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Species-specific injury-induced cell proliferation in the hippocampus and subventricular zone of food-storing and non-storing wild birds

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Abstract

Cells are continuously born and incorporated into the adult hippocampus (HP). Adult neurogenesis might act to increase the total number of cells or replace dead cells. Thus neurogenesis might be a primary factor in augmenting, maintaining, or even recovering functions. In zebra finches, HP injury increases cell proliferation in the HP and stem cell rich subventricular zone (SVZ). It is unknown what effect injury has on a species dependent upon the HP for survival in the wild. In food-storing birds recovery of caches is seasonal, necessary for survival, dependent upon the HP and is concomitant with a peak in HP neurogenesis. During the fall, food-storing black-capped chickadees (BCC) and non-storing dark-eyed juncos (DEJ) were captured and given a unilateral penetrating lesion to the HP one day later. On day 3 birds were injected with the mitotic marker BrdU and perfused on day 10. If unlesioned, more BrdU-labeled cells were observed in the HP and SVZ of BCCs compared to DEJs, indicating higher innate cell proliferation or incorporation in BCCs. If lesioned, BrdU-labeled cells increased in the injured HP of both species, however lesions caused larger increases in DEJs. DEJs also showed increases in BrdU-labeled cells in the SVZ and contralateral HP. BCCs showed no such increases on day 10. Thus, during the fall food-storing season, storers showed suppressed injury-induced cell proliferation, and/or reduced survival rates of these new cells compared to non-storers. These species differences may provide a useful model for isolating factors involved in cellular responses following injury.

Keywords

Avian; Neurogenesis; Traumatic Brain Injury; BrdU; NeuN

Introduction

Traumatic brain injury (TBI) influences neuronal incorporation including cell proliferation, migration, differentiation, and survival in the adult mammalian (Gould and Tanapat, 1997; Chirumamilla, Sun, Bullock and Colello, 2002) and adult avian brain (Barnea and Nottebohm, 1994; Scharff, Kirn, Grossman, Macklis & Nottebohm, 2000; Peterson, 2004; Lee, Fernando, Peterson, Allen & Schlinger, 2007; Peterson et al., 2007). Cellular proliferation is thought to be both neuroprotective and neuroregenerative. Protection occurs via *in situ* glial proliferation forming scar tissue, the removal of dead tissue, and the inhibition of apoptotic events (Peterson et al., 2004; Wynne & Saldana, 2004). Regeneration is hypothesized to largely occur through cell proliferation and migration from neurogenic,

stem-cell-rich regions of the subventricular zone (SVZ; Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, et al., 2002; Lee et al., 2007; Peterson et al. 2007). These endogenous adult stem cells have the potential to replace tissue and possibly restore functionality lost due to age, disease, or injury (Gould and Tanapat, 1997; Gage, 2000; Scharff et al., 2000; Dash et al., 2001; Chirumamilla et al., 2002; Monje et al., 2003; Peterson et al., 2004; Merkle et al., 2007). Generally, neurogenesis in the adult brain is thought to be part of the normal neurobiology underlying many forms of learning and memory (Barnea & Nottebohm, 1994; Lee, Miyasato, & Clayton, 1998; Gould, Beylin, Tanapat, Reeves & Shors, 1999; Shors, 2008).

Song birds are an ideal model for the study of adult stem cells in the brain because of innately high levels of telencephalic neurogenesis, including the highly-studied hippocampus (HP; Barnea & Nottebohm, 1994; Peterson et al., 2004; Lee et al., 2007) and the higher vocal center (HVC; Nottebohm, 1985; Alvarez-Buylla, Kirn, & Nottebohm, 1990). Notably, both the HVC and HP are critical for learning and memory tasks in adult birds. HVC lesions impair performance on learned songs (Nottebohm, 1976; Simpson and Vicario, 1990; Scharf et al., 2000), which can recover covariant with neurogenesis (Scharff et al., 2000). HP lesions impair spatial memory, but not working or visual memory (Hampton & Shettleworth, 1996), and impair the ability to find hidden food caches in food-storing birds (Krushinskaya, 1966; Sherry and Vaccarino, 1989). In zebra finches, HP lesions impair memory for a 1-trial associative memory task, increase cell proliferation in the HP and stem cell rich subventricular zone (SVZ), and upregulate the expression of aromatase (estrogen-synthase) in reactive astrocytes and radial glia ipsilateral to the injury (Patel et al., 1997; Peterson et al., 2004; Lee et al., 2007; Peterson et al., 2007). It was unknown, however, what effect injury would have on a species dependent upon the HP for survival in the wild.

In food-storing birds, but not in non-storing birds, neurogenesis and total cell count in the HP covaries with the seasons, peaking in fall at the height of food-caching behavior (Barnea and Nottebohm, 1994; Smulders et al., 2000). For example, food-storing black-capped chickadees (*Poecile atricapillus*) show higher levels of endogenous neurogenesis compared to non-storing house sparrows (*Passer domesticus*; Hoshoooley and Sherry, 2007). Presumably the increased neurogenesis in the HP of food-storing birds is related to ecological demands for spatial learning during caching behavior.

It is unknown what effect injury would have on this vital system of food storers precisely when it is needed the most. From an alternative perspective, it is also unknown what effect injury would have in a species with particularly high levels of neurogenic activity. Here, food-storing black-capped chickadees (BCC) and non-storing dark-eyed juncos (DEJ; *Junco hyemalis*) were compared during the fall food-storing season on innate and injury-induced cell proliferation. Overall, we found that innate levels of cell proliferation were much higher in BCCs, whereas, following lesion the species differences reverse such that DEJs showed significantly higher levels of cell proliferation.

Methods

Subjects

Twenty-two black-capped chickadees and 22 dark-eyed juncos were captured during October and November 2005 in coastal Maine field sites including Bowdoin College Coastal Studies Center (Orrs Island, ME). Birds were collected using potter traps and mist nets, then pair-housed in cages (47 cm height × 34 cm length × 26 cm width) and kept in an outdoor aviary. They were provided with black oil seed and water *ad libitum*.

Experimental Design

The experiment was a fully-crossed $2 \times 2 \times 6$ factorial design, Species by Lesion-Type by Brain Region: Two between-subjects variables included Species (BCC versus DEJ) and Lesion-Type (unilateral HP lesion versus unlesioned). Six Brain Regions were examined as within-subjects variables: the ipsilateral HP (iHP), the contralateral HP (cHP), the ipsilateral proximal SVZ (ipSVZ), the contralateral proximal SVZ (cpSVZ), the ipsilateral distal SVZ (idSVZ), and contralateral distal SVZ (cdSVZ). In lesioned subjects, ipsilateral versus contralateral designations are made relative to the location of the HP lesion. In unlesioned birds the terms ipsilateral and contralateral correspond to the right and left hemispheres, respectively, even though no lesion was present. Counts were made of BrdU-immunoreactive cells (BrdU-IR) in all six Brain Regions.

Hippocampal Lesions

One day after capture, all birds in the lesion group received a single unilateral penetrating lesion to the right hippocampus. Food was deprived for 1 hour and anesthesia was achieved with equithesin (0.0032 ml/g, i.m.). Feathers on top of the head were plucked to expose an area of skin above the skull. Birds were then placed in a stereotaxic apparatus under a binocular microscope. An incision was made using a scalpel separating the skin and exposing the scalp above the Y-sinus. A flap of skull was removed over the Y-sinus and the right hippocampus. Lesion placement was determined using stereotaxic coordinates relative to the bifurcation of the Y-sinus (0, 0, 0) using a zebra finch brain atlas (Nixdorf-Bergweiler and Bischof, 2007) as a guide. A small drill burr (0.5 mm O.D.) was used to create a discrete lesion. The burr was lowered 0.5 mm ventrally into the right hippocampus (2.2 A/P, 1.0 M/L) and removed. The incision was closed and then sealed using ethyl cyanoacrylate. See Figure 1 for a typical lesion in a BCC brain and Figure 2 for a typical lesion in a DEJ.

BrdU Administration and Histology

Two days after being captured, all birds received a single injection (0.005 ml/g) of 5-bromo-2'-deoxyuridine (BrdU; 10 mg/mL in dH₂O) in the breast muscle. The mitotic marker BrdU is a thymidine analog that is incorporated into DNA during the S-phase of mitotic division, and thus used to label newly born cells. Birds remained in their outdoor cages an additional 7 days following BrdU administration. Birds were given an overdose of equithesin (0.08 ml/g, i.m.) and transcardially perfused with a 0.1 M phosphate-buffer solution (PB) followed with 4% paraformaldehyde (v/v). Following standard histology procedures, brains were immediately removed, postfixed in 4% paraformaldehyde (v/v) for 24 hours, cryoprotected in a 30% sucrose solution, and embedded in 8% gelatin. Coronal sections were obtained through the entire telencephalon (40 μ m thick) with a vibratome (Vibratome 3000 Plus, The Vibratome Company, St. Louis, MO). Five equivalent sets of adjacent slices were separated for processing. One set was Nissl stained and one set underwent BrdU immunohistochemistry (IHC). All other sets were cryoprotected (Watson et al., 1986) and stored at -20°C for future processing.

BrdU IHC (Peroxidase-Based)

Free-floating sections were washed for 3×15 min in 0.1 M PB, pH 7.4 to remove residual sucrose and aldehydes. The tissue underwent DNA denaturation in 3 N HCl, and 3×10 min washes in 0.1 M PB, pH 7.4, followed by immersion in 0.036% H₂O₂ for 15 min in order to neutralize endogenous peroxidases. Sections were then treated with 10% normal horse serum in 3% Triton X-100 in 0.1 M PB, pH 7.4, (PBT), for 60 min at room temperature. For primary antibody treatment, sections were incubated in 1:500 BrdU (Roche Diagnostics, Indianapolis, IN; Cat. No. 11299964001) for 24 hours at 4°C. After primary incubation, sections were washed for 3×15 min with 0.1% PBT and treated with a 1:200 biotinylated

horse anti-mouse IgG (Vector Labs, Burlingame, CA; Cat No. BA2000) in 0.3% PBT for 60 min. Additional washes (3×10 min) were performed in 0.1% PBT, and a 1:200 avidin-biotin complex (Vector Labs; Cat No. PK6100) in 0.3% PBT was administered for 90 min at room temperature. After subsequent washing (3×15 min) in 0.1% PBT, immunoproducat was visualized using diaminobenzidine (DAB; Sigma; Cat No. D5905) to stain it brown. Tissue was then immersed overnight in 0.1 M PB, pH 7.4, at 4°C. The following day, slices were mounted on gelatinized slides, coverslipped, and allowed to dry for at least four days.

BrdU-IR Cell Counts

Immunoreactive cells were counted using procedures described in Lee et al. (2007). Cells were visualized using DIC illumination on a Nikon E-800 microscope using NeuroLucida software (MBF Bioscience, Inc.). Brains were assigned a unique histology number by one researcher and counted by another to ensure that all counts were done blind to the conditions of the experiment. For each brain, approximately 12 coronal tissue slices containing the hippocampus were counted. In order to obtain cell counts, the brain regions of interest were established by drawing contour lines around their outer boundaries for all six regions: iHP, cHP, ipSVZ, cpSVZ, idSVZ & cdSVZ (see Figures 1 & 2). Regions included the ipsilateral and contralateral hemispheres of the hippocampus (iHP, cHP) and adjacent proximal SVZ (ipSVZ, cpSVZ) forming its ventral border. Contour lines for the nonadjacent distal SVZ (idSVZ, cdSVZ) encompassed the hemispheres of the lateral ventricle extending ventral from the ventromedial boundary of the hippocampus to the ventral-most aspect of the ventricle.

In unlesioned birds, an additional region was counted in the telencephalon within the nidopallium, to test whether or not differences in cell proliferation between species reflects a generalized process throughout the telencephalon, or may be more specific to the HP and SVZ. Three slices were chosen per brain; all of which included the hippocampus and septum. The “control” telencephalic region was delineated bilaterally by a 500×500 μm contour box. The dorsal-medial corner began 500 - 1000 μm lateral to the wall of the ventricle. The borders were contained by the lamina mesopallialis (LaM) dorsally and the lamina pallio-subpallialis (LPS) ventrally (Reiner et al., 2004).

BrdU-IR cells falling within contours were counted if (1) greater than 3 μm in diameter; and (2) darkly stained and round or oval in appearance following the morphological criterion of Gould et al. (1999). Positively-labeled BrdU-IR cells appear brown and roundish (see Figure 3). Cells falling within 50 μm of the internal lumen of the lateral ventricles were designated as SVZ cells. The areas under investigation were clearly delineated and all BrdU-IR cells lying within those areas were counted rather than estimated using random sampling procedures. This form of counting has proven to be appropriate (Tramontin et al., 1998), even when compared to unbiased stereological methods of estimation. Since absolute size differed between regions and species, the areas of the brain regions surrounded by the contour lines were obtained in order to calculate the densities of BrdU-IR cells per unit area.

Double-Labeling Fluorescence IHC for BrdU & NeuN

A small subset of the tissue was used to assess the proportion of new cells that were double-labeled with the neuronal-specific protein NeuN (Mullen et al., 1992; Kempermann et al., 2004). Free-floating brain slices were washed for 3×15 min in 0.1M PB, pH 7.4 to remove residual sucrose and aldehydes. Tissue then underwent DNA denaturation in 1N HCl for 30 min and washed for 3×5 min in 0.1M PB. Tissue was immersed in 0.5% H_2O_2 to neutralize endogenous peroxidases and washed for 3×15 min in 0.1MPB. Slices were incubated in 10% normal donkey serum in 0.3% PBT for 60 min and washed for 5×15 min in 0.1% PBT. Slices were then incubated in a 1:500 concentration of the primary antibody, anti-

BrdU (Roche Diagnostics), in 0.3% PBT overnight. The following day slices were washed for 3×15 min in 0.1% PBT and immersed in 1:200 biotinylated donkey anti-mouse IgG (Jacksonimmuno, West Grove, PA; Cat. No. 715-065-150) in 0.3% PBT for 60 min. After washing for 3×10 min in 0.1% PBT, the tissue was treated with Streptavidin-conjugated AlexaFluor 488 (Invitrogen, Eugene, OR; Cat No. S-11223) with immersion in a 1:500 concentration in 0.3% PBT for 60 min and washed 3×15 min in 0.1% PBT, and stored at 4°C overnight. To make certain all epitopes were occupied before applying the NeuN primary antibody, tissue was immersed in a 1:500 concentration of mouse IgG (Jacksonimmuno; Cat. No. 015-000-002) in 0.3% PBT for 60 min; washed 5×15 min in 0.1% PBT; incubated in 40 µg/ml Fab donkey anti-mouse IgG (Jacksonimmuno; Cat. No. 715-007-003) for 60 min; and washed 5×15 min in 0.1% PBT. Tissue was immersed in 4% paraformaldehyde for 1 hr to fix BrdU labeling, washed for 5×15 min in 0.1% PBT; immersed in 10% donkey serum in 0.3% PBT; and washed for 5×15 min in 0.1% PBT. Primary incubation for NeuN was accomplished with a 1:100 concentration of mouse anti-NeuN (Millipore, Temecula, CA; Cat. No. MAB377) in 0.3% PBT overnight and washed for 3×15 min in 0.1% PBT. Alexa594 donkey anti-mouse IgG (Invitrogen, Eugene, OR; Cat. No. A-21203) was then applied at a 1:100 concentration in 0.3% PBT for 1 hr and washed for 3×15 min in 0.1M PB. Slices were mounted on gelatin-coated slides and left to dry overnight in a darkroom. Slides were coverslipped with PROLONG (Invitrogen; Cat. No. P36934) and Krystalon (Fisher Scientific; Cat. No. 64969/71) was applied to seal the coverslips.

Fluorescence imaging was performed with a confocal microscope (Olympus Fluoview 1000) equipped with excitation lines at 488 nm (argon ion laser), 559 (green/yellow diode laser) and a 405 nm (blue diode laser). A z-series of images taken from the HP, pSVZ and dSVZ were acquired with a digital camera at 20× and saved for offline analysis. Cell counts and z-series analyses for double-labeling of BrdU and NeuN were performed using ImageJ software (NIH). Cells were counted as double-labeled if a BrdU-IR cell was centered within a NeuN-IR cell, and appeared yellow throughout the z-series. These cells were considered to be newly generated neurons (Mullen et al., 1992; Kempermann et al., 2004). Image files were assigned a unique number by one researcher and counted by two different researchers to ensure that all counts were done blind to the conditions of the experiment, and to assess inter-rater reliability.

Statistical Analysis

To correct for variation in individual brain sizes between regions and species, all BrdU-IR cell counts were expressed as the number of positive cells divided by the area (mm²) measured within the contour lines used to delineate counting regions. Effect sizes of lesion-induced cell proliferation between BCCs and DEJs were calculated by comparing lesioned and unlesioned groups based on Cohen's d (Cohen, 1988). Double-labeling counts were expressed as a percentage calculated as the number of double-labeled BrdU-IR cells in a given brain region divided by the total number of BrdU-IR cells found in that same region. ANOVAs and paired t-tests were conducted using SPSS 15.0 and 16.0 software for Windows (SPSS, inc.) to test for significant differences. Analyses were determined to be significant if $p < 0.05$ and notable trends were identified as $p < 0.10$.

Results

Due to tissue quality, the final sample sizes were nine subjects in the lesioned and unlesioned DEJ groups, and the lesioned BCC group. The unlesioned BCC group had ten subjects. In lesioned subjects, damage was restricted to the HP region and did not penetrate into the SVZ (Figures 1 & 2). BrdU-IR cells were found in all regions investigated: the HP, pSVZ, and dSVZ (Figures 1 - 3). Notably, BrdU-IR cells were also observed in the septum

of both species, but were not systematically explored for this article. All inferential statistical analyses below were based on cell density (per mm² of tissue; see Materials and Methods).

Main Effects of Species and Lesion

Combining all groups, an overall analysis of the effects of species and lesion condition revealed several main effects. Unilateral hippocampal lesions caused a significant increase in BrdU-IR cells, $F(6,28) = 8.987$, $p < 0.001$. Cell counts of BrdU-IR cells eight days following BrdU administration does not merely reflect proliferation rates however. Thus, an increase in the number of BrdU-IR cells demonstrates that the brain responded to injury with an upregulation of cell incorporation, certain to be the net result of many different processes including cell proliferation, migration, differentiation, and survival. Additionally, the number of BrdU-IR cells was dependent on species, $F(6, 28) = 4.639$, $p < 0.01$. Notably, food-storing BCCs had greater numbers of BrdU-IR cells in the SVZ regions; whereas non-storing DEJs had more cells in the iHP (see Table 1). However, a significant interaction effect of species and lesion, $F(6,28) = 3.866$, $p < 0.01$, suggests that the brains of BCCs and DEJs respond differently to injury, explored further below.

Main Effects of Brain Region and Hemispheres

The number of BrdU-IR cells was significantly different between the three brain regions (HP, pSVZ and dSVZ), $F(2, 66) = 39.45$, $p < 0.001$. The highest to lowest levels were observed in the dSVZ, pSVZ and then the HP, respectively, as can be clearly seen in Table 1. There was also a brain region by species interaction, $F(2,66) = 5.504$, $p < 0.01$, indicating the relationship of cell incorporation rates in the three brain regions depends on the species examined. Mostly, the levels of cell proliferation in the BCC SVZ are particularly high in relation to the HP.

There were significantly more BrdU-IR cells in the hemisphere ipsilateral to the lesion site, compared to the contralateral side, $F(1, 33) = 19.20$, $p < 0.001$. That is, in all brain regions, and in both species, the hemisphere on the same side of the lesion had the highest numbers of BrdU-IR cells. This indicates that lesion-induced cytogenesis has a greater effect on the lesioned hemisphere as a whole. There was also a significant hemisphere by lesion effect, $F(1,33) = 6.559$, $p < 0.05$, a hemisphere by species effect, $F(1,33) = 4.452$, $p < 0.05$, and a hemisphere by lesion by species effect, $F(1,33) = 7.956$, $p < 0.01$. These effects primarily reflect the fact that there were no hemisphere effects in unlesioned birds (see below), and that BCC and DEJ brains responded differently to lesions. No other main effects were observed (p 's > 0.10).

Innate (Unlesioned) Cell Proliferation Levels: Species-Specific Rates

Here an analysis was made of only those birds that did not receive lesions in order to test for differences in innate cell incorporation rates between food-storing BCCs and non-storing DEJs. Notably, in unlesioned birds there were no significant differences observed between hemispheres, no hemisphere by species interaction, no brain region by hemisphere interaction and no brain region by hemisphere by species interaction (p 's > 0.1). This suggests that there is no hemisphericity involved with innate levels of adult cell proliferation rates in these avian brains.

Most notably there was a significant main effect of species on the innate levels of BrdU cells observed, $F(1, 17) = 5.634$, $p < 0.05$. In the HP, pSVZ and dSVZ, food-storing BCCs had more BrdU-IR cells than non-storing DEJs (Figure 4). Consistent with the overall results, there was a main effect of brain region in unlesioned birds, $F(2,34) = 19.588$, $p < 0.001$.

Again, the highest to lowest levels of BrdU-IR cells were in the dSVZ, pSVZ and HP respectively (Figure 4).

To test whether this effect is due to a generalized increase in cell proliferation throughout the telencephalon, counts were made in a region of the telencephalon outside the hippocampal system within the nidopallium. We found no significant differences in BrdU-IR densities (per mm²) between BCCs ($M = 5.13 \pm 1.45$ SEM) and DEJs ($M = 5.56 \pm 1.33$ SEM) in the telencephalon, $t(17) = -0.212$, $p = 0.834$. This suggests that differences in BrdU-IR densities between BCCs and DEJs found in the HP and SVZ does not reflect a generalized increase in cytogenesis throughout the telencephalon.

Lesion-Induced Cell Proliferation Levels: Species-Specific Rate Responses

A separate analysis was made only of those birds that received a unilateral hippocampal lesion in order to examine differences in injury-induced cell incorporation rates between food-storing BCCs and non-storing DEJs. Overall, in lesioned birds, there was a significant effect of hemisphere, with the ipsilateral (lesion site) showing the highest levels of BrdU-IR cells, $F(1,16) = 22.130$, $p < 0.001$. There was also a significant hemisphere by species interaction, $F(1,16) = 11.162$, $p < 0.01$. In BCCs there was a significant difference between the ipsilateral and contralateral HP, $t(8) = 3.715$, $p < 0.01$, but not between the hemispheres of the proximal or distal SVZ (p 's > 0.10 ; Figure 5). Comparatively, DEJs showed significant differences between the ipsilateral and contralateral hemispheres in all three brain regions (Figure 6): the HP, $t(8) = 5.919$, $p < 0.01$; the pSVZ, $t(8) = 2.734$, $p < 0.05$; and the dSVZ, $t(8) = 3.319$, $p < 0.05$. This indicates that injury is having a localized effect in BCCs, but a more wide-spread effect in DEJs.

The differences between BCC and DEJ responses to injury is particularly clear when the data is transformed to effect sizes (see Materials and Methods) comparing lesioned and unlesioned birds (Figure 7). In BCCs only the lesion site, the iHP, shows a positive effect size that was large by convention (greater than 0.8). However, all other brain regions in BCCs had *negative* small to medium (less than 0.8) effect sizes. That is, the non-lesioned areas of the BCCs did not show increased rates of cell incorporation following injury. In DEJs, however, both the ipsilateral and contralateral HP and pSVZ had large or very large effect sizes. Moreover, all regions in DEJs had a positive effect size. This is especially notable suggesting the non-storing DEJs have both a greater response to injury at the lesion site, and a greater response in brain regions not directly damaged by the lesion—that is, a generalized increase in cell incorporation rates.

Proportion of Cells with Dual Labeling for BrdU and NeuN after Lesions

A small subset of the tissue ($n = 4$, 2 from each species in the lesioned group) underwent fluorescence labeling for BrdU and NeuN to examine the proportion of new cells that are neurons in each brain region. See Figure 8 for an example of the confocal images used to determine dual labeling. Confocal images were counted by two independent researchers (inter-rater reliability = 0.959, $p < 0.001$). BrdU-IR cells that have begun to differentiate and express NeuN are considered fated to become neurons, whereas cells that only express BrdU may have differentiated into glia, or have an indeterminate fate and may eventually differentiate into glia or neurons. In both species, cells were found that were dual-labeled for BrdU and NeuN, located almost exclusively in the HP; see Table 2. Consistent with counts from peroxidase labeling, overall BrdU counts were higher (more than 3 times) in lesioned DEJs compared to BCCs. Notably, BCCs had twice the proportion of BrdU-IR cells expressing NeuN. Also note that a few cells were dual-labeled in the pSVZ of the BCC. Since it is unlikely that cells found in the SVZ would be expressing neuronal markers, this may be due to the fact that operationally, pSVZ cells were counted extending up to 50 μ m

into the tissue towards the HP. A small number of cells positioned at the outermost extent of that 50µm could be pSVZ cells that have begun migrating into the HP thus they could already be expressing a neuronal phenotype.

Discussion

Summary of Main Findings

Wild birds were captured and their brains were examined for BrdU-IR cell incorporation levels following a unilateral hippocampal lesion or no lesion. Two species were examined: food-storing black-capped chickadees (BCCs) and non-storing dark-eyed juncos (DEJs). Several interesting results emerged. First, in unlesioned birds, food-storing BCCs showed higher levels of cell incorporation in the HP, pSVZ and dSVZ compared to non-storing DEJs. Second, lesions caused an increase in cell incorporation at the lesion site in both species. Third, surprisingly, DEJs had a *greater* increase in cell incorporation levels after lesions compared to BCCs. Notably, only DEJs showed increases in cell incorporation levels in the HP contralateral to the lesion site and increased levels of cells in the SVZ, unlike BCCs. Fourth, roughly a third of BrdU-IR cells in the BCC HP and a fifth of BrdU-IR cells in the DEJ HP were dual-labeled for NeuN and BrdU; very few cells were dual-labeled in the SVZ in either species.

Innate Levels of Cell Proliferation in Food-Storing BCCs versus Non-Storing DEJs

Based on previous research (Barnea and Nottebohm, 1994, 1996), it was hypothesized that during the fall, when food-storing behavior is at its maximum, unlesioned food-storing BCCs would show more BrdU-IR cells in the HP and SVZ when compared to unlesioned non-storing DEJs. The HP generates new cells throughout adulthood and is involved in spatial learning critical to food-storing behavior (Sherry and Vaccarino, 1989; Hampton and Shettleworth, 1996). Here the SVZ was also examined because it is a stem-cell rich region believed to be responsible for the production of new cells throughout development as well as adulthood, and is a good indicator of stem-cell activity in the adult brain. Additionally, these cells are thought to migrate to, and incorporate themselves into HP. The present results and those of a previous study (Hoshooley and Sherry, 2007) are consistent with the hypothesis that food-storing species have higher levels of cell incorporation than non-storing species. These species-specific differences possibly reflect an adaptive specialization in food-storing species that enhances spatial learning critical to survival, and contributes to the success of caching behavior.

Injury-Induced Cell Proliferation in the Adult Brain

This lab has previously demonstrated that unilateral hippocampal lesions induce significant increases in cell proliferation in vivarium-housed zebra finches (Lee et al., 2007; Peterson et al., 2007). The current data indicates that this is also true of BCCs and DEJs captured in the wild, however the extent of the effects differs rather dramatically in the food-storing BCC.

When comparing the hemispheres, lesioned DEJs had more BrdU-IR cells in all regions investigated—HP, pSVZ, and dSVZ—in the hemisphere ipsilateral to the injury, thus precisely replicating effects seen in zebra finches. Lesioned DEJs also showed more BrdU-IR cells in the contralateral HP and pSVZ when compared to unlesioned DEJs. These results are consistent with research in non-storing zebra finches (Law et al., 2006; Velazquez et al., 2008). DEJs and zebra finches respond to injury in the HP and pSVZ in both the ipsilateral and contralateral hemispheres. This suggests that perhaps the same injury-response mechanism is present in DEJs and zebra finches, a non-storing wild bird and a non-storing semi-domesticated bird raised in the laboratory. Although the exact function of this injury-induced response has yet to be determined, evidence is accumulating that it may be part of

the brain's attempt to repair itself and recover lost functions (Peterson, et al., 2004; Lee et al., 2007; Peterson et al., 2007).

It was also expected that, although the number of new cells might differ, the lesioned BCCs would exhibit essentially the same pattern of injury-induced cell proliferation or incorporation. However, results clearly showed that unilateral hippocampal injury resulted in an increase in new cell incorporation only in the lesioned hippocampus itself. In striking contrast to the results found in the lesioned DEJs and previous research on lesioned zebra finches (Peterson et al., 2004; Law et al., 2006; Lee et al., 2007; Peterson et al., 2007; Velazquez et al., 2008), there is no increase in the number of new cells as a result of injury in the stem-cell rich subventricular zone (pSVZ or dSVZ) of food-storing BCCs or in the contralateral hippocampus. These results indicate that BCCs differ in their response to TBI by perhaps restricting the response to the immediate area of injury. When compared to their lesioned counterparts, unlesioned BCCs actually had slightly higher cell densities in the SVZ, although these differences were not significant (see Figure 7).

For food storers during food-storing season, this could indicate several possibilities. First, cell proliferation and incorporation rates may already be at maximum levels and cannot be further increased by TBI. This possibility is not consistent with the fact that there were increases in BrdU-IR counts in the ipsilateral HP, though it could be true in other brain regions. Second, injury may act to suppress stem-cell division rather than enhance it. This scenario suggests that BCCs have a suppression of what would normally be an enhancement of new cells, as seen in both DEJs and zebra finches. Third, newly born cells in the SVZ may more rapidly migrate out and/or die. It may be the case that in food-storing species it is advantageous to restrict the incorporation of new cells, possibly to preserve recently acquired spatial memories.

Studying Factors Mediating Response to TBI

Beyond the neuroecological relevance of these findings, comparisons of these species provide a unique model for understanding the factors involved in regulating new cells in the adult brain. The differences in cell proliferative and incorporative responses to TBI in the species provides an opportunity to further study biological factors involved in suppressing and/or enhancing the birth and survival of new cells in the adult brain. For example, it would be worth knowing whether these species differ in their levels of aromatase following injury, and if these differing levels correlate with the levels of new cells in the HP and SVZ (Peterson, et al., 2004; Lee et al., 2007; Peterson et al., 2007).

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References

- Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. *The J Neurosci.* 2002; 22(3):629–634.
- Alvarez-Buylla A, Kirn JR, Nottebohm F. Birth of projection neurons in adult avian brain may be related to perceptual or motor learning. *Science.* 1990; 249(4975):1444–1446. [PubMed: 1698312]
- Alvarez-Buylla, Nottebohm F. Migration of young neurons in adult avian brain. *Nature.* 1988; 335:353–354. [PubMed: 3419503]
- Barnea A, Nottebohm F. Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. *Proc Natl Acad Sci.* 1994; 91:11217–11221. [PubMed: 7972037]

- Barnea A, Nottebohm F. Recruitment and replacement of hippocampal neurons in young and adult chickadees: An addition to the theory of hippocampal learning. *Proc Natl Acad Sci.* 1996; 93:714–718. [PubMed: 11607626]
- Chirumamilla S, Sun D, Bullock MR, Colello RJ. Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. *J Neurotrauma.* 2002; 19:693–703. [PubMed: 12165131]
- Cohen, J. Statistical power analysis for the behavioral sciences. New York: Academic Press; 1988.
- Dash PK, Mach SA, Moore AN. Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res.* 2001; 63:313–319. [PubMed: 11170181]
- Gage FH. Mammalian neural stem cells. *Science.* 2000; 287:1433–1438. [PubMed: 10688783]
- Goldman SA, Nottebohm F. Neuronal production, migration and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci.* 1983; 80:2390–2394. [PubMed: 6572982]
- Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci.* 1999; 2:260–265. [PubMed: 10195219]
- Gould E, Tanapat P. Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult rat. *Neuroscience.* 1997; 2:427–436. [PubMed: 9284346]
- Hampton RR, Shettleworth SJ. Hippocampal lesions impair memory for location but not color in passerine birds. *Behav Neurosci.* 1996; 110(4):831–835. [PubMed: 8864273]
- Hoshooley JS, Phillmore LS, Sherry DF, MacDougall-Shackleton SA. Annual cycle of the black-capped chickadee: Seasonality of food-storing and the hippocampus. *Brain Behav Evol.* 2007; 69:161–168. [PubMed: 17106193]
- Hoshooley JS, Sherry DF. Greater hippocampal neuron recruitment in food-storing than in non-food-storing birds. *Dev Neurobiol.* 2007; 4:406–414. [PubMed: 17443797]
- Kempermann G, Jessberger S, Steiner K, Kronenberg G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* 2004; 27(8):447–452. [PubMed: 15271491]
- Krushinskaya N. Some complex forms of feeding behavior of nutcracker caryocaractes, after removal of old cortex. *Zhurnal Evoluzionni Biochimii y Fisiologgia.* 1966; 2:563–568.
- Law, LM.; Lee, DW.; Menjivar, J.; Chapleau, MN.; Velazquez, R. Injury-induced cell proliferation in the adult zebra finch hippocampus: Sex differences and time course comparisons. Society for Neuroscience Annual Meeting; Atlanta, GA. 2006. Program Number 658.22
- Lee DW, Fernando G, Peterson RS, Allen TA, Schlinger BA. Estrogen mediation of injury-induced cell birth in neuroproliferative regions of the adult zebra finch brain. *Dev Neurobiol.* 2007; 67:1107–1117. [PubMed: 17565708]
- Lee DW, Miyasato LE, Clayton NS. Neurobiological bases of spatial learning in the natural environment: Neurogenesis and growth in the avian and mammalian hippocampus. *Neuroreport.* 1998; 9:15–26. [PubMed: 9592040]
- Merkle FT, Mirzadeh Z, Alvarez-Buylla A. Mosaic organization of neural stem cells in the adult brain. *Science.* 2007; 317:381–384. [PubMed: 17615304]
- Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science.* 2003; 302:1760–1764. [PubMed: 14615545]
- Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development.* 1992; 116:201–211. [PubMed: 1483388]
- Nottebohm F. Neural lateralization of vocal control in a passerine bird. *J Exp Zool.* 1976; 177:229–262. [PubMed: 5571594]
- Nottebohm F. Neuronal replacement in adulthood. In: Hope for a new neurology. *Ann NY Acad Sci.* 1985; 457:143–161. [PubMed: 3913361]
- Nixdorf-Bergweiler, BE.; Bischof, HJ. A stereotaxic atlas of the brain of the zebra finch, *Taeniopygia guttata*, with special emphasis on telencephalic visual and song system nuclei in transverse and sagittal sections. Bethesda, MD: National Library of Medicine (US), National Center for Biotechnology Information; 2007.
- Nottebohm F. A brain for all seasons: Cyclical anatomical changes in song control nuclei of the canary brain. *Science.* 1981; 214:1368–1370. [PubMed: 7313697]

- Patel SN, Clayton NS, Krebs JR. Hippocampal tissue transplants reverse lesion-induced spatial memory deficits in zebra finches (*Taeniopygia guttata*). *J Neurosci*. 1997; 17:3861–3869. [PubMed: 9133404]
- Peterson RS, Fernando G, Day L, Allen TA, Chapleau JD, Menjivar J, Schlinger BA, et al. Aromatase expression and cell proliferation following injury of the adult zebra finch hippocampus. *Dev Neurobiol*. 2007; 67:1867–1878. [PubMed: 17823932]
- Peterson RS, Lee DW, Fernando G, Schlinger BA. Radial glia express aromatase in the injured zebra finch brain. *J Comp Neurol*. 2004; 475:261–269. [PubMed: 15211466]
- Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, et al. Revised nomenclature for avian telencephalon and some related brainstem nuclei. *J Comp Neuro*. 2004; 47:377–414.
- Scharff C, Kirn JR, Grossman MR, Macklis JD, Nottebohm F. Targeted neuronal death affects neuronal replacement and vocal behavior in adult songbirds. *Neuron*. 2000; 25:481–492. [PubMed: 10719901]
- Sherry DF, Vaccarino AL. Hippocampus and memory for food caches in black-capped chickadees. *Beh Neurosci*. 1989; 103(2):308–318.
- Shors TJ. From stem cells to grandmother cells: How neurogenesis relates to learning and memory. *Cell Stem Cell*. 2008; 3:253–258. [PubMed: 18786413]
- Simpson HB, Vicario DS. Brain pathways for learned and unlearned vocalizations differ in zebra finches. *J Neurosci*. 1990; 10(5):1541–1556. [PubMed: 2332796]
- Smulders TV, Shiflett MW, Sperling AJ, DeVoogd TJ. Seasonal changes in neuron number in the hippocampal formation of a food-hoarding bird: The black-capped chickadee. *J Neurobiol*. 2000; 27:15–25. [PubMed: 7643072]
- Tramontin AD, Smith GT, Breuner CW, Brenowitz EA. Seasonal plasticity and sexual dimorphism in the avian song control system: Stereological measurement of neuron and density number. *J Comp Neurol*. 1998; 396:186–192. [PubMed: 9634141]
- Velazquez, R.; Menjivar, J.; Drumheller, K.; Lee, DW. Hippocampal damage induces cell proliferation in the septo-hippocampal system. Society for Neuroscience Annual Meeting; Washington DC. 2008. Program Number 122.5
- Watson RE, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides*. 1986; 7:155–159. [PubMed: 3520509]
- Wynn RD, Saldanha CJ. Glial aromatization decreases neural injury in the zebra finch (*Taeniopygia guttata*): Influences on apoptosis. *J Neuroendocrinol*. 2004; 16:676–683. [PubMed: 15271060]

Figure 1.

Example of a lesion to the right HP in a black-capped chickadee brain. A. Nissl-stained coronal section from a BCC showing a unilateral lesion to the right hippocampus, calibration bar = 1 mm. B. Magnified view of the HP region, including the pSVZ, shown in panel A, calibration bar = 250 μ m. C. Magnified view of the dSVZ shown in panel A, calibration bar = 250 μ m. D. Adjacent section of coronal section shown in panel A, stained with DAB for BrdU-IR cells, calibration bar = 1 mm. E. Magnified view of the HP region, including the pSVZ, shown in panel D, calibration bar = 250 μ m. F. Magnified view of the dSVZ shown in panel D, calibration bar = 250 μ m. Panels B, C, E & F delineate the regions of interest for counting BrdU-IR cells. Abbreviations: cHP, contralateral hippocampus; iHP, ipsilateral hippocampus; cpSVZ, contralateral proximal subventricular zone; ipSVZ, ipsilateral proximal subventricular zone; and dSVZ, distal subventricular zone.

Figure 2.

Example of a lesion to the right HP in a dark-eyed junco brain. A. Nissl-stained coronal section from a DEJ showing a unilateral lesion to the right hippocampus, calibration bar = 1 mm. B. Magnified view of the HP region, including the pSVZ, shown in panel A, calibration bar = 250 μ m. C. Magnified view of the dSVZ shown in panel A, calibration bar = 250 μ m. D. Adjacent section of coronal section shown in panel A, stained with DAB for BrdU-IR cells, calibration bar = 1 mm. E. Magnified view of the HP region, including the pSVZ, shown in panel D, calibration bar = 250 μ m. F. Magnified view of the dSVZ shown in panel D, calibration bar = 250 μ m. Panels B, C, E & F delineate the regions of interest for counting BrdU-IR cells. All images were acquired as montages with a 20 $\times$ objective, and resized for presentation purposes. Abbreviations: cHP, contralateral hippocampus; iHP, ipsilateral hippocampus; cpSVZ, contralateral proximal subventricular zone; ipSVZ, ipsilateral subventricular zone; and dSVZ, distal subventricular zone.

Figure 3.

Examples of BrdU-IR cells in the HP and dSVZ. A. BrdU-IR cells surrounding the ventral portion of the HP lesion site (denoted by an asterisk) in a BCC. B. BrdU-IR cells along the walls of the dSVZ in a BCC. C. BrdU-IR cells surrounding the ventral portion of the HP lesion site (denoted by an asterisk) in a DEJ. D. BrdU-IR cells along the walls of the dSVZ in a DEJ. In all panels the arrows indicate sample BrdU-IR cells among several positively-labeled cells. Calibration bar = 100 μ m.

Figure 4.

Comparison of BrdU-IR cell densities in unlesioned BCCs and DEJs. In all regions examined unlesioned BCCs had significantly more BrdU-IR cells than unlesioned DEJs. Asterisks denote $p < 0.05$. Abbreviations: BCC, black-capped chickadee; DEJ, dark-eyed junco HP; dSVZ, distal subventricular zone; HP, hippocampus; pSVZ, proximal subventricular zone.

Figure 5.

Comparison of BrdU-IR cell densities in lesioned BCC by hemisphere. In the HP, there were more BrdU-IR cells ipsilateral to the lesion site compared to the contralateral side in BCCs. Asterisks denote $p < 0.05$. There were no differences in the ipsilateral and contralateral hemispheres in the dSVZ or the pSVZ, p 's > 0.1 . Abbreviations: dSVZ, distal subventricular zone; HP, hippocampus; pSVZ, proximal subventricular zone.

Figure 6.

Comparison of BrdU-IR cell densities in lesioned DEJ by hemisphere. In all regions examined there were more BrdU-IR cells ipsilateral to the lesion site compared to the contralateral side in DEJs. Asterisks denote $p < 0.05$. Abbreviations: dSVZ, distal subventricular zone; HP, hippocampus; pSVZ, proximal subventricular zone.

Figure 7.

Comparison of lesion effects in BCCs and DEJs. Lesions had larger effects on cell proliferation in DEJ than BCC. In the iHP, the effect size was nearly twice as large in DEJs than BCC. Notably, for all other regions (cHP, ipSVZ, cpSVZ, idSVZ and cdSVZ) DEJs had positive effect sizes whereas BCCs had small negative effect sizes. See Eq. (4) for the calculations used to determine effect sizes. Asterisks denote $p < 0.05$. No asterisk indicates that there were no significant differences, $p > 0.1$. Abbreviations: BCC, black-capped chickadees; DEJ, dark-eyed juncos; dSVZ, distal subventricular zone; HP, hippocampus; pSVZ, proximal subventricular zone.

Figure 8.

Dual-labeling fluorescence for BrdU and NeuN. The top row shows an example of the HP in a lesioned DEJ in which two cells are BrdU-IR, but not co-localized with NeuN. The upper left panel shows 2 BrdU-IR cells. The upper middle panel shows the same tissue imaged for the neuronal-specific protein NeuN. The upper right panel shows the two panels merged in which the BrdU-IR cells remain only green. The bottom row shows an example of the HP in a lesioned DEJ (same subject as top row) in which a BrdU-IR cell is co-localized with NeuN. The lower left panel shows the BrdU-IR cell. The lower middle panel shows the same tissue imaged for NeuN. The bottom right panel shows the two panels merged in which the BrdU-IR cell appears yellowish (white arrow). All panels shown are from a single z-plane. A confocal z-series was always used to identify whether or not a BrdU-IR cell was co-localized with NeuN. Calibration bar = 25 μ m.

Table 1
BrdU-IR Cells by Brain Region and Species (Combining Lesion Groups)

Species	Brain Region [†]				
	iHP ^{**}	cHP	ipSVZ [*]	cpSVZ ^{**}	idSVZ [*]
BCC	6.39 ± 1.57	2.65 ± 0.48	82.81 ± 13.88	80.10 ± 13.96	188.04 ± 29.75
DEJ	13.70 ± 1.61	2.68 ± 0.50	37.00 ± 14.25	19.94 ± 14.32	99.92 ± 30.52

[†]Numbers represent the mean ± 1SEM of BrdU-IR cells per mm².

* p < 0.05,

** p < 0.01

Table 2
Proportion of Dual-Labeling for BrdU and NeuN by Brain Region

Species	Brain Region		
	HP	pSVZ	dSVZ
BCC	37%	7%	<1%
DEJ	17%	0%	0%