A TIME COURSE ANALYSIS OF STEM CELL ACTIVITY FOLLOWING BRAIN INJURY IN THE FOOD-STORING BLACK-CAPPED CHICKADEE (*POECILE ATRICAPILLUS)*

A THESIS

Presented to the Department Psychology

California State University, Long Beach

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science in Interdisciplinary Studies

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B.A., 2007, California State University, Long Beach

June 2010

CHAPTER I

INTRODUCTION

Neural stem cell biology is the foundation of neurogenesis, the birth of new neurons, and gliogenesis, the birth of new glia, in developing and adult brains. Once believed to be restricted to periods of early brain development, adult neurogenesis is now known to occur throughout the avian telencephalon (including the hippocampus and song nuclei) as well as the mammalian hippocampus and olfactory bulb (e.g., Gage, 2002; Nottebohm, 1985). Because the hippocampus is important in learning and memory, adult hippocampal neurogenesis in this area may play a role in the extraordinary ability of food-storing birds to remember numerous cache locations.

Remarkably, food-storing birds -- specifically scatter-hoarding birds -- place 1 to 2 seeds in many hundreds of spatially distinct locations or "caches". Memory is forged on a single and often brief visit to a cache location and is retained over many hours to even months (Vander Wall, 1990). For these birds, finding caches is essential to survival during periods when food is otherwise unavailable. Krebs (1990) suggests that the survival value of hippocampal-dependent recall for cache locations may have created an evolved specialization in the brain of these species. In support, hippocampal volume in food-storing birds is larger than that of non-storers (Krebs, Sherry, Healy, Perry, & Vaccarino, 1989; Sherry, Vaccarino, Buckenham, & Herz, 1989).

Seasonal food-storing, a hippocampal-dependent activity, typically begins in the fall in preparation for harsh winter weather. While Smulders, Sasson, and DeVoogd (1995) reported a peak in hippocampal volume in black-capped chickadees (*Poecile atricapillus*) during the fall coinciding with a peak in food-storing behavior, other researchers have found increases in hippocampal volume in the spring (Hoshooley & Sherry, 2007; Mitterling, Law, Gardner, Ramus, & Lee, 2007).

Despite these contrary results, volume increases and decreases may result from a variety of influences. For example, the hippocampus may get larger because of the proliferation of new cells; proliferation is assessed by looking at a very narrow time window of new cell birth typically within 24 hours of mitosis. Volume changes may also be due to the incorporation of cells; this is defined much more broadly and is a measure of the birth, migration, differentiation, survival, and death of new cells assessed over periods greater than 24 hours following mitosis. Therefore, it is possible to have an increase in cell proliferation without an increase in incorporation and vice versa.

Fall peaks in neuronal incorporation into the hippocampus have been observed. For example, looking 7 days post-mitosis, food-storing chickadees have significantly more newly incorporated cells during the fall in the hippocampus and adjacent stem cell rich subventricular zone (SVZ) compared to non-storing dark-eyed juncos (*Junco hyemalis*; Law, Gardner, Allen, & Lee, 2010). Barnea and Nottebohm (1994) also found a significant increase in neuronal incorporation in the hippocampus of chickadees during the fall. As defined above, neuronal incorporation may be due to enhanced neuronal survival, not necessarily an increase in neuronal proliferation. This could

explain why Hoshooley and Sherry (2004) found no seasonal differences in neuronal proliferation as measured at 24 hour survival times in food-storing chickadees.

An increase in cell incorporation is also observed following lesions to the hippocampus in chickadees and juncos during the fall food-storing season (Law, et al., 2010), as well as in semi-domesticated non-storing zebra finches (*Taenopygia guttata*; Lee, Fernando, Peterson, Allen, & Schlinger, 2007). However, chickadees had significantly fewer newly incorporated cells in the injured hippocampus compared to juncos (Law et al). An injury-induced increase in newly born cells is also found in the adjacent SVZ of juncos and finches, but not in chickadees (Law et al.; Lee et al.).

Since chickadees had a higher rate of cell incorporation in the intact hippocampus and SVZ than juncos, these results raise issues concerning stem cell activity following a hippocampal lesion in food-storing birds. Because of the use of 7-day survival times, there are at least two competing explanations of the results. First, it is possible that chickadees simply have a weaker cell proliferation response to injury. Lower proliferation rates could result in fewer newly born cells 7 days later. Second, chickadees could be exhibiting a much more rapid turnover rate -- both birth and death -- of new cells. That is, the process of stem cell division in chickadees may be followed by migration out of the SVZ, incorporation into the hippocampus, and subsequent pruning via cell death much more rapidly. If so, looking at only one time point post mitosis (7 days) would not reveal this. To tease apart these possibilities, this thesis compared both short (proliferation) and long (incorporation) post-mitosis survival times.

To understand these issues more fully, this thesis will summarize what is known about avian food-storing behavior, the avian hippocampus, adult hippocampal neurogenesis, injury-induced neurogenesis, and the role that both seasonal variation and injury-induced cell birth have on the hippocampus of food-storing and non-storing birds.

Avian Food-Storing Behavior

Species face long standing problems that create evolutionary pressure to adapt in order to survive and reproduce. Harsh weather conditions and variability in food supply throughout the year create an adaptive challenge for some species. Solutions to that problem may include migration, torpor, hibernation, loss of body mass, or food-storing (aka food-hoarding; Vander Wall, 1990). The latter has evolved as an optimal solution for several species because of the flexibility of its problem solving approach. While supply of food temporarily exceeds immediate demand, birds in the Passerine order (e.g., *Corvidae* and *Paridae*), cache (or hide) seeds in order to have sustenance during periods when food is otherwise unavailable. Depending on the species, these caches comprise 60-100% of the diet during winter months, thus affording the species the opportunity to remain in its current ecological environment instead of migrating.

Food-storing behavior is triggered by seasonal cues that indicate the most optimal time to cache. Photoperiod, the number of hours of light per day, may be the initial predictive cue triggering food-storing behavior (e.g., Shettleworth, Hampton, & Westwood, 1995). In general, when day length begins to shorten at the autumnal equinox, food-storing birds are likely to begin storing and will continue to do so through the winter solstice. However, other environmental factors may ultimately

determine whether species store food (e.g., ambient temperature and food availability; Vander Wall, 1990).

In order to ensure cache recovery, food-storing species must deter competitors from consuming or contaminating the supply (Vander Wall, 1990). Deterrents include many forms of food manipulation including preparation (e.g., removing husks from walnuts); transportation (with distances ranging from centimeters to kilometers); strategic placement (e.g., holes in trees created by wood-peckers); or concealment (e.g., covering food with various materials; Vander Wall). Many of these methods are used in conjunction with one another. For example, preparing food may make it smaller and easier for the animal to transport it. In this instance, preparing food is not as much of a disguise mechanism as it is transportation. In this sense, transporting food away from a location where it is easily spotted would prevent theft.

Although species may prefer certain types of cache sites (e.g., marsh tits, *Poecile palustris*, prefer to cache seeds in moss covered areas), food-storers rarely use the same site more than once (Vander Wall, 1990). The success of storing activity depends upon the employment of new cache locations in order to avoid discovery by pilferers. Although laboratory experiments have demonstrated site preferences, it is likely that these preferences are due to the unnatural conditions of the experiment itself. Important variables, such as the presence of food competitors, are unaccounted for during these experiments (Shettleworth & Krebs, 1982).

The primary problem for food-storing birds is the challenge of relocating and retrieving caches (Sherry, 2006). Research has demonstrated that olfactory cues are relatively unimportant during cache recovery and, in the wild, recovery rates are much

too accurate (50-99%) to be explained by random search (Vander Wall, 1990). It does not appear that food-storing birds use path integration, fixed paths, or 'snapshots' of a particular environment in order to relocate caches (Kamil, Balda, & Good, 1999). Moreover, birds do not appear to leave marks next to cache locations in order to assist in later recovery. The potential benefits of leaving personal landmarks are quickly erased by a variety of environmental occurrences including wind, rain, and snow cover. Research also indicates that food-storing birds do not employ position rules (e.g., always store 3 feet from the base of a tree). These techniques are more susceptible to pilfering by other species or other birds that can learn a competitor's position rules (Gallistel, 1993).

Instead, spatial memory is used to retrieve stored food (e.g., Balda, 1980; Brodbeck, Burack, & Shettleworth, 1992; Kamil & Balda, 1985; Sherry, Krebs, & Cowie, 1981; Shettleworth & Krebs, 1982). By employing ostensibly arbitrary cache locations, and remembering cues about these locations, these birds make it difficult for competitors to discover cache locations. It is not surprising, therefore, to discover that food-storing birds tend to outperform non-storers on tasks of spatial, but not nonspatial, memory (e.g., Brodbeck et al.; Hampton & Shettleworth, 1996; Olson, Kamil, Balda, & Nims, 1995).

Food-storing birds demonstrate an ability to recall by employing a number of variables including the time of storage, the type of food stored, and the relationship of one stored cache to another (Clayton & Dickinson, 1998; Sherry, 2006). They also have the ability to learn vectors (Vander Wall, 1982), map territory by reference to

landmarks (Kamil & Jones, 1997), and use the position of the sun (Wiltschko, Balda, Jahnel, & Wiltschko, 1999).

Despite the fact that these birds store their food before winter (i.e., during fall), they are nonetheless able to locate the caches after it has snowed (i.e., during winter). In other words, the geographic landscape has changed dramatically by the time birds relocate stored caches. Cues that may have indicated cache location during the fall could now be covered in snow. Thus, birds rely more heavily on global cues (e.g., position of sun, mountain ranges, etc) rather than local cues (e.g., rocks) to retrieve caches (Gould-Beirele & Kamil, 1996). While studying cache recovery mechanisms is truly fascinating, and may even suggest the use of cognitive mapping in birds, the specific details of these studies are beyond the scope of this paper.

A successful food-storing strategy depends upon an ability, not merely to remember where food is stored, but also to keep track of which sites have already been emptied, pilfered, or still await cache recovery. Food-storing birds have demonstrated all of these aspects of memory (Sherry, 1984). Moreover, food-storing birds do not rely on serial position in order to retrieve caches. In other words, these birds are unlikely to recover seeds in the order in which they cached them. Additionally, the last food cached is not necessarily the first to be retrieved (i.e., there are no recency effects). Caches are not recovered linearly because the birds prioritize which caches are visited first based upon preferences for the food stored and its perishability (Clayton, Emery, & Dickinson, 2006; Sherry; Vander Wall, 1990). This cache recovery behavior indicates that food-storing birds distinguish the type of food cached and its location. That is, they distinguish what is stored where, and when, indicating that food-storing birds may be

demonstrating episodic-like memory (Clayton & Dickinson, 1998). Thus, research has revealed an impressive capacity for spatial memory among food-storing birds.

It should be mentioned, however, that corvids and parids differ in the amount of food stored each year. For example, under favorable conditions, some species of corvids are known to store up to thousands of seeds, each in a separate location, and are capable of remembering the location of each seed for approximately one year (Vander Wall & Hutchins, 1983). On the other hand, parids store hundreds of seeds, each in a different location, and recall locations up to 28 days later (Hitchock & Sherry, 1990). Regardless of the number of seeds stored each year, both corvids and parids rely on spatial memory to retrieve caches (e.g., Vander Wall, 1990).

To summarize, food-storing birds preferentially rely on spatial memory to retrieve stored food. As a result, these food-storing birds outperform non-storers on tasks of spatial, but not non-spatial, memory (e.g., Brodbeck et al., 1992). Since spatial memory is believed to be highly dependent on hippocampal functioning, the following section will examine the avian hippocampus.

The Avian Hippocampus

Cytoarchitecture

The hippocampus governs many forms of learning and memory formation in birds and mammals. It is critical for processing spatial memory -- thus playing a pivotal role in the retrieval of food caches -- a fact that will be discussed in detail later in this section (e.g., Hampton & Shettleworth, 1996; Brodbeck et al., 1992). In birds, the hippocampus is part of the telencephalon, is located in the dorsomedial cortex, and extends over approximately 67% of the anterior-posterior axis (e.g., Clayton, 1995; Lee,

Miyasato, & Clayton, 1998). The SVZ, a stem cell rich layer of the lateral ventricles, lies along the lateral and ventral boundaries of the hippocampus. The meninges lie along the dorsal and caudal boundaries of the hippocampus, while the ventral-most boundary lies adjacent to the septum indicated by a clear change in cell density.

Although some have suggested more subdivisions (e.g., Atoji, Wild, Yamamoto, & Suzuki, 2002), the avian hippocampus is generally divided into two internal subdivisions: (1) the parahippocampus (the lateral-most boundaries) and (2) the hippocampus proper (the V-shaped ventral area; Butler & Hodos 2005; Karten & Hodos, 1967; see Appendix A, Figure 1). The hippocampus proper consists of densely packed neurons and pyramidal cells while the parahippocampus contains more diffusely scattered neurons and granule cells (Butler & Hodos; Karten & Hodos; Lee et al., 1998). Evidence also suggests clear differences between the two subdivisions in terms of neurochemistry, connectivity, and cell morphology. Karten and Hodos (1967) further subdivide the parahippocampus into dorsolateral and dorsomedial regions while the hippocampus proper is subdivided into ventral lateral, ventral medial, and ventral core regions (also see Hough, Pang, & Bingman, 2002). The function(s) of these subdivisions has not yet been investigated, but studies attempting to parse out one from the other could lead to a substantial increase in our understanding of this system.

Evidence from both field and laboratory research indicates that the enhanced ability to recall the precise location of stored food relies on spatial memory and an intact hippocampus (e.g., Kamil & Balda, 1985; Sherry et al., 1981; Shettleworth & Krebs, 1982). Krebs (1990) suggests that the survival value of hippocampal-dependent recall for cache locations may have created an evolved brain specialization in food-

storers. In fact, avian food-storing behavior may provide the first example of an evolved specialization in the brain as a result of enhanced memory processing. Therefore, the following section will examine the hippocampus of food-storing birds.

The Hippocampus of Food-Storing Birds

In the intact avian brain, hippocampal volume in food-storing birds is larger than that of non-storing birds (e.g., Krebs et al., 1989; Sherry et al., 1989). This increase may be related to the fact that food-storing birds require greater processing capacity due to their reliance on spatial memory to recover stored food during the fall caching season. In support, neuroanatomical studies have determined that the amount and duration of food storage positively correlates with hippocampal volume (Basil, Kamil, Balda, & Fite, 1996; Hampton, Sherry, Shettleworth, Khurgle, & Ivy, 1995; Healy & Krebs, 1992).

Furthermore, lesions to the hippocampus disrupt cache recovery in Clark's nutcrackers (*Nucifraga columbiana*) and chickadees (Krushinskaya, 1966; Sherry & Vaccarino, 1989). Sherry and Vaccarino gave individual birds 10 minutes to eat or cache seeds in an open flight aviary. The birds were then removed for 3 hours after which they were returned to the aviary and given 10 minutes to recover their caches. After five trials, the subjects were divided into three groups: hippocampal lesion, unlesioned control, and hyperpallium appicale lesion (a region not involved in food-storing behavior, thus a telencephalic lesion control). After five additional cache and recover trials were conducted, Sherry and Vaccarino found deficits in memory for spatial location and working memory (repeated visits to previously retrieved cache locations during a single trial) following hippocampal lesions. Only memory for cache

sites was impaired; lesions had no effect on storing behavior or searching for stored food.

Clayton and Krebs (1994) further demonstrated that birds deprived of storing experience until different ages, then given various amounts of experience storing and retrieving seeds show an increase in the number of neurons and relative volume of the hippocampus, regardless of age. Absence of experience resulted in a cumulative loss of cells and a decrease in hippocampal volume. To determine whether caching experience can induce neuronal proliferation directly, Patel, Clayton, and Krebs (1998) compared food-storing juvenile marsh tits given a varied number of trials of storing and retrieving. Autoradiographic analysis of tritiated thymidine (a mitotic marker) indicated that after only three trials of storing and retrieval, experienced birds showed a significantly higher rate of cell proliferation in the stem cell rich SVZ. After eight trials, experienced birds showed a significantly higher total cell count and neuron count in the hippocampus. Adult cell proliferation in the hippocampus will be discussed in greater detail later in this paper.

Homology

Embryological, anatomical, physiological, and neurochemical evidence suggest a structural and functional homology between the avian and mammalian hippocampus (Lee et al., 1998). Both the mammalian and avian hippocampus derive from the reptilian dorsomedial cortex (Lee et al., 1998; Macphail, 1993). Throughout evolution, non-trivial differences emerged: (1) while the avian hippocampus remained in its original position, the mammalian hippocampus migrated ventro-medially; (2) the avian hippocampus lacks a distinct mossy fiber system (Bingman, Bagnoli, Ioalé, & Casini,

1989); (3) the avian hippocampus lacks a well-defined trisynaptic pathway; and (4) the avian hippocampus lacks structures similar in appearance to the dentate gyrus and Ammon's horn. However, immunocytochemical evidence (Erichsen, Bingman, & Krebs, 1991) and internal connectivity (Kahn, Hough, TenEyck, & Bingman, 2003) suggest that the parahippocampus may correspond to the dentate gyrus.

Embryologically, the avian and mammalian hippocampus both emerge from the same portion of the telencephalon (Källén, 1962; Lee et al., 1998). Both share many of the same cell types including pyramidal and granule cells (Butler & Hodos, 2005; Lee et al.; Mollá, Rodriques, Calvet, & Garcia-Verdugo, 1986; Montagnese, Krebs, Szekely, & Csillag, 1996). Although the precise organization of the substances differ, neurotransmitters and neuropeptides such as acetylcholine, catecholamine, gamma aminobutyric acid, and serotonin are found in both avian and mammalian hippocampi (Butler & Hodos, 2005; Erichesen et al., 1991; Krebs, Erichsen, & Bingman, 1991). Both avian and mammalian hippocampi show similar connectivity to areas such as the septum, hypothalamus, brain stem nuclei, and sensory processing areas, although some differences exist (Casini, Bingman, & Bagnoli, 1986; Montagnese et al.). Furthermore, both structures show long-term enhancement of synaptic responses (e.g., Bliss & Lomo, 1973; Shapiro & Wieraszko, 1996; Wieraszko & Ball, 1991, 1993).

Perhaps most importantly, evidence also suggests that the avian and mammalian hippocampi share functional similarities. Birds and mammals both rely on hippocampal-dependent spatial memory (e.g., Sherry & Vaccarino, 1989; Kleindienst, McGinn, Harvey, Colello, Hamm, & Bullock, 2005). As will be described later, the hippocampus of both responds to seasonal and photoperiodic changes (Barnea &

Nottebohm, 1994; Jacobs, Gaulin, Sherry, & Hoffman, 1990; Smulders et al., 1995, Smulders, Shiflett, Sperling, & DeVoogd, 2000; Yaskin, 1984). And the most striking similarity is that post-developmentally, both demonstrate neurogenesis (e.g., Altman & Das, 1965; Barnea & Nottebohm, 1994); experience-dependent growth (e.g., Clayton & Krebs, 1994; Rosenzweig, Krech, Bennett, & Diamond, 1962); and experience-dependent neurogenesis (e.g., Altman & Das, 1964; Kempermann, Kuhn, & Gage, 1997, Kempermann, Kuhn, & Gage, 1998; Patel et al., 1998). It is this fascinating capability for adult neurogenesis in birds that forms the basis of this thesis.

Adult Neurogenesis

It was once believed that neurogenesis occurred only in the developing brain. Past paradigms compared brain functioning to that of a computer, with fixed circuitry. The idea that the computer could add components to itself did not fit the overall view. Neurons were known to be post-mitotic, incapable of dividing and forming new neurons. At best, central nervous system stem cells were believed to be vestigial, having no function after brain development. However, Altman (1962, 1963, 1966, 1969) and Altman and Das (1964, 1965, 1966) discovered adult cell proliferation in the rat and cat olfactory bulb and hippocampus. Fifteen years later, Kaplan examined the structure of these newly born cells and discovered that they were indeed new neurons (Kaplan, 1981; Kaplan, 1985; Kaplan & Bell, 1984; Kaplan & Hinds, 1977). Since then, adult neurogenesis has been observed in every species examined including fish (Zupanc, 2006), reptiles (Lopez-Garcia, Molowny, Garcia-Verdugo, & Ferrer, 1988), tree shrews (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997), marmosets (Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998), macaques (Gould, Reeves, Fallah, Tanapat,

Gross, & Fuchs, 1999), humans (Eriksson, Perfilieva, Bjork-Eriksson, Alborn, Nordborg, Peterson, & Gage, 1998) and birds (Barnea & Nottebohm, 1994, 1996; Nottebohm, 1985, 1989). Adult neurogenesis is not only possible, it is highly conserved across species.

Adult neurogenesis occurs in the SVZ, olfactory bulb, subgranular layer of the dentate gyrus, and perhaps in the injured neocortex of mammals (e.g., Gage, 2002). In birds, however, neurogenesis occurs throughout the telencephalon, most notably the high vocal center of birds, hippocampus, and SVZ (e.g., Nottebohm, 1985). For the purpose of this thesis, discussion will be limited to adult hippocampal neurogenesis.

Adult Hippocampal Neurogenesis

Adult hippocampal neurogenesis is a multi-step process that begins with stem cell birth in the SVZ of the lateral ventricles (e.g., Alvarez-Buylla, 1990; Alvarez-Buylla, Garcia-Verdugo, Mateo, & Merchant-Larios, 1998; Alvarez-Buylla & Nottebohm, 1988; Kempermann, Wiskott, & Gage, 2004). Adult neural stem cells are characterized by 3 properties: (1) they are unspecialized or undifferentiated; (2) they have the capacity for unlimited self-renewal via cell division; and (3) they are multipotent, maintaining the capacity to generate at least 2 different cell types (Kempermann, 2006).

Neural stem cells give rise to progenitor cells via asymmetric cell division where one daughter cell leaves the cell cycle, resulting in a more limited capacity for self-renewal, while the other reenters the cell cycle. These progenitor cells generate differentiated neurons or glia as they migrate toward their final destination

(Kempermann, 2006; Kriegstein & Alvarez-Buylla, 2009). Thus, glial and neuronal origins are not as separated as once believed.

Vitally important during brain formation, radial cells (aka radial glia) are positioned with their cell bodies anchored in the SVZ, and their long processes radiating outward. During developmental neurogenesis, these radial cells act as scaffolding, upon which new cells attach then migrate to their ultimate destinations thus forming the various structures of the brain (Rakic, 1972). Although both are located in the SVZ, stem cells and radial cells have long been assumed to be two distinct cell classes. However, in addition to their role in migration, radial cells can also act as stem cells (Kriegstein & Alvarez-Buylla, 2009; Noctor, Flint, Weissman, Dammerman, & Kriegstein, 2001). Research designed to determine how the two cell types differ actually determined that they did not differ at all. Stem cells show the same morphological characteristics of radial cells and express the same radial cell markers such as glial fibrillary acidic protein; thus, they may in fact be radial cells (Kempermann, Jessberger, Steiner, & Hronenberg, 2004; Kriegstein & Alvarez-Buylla; Noctor, Flint, Weissmann, Wong, Clinton, & Kriegstein, 2002; Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Because radial cells are abundant in the adult avian brain, it is possible that they serve the same purpose throughout the lifespan. That is, they may be acting as stem cells giving birth to new brain cells, as well as providing the scaffolding upon which those new cells attach and migrate to their final destinations.

Although the fast proliferating progenitors may express immature neuronal markers such as doublecortin and the polysialated form of neural cell adhesion molecule as they begin to migrate toward their final destination (e.g., Ehninger & Kempermann,

2006), once near their destination, these newly born cells invariably express markers of mature neurons, such as neuronal nuclei protein (NeuN; Kempermann et al., 2004). At this time, these newly born cells exit the cell cycle, coinciding with an increased expression of calcium-binding protein calretinin (e.g., Ehninger & Kempermann). Once a neuronal lineage occurs, the process of functional integration begins. Stanfield and Trice (1988) were the first to demonstrate the integration of newly born cells in the mammalian hippocampus via extension of axons and dendrites. In birds, it can take 20-40 days for daughter cells to complete migration, mature into neurons, and functionally integrate into the hippocampal parenchyma (Alvarez-Buylla & Nottebohm, 1988).

Although the SVZ generates a surplus of daughter cells initially, only a fraction will survive and mature into neurons (Kempermann et al., 2004). Of these cells, 73.1% mature into fully functioning neurons in the avian hippocampus (Alvarez-Buylla et al., 1998). Selection for survival occurs when newly born cells express a neuronal lineage within the first 2 weeks of the initial stem cell division (Kempermann et al). Those cells that succumb to selective pressures presumably do so through programmed cell death, apoptosis (Kempermann et al.), rather than by marker dilution (e.g., Cameron, Woolley, McEwen, & Gould, 1993; Gould, Reeves, Fallah, Tanapat, Gross, & Fuchs, 1999). Thus, cell proliferation (i.e., operationally defined as birth within the first 24 hours of marker administration) is only one indicator of potential neurogenesis. Subsequent incorporation of new cells -- migration, differentiation, and cell death -- needs to be considered when estimating net neurogenesis in any region.

Intriguingly, survival of newly formed neurons is enhanced by cognitive stimulation including living in an enriched environment (e.g., Kempermann et al.,

1997), physical activity (van Praag, Christie, Sejnowski, & Gage, 1996), and ecologically relevant behaviors reliant upon the hippocampus, such as food-storing (e.g., Barnea & Nottebohm, 1994).

Adult Hippocampal Neurogenesis in Food-Storing Birds

Barnea and Nottebohm (1994) examined the rate of neurogenesis in both free-range and captive food-storing chickadees. Chickadees were caught throughout the year and given one injection of tritiated thymidine at the time of capture in order to label mitotic cells. Half of these birds were released back into the wild while the other half remained in captivity. The free-range birds were recaptured and perfused 6 weeks later to allow sufficient time for newly born cells to migrate toward their final destination and differentiate into neurons. New cells were counted in the hippocampus and hyperpallium appicale (telencephalic control region not involved in food-storing behavior).

New cells were determined to be neurons based on observable morphology and were found in the hippocampus of both groups during all times of the year (Barnea & Nottebohm, 1994). Neurogenesis peaked during October, coinciding with a peak in food-storing behavior. However, captive birds had significantly fewer new neurons compared to free-range birds. Seasonally induced changes in neurogenesis were not observed in the hyperpallium appicale indicating that increases in the hippocampus did not result from a general seasonal increase in cell birth in the telencephalon. Therefore, Barnea and Nottebohm suggested that the increases observed were due to enhanced survival or incorporation of newly born neurons in order to forge new hippocampal-dependent long-term memories during the food caching season. Since new neurons are

added to areas of the avian brain throughout adulthood, these results reflect the possibility that seasonally mediated food-storing may (1) induce neuron proliferation, (2) promote survival of pre-existing cells, (3) promote survival of new cells, or (4) a combination of these factors.

Seasonal Changes in the Hippocampus of Food-Storing Birds

As reviewed above, the hippocampus plays a role in the accurate retrieval of cached food in food-storing birds, is larger in food-storers than their non-storing cohorts, and shows adult neurogenesis. Since food-storing occurs largely during the fall season, it has been suggested that the adult avian hippocampus may change across seasons in both size and neurogenesis.

Smulders et al. (1995; 2000) examined seasonal fluctuations in hippocampal volume and cell count at six different times of the year. They found that both hippocampal volume and cell count fluctuate seasonally, peaking in October (in New York) when fall storing behavior commences. They speculated that both increases resulted from increased demands placed upon the hippocampus in the fall. Specifically, food-storing birds must remember the location of hundreds of separately stored caches. Because this is a hippocampal-dependent activity, an increase in hippocampal volume and cell number may be required in the fall to process this large amount of information. These results are consistent with the fall increase in neuronal survival in storing chickadees observed by Barnea and Nottebohm (1994).

While Smulders et al. (1995; 2000) provided compelling arguments, others have found conflicting results with regard to hippocampal volume (See Appendix B, Table 1). Hoshooley and Sherry (2007) captured storing chickadees and non-storing house

sparrows (*Passer domesticus*) throughout the year, and injected them with the mitotic marker 5-bromo-2'-deoxyuridine (BrdU). They found an increase in hippocampal volume in spring months, not fall. Rather than relying on the identification of new neurons using cell morphology alone, immunohistological procedures were used to identify new cells (positive for BrdU), existing "old" neurons (positive for NeuN), and new neurons (positive for both BrdU and NeuN). An estimate of the influx of new neurons into the hippocampus, neuronal incorporation, was calculated by counting cells that were positive for both markers. While chickadees had more hippocampal neuronal recruitment compared to sparrows, no seasonal differences were observed in either species. Hoshooley and Sherry speculated that in their chickadees, hippocampal neuronal incorporation may have increased during the midwinter period not examined, potentially causing a volumetric increase during the spring, not the fall. This discrepancy may also be explained by differences in local temperatures, food availability, or the precise capture times employed by Hoshooley and Sherry.

Similar results were observed by Mitterling et al. (2007). Food-storing chickadees and non-storing juncos were captured in the fall and spring. While chickadees had larger hippocampal volumes compared to juncos, hippocampal volume in both species increased during the spring, partially confirming the findings by Hoshooley and Sherry (2007). Thus, volume comparisons may not prove to be a particularly reliable measure of seasonal changes in the hippocampus related to food-storing activities. Indeed, food-storing and volume changes during adulthood may not be related at all, and observations of seasonal fluctuations may be due to some other factor(s) in the natural environment. Because results are still equivocal, methods used

in determining volumes should be scrutinized much more closely, especially with regard to the specific timing of actual commencement and cessation of food-storing for individual species in their respective geographical regions (e.g., Pravosudov & Clayton, 2002; Roth & Pravosudov, 2009).

Mitterling et al. (2007) also looked at seasonally-mediated hippocampal cell incorporation in chickadees and juncos captured during the fall and spring. This study was the first to look specifically in the area thought to be responsible for adult neurogenesis, the SVZ, and compare cell birth in the SVZ of both food-storers and non-storers. Compared to non-storing juncos, food-storing chickadees had more newly born cells in the hippocampus and SVZ 7 days after administration of BrdU, regardless of season. Fall-caught chickadees, but not juncos, also had significantly more new cells in the hippocampus than those caught in the spring; the SVZ showed no such seasonal effect in either species. Seven days may in fact be long enough for cells born into the SVZ to migrate out of the SVZ and into the hippocampus, thus accounting for the increased hippocampal incorporation especially during the fall when hippocampal cells would be in high demand for storers. Taken together, results suggest that cell incorporation is enhanced during the fall in food-storing chickadees, but not in juncos, and may reflect a selective adaptation in the brain due to the increase in food-storing behavior and hippocampal demand during that season.

Thus far, this paper has examined normal hippocampal functioning in food-storing and non-storing species. Hippocampal volume, cell proliferation, and cell incorporation fluctuate according to degree of food-storing specialization and possibly with seasonal variations. Because of the extraordinary levels of plasticity in the

hippocampus of food-storers during any season, research ultimately led to questions regarding various functions for the new neurons. For example, are new neurons increasing the processing capacity and/or efficiency of the hippocampus? If so, we could argue that neurogenesis would be seasonal in food-storing birds, not necessarily a year-round phenomenon. Alternatively, are new neurons part of a neuronal turnover mechanism, continually being used to replace dead or dying neurons? As demand increases during the fall food-storing season, more neuronal death may occur and more new cells would be born to replace them. In that case, we should -- and do -- see year-round incorporation of new cells with a peak during food-storing season. Is neurogenesis then a mechanism for chronic repair/replacement of dead or dying neurons? And if so, could new neurons also be involved in repair/replacement of cells lost following acute injury? Considerable research has shown that neurons are indeed born as a result of acute traumatic brain injury (TBI), leading to the possibility that, under the right set of circumstances, the brain engages in self-repair.

Responses to TBI

Following injury, the mammalian and avian brain increase glia and neuron proliferation. This injury-induced increase in cell birth has been shown following fluid percussion injury (Chirumamilla, Sun, Bullock, & Colello, 2002), chromophore-targeted neuronal degeneration (Magavi, Leavitt, & Macklis, 2000; Scharff, Kirn, Grossman, Maclism & Nottebohm, 2000), endotoxin-induced inflammation (Ekdahl, Claasen, Bonde, Kodaia, & Lindvall, 2003; Monje, Toda, & Palmer, 2003), irradiation-induced inflammation (Monje et al.), and penetrating injury (Gould & Tanapat, 1997; Lee et al., 2007; Zhu, Umegaki, Shinkai, Kurontani, Suzuki, Endo, & Iguchi, 2003).

Following injury, glia rapidly proliferate, reactive astrocytes are upregulated, microglia are activated, and scar tissue forms (Almi & Finger, 1992; Amat, Ishiguro & Nakamura, 1996; Kempermann & Neumann, 2003). This reactive gliosis facilitates recovery by establishing a glial scar around the area of injury. This scar barricade allows glia to remove cellular debris, restrict inflammation, and prevent further neuronal death. Astrocytes, glial precursors, and microglia take part (Alonso, 2005; Sofroniew, 2005).

It is commonly believed that the injury-induced activation of microglia interferes with and even *prevents* neurogenesis when the response is systematically induced and not confined to a specific brain region (e.g., Ekdahl et al., 2003; Monje et al., 2003). However, when injury is localized to one structure, neurogenesis is enhanced (e.g. Gould & Tanapat, 1997; Scharff et al., 2000). This difference may be the result of a relative sparing of neuronal stem cells in localized injuries, compared to stem cell damage in more global, systemic inflammation and injury. Furthermore, microglia secrete brain-derived neurotrophic factor, which appears to influence injury-induced neurogenesis and synaptogenesis, possibly aiding in recovery of function (e.g., Madinier et al., 2009). Therefore, the true role that glia play may be a complicated interplay between protection of the injured area and proliferation of new "replacement" cells.

Because of the glial scar, new neurons must be born either at the injured site directly, or in distal regions then migrate through the scar if they are to be effective. For example, new neurons would need to be born directly in the injured hippocampus, or in the stem cell rich SVZ then migrate into the injury. In adult zebra finches, unilateral hippocampal injury increases the number of BrdU immunoreactive (BrdU-IR)

cells both proximal (ipsilateral hippocampus and adjacent SVZ; pSVZ), and distal (ipsilateral but non-adjacent SVZ; dSVZ) to the injury (Lee et al., 2007; See Appendix C, Figure 2). SVZ-generated cells in birds predominately become neurons that migrate into surrounding structures (Alvarez-Buylla, 1990; Alvarez-Buylla et al., 1998; Alvarez-Buylla & Nottebohm, 1988). Therefore, many of these new injury-induced cells may be fated to take on a neuronal profile. In females, the proliferative response is accompanied by upregulation of aromatase expression (Peterson, Lee, Fernando, & Schlinger, 2004; Peterson, Fernando, Day, Allen, Chapleau, Menjivar, & Schlinger, 2007; Lee et al., 2007).

Upregulation of Aromatase Expression Following Injury

In female zebra finches, expression of aromatase, the critical enzymatic step in the metabolic pathway to convert testosterone into estrogen, dramatically increases in reactive astrocytes and radial cells proximal to the injured hippocampus. New cells do not appear to express aromatase themselves, nor do neurons. However, they are born into this rich bed of aromatase-expressing glia. Notably, aromatase-positive radial cells can be seen to originate in the SVZ and project towards the injury. BrdU-IR cells are in close association with these radial cells and appear to be in contact with the aromatase-positive fibers (Peterson et al., 2004; 2007).

As mentioned in an earlier section, radial cells are known for their role in guiding new neurons along their migratory path to brain regions during development, and are quite likely the actual stem cells themselves. This is perhaps the most striking finding and provides substantial support to the idea that a mechanism exists for SVZ-generated new replacement neurons that may then migrate along radial processes into

the injured site to enact a repair. Additional studies determined that estrogen removal suppresses injury-induced cell proliferation, whereas estrogen replacement restores it to normal levels (Lee et al. 2007; Peterson et al). Thus, this putative repair mechanism is believed to be estrogen-mediated.

Radial cells are known to exist in the adult avian brain, and are now known to be involved in acute injury by expressing aromatase. Following mammalian brain development, however, radial cells transform into post-mitotic, non-proliferating astrocytes. Thus, it was logical to believe that the adult mammalian brain was incapable of adult neurogenesis (no stem cells) and/or adult neuronal migration (no radial fibers). However, recent research has revealed that, in the mammalian brain, reactive astrocytes acquire stem cell properties after TBI (Buffo, Rite, Tripathi, Lepier, Colak, Ana-Paula Horn, et al., 2008), and are known to revert back into radial cells and become neuroproliferative (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Seri et al. 2001). Thus, radial cells may be responsible for both neuron proliferation and migration in all vertebrates.

Injury-Induced Changes in the Hippocampus of Food-Storing Birds

Law et al. (2010) is the only study to have addressed the neurogenic effects of lesions to the hippocampus of food-storing birds. Though lesions to the hippocampus are known to affect the retrieval of stored food (Krushinskaya, 1966; Sherry & Vaccarino, 1989), the cellular responses to lesions had not been investigated. Food-storing birds rely heavily upon their caches during the fall and winter. Therefore, damage to the brain structure upon which retrieval accuracy depends may result in a different cytogenic response than it would in non-storing birds especially during the

fall. Because food-storers show higher levels of neuronal incorporation in the intact brain, they may also show an enhanced ability to repair an injured hippocampus. Additionally, new cells may need to be incorporated into the hippocampus more rapidly in order to replace dead and dying cells. Since hippocampal injury may be unlikely under natural conditions, an injury-induced increase in cell proliferation in storers compared to non-storers may reflect a beneficial by-product rather than a direct adaptation.

During the fall, food-storing chickadees and non-storing juncos were captured in coastal Maine and given either no lesion, or a single penetrating lesion to the right hippocampus, followed by one injection of BrdU 24 hours after the lesion (Law et al., 2010). Birds were perfused 7 days following BrdU. BrdU-IR cells were counted in the hippocampus and SVZ then cell densities, defined as cells per mm^2, were calculated in order to control for species differences in brain sizes. If unlesioned, more BrdU-IR cells were observed in the hippocampus and SVZ of chickadees compared to juncos, indicating higher innate cell proliferation or incorporation rates in chickadees.

For both species, whether lesioned or unlesioned, the adjacent SVZ demonstrated a higher density of BrdU-IR cells compared to the hippocampus. This more pronounced effect in the SVZ is not surprising because, as the stem cell layer, the SVZ constantly generates new cells with the potential to migrate toward the hippocampus. As expected, hippocampal lesions resulted in an increase in BrdU-IR cells in the hippocampus of both species (Law et al., 2010). BrdU-IR cells increased in the injured hippocampus; however, lesions caused larger increases in juncos. Surprisingly, chickadees had significantly fewer BrdU-IR cells in the injured

hippocampus compared to juncos. Perhaps most unexpected, however, was the finding that the SVZ of chickadees did not show an injury-induced increase in BrdU-IR cells. Similar to previous results in zebra finches (Lee et al., 2007), juncos showed increases in BrdU-IR cells in the SVZ as well as the contralateral hippocampus. Chickadees showed no such increases.

Thus, during the fall food-storing season, the food-storing chickadee showed less of a response to injury. Interestingly, Law et al. (2010) also examined the percentage of cells double-labeled for BrdU and NeuN. While double-labeled cells were observed in the injured hippocampus of both species, chickadees had twice the number as juncos. At 7 days following BrdU incorporation, chickadees showed fewer new injury-induced cells; however, more of those cells became neurons with the net result being higher neuronal incorporation.

Higher neuronal incorporation with lower new cell counts could be explained using a number of distinct scenarios; however, only one will be investigated in the proposed study. First, because food storing is seasonal, food-storers may also show seasonal differences in their response to injury. For example, they may respond with less cell proliferation during the fall food-storing season as shown by Law et al. (2010), but more proliferation during the spring bringing the levels closer to those seen in non-storers. Comparing injury-induced cell birth in food-storing birds captured during the fall and spring would address that possibility; however, performing that experiment is beyond the scope of this thesis. Second, neuronal turnover could be considerably faster in chickadees than their non-storing cohorts leading to more rapid proliferation and

death of non-neuronal cell lines. As carried out in this thesis, investigating injury-induced cell birth at various times post-injury addressed this second alternative.

Summary and Hypotheses

To summarize: (1) TBI induces birth of both neurons and glia; (2) in zebra finches, TBI induces estrogen-mediated cell birth in the hippocampus and stem cell rich SVZ; (3) TBI results in a dramatic increase in aromatase expression in reactive astrocytes and radial cells indicating that a potential migratory pathway exists and may be able to direct new cells migrating from the SVZ into the injured site; (4) recovery of caches is seasonal, necessary for survival, dependent upon an intact hippocampus, and concomitant with a peak in hippocampal neurogenesis in food-storing birds leading to an expectation of enhanced cell birth following TBI; yet (5) TBI results in similar responses in zebra finches and juncos, but dissimilar and reduced responses in food-storing chickadees.

Thus, this thesis expanded upon the evidence and evaluated the differing responses of food-storing chickadees following TBI. The main question addressed by this thesis was whether food-storing birds differ in the speed of cellular turnover following TBI. To answer this question groups of food-storing chickadees differed in BrdU injection times post injury, and survival times post-BrdU. All birds received a unilateral penetrating lesion to the right hippocampus followed by BrdU injection immediately or 24 hours post-lesion, and 2 or 168 hour survival times post-BrdU. BrdU-IR cells in the hippocampus and SVZ were compared across time.

If speed of cellular turnover explains the results of Law et al. (2010), the chickadee hippocampus should respond with a higher production of cells immediately

following injury, rapidly diminishing over time. Six specific hypotheses were tested stemming from that proposal. Hypotheses 1 and 2 were designed to replicate key results shown in Law et al. (2010), establishing that some of the basic phenomena are indeed true. Hypotheses 3 through 6 explored the temporal characteristics of injury-induced cell birth and incorporation.

Hypothesis 1 predicted that injury would increase cell proliferation in the hippocampus and SVZ. Hypothesis 2 predicted that all birds would have a higher density of BrdU-IR cells in the SVZ compared to the hippocampus, a result which would replicate that observed by Law et al. (2010). Hypothesis 3 predicted that chickadees would have higher cell proliferation rates in the hippocampus (short survival times) when injected with BrdU immediately following lesion compared to those injected 24 hours post-lesion. Hypothesis 4 predicted the same for the SVZ. Hypothesis 5 predicted that chickadees would have lower rates of new cell incorporation (long survival times) in the hippocampus 7 days post-BrdU injection compared to 2 hours post-BrdU injection. Hypothesis 6 predicted the same for the SVZ.

CHAPTER II

METHODS

Subjects

Twenty four adult black-capped chickadees (*Parus atricapillus*) were captured in the fall between October 2009 and November 2009. These capture dates replicated those employed by Law et al. (2010). All birds were captured at the Coastal Studies Center in Orr's Island, Maine. Collecting occurred over a relatively short range of day length – 9:55 to 10:53 -- minimizing potential photoperiodic effects. Analyses conducted using photoperiod as a possible factor in cell proliferation and incorporation yielded nonsignificant results.

Following capture, target birds (i.e., chickadees) were randomly assigned to one of 3 groups differing in (1) injection time of BrdU relative to time of lesion; and (2) length of survival time following that BrdU injection. Final sample size per group was 9, 9, and 6 chickadees. See Appendix D, Table 2 for experimental manipulations. All nontarget birds were released.

Birds were trapped using potter traps and were pair housed in cages contained in an outdoor aviary to ensure they experience natural environmental fluctuations in daylight and weather. These birds were provided water and food *ad libitum.* Chickadees weighed approximately 10-13 grams. Because chickadees are not sexually

dimorphic, sex was determined by locating and removing the gonads postmortem. Age -- whether adult or juvenile -- was determined based on the ossification of the outer skull. Since stress is a known factor in neurogenesis, every effort was made to minimize stress whenever possible. When stress was inevitable, such as when handling for injections, all birds were treated equivalently.

Hippocampal Lesions

One day after capture, all birds received a single unilateral penetrating lesion to the right hippocampus. Unilateral hippocampal lesions enabled the use of the contralateral hemisphere as a within-subjects control (Law et al., 2010). Following one hour of food deprivation to minimize illness during surgery, birds received one injection of the anesthetic Equithesin at a dose of 0.0032 ml/g body weight intramuscularly into the breast muscle (i.m.). Birds were wrapped in a flannel "coat" to keep them warm and safe from injury during surgery. The inhalant isoflurane was given only when needed to gently boost/extend the anesthesia effect. Isoflurane was delivered by placing 2 saturated q-tips directly in front of the external nares. Feathers at the top of the head were plucked to expose an area of skin above the skull which was then swabbed with betadyne. Birds were then placed in a stereotaxic apparatus under a binocular dissecting microscope. To assure accurate placement of the lesion, coordinates of the hippocampus were obtained relative to the bifurcation of the Y-sinus using the zebra finch atlas of Nixdorf-Bergweiler and Bischof (2007) as a guide. A small incision (~3mm) was made with a scalpel into the skin covering the skull in order to expose the scalp above the Y-sinus. A flap of skull was removed above the Y-sinus and the right hippocampus. A small drill burr (0.5mm diameter) was lowered 0.5mm deep, 2.2mm

anterior, and .5mm lateral from the bifurcation of the Y-sinus in order to create a discrete lesion. Following lesioning, the incision was closed and sealed with the skin bonding agent ethyl cyanoacrylate.

Using our coordinates, the hippocampus is damaged but the underlying SVZ is spared, therefore the procedure yields highly consistent results and optimally stimulates cell proliferation.

BrdU Administration

BrdU (5-bromo-2'-deoxyuridine) is a thymidine analog that is selectively taken into the DNA of dividing cells (during S Phase) instead of thymidine, which is present in less quantity. Thus, daughter cells become permanently labeled with BrdU. Although the exact saturating dose of BrdU has yet to be determined in birds, a single injection of BrdU yields a more temporally accurate picture of mitotic activity than multiple injections, which can be difficult to interpret due to the mixed "age" of cells. Therefore, all birds received one intramuscular injection with 0.005ml/g body weight BrdU either immediately following a lesion, or 24 hours post-lesion. This dose of BrdU is standard in our laboratory (e.g., Law et al., 2010, Lee et al., 2007).

Birds that received a BrdU injection immediately following a lesion were perfused 2 hours post-injection (See Appendix D, Table 2). Birds that received a BrdU injection 24 hours post-lesion were perfused either 2 hours following injection or 168 hours (7 days) following injection. Thus, 3 groups differed in survival and BrdU injection times. In group 1 (IMM-2), chickadees received a unilateral penetrating lesion to the right hippocampus followed by an immediate (within 2 minutes) injection of BrdU. They were euthanatized 2 hours following injection. This allowed us to examine

injury-induced cell proliferation in the SVZ and hippocampus by looking at the earliest point in time following injury. Euthanasia occurred 2 hours following injection because the estimated maximum bioavailability of BrdU is 2 hours after injection (Kempermann, 2006; Takahashi, Nowakowski, & Cavines, 1992; Packard, Menzies, & Skalko, 1973).

In group 2 (24-2), chickadees received a unilateral penetrating lesion to the right hippocampus followed by one injection of BrdU 24 hours post-lesion and were euthanatized 2 hours post-injection. This gave us information on the injury-induced effect of cell proliferation after a short delay. In group 3 (24-168), chickadees received a unilateral penetrating lesion to the right hippocampus followed by one injection of BrdU 24 hours post-lesion and were euthanatized 168 hours (7 days) post-injection. This last group was employed to confirm previous findings of Law et al. (2010). These three survival times were chosen because previous research demonstrated injury-induced cell proliferation in the hippocampus and SVZ of adult zebra finches euthanized 2 hours, 24 hours (1 day), and 168 hours (7 days) post-BrdU injection (Lee et al., 2007).

There has been much debate as to whether BrdU would not only label cell proliferation but cell death as well (e.g., Rakic, 2002). During cell death, DNA may become fragmented or the cell may induce an abortive attempt to enter into cell division prior to apoptosis (e.g., Copani, Uberti, Sortino, Bruno, Nicoletti, & Memo, 2001). Either of these situations may allow for the incorporation of BrdU. Kuan, Schloemer, Lu, Burns, Weng, Williams, et al. (2004) suggest that these "terminally sick" cells would be incapable of living through experiments with long survival times. Similarly,

Cooper-Kuhn and Kuhn (2002) employed BrdU and TUNEL (apoptotic cell marker) double labeling and found that in no instance were cells double labeled. Similar results have been observed by Gould and Tanapat (1997) following injury to the hippocampus.

Perfusion and Tissue Preparation

At the time of perfusion, birds were anesthetized with a lethal intramuscular injection of equithesin (0.05ml) and transcardially perfused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde. The preserved brains were removed from the skull and assigned a unique histology number in order to ensure that the primary researcher remained blind to the conditions of the experiment. Brains were postfixed in 4% paraformaldehyde for 24 hours, transferred to 0.1 M phosphate buffer (PB), and embedded in 8% gelatin. After being embedded, brains were then cut into 5 equivalent sets (approximately 48 slices) of 40µm thick coronal sections using a vibratome. The first set was Nissl stained to determine the extent of the lesion. The second set was used to visualize new cells using BrdU immunohistochemistry (IHC); all other sets were immersed in a cryoprotection fluid (Watson, Wiegand, Clough, & Hoffman, 1986) and stored at -20°C.

Single Label BrdU IHC

To employ BrdU IHC, tissue slices from set two were washed 3 times for 15 minutes each (3x15 min) in 0.1 M phosphate buffer (PB) in order to remove left over aldehydes. The tissue was then incubated in 1 N HCl for 30 minutes in order to denaturate the DNA. Following denaturation, tissue was washed 3x15 min in 0.1M PB, followed by immersion in 0.5% H_2O_2 for 30 minutes. Hydrogen peroxide neutralized endogenous peroxidases and decreased background staining. Following 3 additional 15

minute washes in 0.1 M PB, tissue was treated with 10% normal horse serum in 0.3% Triton X-100 for 60 minutes. For primary antibody treatment, sections were incubated in 1:500 anti-BrdU (Roche Diagnostics) for 24 hours. After primary incubation, sections were washed 3x15 min with 0.1% PBT and then treated with 1:200 biotinylated horse anti-mouse IgG (Vector Labs) in 0.3% PBT for 60 minutes. Three additional 15 minute washes in 0.1% Triton-X PB (PBT) were administered prior to incubation in 1:200 avidin-biotin complex (Vectastain) in 0.3% PBT for 90 minutes. The avidin binds to the secondary antibody (which is bound to the primary antibody) in order to amplify the response. Following 3 more 15 minute washes in 0.1% PBT, the immunoproduct was visualized using diaminobenzidine (DAB; Sigma). The tissue was then washed 3x15 min in 0.1 M PB. Finally, tissue was mounted to gelatinized slides and dried overnight. Once dried, slides were placed in staining racks, dehydrated in alcohols, soaked in histoclear, and coverslipped.

BrdU-IR Cell Counting

Cells visualized using BrdU IHC were counted using differential interference contrast (DIC) illumination on a Nikon E-800 microscope using NeuroLucida software (MBF Bioscience) following procedures described in Lee et al. (2007) and Law et al. (2010). In order to count cells labeled with BrdU, four cell counting areas were established by drawing contour lines around their outer boundaries (See Appendix E, Figure 3). For each brain, contour lines were drawn for approximately 12 coronal sections, which included the majority of the hippocampus and telencephalon. BrdU-IR cells were defined as being larger than 3μm in diameter, darkly stained, and somewhat

rounded, following the morphological criterion of Gould, Reeves, Graziano, and Gross (1999).

BrdU-IR cells were counted within the contour lines bordering the right (ipsilateral) and left (contralateral) hippocampus as well as the right (ipsilateral) and left (contralateral) SVZ. Cells falling within 50µm of the internal lumen of the lateral ventricles were designated as SVZ cells. Our laboratory usually divides the SVZ into two sections based on its location. The portion of the SVZ that lies adjacent to the hippocampus is referred to as the proximal SVZ (pSVZ) and the portion that does not lie adjacent to the hippocampus is referred to as the distal SVZ (dSVZ). Only pSVZ cells were counted because of their potential to migrate toward the hippocampus. Distal SVZ cells may indicate general stem cell activity and could be counted in subsequent experiments.

Thus, BrdU-IR cells counted within the hippocampal contour line were referred to as hippocampus cells whereas BrdU-IR cells counted within the pSVZ were referred to as pSVZ cells. The final result yielded a total of 4 different BrdU-IR cell counts: ipsilateral (lesioned) hippocampus, contralateral (unlesioned) hippocampus, ipsilateral pSVZ, and contralateral pSVZ. All cells meeting the criteria and falling within a given contour were counted rather than relying on estimates. This method of counting has proven to be appropriate (Tramontin, Smith, Breuner, & Brenowitz, 1998), even when compared to unbiased stereological methods of estimation.

<u>Statistical Analysis</u>

Using ANOVA, the following variables were analyzed: hemisphere (ipsilateral, contralateral) and brain region (hippocampus, pSVZ). Independent *t*-tests compared

BrdU injection times post-lesion (immediate, 24 hr) and survival times (2 hr, 168 hr). The dependent variables were the density of BrdU-IR cells located in the ipsilateral hippocampus, contralateral hippocampus, isilateral pSVZ, and contralateral pSVZ. Cell densities (defined as the number of cells per mm^2) were calculated to replicate Law et al. (2010) by dividing the number of BrdU-IR cells falling within a given contour line by the area bound by that contour line. Data was analyzed in two ways: (1) by examining mean density of BrdU-IR cells derived from 12 slices and (2) by examining mean density of BrdU-IR cells derived from 1 slice only (the lesion slice). Because these analyses yielded similar results, this thesis will report only the analyses using mean density of BrdU-IR cells derived from 12 slices.

Analyses were conducted using the Statistical Package for the Social Sciences version 15.0 for Windows. Post hoc analyses were performed on main factors using Fisher's Least Squared Means (LSMs). An analysis was considered significant if $p<.05$. All prior work in this laboratory -- using similar procedures in zebra finches, chickadees, and juncos -- has shown that (1) injury increases cell birth, never decreases it; and (2) the pSVZ has more BrdU-IR cells than the hippocampus (e.g., Law et al., 2010; Lee et al., 2007; Peterson et al., 2004). Thus, a 1-tailed level of significance can be utilized when conducting planned comparisons between these variables. ANOVAs were interpreted using the conservative 2-tailed approach; however, subsequent post hocs and planned comparisons made use of the 1-tailed level of significance.

CHAPTER III

RESULTS

BrdU-IR cells were observed in all groups in both the ipsilateral and contralateral hippocampus as well as the ipsilateral and contralateral pSVZ. Photomicrographs depicting BrdU-IR cells in the hippocampus and pSVZ are presented in Figures 4 and 5 (Appendices F and G, respectively). Six specific hypotheses were tested for this thesis. Analyses and results bearing on each are detailed below. Mean and standard error density measures for all analyses are summarized in Appendix H, Table 3.

Hypothesis 1

Hypothesis 1 predicted an injury-induced increase in cell proliferation in the hippocampus and pSVZ of food-storing chickadees. In order to test this as well as Hypothesis 2 below, a 2 (brain region) x 2 (hemisphere) repeated measures ANOVA was employed.

Main Effect of Hemisphere

A main effect of hemisphere was predicted such that the ipsilateral hemisphere was expected to have a higher density of BrdU-IR cells compared to the contralateral hemisphere, regardless of brain region. ANOVA results indicated that there was a nonsignificant trend in the data ($F(1,23) = 4.167$, $p=.053$). Upon closer examination, a

post hoc LSM determined that the hippocampus, but not the pSVZ, showed an injury-induced increase in BrdU-IR cells ($t(23) = 1.775$, $p<.05$; Figure 6, Appendix I).

To isolate injury-induced cell proliferation in the hippocampus of each group, planned comparisons employed independent *t*-tests. At the shortest time point (IMM-2), the ipsilateral and contralateral hippocampus did not differ significantly. At both longer time points (24-2 and 24-168) the ipsilateral hippocampus had a significantly higher density of BrdU-IR cells than the contralateral hippocampus ($t(8) = 2.133$, $p<.05$; ; $t(5) = 2.708$; $p<.05$, respectively) indicating that injury stimulated cell birth and incorporation at these times. Strikingly, no post hoc nor planned comparison between pSVZ hemispheres of any group was found to be significant. Thus, the pSVZ showed no significant injury-induced increase in cell birth. As can be seen in Appendix J, Figure 7 results confirm and extend the findings of Law et al. (2010), indicating that chickadees respond to hippocampal injury with a local upregulation of cell proliferation in the hippocampus itself but not in the stem cell rich pSVZ.

Hypothesis 2

Hypothesis 2 predicted that there would be a significantly higher density of BrdU-IR cells in the pSVZ compared to the hippocampus. This prediction was tested in the aforementioned 2 (brain region) x 2 (hemisphere) repeated measures ANOVA, looking at the main effect of brain region.

Main Effect of Brain Region

Based upon the findings of Law et al. (2010), Lee et al. (2007), and Peterson et al. (2004), it was hypothesized that there would be a significant main effect of brain region. Specifically, the pSVZ was expected to have a higher density of BrdU-IR cells

compared to the hippocampus, regardless of hemisphere. As predicted, Appendix I, Figure 6 shows that the pSVZ has a higher density of BrdU-IR cells than the hippocampus ($F(1,23) = 5.316$, $p<.05$). As the stem cell layer, the pSVZ constantly generates new cells with the potential to migrate toward the hippocampus. It is not surprising, therefore, that this brain region would demonstrate more cell proliferation than the hippocampus.

Planned comparisons employed independent *t*-tests to determine whether the pSVZ of each survival group had greater BrdU-IR cell densities than the hippocampus. However, only the 24-2 group showed clear significance ($F(1) = 9.241$, $p<.05$); both of the other groups yielded non-significant trends (IMM-2 $F(1) = 2.652$, $p = .07$; 24-168 $F(1) = 3.141$, $p = .06$). The lack of robust significance when separating out the individual groups may be a function of the sizeable variability (see Appendix K, Figure 8).

Hypothesis 3

While Law et al. (2010) found that chickadees respond with an increase in injury-induced cell proliferation in the hippocampus, chickadees had fewer BrdU-IR cells in the injured hippocampus than juncos. In contrast, unlesioned chickadees had more BrdU-IR cells in the hippocampus than juncos. This result was unexpected and raised issues concerning the temporal aspects of cell birth and migration following hippocampal injury. It is possible that Law et al. may have "missed" the injury-induced response by investigating only a 7 day survival time. If cell proliferation, migration, and death occur faster in chickadees than juncos, speed of cellular turnover may be an aspect of the adaptive specialization in the hippocampus of food-storing birds.

Hypotheses 3 through 6 were chosen to investigate this possibility (see Appendix L, Figure 9). Specifically, Hypotheses 3 and 4 compared short cell survival times (less than 24 hours), allowing for a time course analysis of cell proliferation. Hypotheses 5 and 6 compared longer cell survival times (greater than 24 hours), allowing for a time course analysis of cell incorporation resulting from the net effect of birth, migration, survival, and death.

Hypothesis 3 predicted significantly higher rates of cell proliferation in the ipsilateral hippocampus in IMM-2 chickadees compared to 24-2 chickadees. Since birds injected with BrdU either immediately or 24 hours post-lesion were all perfused 2 hours later, this hypothesized result would have indicated that the production of new cells began immediately following injury then decreased over time.

To test whether chickadees demonstrated this difference in hippocampal cell proliferation, an independent samples *t*-test was used to compare the density of BrdU-IR cells in the ipsilateral (lesioned) hippocampus at the earliest time points employed by this study (see Appendix D, Table 2 and Appendix L, Figure 9). As shown in Appendix M, Figure 10, no significant difference was found between these groups. Results suggest that the rate of hippocampal cell proliferation (as oppose to cell survival/turnover – see hypotheses 5 and 6) does not decrease significantly within the first 24 hours post-lesion.

Hypothesis 4

Similar to Hypothesis 3, Hypotheses 4 predicted significantly higher rates of cell proliferation in the ipsilateral pSVZ in IMM-2 chickadees compared to 24-2 chickadees. Because cells born in the pSVZ are thought to migrate toward the injured hippocampus,

this hypothesized result would have suggested that a significant number of cells born in the pSVZ leave the pSVZ, migrate toward the hippocampus, or die during the first 24 hours post-injury, resulting in a net decrease.

Results of a single independent samples *t*-test demonstrated no significant difference in the density of BrdU-IR cells in the ipsilateral pSVZ of IMM-2 chickadees and 24-2 chickadees (see Appendix M, Figure 10). Thus, the rate of pSVZ cell proliferation and migration did not decrease significantly within the first 24 hours post-lesion.

<u>Hypothesis 5</u>

Hypothesis 5 sought to compare cell incorporation (i.e., the net result of proliferation, migration, and survival) predicting significantly fewer new cells in the ipsilateral hippocampus of 24-168 chickadees relative to 24-2 chickadees. Kempermann et al. (2004) suggested that initial cell proliferation is not an accurate predictor of net neurogenesis because cellular selection occurs over 7 days, killing most newly born cells. Thus, it was hypothesized that birds should have more cell incorporation when perfused close to the time of injury, compared to those perfused later. This hypothesized result would have been consistent with that observed by Lee et al. (2007) in zebra finches.

In order to determine whether chickadees demonstrated this difference in cell survival in the hippocampus, a single independent samples *t*-test compared the density of BrdU-IR cells in the ipsilateral hippocampus of 24-2 chickadees to 24-168 chickadees (see Appendix D, Table 2 and Appendix L, Figure 9). Surprisingly, results indicated no significant difference in hippocampal cell incorporation between these

groups (see Appendix N, Figure 11), providing further evidence that the rate of hippocampal cell incorporation is relatively constant at least through 168 hours post-lesion.

Hypothesis 6

Hypothesis 6 predicted significantly fewer new cells in the ipsilateral pSVZ of 24-168 chickadees relative to 24-2 chickadees. This expected result would have been consistent with that observed by Lee et al. (2007). A single independent samples *t*-test, determined however, that 24-2 chickadees did not significantly differ in pSVZ cell densities from 24-168 chickadees (see Appendix N, Figure 11). Thus, these results suggest similar incorporation rates over time in the pSVZ. See Appendix O, Table 4 for a summary of the results obtained by this thesis and Law et al. (2010).

CHAPTER IV

DISCUSSION

Law et al. (2010) examined innate and injury-induced cell incorporation in wild-caught food-storing black-capped chickadees and non-storing dark-eyed juncos during the fall food-storing season (see Appendix O, Table 4). While unlesioned chickadees had a higher rate of cell incorporation in the hippocampus and adjacent stem cell rich SVZ compared to non-storing juncos, lesioned chickadees had significantly fewer BrdU-IR cells in the hippocampus compared to lesioned juncos. Only lesioned juncos demonstrated injury-induced increases in cell incorporation in the SVZ, a result similar to that observed in non-storing semi-domesticated zebra finches (e.g., Lee et al., 2007). These results suggest that the processes of stem cell birth, migration, and differentiation varies as a function of injury and species.

While the results obtained by Law et al. (2010) may have indicated that chickadees simply respond to injury with fewer new cells than juncos during the fall, results may have also indicated a much more rapid turnover rate of newly incorporated cells in the chickadee hippocampus. This thesis investigated the possibly of rapid cellular turnover in the chickadee hippocampus and pSVZ by varying the time between injury, mitotic labeling, and survival. By employing short (less than 24 hours) and long (greater than 24 hours) survival times, this thesis was the first to compare temporal

parameters of injury-induced cell proliferation and incorporation in the food-storing chickadee.

Results demonstrated that food-storing chickadees show injury-induced cell proliferation in the hippocampus, but not in the pSVZ, a result which replicates Law et al. (2010; see Appendix O, Table 4). Clearly, chickadees respond to hippocampal injury with a highly localized response. Across all survival times employed by this thesis, the pSVZ failed to demonstrate an injury-induced increase in cell proliferation. Thus, speed of cellular turnover fails to explain the lack of injury-induced cell proliferation in the pSVZ. This lack of injury-induced pSVZ activity is extremely unusual, especially because the pSVZ continuously produces new cells which are thought to migrate to the injured hippocampus.

While this thesis demonstrated a highly localized response to hippocampal injury, injury-induced hippocampal cell proliferation was only observed in chickadees with survival times greater than 24 hours after injury (see Appendix O, Table 4). Chickadees perfused less than 24 hours post-lesion failed to show injury-induced hippocampal cell proliferation. Thus, cell proliferation is not an immediate response to hippocampal injury in chickadees.

Evidently, the mechanism responsible for hippocampal cell proliferation following traumatic brain injury (TBI) in chickadees takes longer than 2 hours, the shortest survival time investigated in this thesis. As mentioned earlier, hippocampal TBI results in both cell death and cell proliferation at the lesion location days following the initial insult. Cell proliferation and apoptosis in the surrounding tissue largely result from the upregulation of cell cycle proteins (e.g., Byrnes & Faden, 2007). Injury-

induced cell cycle activation appears to gain momentum by 24 hours and Jarrad (1986) asserts that the maximal damage occurs within 4 to 5 days following injury. In support, Katano, Masago, Taki, Nakatsuka, Fuse, and Yamada, (2000) and Kobori, Clifton, and Dash (2002) demonstrated a transient upregulation of p21, an S phase inhibitor, between 2 and 24 hours post-injury. Between 24 and 48 hours post-lesion, D-type cyclins (e.g., cyclin D1 and CDK4; which promote entry into G1 of the cell cycle) demonstrate a significant increase in mRNA expression in both neurons and glia (e.g., Byrnes & Faden, 2007; Di Giovanni, Movsesyan, Ahmed, Cernak, Schinelli, Stoica, et al., 2005). Di Giovanni et al. also demonstrated a significant down-regulation of p27, a D-type cyclin inhibitor, 24 hours following TBI. Thus, this highly localized response to hippocampal injury appears to increase over time.

With the survival times employed by this thesis, results indicate that cell proliferation and survival do not change significantly within the first 7 days following hippocampal injury in food-storing chickadees. While data obtained from wild birds are inherently variable, these results suggests that: (1) chickadees may respond to injury with fewer cells compared to juncos during the fall as postulated by Law et al. (2010); or (2) chickadees do, in fact, respond to injury more rapidly than juncos, but not at the time points examined in this thesis. Because food-storing is a seasonally regulated behavior, to address the first possibility, future research could compare injury-induced cell proliferation in the hippocampus of fall-caught and spring-caught chickadees and juncos. If driven by seasonally-related changes in food storing behaviors, chickadees may respond to hippocampal injury with fewer new cells during the fall but "normal" levels of proliferation during the spring. To address the second possibility, future

research could compare injury-induced cell incorporation in chickadees and juncos at precise time points between 24 and 168 hours post-injury.

It remains unclear whether the highly localized response to hippocampal injury observed in chickadees is unique to this species. Future research could compare chickadees to other food-storing birds. Should other food-storing species demonstrate the same highly localized response, local upregulation of new cells could be related to the seasonal food-storing behavior. On the other hand, should other food-storing species demonstrate a similar injury-induced response as juncos and semi-domesticated non-storing zebra finches, it would suggest a species-specific response in chickadees. By examining species differences in response to damage to the hippocampus, a structure vital for long-term memory formation, results could provide important information as to the role behavior plays on the generation of stem cells.

Significance of Research

There is widespread optimism among researchers that brain injury and neurodegeneration are both potentially reparable. However, progress in neural stem cell transplantation has been restricted by both ethical and technical obstacles. Alternatively, neural endogenous precursors may be useful in achieving the same ends while avoiding current ethical constraints. To achieve this objective, a greater understanding of the mechanisms that regulate injury-induced neural endogenous precursors is necessary. With this knowledge, we may yet be able to modulate these processes in the damaged or diseased brain to achieve beneficial, therapeutic results. Our understanding of stem cell biology holds great promise for the repair of damaged brain tissue, not only in birds, but in humans as well. To achieve this goal we must

understand the conditions under which new brain cells are created and delivered. Comparative research will aid in the investigation of methods to enhance recovery following brain injury.

APPENDICES

APPENDIX A

CORONAL SECTION OF NISSL STAINED AVIAN BRAIN

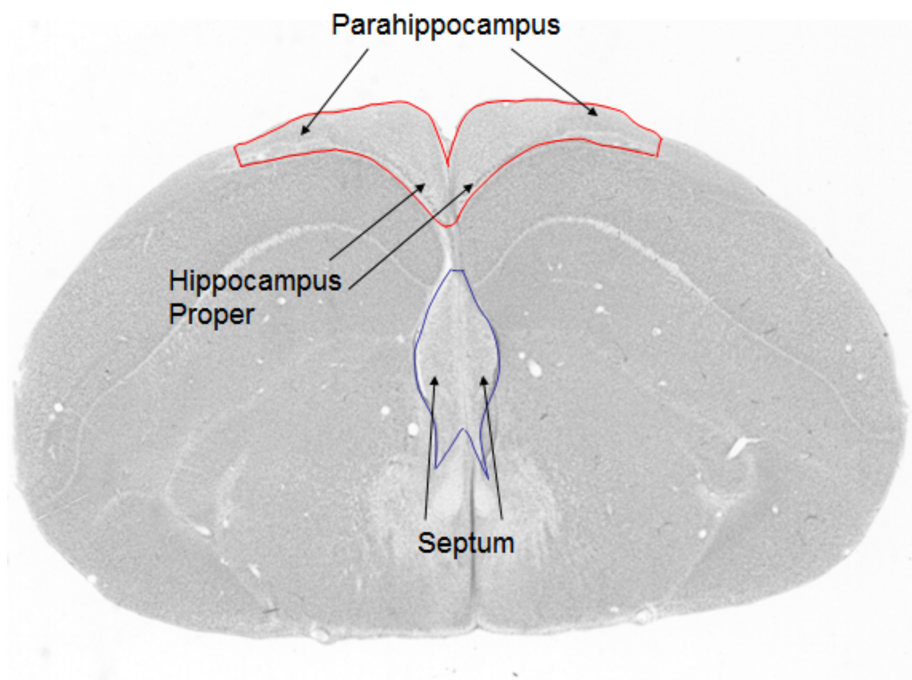

FIGURE 1. Coronal section of Nissl stained avian brain. Hippocampus is outlined in red. Septum is outlined in blue. Internal hippocampal subdivisions are also labeled. The hippocampus proper is the V-shaped ventral area while the parahippocampus is located at the lateral most boundaries.

APPENDIX B

SUMMARY OF RESEARCH EXAMINING SEASONAL DIFFERENCES IN THE AVIAN HIPPOCAMPUS

TABLE 1. Summary of research examining seasonal differences in the avian hippocampus. While some researchers found increases in hippocampal volume (Smulders et al., 1995) and neurogenesis (Barnea & Nottebohm, 1994) during the fall coinciding with peaks in hoarding behavior, others have found spring increases (e.g., Hoshooley & Sherry, 2007, Mitterling et al., 2007).

	Barnea & Nottebohm	**Smulders & DeVoogd**	**Hoshooley & Sherry**	**Lee**
HP Volume	---	Fall > Spring	Spring > Fall	Spring > Fall
HP Cell Counts	No Difference	Fall > Spring	---	---
Newly Born Cells (BrdU-IR)	---	---	---	Fall > Spring
Neurogenesis	Fall > Spring	---	No Difference	---

APPENDIX C

PHOTOMICROGRAPHS ILLUSTRATING CELL PROLIFERATION IN THE ADULT ZEBRA FINCH BRAIN

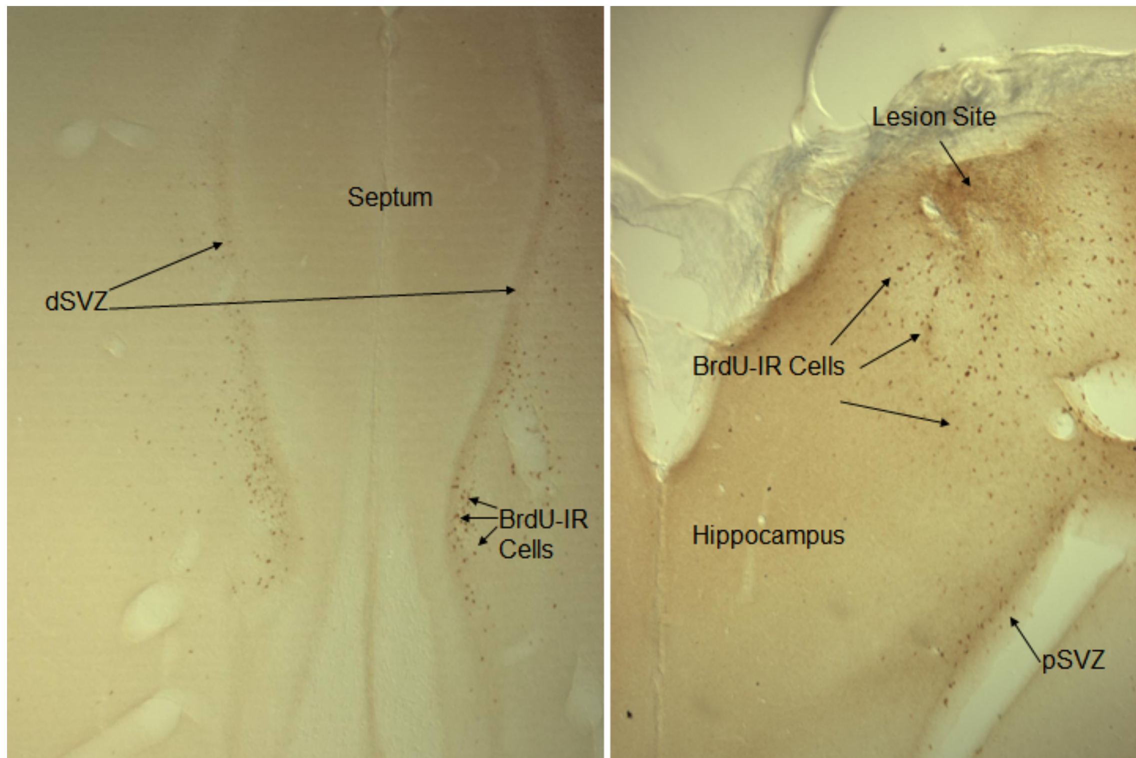

FIGURE 2. Photomicrographs illustrating cell proliferation in the adult zebra finch brain. In this species, hippocampal injury results in an increase in cell proliferation in the hippocampus, proximal SVZ (pSVZ; right) and distal SVZ (dSVZ; e.g., Lee et al., 2007). Brown dots indicate BrdU-IR cells.

APPENDIX D

GROUPS TABLE WITH EXPERIMENTAL MANIPULATIONS

TABLE 2. Groups table with experimental manipulations. This thesis employed 24 black-capped chickadees. Groups were formed to compare BrdU-injection times and survival times, as noted.

GROUP	**LESION**	**BrdU INJECTION POST-LESION**	**SURVIVAL POST-BrdU**	**N**
IMM-2	Unilateral HP	Immediate	2 hrs	9
24-2	Unilateral HP	24 hrs	2 hrs	9
24-168	Unilateral HP	24 hrs	168 hrs	6
			Total N =	24

APPENDIX E

CORONAL SECTION WITH DELINEATED AREAS FOR CELL COUNTING

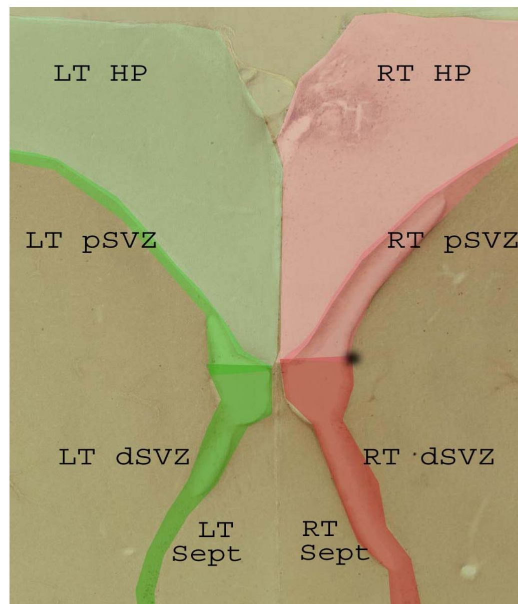

FIGURE 3. Coronal section with delineated areas for cell counting: both hemispheres of the hippocampus (HP) and proximal SVZ (pSVZ). The distal SVZ (dSVZ) and Septum (Sept) are also identified in this slice but were not investigated in this thesis.

APPENDIX F

PHOTOMICROGRAPHS OF CORONAL SECTIONS ILLUSTRATING TYPICAL UNILATERAL HIPPOCAMPAL LESIONS

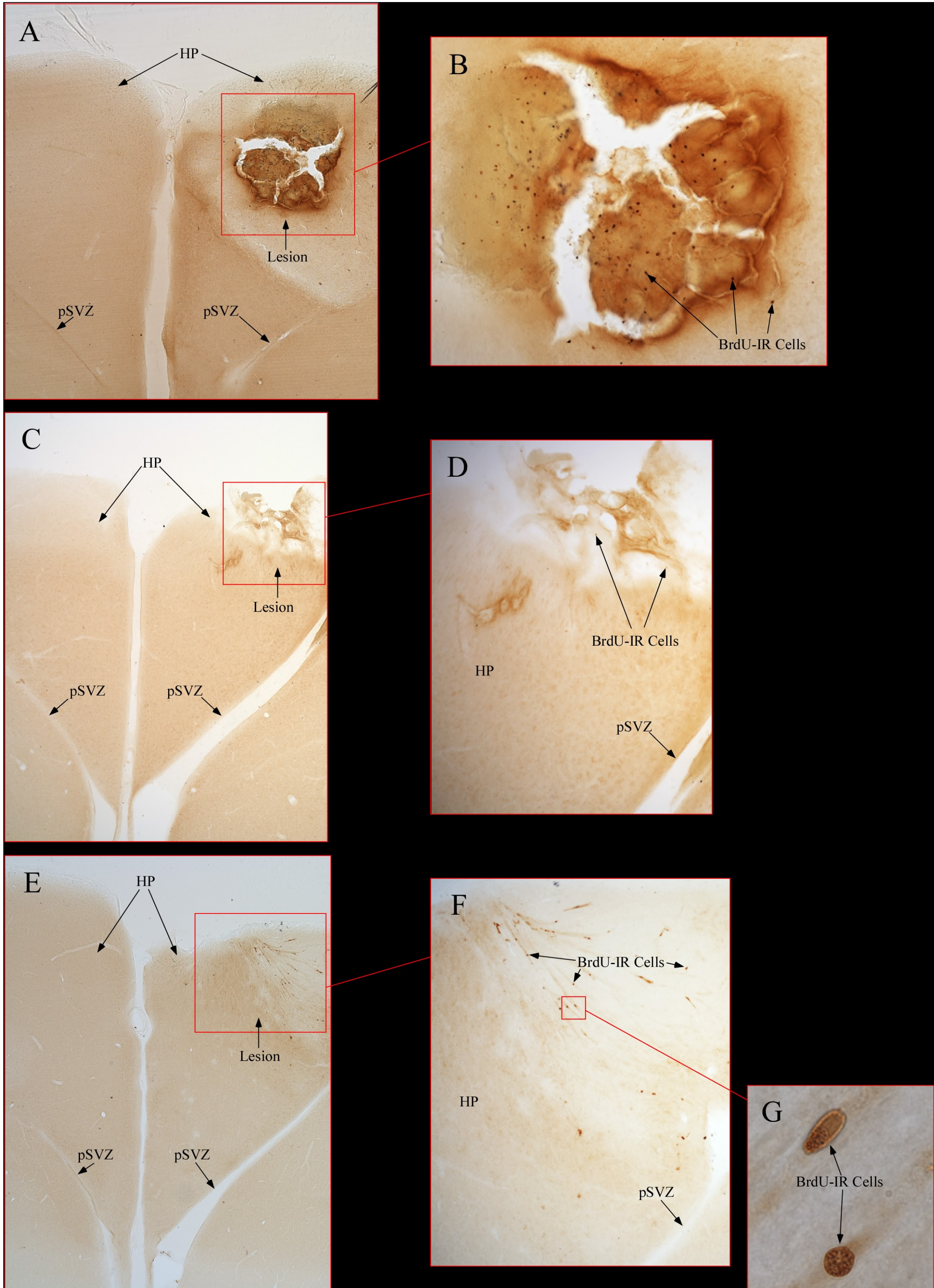
A
HP
Lesion
pSVZ
pSVZ
B
BrdU-IR Cells
C
HP
Lesion
pSVZ
pSVZ
D
BrdU-IR Cells
HP
pSVZ
E
HP
Lesion
pSVZ
pSVZ
F
BrdU-IR Cells
HP
pSVZ
G
BrdU-IR Cells

FIGURE 4. Photomicrographs of coronal sections illustrating typical unilateral hippocampal (HP) lesions. Proximal SVZ (pSVZ) and BrdU-IR cells are denoted. (A) IMM-2 chickadee magnified at 4x, and (B) 10x. (C) 24-2 chickadee magnified at 4x and (D) 10x. (E) 24-168 chickadee magnified at 4x, (F) 10x, and (G) 100x. Brown dots indicated BrdU-IR cells

APPENDIX G

PHOTOMICROGRAPHS OF CORONAL SECTIONS ILLUSTRATING PROXIMAL SUBVENTRICULAR ZONE

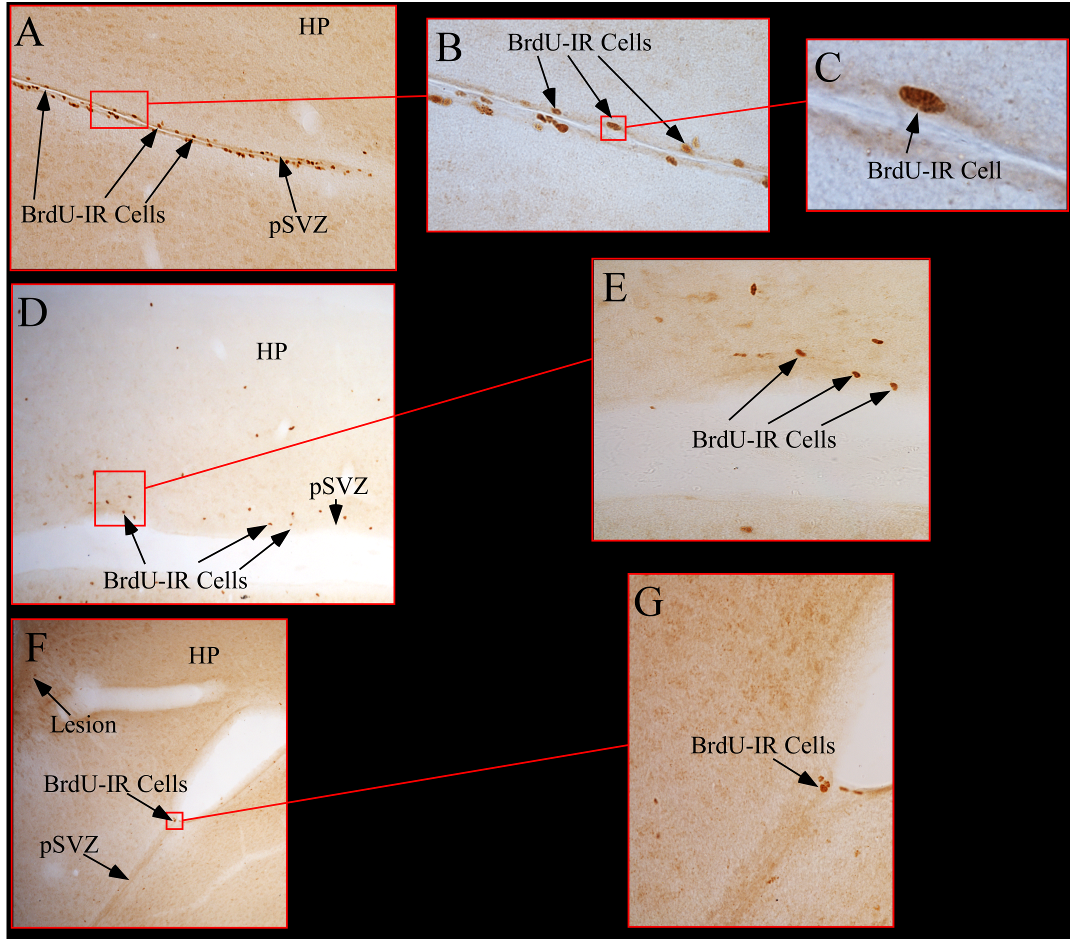

FIGURE 5. Photomicrographs of coronal sections illustrating typical BrdU-IR cells located within the proximal SVZ (pSVZ). (A) IMM-2 chickadee at 10x, (B) 40x, and (C) 100x. (D) 24-2 chickadee at 10x and (E) 40x. (F) 24-168 chickadee at 10x and (G) 100x. Brown dots indicate BrdU-IR cells.

APPENDIX H

TABLE SHOWING MEANS AND STANDARD ERRORS OF DENSITY OF BRDU-IR CELLS IN THE IPSILATERAL AND CONTRALATERAL HIPPOCAMPUS AND PSVZ (HYPOTHESES 1 & 2)

TABLE 3. Means ($\overline{X}$) and standard errors (SE) of density of BrdU-IR cells in the ipsilateral and contralateral hippocampus (iHP; cHP) and pSVZ (ipSVZ; cpSVZ).

GROUP	N	iHP		cHP		Total HP		ipSVZ		cpSVZ		Total pSVZ	
		$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE
IMM-2	9	1.24	1.05	0.34	0.29	1.58	1.03	36.89	13.87	36.65	14.37	73.56	43.99
24-2	9	3.67	1.05	1.41	0.29	5.09	1.73	10.90	13.87	10.40	14.37	21.30	29.56
24-168	6	1.70	1.28	0.66	0.36	2.36	0.87	15.65	16.99	13.91	17.69	6.66	16.09
All	24	2.27	0.65	0.82	0.20	1.55	0.35	21.83	0.48	21.12	8.78	21.48	6.04

APPENDIX I

BAR GRAPH ILLUSTRATING RESULTS FOR HYPOTHESES 1 AND 2

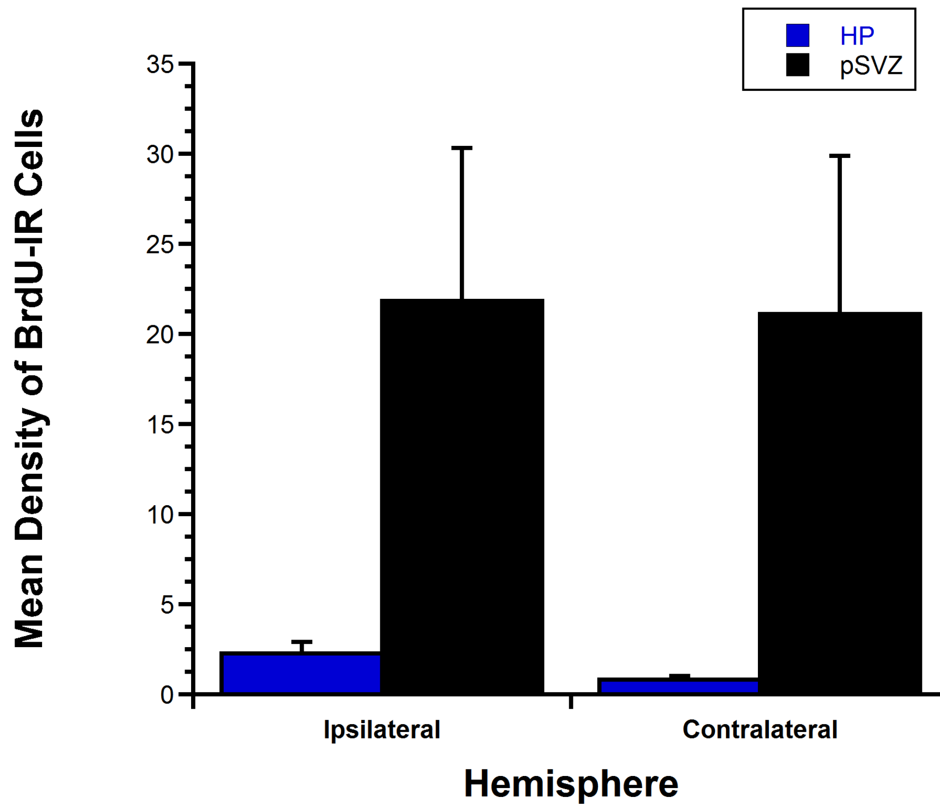

FIGURE 6. Mean differences in density of BrdU-IR cells within the ipsilateral and contralateral hippocampus (HP) and proximal subventricular zone (pSVZ). Injury induced a moderate increase in new cells in the hippocampus only, not in the pSVZ (Hypothesis 1). The pSVZ had more new cells regardless of hemisphere (Hypothesis 2). BrdU injection time and survival times were collapsed. Error bars reflect SEMs.

APPENDIX J

BAR GRAPH ILLUSTRATING RESULTS OF PLANNED COMPARISONS FOR HYPOTHESIS 1

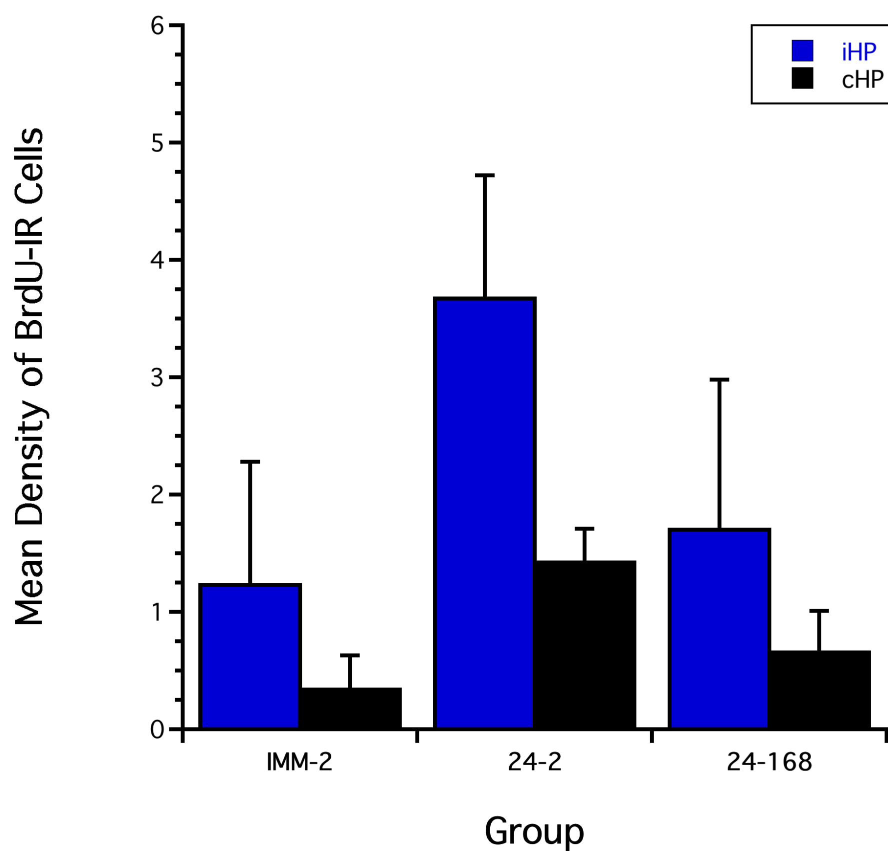

FIGURE 7. Mean density of BrdU-IR cells in the ipsilateral and contralateral hippocampus (iHP, cHP, respectively) by group (Hypotheses 1). Significant injury induced cell birth and incorporation occurred at the longer time points (24-2. 24-168) only. Error bars reflect SEMs.

APPENDIX K

BAR GRAPH ILLUSTRATING RESULTS OF PLANNED COMPARISONS FOR HYPOTHESIS 2

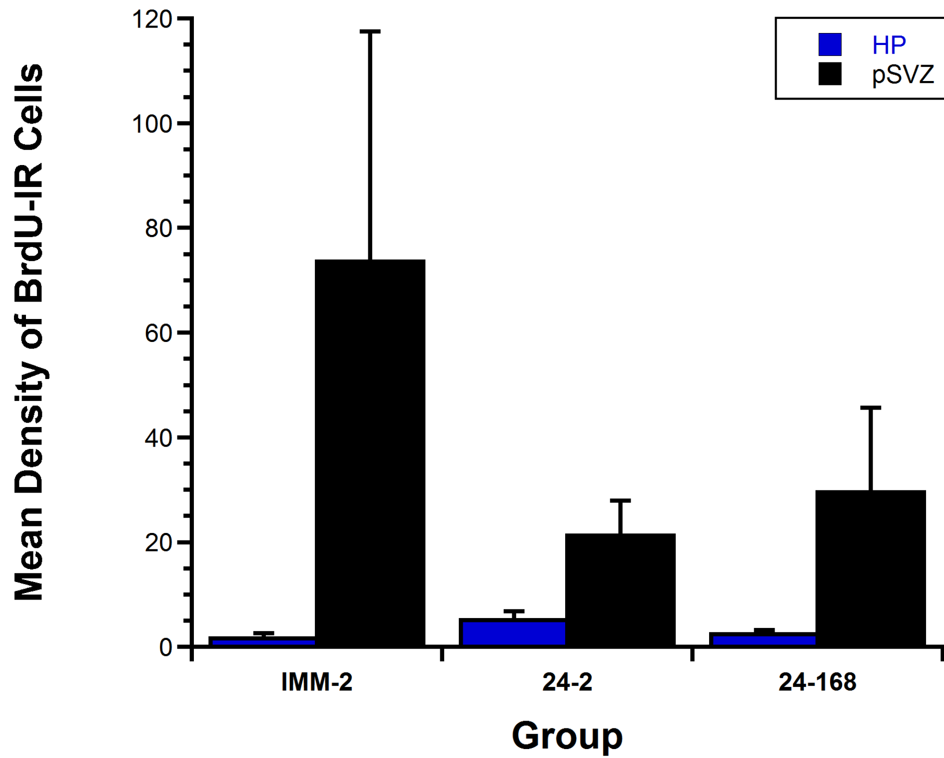

FIGURE 8. Mean density of BrdU-IR cells in the hippocampus (HP) and pSVZ by group (Hypotheses 2). The SVZ had significantly more new cells than the hippocampus in the IMM-2 group; all other group comparisons showed the same, but nonsignificant, trend. Error bars reflect SEMs.

APPENDIX L

DIAGRAM DEPICTING PLANNED COMPARISONS FOR HYPOTHESES 3, 4, 5 AND 6

Planned Comparisons

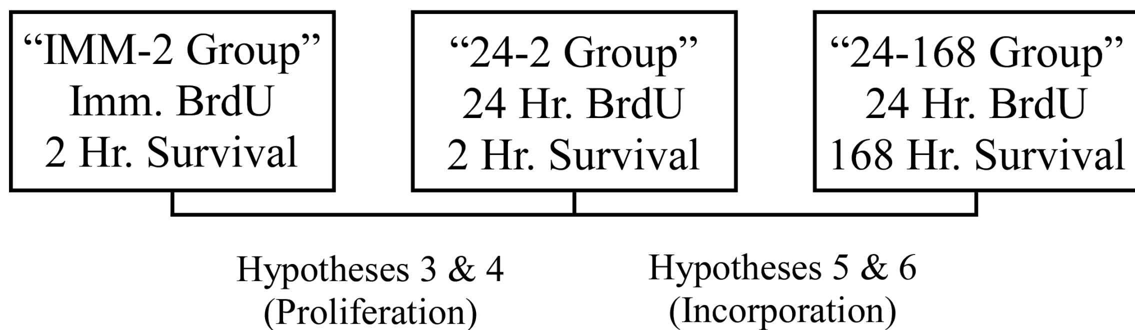

FIGURE 9. Planned comparisons for Hypotheses 3 and 4, as well as Hypotheses 5 and 6. All analyses employed density of BrdU-IR cells in the ipsilateral hemisphere of either the hippocampus (Hypotheses 3 & 5) or pSVZ (Hypothesis 4 & 6). Hypotheses 3 and 4 examined cell proliferation at the short survival times employed. Hypotheses 5 and 6 examined cell incorporation at the long survival times employed.

APPENDIX M

BAR GRAPH ILLUSTRATING RESULTS FOR HYPOTHESES 3 AND 4

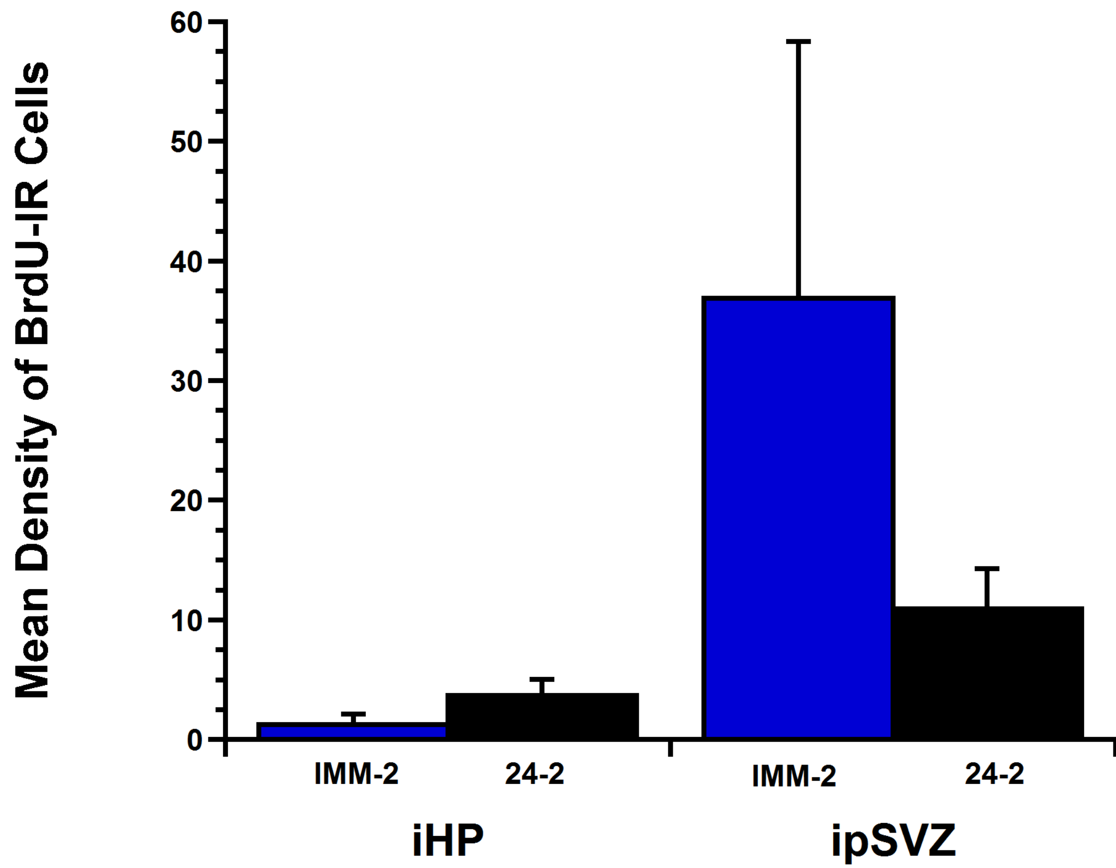

FIGURE 10. Mean differences in density of BrdU-IR cells within the ipsilateral hippocampus (iHP) and ipsilateral proximal subventricular zone (ipSVZ) of IMM-2 and 24-2 chickadees (Hypotheses 3 & 4). A significant difference was not obtained between groups in either structure. Error bars reflect SEMs.

APPENDIX N

BAR GRAPH ILLUSTRATING RESULTS FOR HYPOTHESES 5 AND 6

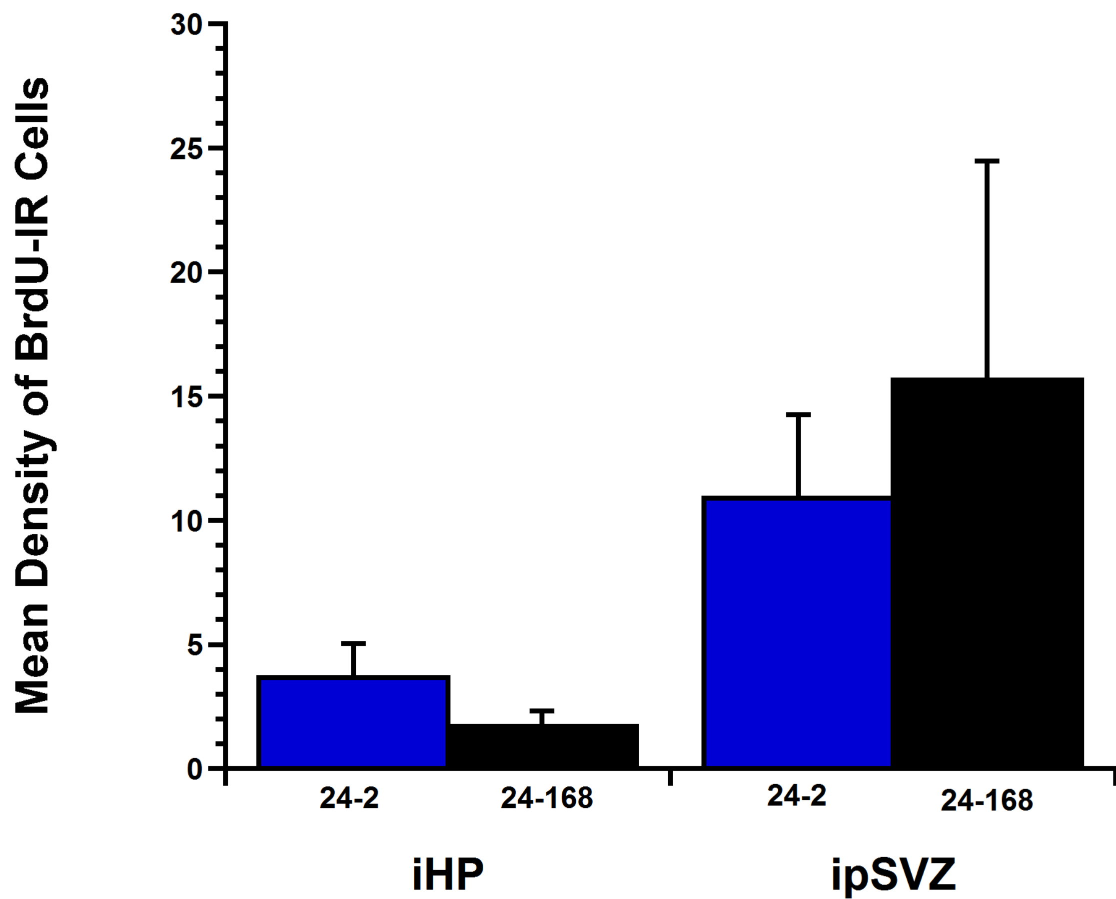

FIGURE 11. Mean differences in density of BrdU-IR cells within the ipsilateral hippocampus (iHP) and ipsilateral proximal subventricular zone (ipSVZ) of 24-2 and 24-168 chickadees (Hypotheses 5 & 6). A significant difference was not obtained between groups in either structure. Error bars reflect SEMs..

APPENDIX O

SUMMARY OF RESEARCH EXAMINING INNATE AND INJURY-INDUCED CELL PROLIFERATION AND INCORPORATION IN CHICKADEES AND JUNCOS

TABLE 4. Summary of research examining innate (uninjured) and injury-induced cell proliferation and incorporation in the hippocampus (HP) and proximal subventricular zone (pSVZ) food-storing black-capped chickadees (BCCs) and non-storing dark-eyed juncos (DEJs).

	Law et al. (2010)	**Gardner Thesis**
Innate HP	BCC > DEJ	----
Innate pSVZ	BCC > DEJ	----
Injury-Induced HP Cell Incorporation	24-168 BCCs 24-168 DEJs	24-2 BCCs 24-168 BCCs
	DEJ > BCC (24-168)	----
Injury-Induced HP Neurogenesis	BCC > DEJ	----
Injury-Induced pSVZ Cell Incorporation	ipSVZ = cpSVZ 24-168 BCCs	ipSVZ = cpSVZ all BCCs
	ipSVZ > cpSVZ 24-168 DEJs	----
Brain Regional Differences	pSVZ > HP	pSVZ > HP
Speed of Cell Proliferation in HP	----	IMM-2 = 24-2
Speed of Cell Proliferation in pSVZ	----	IMM-2 = 24-2
Speed of Cell Incorporation in HP	----	24-2 = 24-168
Speed of Cell Incorporation in pSVZ	----	24-2 = 24-168

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