either the existence of a ceiling effect or an antagonism between the two when combined (2009).

Findings show that females in the proestrus stage have higher rates of cell proliferation than do males. Under standard laboratory conditions, however, a greater number of these cells were seen to degenerate in females than in males. Furthermore, overall numbers of cells in the dentate gyrus were examined and no sex differences were observed (Tanapat, Hastings, Reeves, & Gould, 1999). Other research has corroborated findings suggesting that females have higher levels of both cell proliferation and cell death than do males, ultimately resulting in a lack of

gender differences in neurogenesis (Abrous, 2005). In neonatal male and female Sprague-Dawley rats, exogenous oestradiol exposure was found to promote cell proliferation and survival in females, but not in males. However, cell proliferation in males was found to be reduced when endogenous oestradiol is antagonized (Bowers, Waddell, & McCarthy, 2010).

Differences in reproductive experience from sex and motherhood have also been found to influence neurogenesis (Amrein et al., 2009). Pregnant females, for example, have decreased depressive-like behavior, lower corticosterone levels, and increased anxiety-like behaviors during late pregnancy as compared to virgin females, all of which have been studied in connection to neurogenesis (Pawluski et al., 2011). Furthermore, maternal deprivation has been studied for differential effects on males and females such that maternal deprivation led to an increase in neurogenesis in male rats and a decrease in neurogenesis in female rats, as well as sex differences related to neuronal differentiation. Baseline sex differences in the juvenile Wistar rats used for this study also indicated that control males generally had a higher proliferation rate and increased survival of cells than control females, while control females demonstrated more astrocytes than control males (Oomen et al., 2009).

Sex differences have also been found in tests of depression and anxiety-like behaviors. In the elevated plus maze, females are overall more active than males and spend a greater percent of their time in an open arm, therefore indicating that male rats may have a more anxious-like profile on the elevated plus maze than do females. In the open field test, females of the Sprague-Dawley and Long-Evans strains displayed both greater distance moved and rearing behavior than did males, again suggesting lower levels of anxiety. In studies of learned helplessness behavior, researchers have found that most males previously exposed to uncontrollable stress did not learn to escape when placed in a situation in which escape was possible. Most females, however, did

learn to escape regardless of previous exposure, demonstrating that male rats are more prone to learned helplessness behaviors. Finally, in the forced swim test, females have shown an overall lower duration of immobility and greater time spent climbing or swimming than male rats, suggesting that males have a greater propensity to display depression behaviors (Simpson & Kelly, 2012).

Female and male rats have shown differences in patterns of eating. Male rats, for example, typically eat more, weigh more, and grow faster than female rats. Both genders, however, seem to respond similarly to food deprivation (Andrews, 1996). Stress has also been found to have differential effects on eating behaviors based on gender with males being more sensitive to the effects of stress than are females (Faraday, 2002). In terms of the development of the brain, subtle sex differences have been found in the volume and the shape of cells in the hippocampus. The granule cells in the dentate gyrus of males have also been shown to develop faster than those in females, suggesting the presence of a sex difference in cell proliferation (Bowers et al., 2010).

Current Study

The current study seeks to bridge the gap in the known literature concerning neurogenesis, gender, and strain. In particular, the goal is to determine if there are significant differences in levels of cell proliferation between two commonly used strains of rats, Long-Evans and Sprague-Dawley, and both genders. Specifically, it is predicted that Long-Evans rats will display higher levels of cell-proliferation than Sprague-Dawley rats. It is also expected that male rats will display higher levels of cell-proliferation than female rats. Finally, an interaction between the two variables is expected such that male Long-Evans rats will display the highest

levels of cell-proliferation and female Sprague-Dawley rats will display the lowest levels of cell proliferation.

Chapter 2: Method

Subjects

Adult (~6 months) male and female Sprague-Dawley (*N*=16) and Long-Evans (*N*=16) rats obtained from Charles River Laboratories (Raleigh, NC) were used. Animals were kept in a temperature and humidity controlled room on a 12:12 hour light-dark cycle with light onset at 7:00 am, and given access to food and water *ad libitum*. At the time of tissue collection, animals were naïve to any experimental manipulations so as to investigate baseline levels of neurogenesis. All procedures were approved by the Radford University Institutional Animal Care and Use Committee (IACUC).

Tissue Collection

Rats were overdosed with sodium pentobarbital (Fatal Plus, Vortech, Pharmaceuticals, 390 mg/ml) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer immediately following 0.1M phosphate buffered saline flush (PBS). Whole brains were extracted and then post-fixed in PFA for 24 hours at 4°C, at which point brains were transferred to PBS and stored until use. Coronal sections of 50 μm thickness were obtained using a vibrating microtome (OTS-4500, Electron Microscopy Sciences, Hatfield, PA) and collected in a 1:8 series through the entire rostral to caudal extent of the limbic cortices. Sections were then kept in cryoprotectant solution at -20°C until use.

Immunohistochemistry

A well of brain tissue from each animal was thoroughly washed in tris-buffered saline (TBS) and then incubated in 0.6% H_2O_2 for 30 minutes. Following the incubation, the tissue was again washed in TBS before a standard sodium citrate incubation at 65°C to aid in antigen retrieval. A third round of TBS wash was performed before the sections were locked in TBS

containing Triton-X 100 and appropriate normal serum. The tissue was then incubated in primary antibodies (mouse anti-Ki67; Vector Labs, Burlingame, CA, 1:200) at room temperature for four days. Following the four-day period, the sections were again washed in blocking solution, incubated in horse anti-mouse secondary antibodies (Vector Labs, Burlingame, CA, 1:200) and normal serum, and then incubated in Avidin-Biotin Complex (ABC) solution (Vector Labs, Burlingame, CA) prior to peroxidase detection using 3,3, Diaminobenxidine Tetrahydrochloride with cobalt chloride, nickel ammonium sulfate, and hydrogen peroxide (DAB; Figure 5; Polysciences, Inc., Warrington, PA). Finally, sections were washed in TBS, mounted onto glass slides, and given time to dry before undergoing a Cresyl Violet counterstain. The Cresyl Violet counterstain consisted of a series of washes over 12 minutes and 30 seconds as follows: 100% ethanol (EtOH), 95% EtOH, 70% EtOH, 50% EtOH, distilled water, Cresyl Violet stain, distilled water, 50% EtOH, 95% EtOH, 100% EtOH, and Xylenes. Upon removal, slides were immediately cover-slipped and set aside to dry before the quantification process could begin.

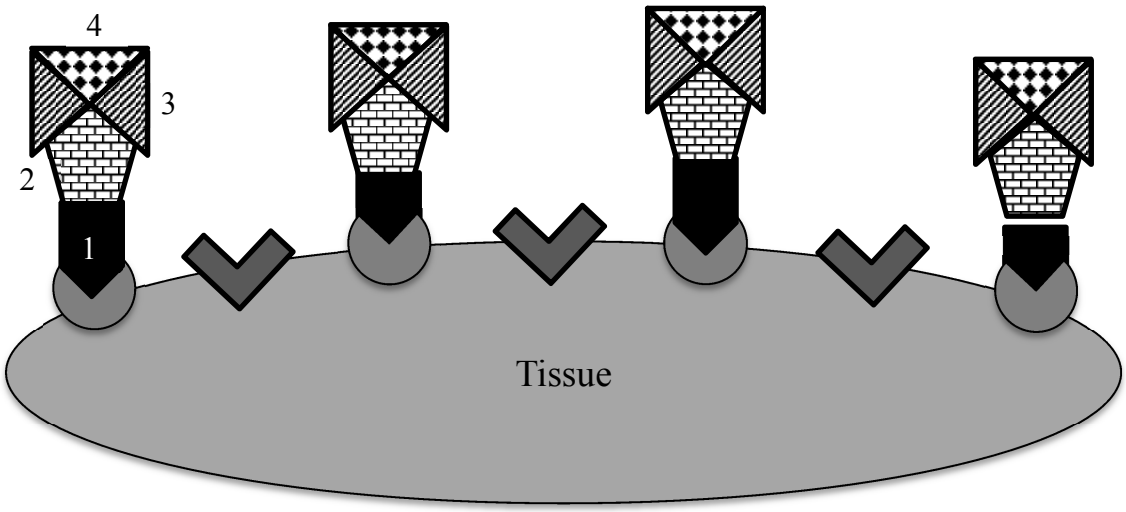

Figure 5: Immunohistochemical Process

Quantification

Cells expressing Ki67, a marker for cell proliferation, are located within the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Cell clusters were located and individual cells were quantified using a profile-counting methodology on a BX43 Olympus Microscope (Waltham, MA) with a 100x oil immersion objective. Cells from both the inferior and superior blades of the dentate gyrus from one hemisphere of each of the six to eight coronal sections throughout the entire rostral to caudal extent of the hippocampus were counted and recorded. Data will be presented as the mean number of Ki67 positive cells per section of tissue for each animal with the standard error of the measurement included. In order to keep the researchers blind to the treatment conditions, animals were randomly assigned to alternative ID's throughout the entire data collection process (Figure 6).

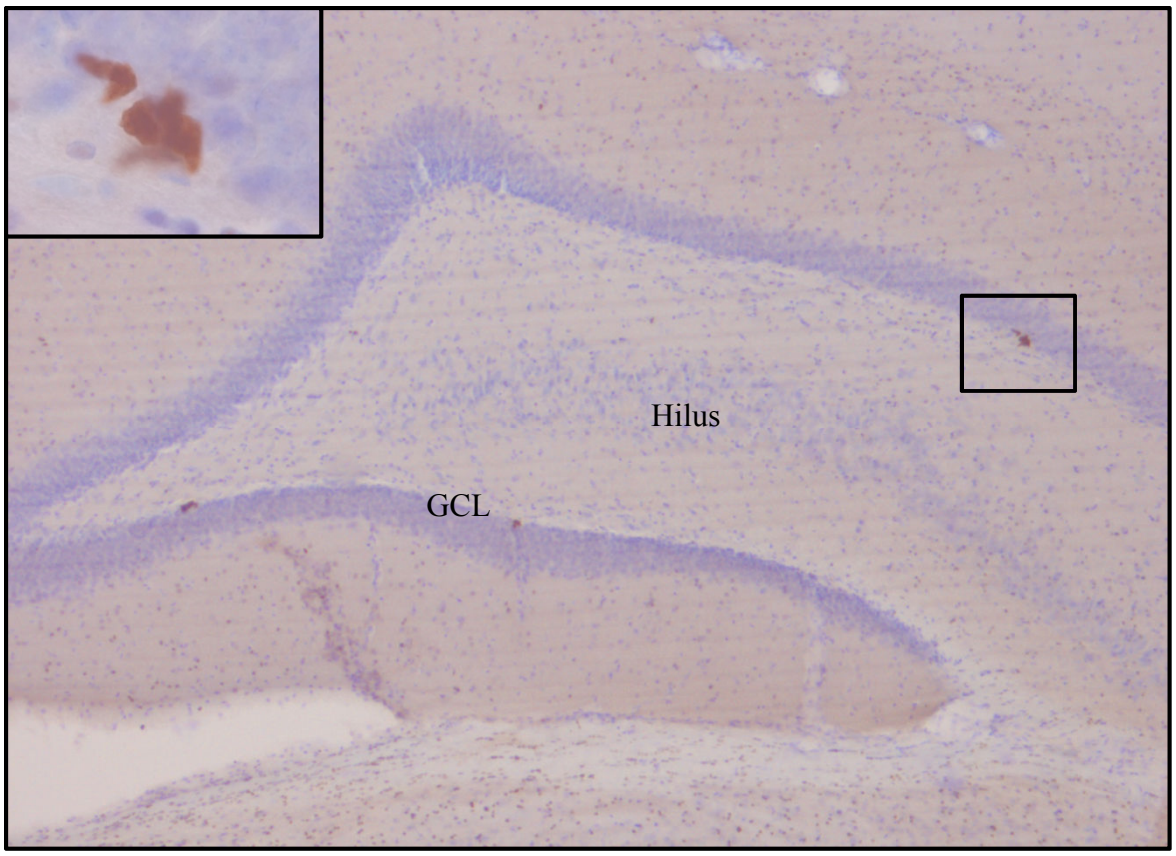

Figure 6: Example of Quantification Process

Chapter 3: Results

A two-way 2 (strain) X 2 (gender) analysis of variance (ANOVA) was conducted in order to determine if there is a main effect of either variable on the number of cells expressing Ki67. It was expected that Long-Evans rats would have higher levels of cell proliferation than Sprague-Dawley rats. However, the ANOVA revealed no significant effects of strain on the number of Ki67-positive cells (p = .161; see Figure 7). Furthermore, it was expected that male rats would have higher levels of proliferation than female rats. Again, the ANOVA determined that gender did not have a significant main effect on the number of Ki67-positive cells (p = .825). Finally, it was predicted that an interaction between the two variables would exist such that male Long-Evans rats would display the highest levels of cell proliferation and female Sprague-Dawley rats would display the lowest levels of cell proliferation. The interaction term of the ANOVA, however, was also shown to be insignificant (p = .624).

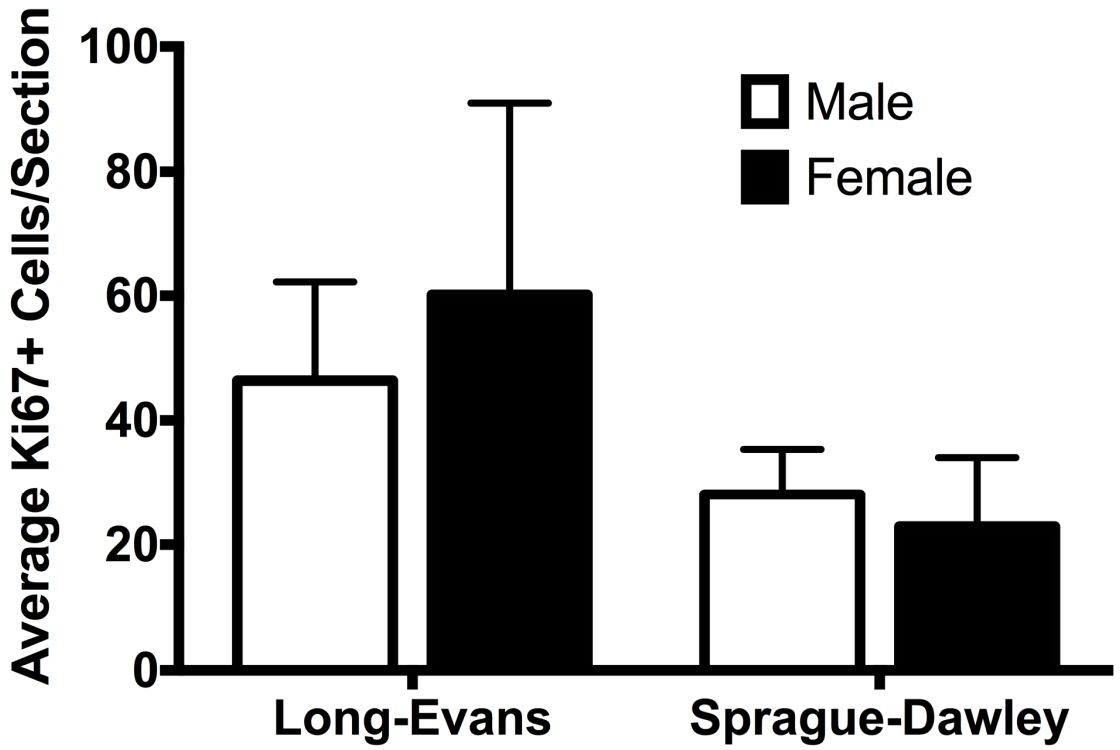

Figure 7: Average Ki67-Positive Cells By Strain and Gender

All animals were naïve to any experimental treatment prior to brain collection and were 6.5 months old on average. Ages were compared in order to look for any potential differences between the groups, and no significant differences were found (p = .080, see Figure 8).

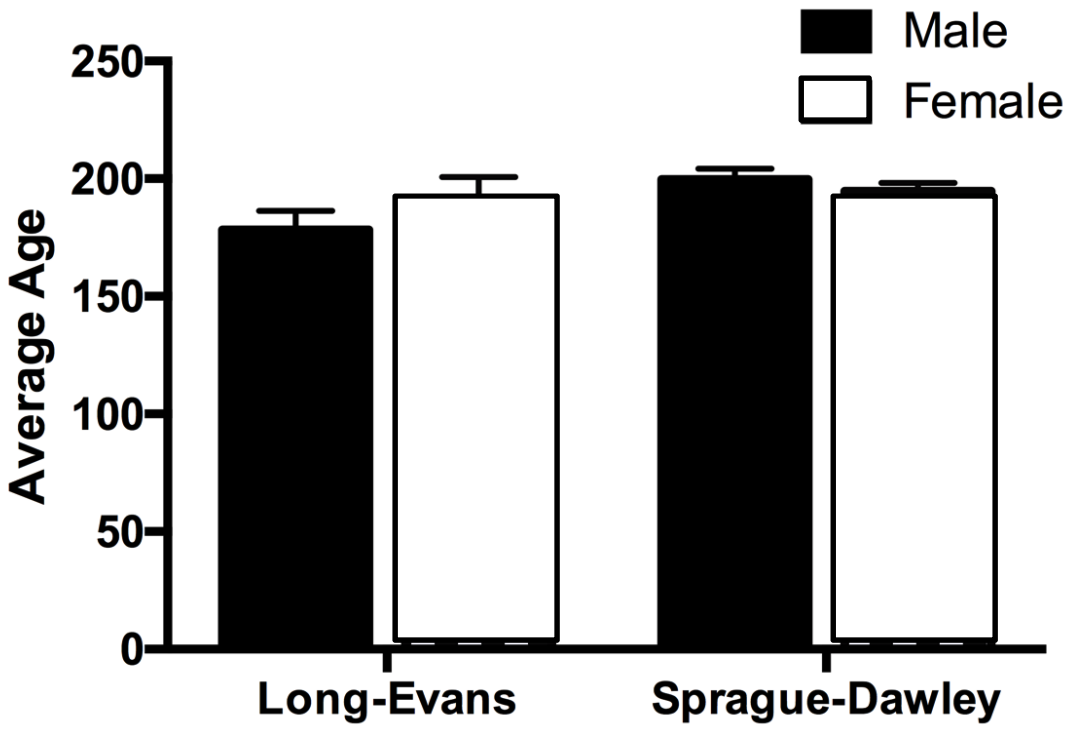

Figure 8: Age Comparison

Furthermore, due to the way the breeding colony at Radford University is managed, three of the male Long-Evans animals were housed individually prior to tissue collection while the rest of the animals were housed in grouped conditions. As previously mentioned, solo housing conditions can cause stress in the animals and therefore reduce neurogenesis (Abrous et al., 2005). In order to determine if this difference had any effect on the level of cell proliferation, an ANOVA was performed including housing condition as one of the variables. Housing condition was not found to have a significant effect on cell proliferation (p = .946; see Figure 9). One interesting result when comparing these animals was the significant difference in body weights. A two-way ANOVA revealed that, unsurprisingly, the male rats (M = 712.89, SD = 109.72)

weighed significantly more than the female rats (M = 397.15, SD = 72.00), $F(1, 20) = 102.42$, $p < .001$. Moreover, the Sprague-Dawley rats (M = 627.42, SD = 209.60) weighed significantly more than the Long-Evans rats (M = 518.05, SD = 152.68), $F(1, 20) = 10.83$, $p = .004$. No significant interaction was observed between gender and rat strain ($p = .193$; see Figure 10).

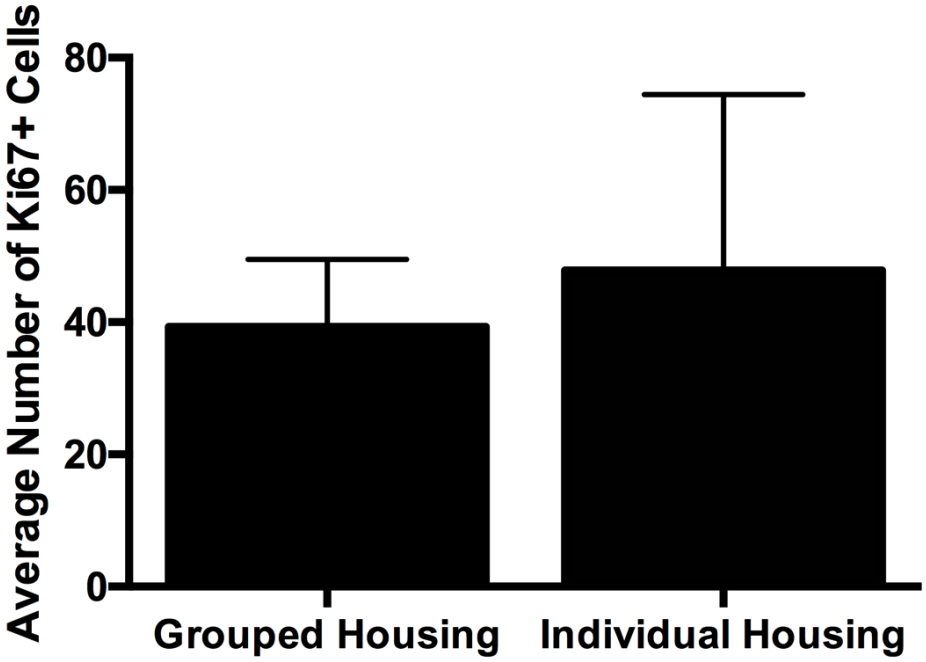

Figure 9: Comparison of Group Housed and Individually Housed Animals

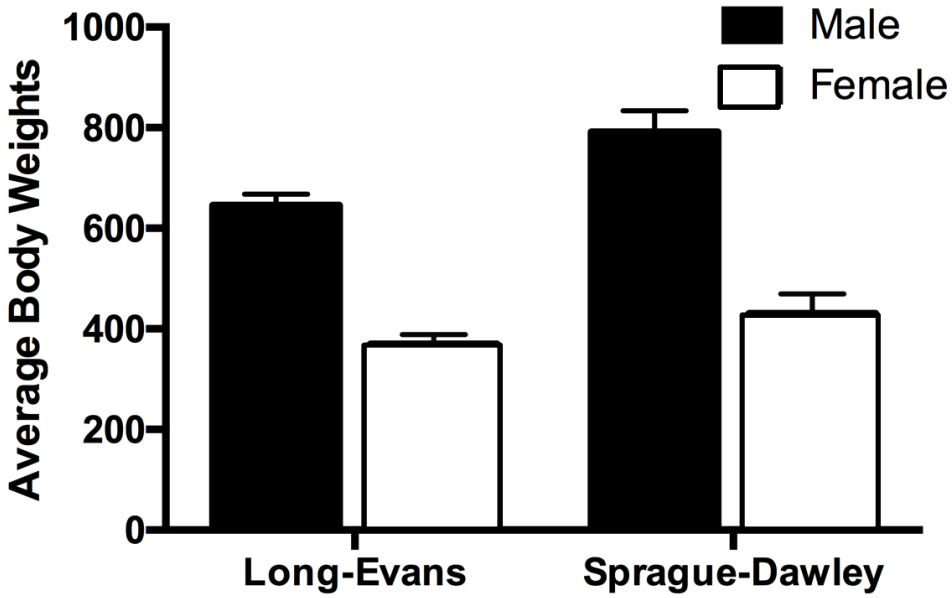

Figure 10: Average Body Weights By Strain and Gender

Chapter 4: Discussion

It was predicted that Long-Evans rats would display higher levels of cell proliferation than Sprague-Dawley rats. It was also predicted that male rats would display higher levels of cell proliferation than female rats. What the results indicate is that there were no significant effects of either variable on the number of Ki67-positive cells counted in the subgranular zone of the hippocampal dentate gyrus. Though this finding is in stark contrast to the hypothesis, several studies have shown similar results. For example, while strain differences have been found between Long-Evans and Sprague-Dawley adolescent rats, these differences in the same study no longer existed beyond young adulthood, about 70 days of age (Epp et al., 2009). However, differences still exist in performance on spatial learning tasks between the two strains well into adulthood. Studies are beginning to show that this might be due to differential activation of neurons between the two strains rather than differences in proliferation (Epp et al., 2011). Finally, findings have indicated that an increased level of proliferation combined with an increase in cell death in females may ultimately result in no fundamental differences between baseline levels of neurogenesis in male and female rats (Abrous, et al., 2005). It is possible that the results seen in the current study are reflecting balanced levels of neurogenesis between males and females because of these natural processes. Therefore, it would be important for future studies to expand on this research using a marker of cell death, such as fluorojade, in combination with Ki67 as a combination of proliferation in order to delve deeper into the natural neurogenesis-regulating processes within the brain.

It is important to note that the rats used in the current study were all adult rats. However, there are a number of differences that occur in neurogenesis throughout the lifespan. For example, middle-aged rats show a drastic decline in neurogenesis by 12-months of age. In a

direct comparison between juvenile rats and middle-aged rats, juvenile rats show levels of neurogenesis at about ten times higher rates than middle-aged rats (McDonald & Wojtowicz, 2005). Furthermore, this age-related decline has been found to be specifically related to a decrease in cell-proliferation (Kuhn et al., 1996). Adult rats, therefore, may not have been the best choice to begin mapping baseline levels of neurogenesis. Rather, a full time course of neurogenesis across the lifespan of rats is a more appropriate way to determine if any rat strain and/or gender differences exist. As such, future studies should be focused on the inclusion of adolescent animals as well as geriatric animals in order to determine if strain and gender differences exist at those age points.

Interestingly, there was a significant difference between the body weights of the Sprague-Dawley rats and the Long-Evans rats such that the Sprague-Dawley rats were significantly heavier than the Long-Evans rats. This could be a cause for concern because it may indicate a difference in exercise levels between the Long-Evans rats and the Sprague-Dawley rats. Exercise has been shown to increase the levels of neurogenesis (Inoue et al., 2015; Lee et al., 2013; Okamoto et al., 2012). Because no significant differences in proliferation were found, body weight and exercise are not likely to be factors in this case. However, this is important to keep in mind for future studies and may indicate a need to monitor and/or yoke food intake. If the Long-Evans rats are indeed consuming smaller amounts of food than the Sprague-Dawley animals, it may be necessary to measure how much the Long-Evans animals are eating and provide only that much food to the Sprague-Dawley animals instead of allowing free access to the food source. Due to the animal housing procedures at Radford University, all animals were housed in similar cages which do not allow for a large amount of exercise. Energy expenditure is therefore

not expected to be different between the groups. However, the addition of exercise as a factor challenging baseline levels of neurogenesis may be important to examine in the future.

Furthermore, as future animal researchers continue to expand their work in the hopes of developing a more inclusive knowledge of female rats and the effects of the estrous cycle, it will be important to compare females in the different stages of estrous to males to look for any potential differences in neurogenesis. While the research in this field is only just growing, studies have begun to show that there is a difference in neurogenesis throughout the stages of the estrous cycle (Farinetti et al., 2015). For example, females in the proestrus stage have higher rates of cell proliferation than they do in other stages of the estrous cycle (Tanapat et al., 1999). For the purposes of the current study, it is not likely that estrous cycle had a significant effect on the results because there is enough natural variability in the estrous cycle between female rats that a majority of researchers in the field consider it to naturally balance. Moving forward, however, it would be beneficial to the field to include the additional step of analyzing which stage of the estrous cycle each female rat is in immediately prior to tissue collection in order to verify that these differences are not significant.

Overall, the current study indicates that there are not significant baseline differences in neurogenesis between Long-Evans male and female rats and Sprague-Dawley male and female rats at approximately 6.5 months old. However, the current selection of animals represents only a small snapshot of the full range of possibilities for comparison. In order to fully investigate this issue, a full lifespan time course must be completed from birth to old age (beyond 12 months of age) as it has already been shown that differences in neurogenesis do occur throughout the lifespan. Furthermore, greater control over basic living conditions, such as housing, food intake, and exercise, may be necessary in order to more precisely pin down any potential differences.

However, as of the current study, any differences found in neurogenesis have been primarily the result of experimental treatment and manipulation, therefore reinforcing the results of previous studies.

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