Seasonal Changes in Cell Neogenesis in the Brain and Pituitary Gland

A Study in the Adult Male Frog, *Rana catesbeiana*

by

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ABSTRACT

Though for most of the twentieth century, dogma held that the adult brain was post-mitotic, it is now known that adult neurogenesis is widespread among vertebrates, from fish, amphibians, reptiles and birds to mammals including humans. Seasonal changes in adult neurogenesis are well characterized in the song control system of song birds, and have been found in seasonally breeding mammals as well. In contrast to more derived vertebrates, such as mammals, where adult neurogenesis is restricted primarily to the olfactory bulb and the dentate gyrus of the hippocampus, neurogenesis is widespread along the ventricles of adult amphibians. I hypothesized that seasonal changes in adult amphibian brain cell proliferation and survival are a potential regulator of reproductive neuroendocrine function. Adult, male American bullfrogs (*Rana catesbeiana*; aka *Lithobates catesbeianus*), were maintained in captivity for up to a year under season-appropriate photoperiod. Analysis of hormone levels indicated seasonal changes in plasma testosterone concentration consistent with field studies. Using the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) as a marker for newly generated cells, two differentially regulated aspects of brain cell neogenesis were tracked; that is, proliferation and survival. Seasonal differences were found in BrdU labeling in several brain areas, including the olfactory bulb, medial pallium, nucleus accumbens and the infundibular hypothalamus. Clear seasonal differences were also found in the pars distalis region of the pituitary

gland, an important component of neuroendocrine pathways. BrdU labeling was also examined in relation to two neuropeptides important for amphibian reproduction: arginine vasotocin and gonadotropin releasing hormone. No cells co-localized with BrdU and either neuropeptide, but new born cells were found in close proximity to neuropeptide-containing neurons. These data suggest that seasonal differences in brain and pituitary gland cell neogenesis are a potential neuroendocrine regulatory mechanism.

DEDICATION

I would like to thank my family who has always supported me, whatever I have wanted to do, even study frog brains.

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SECTION 1

INTRODUCTION

Adult brain cell neogenesis, including adult neurogenesis and gliogenesis, is an emerging field of study with exciting implications about the functioning of the vertebrate brain. For most of the twentieth century, the traditional view was that the cells of adult brain were post-mitotic. This view was, in part, established by the esteemed neuroanatomist Santiago Ramon y Cajal who maintained that the adult brain was fixed, without regenerative capabilities (Colucci-D'Amato, Bonavita, & di Porzio, 2006). This dogma began to be challenged in the nineteen sixties with papers published by Altman and Das (Joseph Altman & Gopal D. Das, 1965; J Altman & G D Das, 1965). These studies used tritiated thymidine and light microscopy to identify newborn neurons in the adult rat brain. Following these discoveries Kaplan used electron microscopy to identify synapses, vesicles, and neuronal processes in tritiated thymidine labeled cells in the adult brain (M. Kaplan & Hinds, 1977). The discoveries of Altman and Kaplan were largely ignored or discounted by the academic community at the time (Colucci-D'Amato, et al., 2006; M. S. Kaplan, 2001). Another blow to the traditional view of a static adult brain was dealt by Fernando Nottebohm in studies the HVC (formerly High Vocal Center) of male canaries, *Serinus canaria*. Nottebohm found that, not only was there adult neurogenesis, but that these newborn neurons integrated into functional circuits, as shown by electrophysiological studies (Nottebohm, 1985).

Interest in mammalian adult neurogenesis was re-ignited in the nineties, in part due to the studies of Elizabeth Gould, who showed strong effects of stress on the birth and survival of newborn neurons in the adult rat hippocampus (Cameron & Gould, 1994; Cameron, Woolley, McEwen, & Gould, 1993). The introduction of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) and cell type specific markers eased the process of identifying newborn cells and differentiating between neurogenesis and gliogenesis (Gould & Gross, 2002; Taupin, 2007).

Following these studies, adult brain cell neogenesis was confirmed as a phenomenon found across vertebrate species (Lindsey & Tropepe, 2006). Proliferation, migration and survival of newborn cells in the central nervous system is now known to occur in animals ranging from fish (Zupanc, 2008), reptiles (Font, Desfilis, Pérez-Cañellas, & García-Verdugo, 2001), amphibians (Simmons, Horowitz, & Brown, 2008), birds (Anat Barnea & Pravosudov, 2011; Nottebohm, 1985), to mammals (Joseph Altman & Gopal D. Das, 1965; Ming & Song, 2011b), including humans (Eriksson et al., 1998). There is also a high degree of variation in the range of adult neurogenesis across species. While adult neurogenesis is widespread throughout the brains of less derived vertebrates such as fish, amphibians and reptiles, in birds and mammals the centers of adult neurogenesis are more localized. This retention and specialization of adult neurogenesis throughout vertebrates suggests that adult neurogenesis has an adaptive function vital to the proper functioning of

the animal (Barker, Boonstra, & Wojtowicz, 2011). However, the function of newborn cells is not well known.

Newborn cells in the adult brain originate in specialized locations referred to as neurogenic niches (Ihrie & Alvarez-Buylla, 2011). In mammals there are two primary neurogenic niches, in the subgranular layer of the dentate gyrus (SG) and the subventricular zone of the lateral ventricle(SVZ) (Ihrie & Alvarez-Buylla, 2011). The subgranular layer of the dentate gyrus is unique in that it is a neurogenic niche that is not adjacent to a ventricle (Ming & Song, 2011a). These niches contain stem cells that divide into progenitor cells, which, in turn, proliferate, migrate, and differentiate into adult cells, both glial and neuronal. Along with neural stem cells, other glial cells in the neurogenic niche support and regulate the proliferation and differentiation of progenitor cells (Ihrie & Alvarez-Buylla, 2011). Regions similar to the neurogenic niche of the SVZ have been identified in the hypothalamus of mammals (Migaud et al., 2010). Similarities also exist between the neurogenic niches of mammals and the proliferative zones of amphibians. In amphibians, adult brain cell proliferation occurs primarily along the ventricles (D'Amico, Boujard, & Coumailleau, 2011; Saito, Talboom, & Orchinik, submitted; Simmons, et al., 2008). Radial glial cells similar to those found in the mammalian neurogenic niches have been identified in the proliferative zones of the African clawed frog, *Xenopus laevis* (D'Amico, et al., 2011). Amphibian

adult proliferative zones are likely regulated similarly to neurogenic adjacent to ventricles in mammals.

Study of adult neurogenesis in mammals is primarily constrained to the hippocampus, where studies have implied roles in learning and memory (Deng, Aimone, & Gage, 2010). Interestingly, adult neurogenesis is also implicated in the regulation of anxiety, (Sahay & Hen, 2007) and the activity of the hypothalamo-pituitary-adrenal (HPA) axis (Snyder, Soumier, Brewer, Pickel, & Cameron, 2011). This indicates that newborn neurons aren't just new neurons for the formation of new memories, but that these neurons affect the function of regions with adult neurogenesis. Adult neurogenesis in the olfactory bulb is also well characterized in rats and roles in olfactory memory and discrimination have been observed (Nissant & Pallotto, 2011). However, outside of these regions relatively little work has been done to determine the range and function of adult neurogenesis. Hypothalamic adult neurogenesis has been described in a number of animals, including rats, but its function remains relatively unexplored (Migaud, et al., 2010). Whereas newborn cells in the hippocampus are predominantly neuronal, less than 50% of newborn cells in the hypothalamus express neuronal markers (Migaud, et al., 2010). This ratio of neurogenesis to gliogenesis is more similar to the ratio found in amphibians (D'Amico, et al., 2011; Saito, et al., Submitted; Simmons, et al., 2008). Mammalian adult brain cell neogenesis in this region important

for neuroendocrine function may retain the same characteristics found in more basal vertebrates, like amphibians.

Neurogenesis in birds is best characterized in the HVC where newborn cells are important for learning new songs. In song birds this adult neurogenesis is regulated by photoperiod (Tramontin, Wingfield, & Brenowitz, 1999) and important for the vital mating behavior of singing. Hippocampal neurogenesis in birds has also been shown to be seasonal, but is thought to be related to foraging behaviors rather than reproduction (A Barnea & Nottebohm, 1994; Anat Barnea & Pravosudov, 2011). Seasonal changes in adult neogenesis have been shown in frogs; both in relation to hibernation (Cerri, Bottiroli, Bottone, Barni, & Bernocchi, 2009) and time of year (Chetverukhin & Polenov, 1993; Saito, et al., Submitted). In common lizards , *Podarcis hispanica*, differential effects of photoperiod and temperature were found on proliferation and migration rates of newborn cells (Ramirez et al., 1997). Adult neurogenesis has also been shown to fluctuate with season in seasonally breeding mammals such as voles, *Microtus pennsylvanicus*, (L. a. Galea & McEwen, 1999), hamsters, *Mesocricetus auratus,* (Liyue Huang, Geert J Devries, & Eric L Bittman, 1998a) and sheep, *Ovis aries,* (Migaud, Batailler, Pillon, Franceschini, & Malpaux, 2011). Season can drastically change the hormonal and behavioral responses of animals. In order to precipitate and accommodate these changes, structural changes occur in the brain. Changes in brain structure size, connectivity and responsiveness change

with season (Tramontin & Brenowitz, 2000). While not all of these changes are dependent on adult cell neogenesis, seasonal changes in in cell proliferation, migration, and maturation could account for many of these changes. In non-seasonally breeding mammals, mating and breeding related changes in neurogenesis have also been observed. In adult rats, sexual activity increases the amount of adult neurogenesis in the hippocampus (Leuner, Glasper, & Gould, 2010). The reproductively important hormone prolactin increases neurogenesis, which mediates offspring recognition (Mak & Weiss, 2010). The mammalian reproductive hormone oxytocin also increases adult neurogenesis (Leuner, Caponiti, & Gould, 2011).

Adult gliogenesis is an under explored area of study in adult brain cell neogenesis. Constitutive adult gliogenesis is found alongside adult neurogenesis in many studies in both mammals (Cameron, et al., 1993; Migaud, et al., 2010) and amphibians (D'Amico, et al., 2011; Saito, et al., Submitted; Simmons, et al., 2008). In rats, adult gliogeneis is differentially induced by learning, suggesting a functional role for adult born glial cells (Rapanelli, Frick, & Zanutto, 2011). Recent studies have shown that glial projections have close associations with synapses and regulate synaptogenesis and synaptic activity (Eroglu & Barres, 2010). Glial cells also release signaling factors (Fields & Stevens, 2000). In Japanese Quail, *Coturnix japonica*, glial processes regulate neuroendocrine signaling in a season dependent manner (Yamamura, Hirunagi, Ebihara,

& Yoshimura, 2004). These factors, along with the high ratio of gliogenesis to neurogenesis in reproductively important brain regions, suggest that adult gliogenesis may also contribute to regulation of brain function.

Pituitary gland hormones are powerful regulators of reproductive function. Cell proliferation in the anterior pituitary gland has been shown to be regulated by the estrus cycle (Oishi, Okuda, Takahashi, Fujii, & Morii, 1993) and by season (Migaud, et al., 2011), which suggest a reproductive role for adult born pituitary gland cells. The greatest percentages of these cells have been shown to differentiate into PRL producing cells, suggesting reproductive relevance to the cell proliferation. Adult cell neogenesis of neurons, glia, and pituitary cells could play vital roles in reproductive functions across vertebrates.

The American Bullfrog, *Rana catesbieana* aka *Lithobates catesbieanus*, is an excellent model for studying these seasonal effects on cell neogenesis throughout the brain and pituitary gland and potential neuroendocrine regulatory functions of the newborn cells. Bullfrogs are seasonal breeders (Licht, Mccreery, Barnes, & Pang, 1983) with widespread adult brain cell proliferation (Simmons, et al., 2008). The neuroendocrinology and hormonal responses of anuran amphibians have been well characterized in terms of breeding responses (Boyd, 1997; Moore, Boyd, & Kelley, 2005). Many of these hormones are identical or homologous to reproductively important hormones in mammals. GnRH release is regulated seasonally and is the primary regulator of gonadal

hormone release in both amphibians (Daniels & Licht, 1980; Mendonça, Licht, Ryan, & Barnes, 1985) and other vertebrates(Maruska, Mizobe, & Tricas, 2007), including mammals (Bernard, Abuav-Nussbaum, Horton, & Turek, 1999) . AVT is a powerful regulator of male typical behaviors in amphibians (Boyd, 1997; Woolley, Sakata, & Crews, 2004), as is its mammalian analogue vasopressin (De Vries & Panzica, 2006; Young, Wang, & Insel, 1998). Seasonal differences in mammalian adult neurogenesis have been found in more basal regions of the brain, such as the hypothalamus (Fowler, Liu, & Wang, 2008; Migaud, et al., 2011), which shows characteristics similar to amphibian neurogenesis. Exploring the effects of season on adult brain cell neogenesis in the bullfrog could help illuminate the function of new born cells in the vertebrate brain, especially those related to reproductive function. We hypothesized that: 1) Season would affect the levels of cell proliferation, maturation, and survival in the brain and pituitary gland of adult, male bullfrogs; and 2) These seasonal changes would occur in brain regions important for the physiological and behavioral regulation of breeding.

SECTION 2

MATERIALS AND METHODS

Frogs

Thirty six adult male American Bullfrogs (*Rana catesbeiana aka Lithobates catesbeianus*) were obtained from Kons Scientific (Germantown, WI) and kept in Animal Care Facilities at Arizona State University (Tempe, AZ). Frogs were group-housed in three raceways (150 cm in diameter, 75 cm in depth), with 7 cm of de-chlorinated water with constant filtration and fresh water drips. Raceways were half shaded and had short lengths PVC pipe to provide shelter. Photoperiod was maintained at season-appropriate lengths (see below). Temperature was kept between 68° and 72° F. Frogs received intraperitoneal (ip) doses of amikacin (0.1 mL) 2 days and 5 days after arrival. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Arizona State University Institutional Animal Care and Use Committee.

Photoperiod, BrdU Injections and Sacrifice

Upon arrival on December 19 (Dec. 19) animals were housed at a photoperiod of 10 hours light and 14 hours dark (10L/14D). Photoperiod was then manipulated as follows: on Feb. 2 to 11 L/13D; on Mar. 3 to 12L/12D; on Apr. 11 to 13L/11D; on May 15 to 14L/10D; on Jul. 7 to 13L/11D; on Sep. 16 to 12L/12D; on Oct. 14 to 11L/13D.

Four groups of frogs (called Pre-Breeding, Early Breeding, Late Breeding and Post-Breeding) each received a total of five ip injections of [25 mg/ml] BrdU dissolved in amphibian Ringer's solution (ARS) at a dose of 50 mg/kg body weight. Frogs were weighed on the first day of injections. Injections were given at approximately 09:00 and 16:00 on two consecutive days and the fifth injection was given at approximately 09:00 on the third day. The frogs were euthanized either 4 hours after the final injection (called 2d animals) or six weeks later (6wk animals) by transcardial perfusion with ~100 mL ice-cold ARS, followed by ~100mL ice cold 4% paraformaldehyde (Sigma-Aldrich Corp; St. Louis, MO) in ARS (PFA). Perfusions were performed with frogs deeply anesthetized with 20% MS-222 in ARS. Initial dates of injection and perfusion were as follows (see Figure 1): 1) Pre-breeding, injection on Jan. 21, 2d perfusion on Jan. 23 (N=5), and 6wk perfusion on Mar. 4 (N=5); 2) Early breeding injection on Mar. 18, 2d perfusion on Mar. 20 (N=5), and 6wk perfusion on Apr. 29 (N=4); 3) Late breeding injection on May 20, 2d perfusion on May 22 (N=4), and 6wk perfusion on Jul. 1 (N=5); Post-breeding injection on Sep. 23, 2d perfusion on Sep. 25 (N=4), and 6wk perfusion on Nov. 4 (N=4).

Tissue Preparation and Handling

After perfusion, brains were removed and post-fixed in 4% PFA overnight (~18 hrs) at 4° C and then moved to 30% sucrose in ARS at 4° C until

brains sunk in solution. Brains were embedded in Optimal Cutting Temperature Compound (Sakura Finetek USA, Inc; Torrance, CA), frozen on dry ice, and stored at -70° C until sectioning. Entire brains were sectioned at 20 microns using a Jung Frigocut 2800 E freezing microtome (Leica; Germany) between -17 C and -22 C. Sections were collected on Superfrost Excel Slides (Thermo Scientific, LLC; Portsmouth, NH) in serial series of six slides and stored in slide boxes at -70° C until use.

BrdU Chromogenic Immunohistochemistry

Slides were brought to room temperature in a desiccator and the sections were circumscribed with ImmEdge Pen (Vector Laboratories, Inc; Burlingame, CA). Sections were washed twice with 0.05 M phosphate buffered saline (PBS) for five min. Antigen retrieval was performed with a 10-minute incubation of slides in 10 mM sodium citrate buffer with 0.05% Tween-20 in Coplin jars immersed in a 95° C water bath. The Coplin jars were moved to room temperature for 20 min and slides were washed twice with PBS with 0.3% Triton X-100 (PBST) for 10 min. Slides were then incubated overnight at 4° C with 1:500 dilution of monoclonal rat anti-BrdU primary antiserum (Abcam; Cambridge, MA) in PBST with 0.1% bovine serum albumin (Sigma-Aldrich Corp; St. Louis, MO). After primary antibody incubation slides were washed three times with PBST for 10 min. Tissue was then incubated with 1:200 dilution of polyclonal goat anti-rat secondary antiserum (Vector Laboratories, Inc; Burlingame, CA) in PBST

for 1 hr. Slides were washed twice with PBST for 10 min and incubated for 1 hr with ABC Reagent diluted to have standard dilution 30 min after preparation in PBS from the Vectastain© ABC Kit (Vector Laboratories, Inc; Burlingame, CA), prepared 30 min prior to use. Slides were washed twice with PBST for 10 min before incubation in SG chromogen solution prepared fresh from Vector SG© Peroxidase Substrate Kit (Vector Laboratories, Inc; Burlingame, CA) for 4 min. Slides were washed twice with PBS for 5 min and dehydrated in an increasing series of ethanol (70% for 30 s, 95% for 30 s, 100% twice for 45 s). Slides were cleared twice for five min with Neo-Clear© Clarification Solution (EMD Chemicals, Inc.; Gibbstown, NJ). Slides were then mounted with Permount© mounting media (Fisher Scientific; Fair Lawn, NJ) and coverslipped. Monoclonal anti-BrdU antibody specificity was validated by pre-incubation with BrdU. Secondary antibody specificity was validated with runs that excluded the primary antibody.

BrdU Immunofluorescent Double Labeling with AVT and GnRH

Slides were brought to room temperature in a desiccator and the sections were circumscribed with ImmEdge Pen (Vector Laboratories, Inc; Burlingame, CA). Sections were washed twice with 0.05 M phosphate buffered saline (PBS) for five min. Antigen retrieval was performed with a 10 min incubation of slides in 10 mM sodium citrate buffer with 0.05% Tween-20 in Coplin jars immersed in a 95° C water bath. The Coplin jars

were moved to room temperature for 20 min and slides were washed twice with PBST for 10 min. Slides were incubated with 10% normal goat serum (Vector Laboratories, Inc; Burlingame, CA) in PBST for 1 hour at room temperature. Slides were then incubated overnight at 4° C with primary antibody cocktail containing 1:250 dilution of monoclonal rat anti-BrdU antibody(Abcam; Cambridge, MA) and either 1:200 dilution of polyclonal rabbit anti-LHRH antibody (ImmunoStar; Hudson, WI) or 1:10,000 dilution of polyclonal rabbit anti-AVT antibody (graciously provided by Matthew Grober). After primary antibody incubation slides were washed three times with PBST for 5 min. Slides were incubated for 1 hr with fluorescent secondary cocktail with 1:500 AlexaFluor568 Goat anti-rat antibody and AlexaFluor488 Highly Cross-Absorbed Goat anti-rabbit antibody (Vector Laboratories, Inc; Burlingame, CA) in PBST. After addition of fluorescent antibodies slides were kept in light proof containers and methods were performed in the dark. Slides were washed twice with PBST for 10 min. Slides were mounted with 90% glycerol (Sigma-Aldrich Corp; St. Louis, MO) in PBS, coverslipped and sealed with clear nail polish. Slides were stored in light tight containers at 4° C until image analysis. Anti-AVT antibody specificity was validated with pre-incubation with argenine vasotocin (Sigma-Aldrich Corp; St. Louis, MO). Anti-LHRH antibody specificity was validated with pre-incubation with mGnRH and cGnRH. Only mGnRH prevented staining. Secondary antibody specificity was validated with runs that excluded the primary antibody.

Image Analysis-Chromogenic BrdU-labeling

BrdU-labeled cells were identified as dark, round cells with staining only in the nuclear region of the cell using an Olympus BX-50 microscope (Olympus America; Center Valley, PA). Background staining in these sections was negligible, so identification of BrdU-labeled cells was non-ambiguous. Cells in each region of interest were counted using an in house bullfrog brain atlas based on previous neuroanatomy studies for consistent identification of regions (T. J. Neary & Northcutt, 1983; Northcutt & Royce, 1975; Opdam, Kemali, & Nieuwenhuys, 1976). Cells were identified as either part of the ependymal layer (defined as adjacent to the ventricle or in contact with a cell directly adjacent to the ventricle) or parenchymal (more than two cell layers from the ventricle). Brain regions counted included: GL, IGL, rMP, DP, LP, DST, VST, MS, LS, NA, POA, cMP, VM, SCV, CTN, LTN, PTN, rVH, rNPV, cVH, cNPV, TEC, TEG. (see abbreviations above) Three regions of the pituitary gland were also analyzed: PD, PI, PN.

Image Analysis- BrdU Immunofluorescent Double Labeling with AVT and mGnRH

A survey of regions with dense neuropeptide labeling was performed to identify co-localization of BrdU with the neuropeptides, AVT and mGnRH. For BrdU/mGnRH double labeling, the septal region and rostral POA were

examined. For BrdU/AVT double labeling, the POA, BN and SCN were examined. Only slides from frogs perfused 6wks post-BrdU injections were used. Slides were examined using an epifluorescent Olympus BX-50 and attached Olympus DP70 camera (Olympus America; Center Valley, PA) microscope with mercury light source. Slides were analyzed using separate filter cubes. AlexaFluor 488 labeled neuropeptides were analyzed using a FITC filter cube and AlexaFluor568 labeled BrdU was analyzed using a TRITC filter cube. Images were taken separately using DP controller software (Olympus America; Center Valley, PA).

Plasma Testosterone Concentration

At the time of perfusion, ~1 mL cardiac blood was drawn and placed in a microcentrifuge tube and stored on wet ice until centrifuged. Blood was centrifuged at 6000 rcf at -5° C in a refrigerated, table-top centrifuge. Plasma was then decanted and stored at -20° C until use. Plasma testosterone concentration was measured using a testosterone EIA Kit (Enzo Life Sciences, Inc; Farmingdale, NY). Samples were run in duplicate at a 1:20 dilution following manufacturer instructions. Plates were read at 405nm with correction at 595nm on an Opsys MR plate reader (Dynex Technologies; Chantilly, VA). Testosterone levels were extrapolated from a standard curve using GraphPad Prism 5 (GraphPad Software, Inc). Manufacturer reported cross reactivity of the kit includes 19-hydroxytestosterone (14.64%), androstendione (7.20%),

dehydroepiandrosterone (0.72%), and estradiol (0.40%). Manufacture reported intra-assay precision is between 7.8% and 10.8% and inter-assay precision is between 9.3% and 14.6%. Close agreement was found between sample run at 1:20 dilution and 1:10 dilution.

Statistical analysis

Statistical analysis of the number of BrdU-labeled cells was performed on nine brain regions (cMP, cNPV, cVH, DST, IGL, NA, POA, rLS and rMP). The regions chosen met the criterion of having a minimum average of 20 BrdU labeled cells/section during at least one season in either proliferation or survival animals. Statistical analysis was also performed on sections from the pars distalis.

Repeated measures ANOVAs with the within subjects variables of brain region and cell layer and the between subjects variable of season were performed for the 2d and 6wk animals using Statistica (StatSoft, Inc). The number of BrdU-labled cells from two randomly selected counts from sections containing a specific region was averaged together. Preliminary analysis indicated that there were no significant differences in the number of BrdU-labeled cells between hemispheres, so cells were averaged across hemisphere. Frogs perfused at 2d and 6wk post-BrdU injections were analyzed separately, except when analyzing differences between time points. Separate repeated-measures ANOVA was performed on the pars distalis using counts from 3 randomly selected counts of number of

BrdU-labeled cells from a section containing the pars distalis as the within-subjects variable and season as the between subjects variable. Significance was set at an alpha value of less than 0.05. Significant main effects and interactions were further investigated using Newman-Keuls post-hoc tests, with an alpha value of less than 0.05.

Plasma testosterone concentrations were grouped by month and analyzed by one-way ANOVA using Statistica 7.1 (StatSoft, Inc). Two animals were removed from analysis due to being determined an outlier by the Grubb's Test (GraphPad Software, Inc). Regression analysis of testosterone and number of BrdU-labeled cells was performed using StatView Software (SAS Institute Inc). A Fisher's r to z analysis was used to determine if the data were linearly related. Significance was set at an alpha value of less than 0.05. Since there was no significant correlation between testosterone concentration and the number of BrdU-labeled cells across the seasons, the relationship between BrdU and [T] without the influence of season, data were analyzed by correlation analysis with centered data; season means of BrdU count and plasma testosterone concentration were subtracted from each individual's BrdU and testosterone values (Enders & Tofighi, 2007; Hallahan & Rosenthal, 2000). Correlations were analyzed with both centered and uncentered data.

SECTION 3

RESULTS

Seasonal Differences in Cell Proliferation, as Indicated by the Number of BrdU-labeled Cells at Two Days following BrdU Injection.

Repeated measures ANOVA of the number of BrdU-labeled cells at the 2d time point showed a significant three-way interaction of season, cell layer and brain region ($F_{(24, 72)}$= 3.27, $p<0.0001$).

Analysis of the three-way interaction of season, brain region and cell layer with a post-hoc test showed seasonal differences in two brain regions. In the ependymal layer of the cNPV, the number of BrdU labeled cells was significantly higher in the late breeding season compared to the pre-breeding season (Figure 2). In the ependymal layer of the NA, there were significantly more BrdU-labeled cells in the post-breeding season compared to the other seasons (Figure 3). There were no seasonal differences in the number of BrdU-labeled cells in the parenchymal layer at the 2d time point.

Seasonal Differences in Cell Proliferation, Migration, and Survival as Indicated by the Number of BrdU-labeled Cells at Six Weeks following BrdU Injection.

Repeated measures ANOVA of the counts of BrdU-labeled cells showed that at the 6wk time point there was a significant three-way interaction of season, cell layer and brain region ($F_{(24,88)}$= 1.7288, $p<0.05$).

A post-hoc test of the three way interaction of season, cell layer and brain region showed seasonal differences in 4 brain regions: the cMP, the cNPV, the NA and the IGL. The pre-breeding season frogs had more BrdU-labeled cells in the parenchymal layer of the cMP than the late-breeding season frogs. There was no seasonal difference in the ependymal layer of the cMP (Figure 4). Similarly, there were more BrdU-labeled cells in the parenchymal layer of the IGL, in the pre-breeding season compared to the early breeding season. There were no seasonal differnces in the ependymal layer of the IGL (Figure 5). In the ependymal layer of the cNPV, there were more BrdU-labeled cells in the late breeding season than in the pre- and post-breeding seasons. There were no significant differences in the parenchymal layer in the cNPV (Figure 6). In the ependymal layer of the NA, there are more BrdU-labeled cells in the late breeding season compared to the early breeding season. There were no seasonal differences in the parenchymal layer in the NA (Figure 7).

The Number of BrdU-labeled Cells Increases in the Parenchymal Layer from the 2d to 6wk Time Point

A repeated measure ANOVA of the number of BrdU-labeled cells across all brain regions analyzed showed that there was a significant interaction between time point following BrdU injection and cell layer on the number of BrdU-labeled cells ($F_{(1, 229)}= 57.81$, $p<0.00001$). Post-hoc tests showed

that there were more BrdU-labeled cells in the parenchymal layer at six weeks than at 2 days after BrdU injection (Figure 8).

Season Affects BrdU-labeling in the Pars Distalis at 6wk, but Not 2d after BrdU Injection

A one-way ANOVA of the BrdU-counts showed that at the two day time point there was no significant seasonal differences on the number of BrdU-labeled cells in the pars distalis ($F_{(3,12)}$= 1.44, p>0.27) (Figure 9). However, at the six week time point there was a significant seasonal difference ($F_{(3,13)}$= 10.5535176, p<0.002) on the number of BrdU-labeled cells. A post-hoc test showed that the early breeding season had the greatest number of BrdU-labeled cells compared to the other seasons (Figure 10).

Plasma Testosterone Concentration Varies Significantly with Month

Plasma testosterone concentration varied significantly with month F(6, 27)=4.6648, p<0.003. A post-hoc analysis showed that frogs had higher plasma testosterone concentrations in April than January, September and November (Figure 11).

Interactions of Plasma Testosterone Concentrations and BrdU-labeling

There was no significant seasonal correlation between plasma testosterone level and BrdU labeled cells in either the brain or pituitary gland of 2d animals. After centering, a positive linear regression was found in the pars distalis at the 2d time point ($R=0.585$, $p=0.02$) (Figure 12).

Neuroendocrine Fate of New Born Cells

A survey of sites of densely labeled neuroendocrine cells in frogs 6wk after BrdU injection found no co-localization between BrdU and neuropeptides. Sections from each frog in each season at the 6wk time point were analyzed. mGnRH-ir cell bodies were analyzed in the septum and rostral POA. AVT-ir cell bodies were analyzed in the POA, SCN, and BN. No cells were co-localized with BrdU and either neuropeptide. Though no co-localized cells were found at the 6wk time point, many BrdU-labeled cells were found in close proximity (<20 μm) to neuropeptide containing cells (Figure 13 and 14).

SECTION 4

DISCUSSION

Frogs maintained under naturalistic lighting conditions displayed seasonal differences in the number of newborn cells in several brain regions and the pituitary gland. Seasonal differences varied between the 2d and 6wk after BrdU injection indicating seasonal differences in both proliferation, and migration and survival of newborn cells. These differences were in regions of the brain known to be important regulators of neuroendocrine function and reproductive behavior. Hormones of the pars distalis (adenohypophysis) have powerful physiological and behavioral effects, including on seasonal reproductive processes. Together, these results suggest that adult cell neogenesis is involved in seasonal regulation of neuroendocrine pathways and responses. I also found that seasonal changes in BrdU-labeling in the brain, consistent with either cell proliferation or survival, did not correlate with plasma testosterone concentrations, suggesting that that androgens are not regulating this difference in bullfrogs, unlike in songbirds (Anat Barnea & Pravosudov, 2011).

While there was detectable BrdU labeling in the nine brain regions studied (see Table 1 and 2), I found seasonal and regional differences in BrdU-labeling at both the 2d and 6wk post-injection time points. Seasonal differences were only present in the ependymal layer at the 2d time point. The proliferative zone for adult born cells in amphibian brains is generally

considered to be in the ependymal layer of the brain (D'Amico, et al., 2011; Simmons, et al., 2008). Therefore, the seasonal differences in BrdU-labeling at the 2d time point is presumably due to differences in cell proliferation rates, and not cell migration or survival. Two regions showed seasonal differences shortly after BrdU injections: the cNPV and the NA. The cNPV is located in the infundibular hypothalamus and showed the highest levels of cell proliferation during the late breeding season, when photoperiod is longest. The NPV serves important neuroendocrine regulatory functions in amphibians (Muske & Moore, 1994). The NA had the highest amount of cell proliferation in the Post-breeding season. Together, this regional and temporal division of seasonal proliferation rates suggests that the mechanisms that regulate brain cell proliferation are site and season specific.

Both regions that showed seasonal differences in BrdU-labeling at the 2d time point, also showed seasonal differences at the 6wk time point. Two additional brain regions showed seasonal differences in BrdU-labeling at the 6wk time point: the cMP and the IGL. Interestingly, both regions with seasonal differences exclusively at the 6wk time point only showed differences in the parenchymal region. The cMP had the highest number of BrdU-labeled cells in parenchyma during the pre-breeding season and the least number of BrdU-labeled cells during the late breeding season, compared to other seasons. The IGL had the most BrdU-labeled cells in the pre-breeding season and the least in the early

breeding season. The cNPV also maintained a similar pattern of BrdU-labeling between the 2d and 6wk time points, with high numbers of BrdU-labeled cells in the late season and low numbers of BrdU-labeled cells in the pre- and post-breeding season. The pattern of BrdU-labeling in the NA shifted from the 2d to the 6wk time point with the late breeding season having the highest levels of BrdU-labeling and the early season having the lowest levels of BrdU-labeling. For both these regions, the effect of season on number of BrdU-labeled remained located to the ependymal layer. The regional, temporal, and cell layer specificity of the seasonal differences in BrdU-labeling indicates that seasonal differences in cell proliferation, migration and survival are regulated by specific site and season mechanisms and that these mechanisms regulate different stages of cell neogenesis.

The number of BrdU-labeled cells at six weeks post-BrdU injection presumably results from several factors, including cell proliferation, migration and survival. More BrdU-labeled cells were present at the 6wk time point compared to the 2d time point. This effect was only significant in the parenchymal layer, so this result supports the idea that newborn cells migrate from the proliferative zones of the ependymal layer lining the ventricles and into the parenchyma. It is unlikely that this increase in cell number at 6wk is caused by BrdU remaining available for incorporation into proliferating cells past the perfusion time of the 2d animals. Following i.p. injection of rats, BrdU is available for incorporation in to cells for only

approximately 2 hours (Taupin, 2007). Even with potential differences in the rate of elimination of BrdU in ectotherms, due to the pattern of BrdU labeling, it is more likely that there is continued division and replacement of neural stem cells in the proliferative zone in the ependymal layer. This would cause the number of BrdU labeled cells in the ependymal layer to remain constant between the 2d and 6wk time point. Because of the semiconservative nature of DNA replication, incorporated BrdU is passed to the daughter cells of BrdU-labeled cells. In rats, over a 24 hour period, the number of BrdU-labeled cells increases due to division of BrdU-labeled cells (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003). Seasonal differences between regions at the 6wk time point could be accounted for by changes in proliferation, migration or survival of newborn cells.

The increase in overall cell number indicated continued proliferation of BrdU-labeled cells over the six week period. Factors affecting this continued proliferation could account for seasonal differences in the number of BrdU-labled cells. Migration of proliferating cells into or out of specific brain regions could also mediate the differences between time points. The specific paths of migration in the amphibian brain are not well known. Radial glial cells are present in the proliferative zones of amphibians and newborn cells migrate along their processes (D'Amico, et al., 2011). However, if long distance migration of cells occurs in amphibians, as it does in the rostral migratory stream of mammals, it has

not been documented. Regulation of cell survival is another potential mechanism that could account for regional differences in the number of BrdU-labeled cells between seasons. In song birds, seasonal differences in neuronal number in the HVC are caused by testosterone dependent changes in survival, not proliferation rates (Rasika, Alvarez-Buylla, & Nottebohm, 1999). Because of the high number of cells in the six week animals compared to the 2d animals it is difficult to determine the role of survival in the seasonal effects found in this study. However, the cells in the parenchyma at the 6wk time point are likely mature and functional cells, and indicative of the fate of cells that proliferated at the time of injection.

Several of the brain regions that showed seasonal differences in BrdU-labeling have neuroendocrine or behavioral functions that relate to reproduction. The areas with significant seasonal differences in the number of BrdU-labeled cells were: the olfactory bulb, the medial pallium, the nucleus accumbens and the infundibular hypothalamus. The olfactory bulb had a higher amount of BrdU-labeling in the pre-breeding season compared to the early breeding season at the six week time point. Although in anurans auditory signaling is more important to reproductive function than olfactory signaling, there are potential reproductive functions to the olfactory bulb (Wilczynski, Lynch, & O'Bryant, 2005). The reproductively important neuropeptides AVT (Boyd & Moore, 1992) and GnRH (D'Aniello et al., 1995) are found in the olfactory bulb. The MP of

amphibians is considered to be homologous to the hippocampus of birds and mammals (Roth & Westhoff, 1999). In anurans the MP is the most connected field of the pallium and integrates ascending sensory information (T. Neary, 1990). The MP also contains efferent projection to many brain regions including the olfactory bulb, the SCN, and the hypothalamus and can exert control over motor functions (T. Neary, 1990). The amount of BrdU-labeling in the MP is lowest during the highest photoperiod season groups, a finding constant with findings in the hippocampus of birds (A Barnea & Nottebohm, 1994) and hamsters (Liyue Huang, G J DeVries, & Eric L Bittman, 1998b). The NA is a part of the basal ganglion in amphibians (Smeets, Marin, & Gonzalez, 2000). It has efferent projections to several areas of reproductive relevance, including the septal region, the POA, and the hypothalamus (Marín, González, & Smeets, 1997). AVT content of the NA mediates calling behavior in male cricket frogs, *Acris crepitans*, (Marler, Boyd, & Wilczynski, 1999). In male Japanese red-bellied newts, *Cynops pyrrhogaster*, dopaminergic signaling in the NA induced by a seasonally variable neurosteroid increases locomotion, a breeding related behavior (Tsutsui et al., 2010). The infundibular hypothalamus is also an important region for neuroendocrine regulation as it is the path of hypothalamic hormones to the median eminence (Ball, 1981). In anurans, this region receives auditory inputs (T. J. Neary & Wilczynski, 1986) and cell proliferation in the infundibular hypothalamus is increased by exposure to conspecific calling in green

treefrogs, *Hyla cinerea* (Almli & Wilczynski, 2012). Lesions of the infundibular hypothalamus inhibit courting behavior in male salamanders, *Triturus cristatus* (Dubé, Clairambault, & Malacarne, 1990). Cells in the infundibular hypothalamus accumulate sex hormones (Morrell, Kelley, & Pfaff, 1975) and it is one of two regions of the brain that expresses both androgen and estrogen receptors (Moore, et al., 2005). Interestingly, in hibernating frogs, *Rana temporaria*, the infundibular hypothalamus has been shown to have inhibitory regulation over GnRH and gonadotropin release (Sotowska-Brochocka & Licht, 1992). Seasonal differences in newborn cells in these regions could regulate their function and affect seasonal neuroendocrine responses and behavior.

Immunofluorochemistry with BrdU and AVT or mGnRH did not find any BrdU-labeled neuroendocrine cells, however BrdU-labeled cells were found within areas of dense neuropeptide labeling. BrdU-labeled cells with close proximity to mGnRH-ir cells were found in the septal and rostral POA regions. BrdU-labeled cells with close proximity to AVT-ir cells were found in the POA, the SCN and the BN. Though these regions did not show seasonal differences in BrdU-labeling, seasonal differences may exist in the subregion distribution of newborn cells or the phenotypic fate of these cells. The cellular identity of these newborn cells is not known; previous studies of bullfrog neogenesis found roughly equal amounts of neurogenesis and gliogenesis (Saito, et al., submitted). These cells could be neurons synapsing onto neuroendocrine cells. It is also possible that

newborn glial cells could help to mediate seasonal differences in glial-neuroendocrine interactions. Seasonal differences in polysialylated form of neural cell adhesion molecule (PSA-NCAM), a molecule that regulates neuron-glial interactions, have been observed with GnRH cells in seasonally breeding animals (Chalivoix, Malpaux, & Dufourny, 2010; Lee, Watanabe, & Glass, 1995). Thus, either newborn neuronal or glial cells could help to modulate neuroendocrine function.

The pars distalis also showed significant seasonal effects on the number of BrdU-labeled cells. The seasonal effects were only present at the 6wk time point and the highest amount of BrdU-labeled cells was in the early breeding period. The pars distalis in anurans is well developed and its function in adults is regulated by hypothalamic hormones through the hypophysial portal system (Ball, 1981). Adult proliferative cells in the pars distalis have been identified in rats (Gleiberman et al., 2008) and seasonal or hormonal cycle changes in cell proliferation have been observed in sheep (Migaud, et al., 2011) and rats (Oishi, et al., 1993). A number of pituitary hormones affect reproductive physiology and behavior in amphibians. The gonadotropins LH and FSH are present in anurans and regulate sex hormone levels (Licht, et al., 1983; a. M. Polzonetti-Magni et al., 1998). Luteinizing hormone receptor mRNA in the brain of male *Xenopus laevis* suggests a direct role for LH in calling behavior (Wilczynski, et al., 2005). Prolactin cells are the most commonly found proliferating cell type in the anterior pituitary gland of rats (Oishi, et al.,

1993) and in frogs PRL increases the release of LH and FSH and induces vitellogenesis in females, an important step in oocyte production (A. Polzonetti-Magni, Carnevali, & Yamamoto, 1995). Growth hormone is seasonally variable and its trophic effects could contribute to reproductive function (A. Polzonetti-Magni, et al., 1995). The seasonal difference in BrdU-labeling could be regulated by a proliferative effect of releasing factors on target cells progenitor lines as is seen in ghrelin and GH cell lines (Nanzer et al., 2004). Interestingly, many pituitary hormones or downstream products under pituitary gland control have been shown to either increase proliferation or survival of adult born neurons, including androgens (Anat Barnea & Pravosudov, 2011; Spritzer & Galea, 2007), estrogens (Fowler, et al., 2008), PRL (Mak & Weiss, 2010) GH (Lichtenwalner et al., 2006) and thyroid hormone (López-Juárez et al., 2012).

Following the naturalistic photoperiod in laboratory conditions we found a seasonal pattern of plasma testosterone concentrations similar to that found in wild male bullfrogs; low androgen in the winter, a sharp increase in the early season, followed by a notable dip, a second increase in androgens late in the season and then a decrease in the fall and winter (Licht, et al., 1983; Mendonça, et al., 1985). Though 5αdihydrotestosterone (DHT) is the main active androgen in bullfrogs, plasma DHT and testosterone concentrations are strongly correlated (Licht, et al., 1983). Previous studies with bullfrogs did not find strong

effects of photoperiod on reproductive fitness (Easley, Culley, Horseman, & Penkala, 1979; Horseman, Smith, & Culley, 1978). However, these studies used laboratory bred bullfrogs kept in constant photoperiods. The bullfrogs used in this study were bred in outside breeding pools and placed on gradually changing naturalistic photoperiods. This could explain the differences seen between this and previous studies of photoperiod. Though there were seasonal changes in both BrdU-labeling and testosterone concentration, there were not significant seasonal correlations between testosterone concentration and BrdU-labeling. This suggests that, unlike in songbirds, seasonal differences in cell proliferation or survival are not dependent on testosterone. After centering the data to remove the effect of season on the number of BrdU-labeled cells and testosterone concentration, a correlation was found between testosterone concentration and the number of BrdU-labeled cells in the pars distalis at the 2d time point. This is not a seasonal effect, but suggests that, in general, frogs with higher testosterone concentration have higher amounts of cell proliferation in the pars distalis.

SECTION 5

SPECULATION AND CONCLUSION

Potential Mechanisms for Regulation of Seasonal Brain Cell Neogenesis

Seasonal differences in newborn adult cell proliferation, migration and survival could be regulated by a number of environmental factors and cellular mechanisms. Some environmental factors that that could cause seasonal changes in BrdU-labeling include photoperiod, temperature and social interactions. Seasonal changes in cell survival in the HVC in songbirds are regulated by photoperiod dependent changes in testosterone (Anat Barnea & Pravosudov, 2011). In hamsters, long photoperiods inhibit BrdU-labeling (Huang, et al., 1998b). In common lizards, long photoperiods increase adult brain cell proliferation (Ramirez, et al., 1997). As previously noted, photoperiod has not been shown to have a strong effect on bullfrog reproductive status(Easley, et al., 1979; Horseman, et al., 1978). However, bullfrogs in the wild do show seasonal changes in reproductive condition (Licht, et al., 1983; Mendonça, et al., 1985). These effects are possibly due to temperature (Gibbs & Breisch, 2001). Temperature affects the migration of newborn cells in the lizard brain (Ramirez, et al., 1997). There is an increase in the amount of both cell proliferation and death in the brain of hibernating frogs, but whether this a seasonal or an activity dependent effect is unknown (Cerri, et al., 2009). However, the frogs in the study presented here were kept in a

controlled condition, with temperatures between 68° and 72° F. Social interaction could also mediate some of the seasonal differences in adult cell neogenesis. Exposure to calling increases BrdU-labeling in the infundibular hypothalamus and POA independent of gonadal steroids in treefrogs (Almli & Wilczynski, 2012). Sexual experience increase adult neurogenesis in rats (Leuner, et al., 2010). Exposure to males causes region specific changes in adult neurogenesis in sexually receptive female prairie voles, *Microtus ochrogaster* (Fowler, Liu, Ouimet, & Wang, 2002). Clasping behavior was observed in the study animals, but it is not known if this behavior affects cell proliferation in amphibians. It is unknown which social or environmental factors regulate the observed seasonal effects on BrdU-labeling. Beyond environmental factors, it is important to understand the physiological and cellular mechanism that could regulate seasonal changes in adult brain cell neogenesis.

Many hormones control adult brain cell proliferation and survival and also hormone levels vary with season. Gonadal steroids levels vary seasonally in many vertebrates. Seasonal brain cell neogenesis changes with the level of these hormones. In meadow voles there are seasonal differences in neurogenesis in breeding and non-breeding females and this effect is thought to be mediated by estrogen (Fowler, Johnson, & Wang, 2005; L. a. Galea & McEwen, 1999; Ormerod & Galea, 2001). Testosterone mediates the seasonal effect in cell survival in the songbird HVC (Anat Barnea & Pravosudov, 2011). Testosterone and estrogen

have also been shown to increase adult neurogenesis in non-seasonally breeding mammals (Spritzer & Galea, 2007; Tanapat, Hastings, Reeves, & Gould, 1999). Adrenocortical steroids inhibit amphibian reproduction (Wilczynski, et al., 2005) and adult neurogenesis (Schoenfeld & Gould, 2012). Prolactin and GH concentrations vary seasonally in frogs (A. Polzonetti-Magni, et al., 1995) and increase adult neurogenesis (Lichtenwalner, Forbes, Sonntag, & Riddle, 2006; Mak & Weiss, 2010). Thyroid hormone concentration varies seasonally in frogs(Kühn, Darras, & Verlinden, 1985) and has been shown to regulate adult neurogenesis (López-Juárez, et al., 2012). While some hormones have direct effects on proliferating cells, other hormonal effects are mediated by site specific mechanisms, such as signaling by growth factors.

Neuronal growth factors differ from hormones in that they are locally synthesized and are therefore well suited to mediate site specific effects. Brain derived neurotrophic factor (BDNF) is a growth factor that increases adult neurogenesis (Lledo, Alonso, & Grubb, 2006). The effect of testosterone on cell survival in songbirds and rats is mediated by BDNF (L. a. M. Galea, Spritzer, Barker, & Pawluski, 2006; Rasika, et al., 1999). The seasonal and social effects of estrogen in voles are also likely due to BDNF (Fowler, et al., 2002) Another growth factor, basic fibroblast growth factor (bFGF), induces cell proliferation in the third ventricle of the rat, a region with low levels of cell proliferation (Gould, 2007), more effectively than BDNF or epidermal growth factor (EGF) (Xu et al., 2005). This could

be relevant because many regions with neuroendocrine functions lie along the third ventricle and the amount of cell proliferation in the third ventricle is a prominent distinction in neurogenesis between birds and mammals, and less derived vertebrates (Barker, et al., 2011). Ciliary neurotrophic factor (CNTF) has also been show to induce increased neurogenesis in the mammalian hypothalamus (Kokoeva, Yin, & Flier, 2005). Along with growth factors, neurotransmitter and neuromodulator activity can be a strong site specific regulator of cell proliferation and survival.

Seasonal differences in adult brain cell neogenesis could be caused by seasonal differences in the amount of activity in different brain regions. Both glutamate and gamma-Aminobutyric acid (GABA) regulate levels of adult neurogenesis. Glutamate signaling, especially N-Nitrosodimethylamine (NMDA) activation correlates with high levels of neurogenesis in the mammalian hippocampus (Ma et al., 2009). GABA is excitatory in newborn neurons an enhances proliferation and integration of new born neurons (Ge, Pradhan, Ming, & Song, 2007). Acetylcholine also increases hippocampal neurogenesis (Mohapel, Leanza, Kokaia, & Lindvall, 2005). Monoamine signaling also regulates adult neurogenesis. Both serotonin and norepinephrine have been correlated with increased proliferation and survival of newborn cell in the mammalian hippocampus (Zhao, Deng, & Gage, 2008). In red spotted newts, *Notopthalmus viridescens*, dopamine has been shown to regulate the production of dopaminergic neurons in the adult brain (Berg, Kirkham, Wang, Frisén, &

Simon, 2011). These activity dependent mechanisms could ensure that regions that mediate season dependent behaviors have increased cell numbers in a season dependent manner.

Potential Functions of Seasonal Brain Cell Neogenesis

Season dependent adult brain cell neogenesis could modulate neuroendocrine regulation through a number of mechanisms. The most direct way for newborn cells to affect the neuroendocrine system is to develop into neuroendocrine cells. The number of AVT producing cells changes with season in green treefrogs (Bryant & Wilczynski, 2010) and AVT and GnRH cell number change with season in halfspotted goby, *Asterropteryx semipunctata* (Maruska, et al., 2007). However, it has not been demonstrated that these are adult born cells. In ring doves, *Streptopelia risoria*, season dependent maturation of newborn cells into GnRH producing cells following lesion of the POA has been shown (Cheng, Alexander, Zhou, Bonder, & Chuang, 2011). Despite finding migration of BrdU-labeled cells into neuroendocrine areas, I did not find evidence of maturation of these cells into either AVT or mGnRH producing cells. However, newborn cells could modulate the function of neuroendocrine cells in a number of ways.

When considering the role of season dependent adult neurogenesis and gliogenesis in neuroendocrine regulation, the function of adult cell proliferation in general should be discussed. In constrat to mammals,

amphibians grow over their entire lifespan and the total number of cells in the brain of amphibians increases. Widespread adult brain cell neogenesis in amphibians may be a mechanism for facilitating these changes (Barker, et al., 2011). Seasonal changes in brain cell neogenesis may be related to seasonal in changes trophic hormone concentrations and body weight. However, even more derived vertebrates that don't display this lifetime increase in body size have retained constituative adult neurogenesis. In part this can be explained by the special properties of newborn neurons. Newborn neurons have a resiliance to GABAergic inhibition and show increased synaptic plasticity (Ming & Song, 2011a). These properties of newborn neurons may be important in learning and memory (Anat Barnea & Pravosudov, 2011; Deng, et al., 2010). Seasonal changes in brain cell neogenesis may be important for encoding new memories in a changing environment. Another important function of adult neurogenesis that is emerging is regulation of the hypothalamo-pituitary-adrenal (HPA) axis (Snyder, et al., 2011). Adult neurogenesis deficient mice have blunted negative feedback of the HPA axis following an accute stressor (Snyder, et al., 2011). This suggests that the special properties of newborn neurons can regulate neuroendocrine responses. Frogs may be using these special properites to mediate the seasonal responsiveness of brain regions with seasonal differences in brain cell neurogenesis. Adult gliogenesis is less well studied but could also serve to regulate neuroendocrine function. Glial

cells in close proximity to neuroendocrine cells or their targets could also regulate neuroendocrine function as part of the “tripartite synapse” (a Araque, Parpura, Sanzgiri, & Haydon, 1999). Glial processes can modulate synaptic neurotransmission (Alfonso Araque & Perea, 2004), synaptic connectivity (Eroglu & Barres, 2010) and produce their own signaling molecules (Fields & Stevens, 2000). Glial processes have also been shown to regulate release of neuropeptides in a season dependent manner by glial-terminal interactions at the median eminence (Yamamura, et al., 2004). These interactions with glial cells could either enhance signaling through increased synaptogenesis and signaling activity or inhibit signaling by interposing glial processes into synaptic regions. Whether neurogenesis or gliogenesis, seasonal changes in brain cell neogenesis could contribute to regulation of neuroendocrine function.

Future Studies

In order to elucidate the mechanisms and functions of season dependent adult cell neogenesis a variety of studies could be performed. To determine the environmental factors that regulate season dependent cell neogenesis, studies that control for photoperiod and temperature could be performed. Studies of photoperiod should examine constant long photoperiod and short photoperiod as well as transitions from long to short and short to long photoperiod. Temperature studies could be combined with photoperiod, to create “summer” and “winter” like effects as seen in

Font, Desfilis et al. 2001. To test if social interactions mediate the seasonal effects frogs could be housed singlely or socially. The effects of housing with females verses males could be examined. Individual housing may be problematic as exposure to calling may affect cell proliferation so frogs would have to be accoustically isolated (Almli & Wilczynski, 2012). To test the effects of gonadal steroids, gonad ectomies and hormonal replacements with implants could be performed with any of the above studies. Also, plasma samples could be taken at regular intervals between time of injection with BrdU and sacrifice to better understand any potential effects of hormone levels on cell proliferation and survival. To determine if BDNF regulates seasonal changes in cell proliferation and survival, either immunohistochemical staining for BDNF could be used, or Western blot analysis could be used to determine seasonal changes in BDNF production. Brains may have to be microdissected in order to determine regional differces in BDNF levels. In any of the above studies immunohistochemistry for Proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining could be used to determine the effects of cell proliferation and survival. PCNA labels proliferating cells and TUNEL staining identifies apoptotic cells. However, this method does not allow for the identication of the phenotypic fate of newborn cells, so BrdU-labeling would still be required.

Identifying the cell fate of newborn cells in close proximity to neuroendocrine cells could help to understand their function. Immunohistochemical triple-labeling with BrdU, the neuropeptide of interest and a neuronal or glial marker would demonstate the identity of these cells. If the cells are neuronal, determination of the nature of these neurons with a marker like glutamic acid decarboxylase (GAD) for GABAergic cells should be performed. If these cells are glial, then investigation of possible neuronal-glial interactions should be performed. Triple-labeling for BrdU, the neuropeptide and PSA-NCAM could show if seasonal differences in neuroendocrine-glial interactions are in part mediated by newborn cells.

As well as identifying the nature of newborn cells in close proximity to neuroendocrine cells, the location and nature of newborn cells in at the targets of neuroednocrine cells should be identified. Along with being released from the brain, neuropeptides also have function within the brain. Using immunohistochemistry to double label with the neuropeptide of interest and BrdU, newborn cells that are in terminal fields of neuropeptide release in the brain can be identified. The cells in these terminal fields could either be targets of the neuropeptides, or modulating the responsiveness of target cells. Triple labeling or labeling of adjacent sections could be used to determine the nature of newborn cells in the terminal fields. Double labeling could also be used to see if newborn cells express receptors for the neuropeptide in question. These studies would

help determine the nature and possible function of season dependent newborn cells.

A large seasonal differnce in cell neogenesis was seen in the par distalis. Identification of the phenotype of the newborn pituitary gland cells could help to understand the role of cell proliferation in the pituitary gland and the role of different pituitary hormones in reproduction. Identification of these cells would only require immunofluorescent double-labeling against BrdU and the pituitary hormone of interest. Prime canidates for study are LH and FSH as there was a non-seasonal correlation between plasma testosterone concentration and cell proliferation in the pars distalis. Prolactin is also of interest as it regulates reproductive function in amphibians and is the most commonly adult born pituitary gland cell in rats (Oishi, et al., 1993; A. Polzonetti-Magni, et al., 1995). Less important but possible targets of interest are GH and TSH.

In order to definatively study the seasonal effects of adult brain cell neogenesis on reproductive function, ablation of neurogenesis would be optimal. However, genetic models for ablating neurogenesis are not available in amphibians. An alternative method for ablation of adult brain cell proliferation is the use of X-rays to kill mitotic cells (Santarelli et al., 2003). However, this requires specialized equipment and may not be feasible. Correlational studies as outline above, will help to confirm seasonal differences in brain cell neogenesis and suggest the function of these newborn cells.

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FIGURES

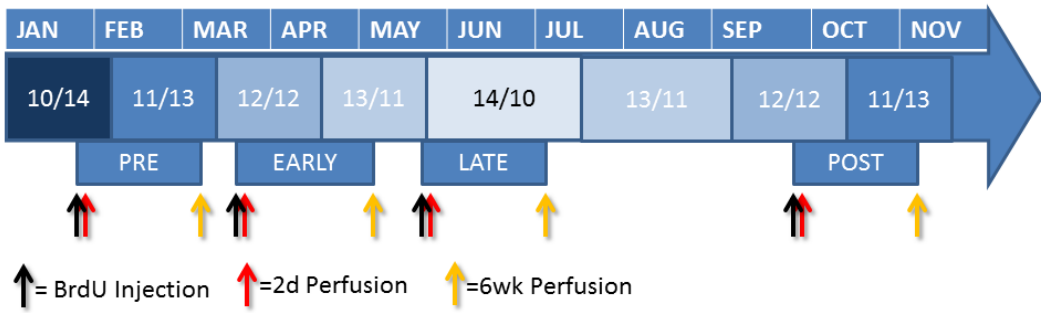

Figure 1. Timeline of Study. Month, photoperiod, season groups, and injection and perfusion times are shown. Black arrows indicate beginning of BrdU injections. Red arrows indicate 2d perfusions and yellow arrows indicate 6wk perfusions.

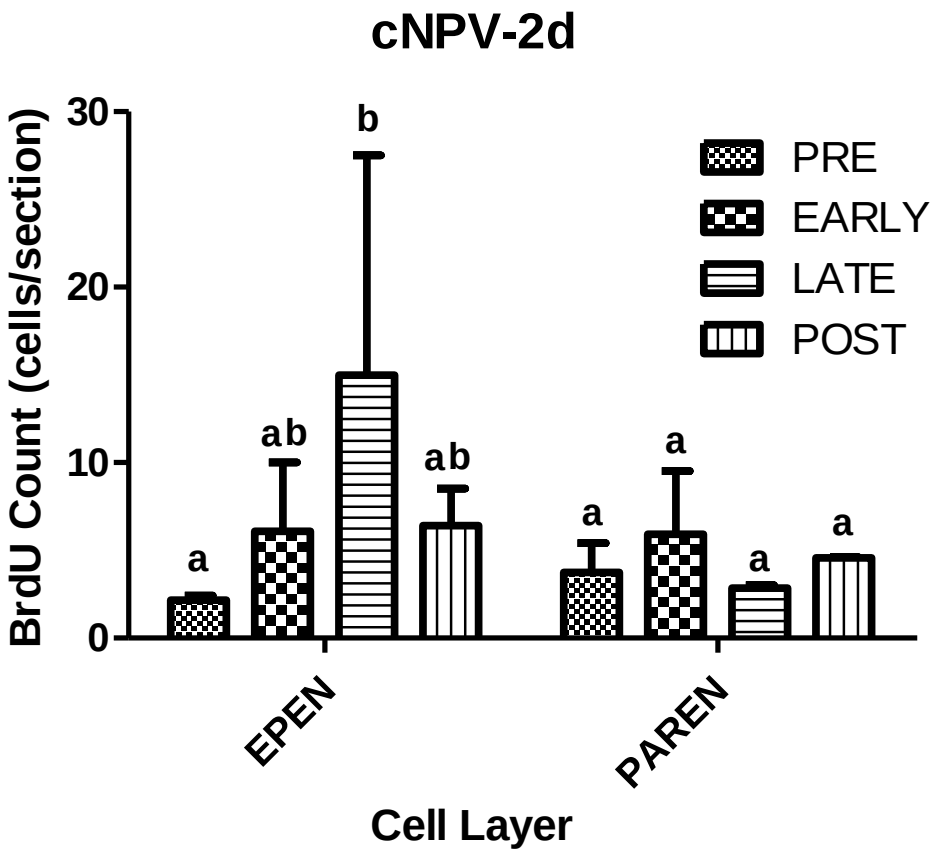

Figure 2. Average number of BrdU-labeled cells in the cNPV at 2d varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

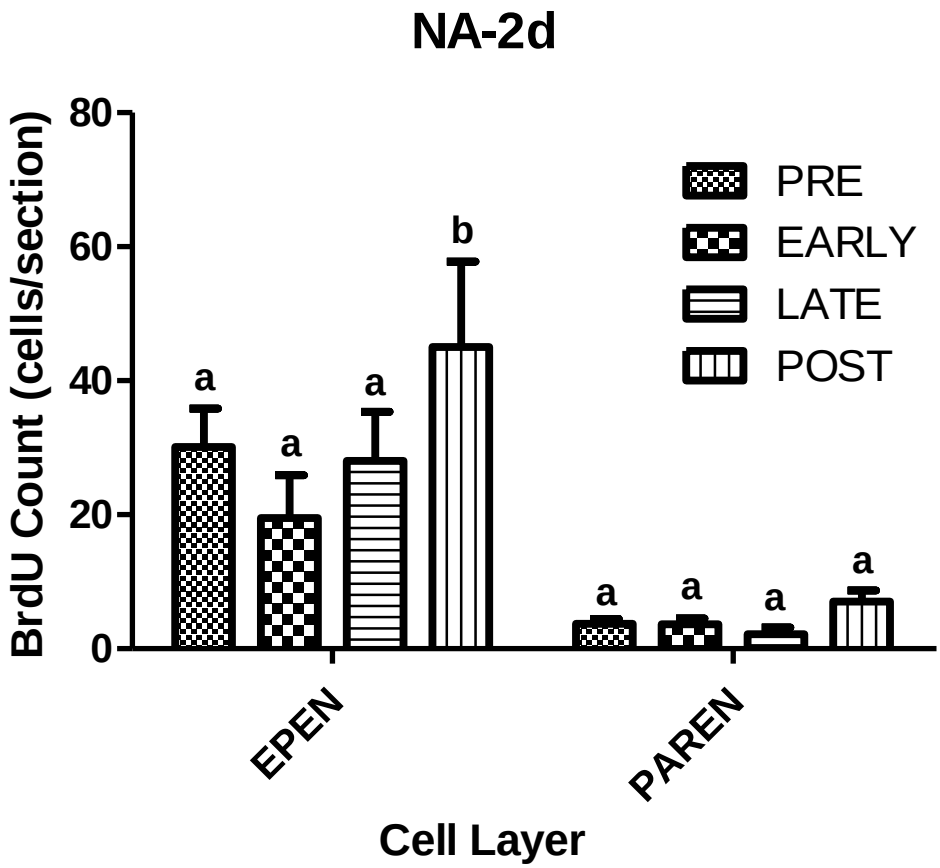

Figure 3. Average number of BrdU-labeled cells in in the NA at 2d varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

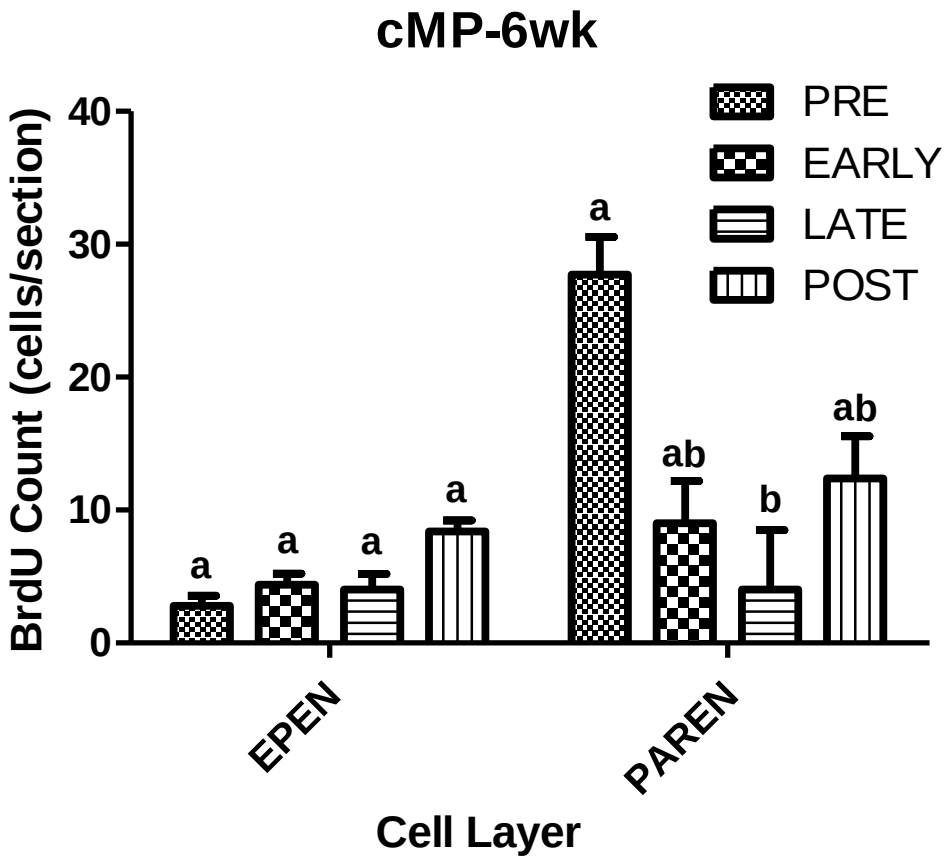

Figure 4. Average number of BrdU-labeled cells in in the cMP at 6wk varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

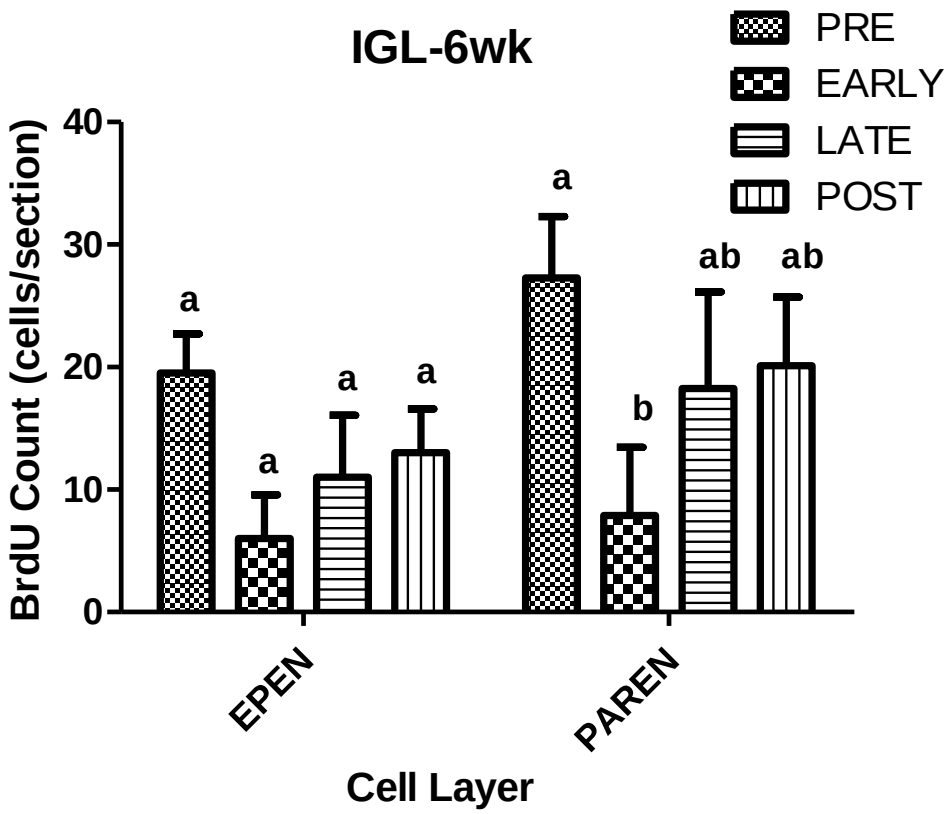

Figure 5. Average number of BrdU-labeled cells in in the IGL at 6wk varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

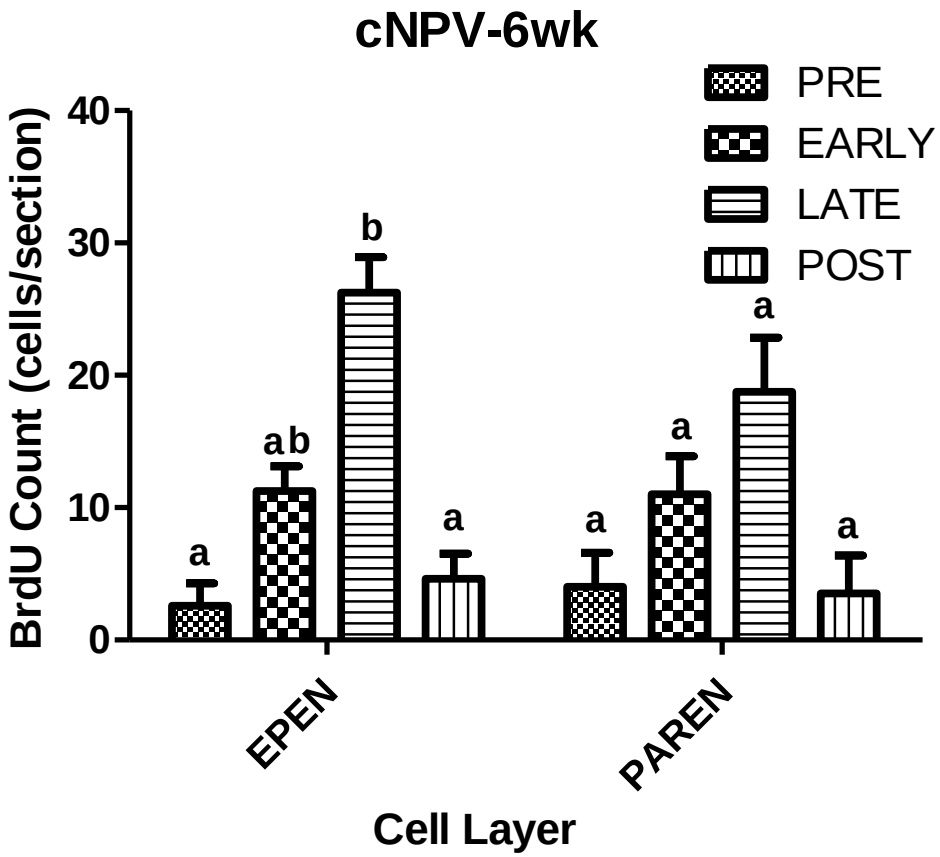

Figure 6. Average number of BrdU-labeled cells in in the cNPV at 6wk varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

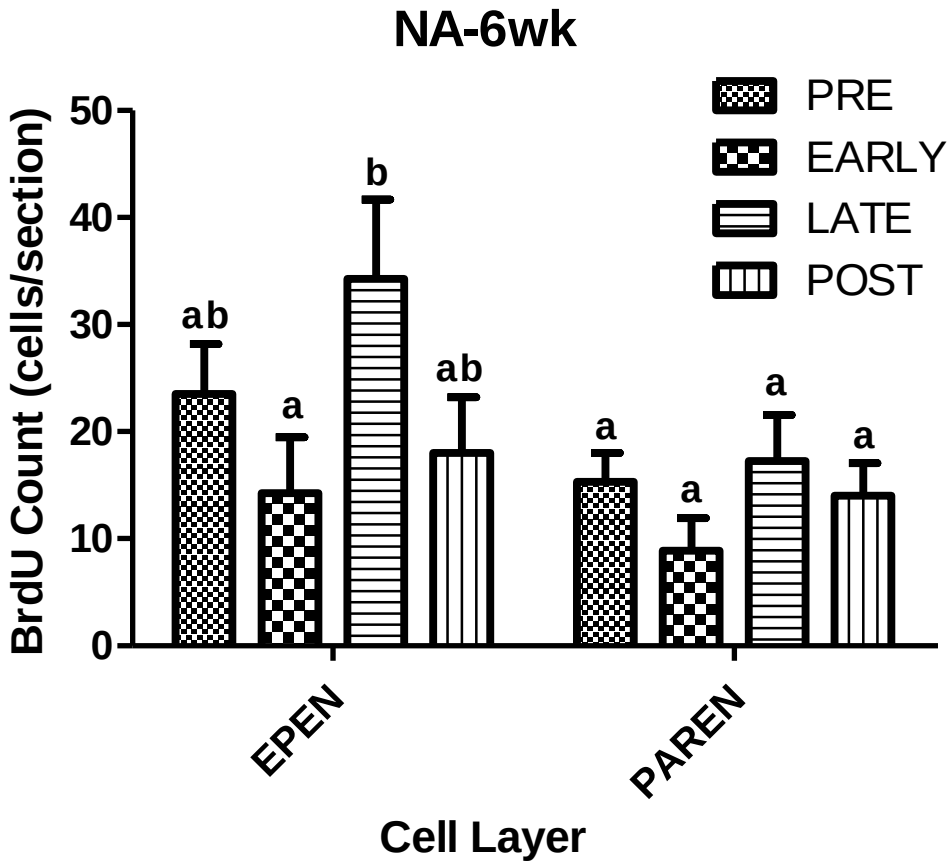

Figure 7. Average number of BrdU-labeled cells in in the NA at 6wk varies with season and cell layer. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across season, within the same cell layer.

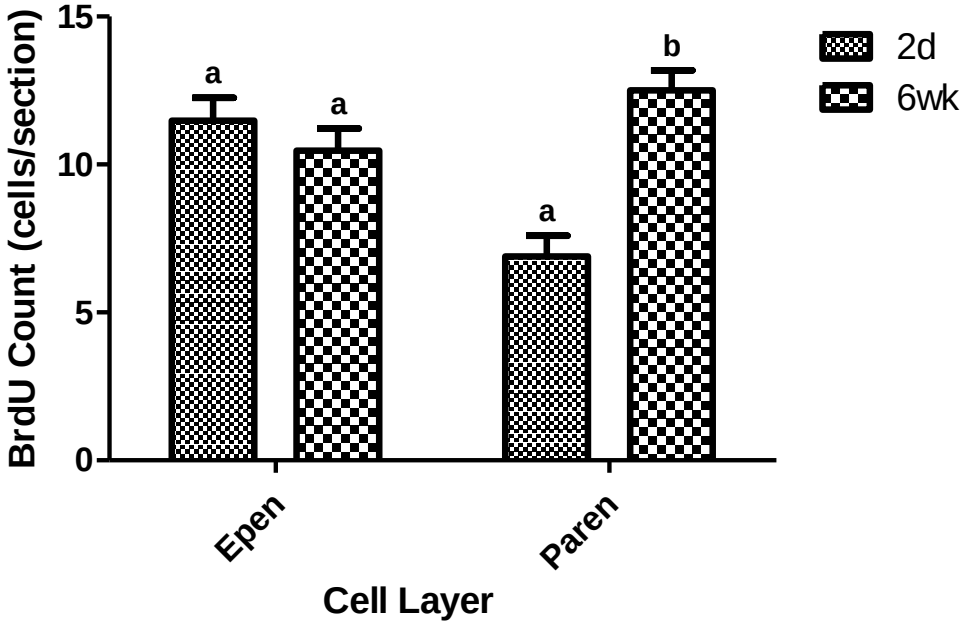

Figure 8. Average number of BrdU-labeled cells in the parenchymal, but not ependymal layer, increases between 2d and 6wks. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM. Significant differences are only shown across timepoint, within the same cell layer.

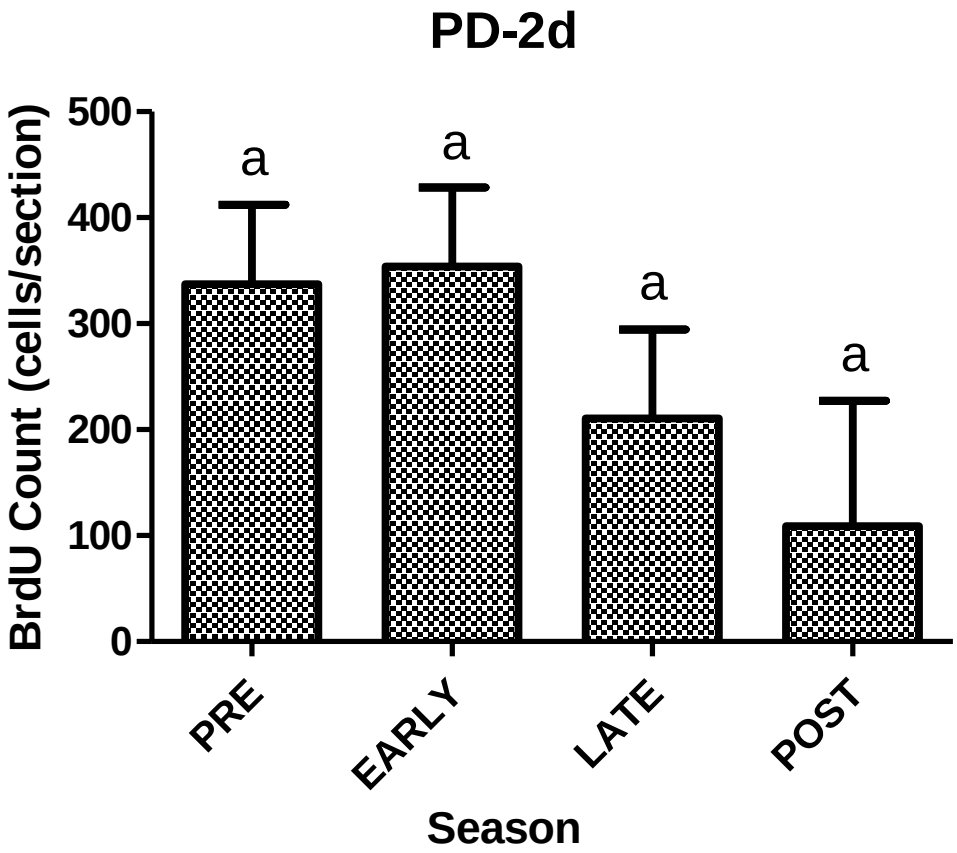

Figure 9. Average number of BrdU-labeled cells in the PD at 2d does not vary with season. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM.

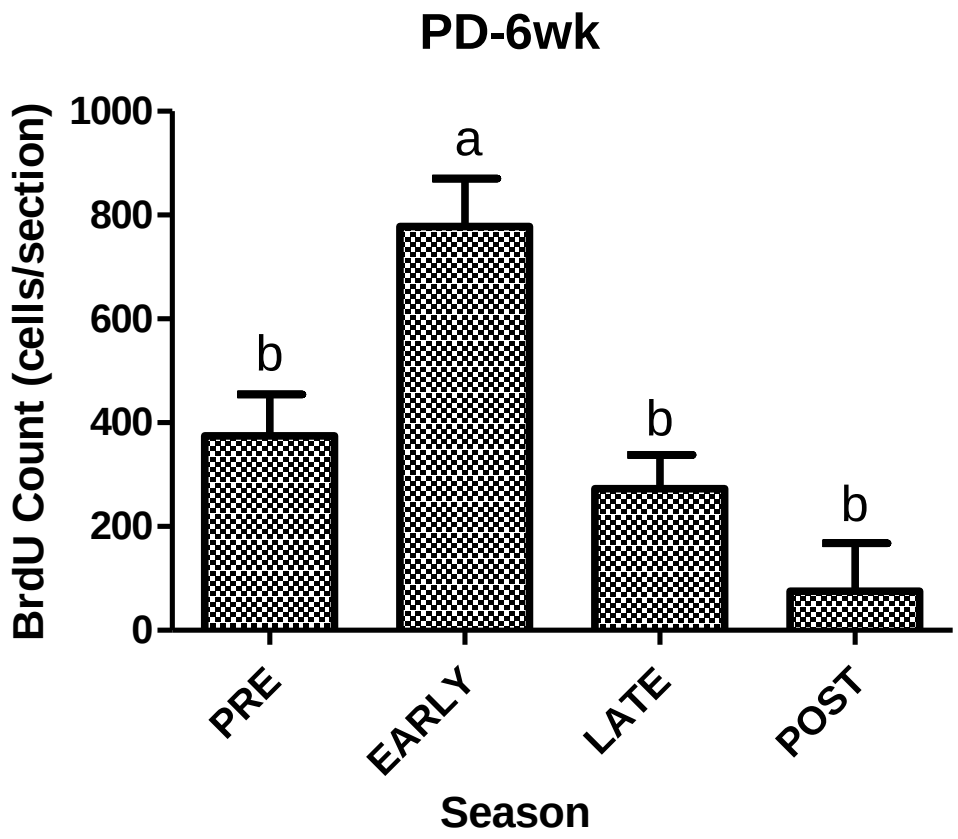

Figure 10. Average number of BrdU-labeled cells in the PD at 6wk varies with season. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis. Error bars indicate SEM.

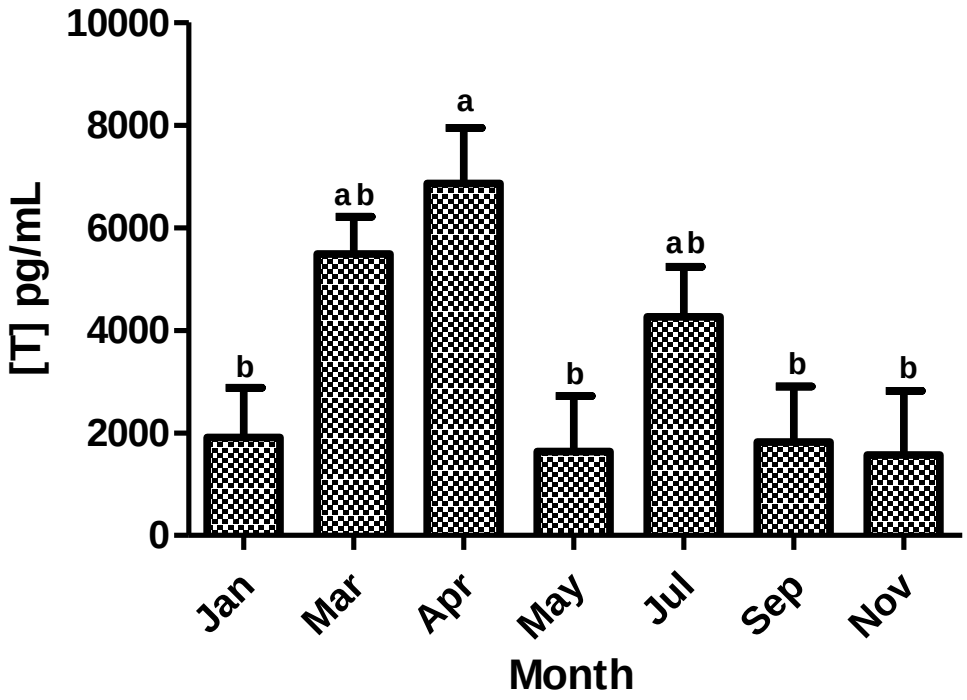

Figure 11. Average plasma testosterone concentration by month varies with month. Bars with different letters indicates significant difference ($p<0.05$) based on a Newman-Keuls post-hoc analysis Error bars indicate SEM.

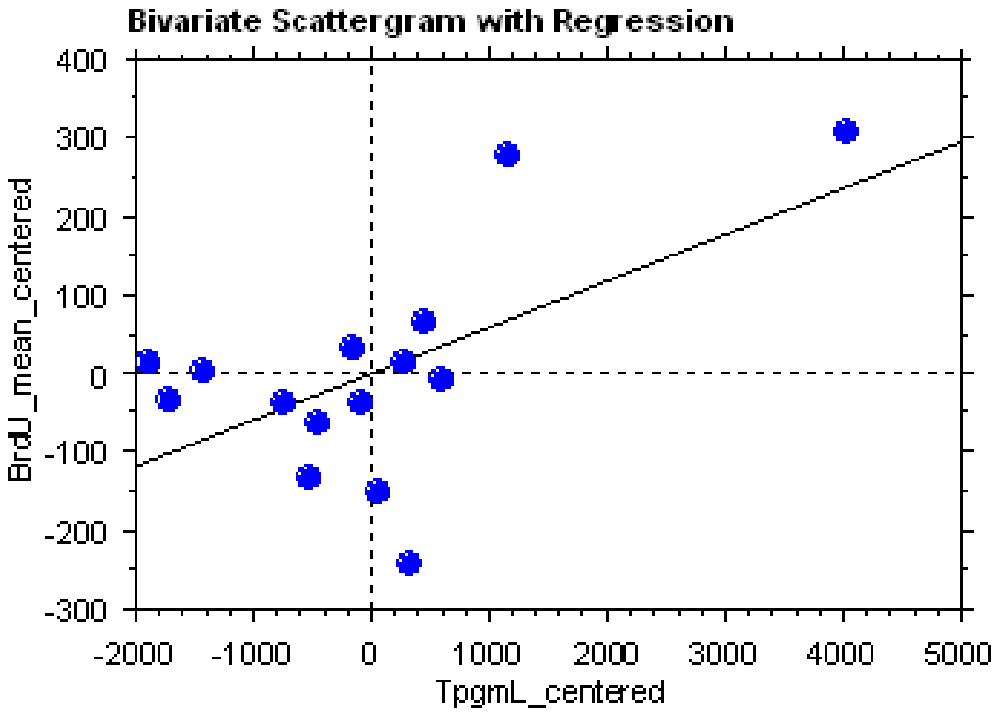

Figure 12. Centered Plasma [T] and BrdU counts correlate in the PD of 2d animals.

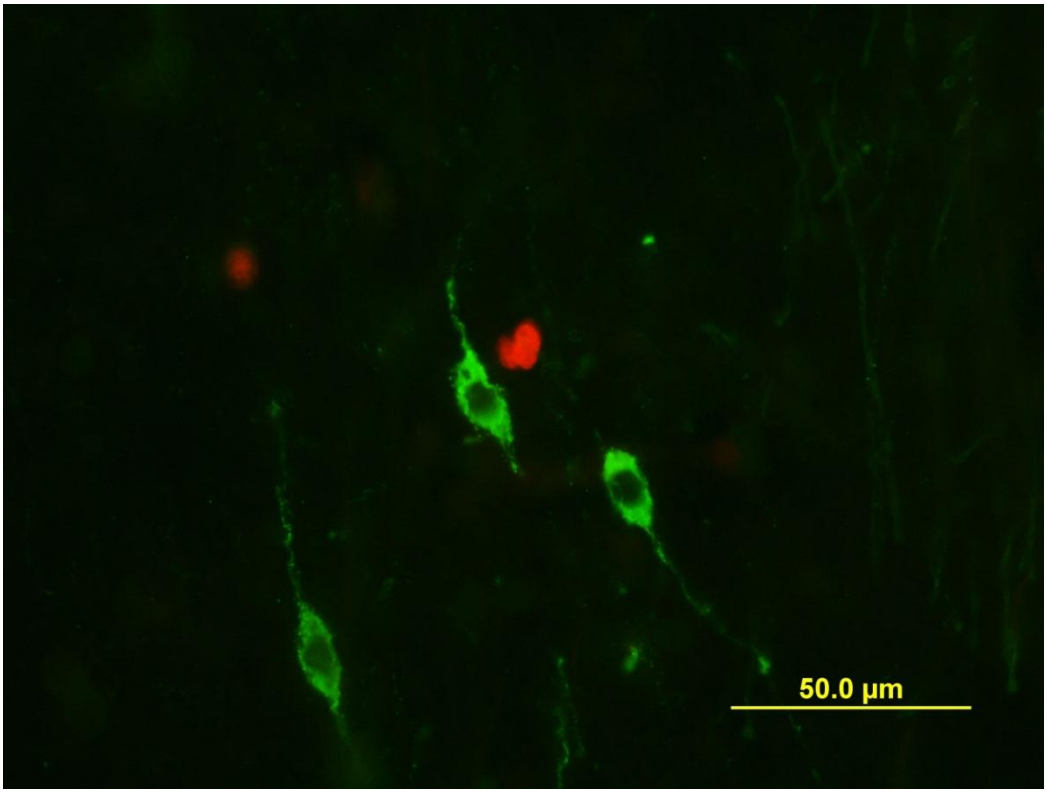

Figure 13. BrdU-labeled cell (red) in close proximity to mGnRH-ir cells in the septum. 40x objective.

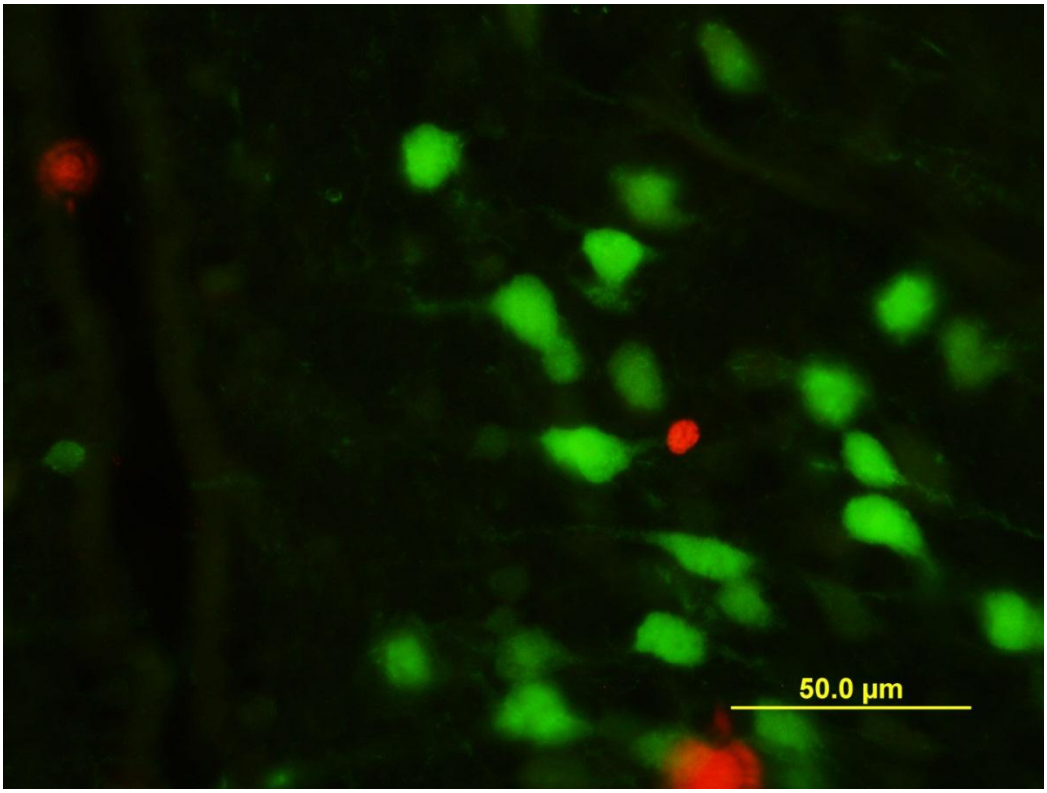

Figure 14. BrdU-labeled cell (red) in close proximity to AVT-ir cells (green) in the POA. 40x Objective.

TABLES

		Number of BrdU-labeled Cells per Section @ 2d								
Region	Cell Layer	PRE	SEM	EARLY	SEM	LATE	SEM	POST	SEM	Sig.
cMP	EPEN	3.50	0.98	2.63	1.10	2.00	1.26	15.00	2.19	-
cMP	PAREN	11.70	2.92	10.88	3.26	11.33	3.76	10.50	6.52	-
cNPV	EPEN	2.40	1.93	10.00	2.16	27.50	2.49	8.50	4.31	*
cNPV	PAREN	5.40	2.06	9.50	2.30	3.00	2.66	4.50	4.61	-
cVH	EPEN	2.60	2.36	3.25	2.63	19.00	3.04	4.50	5.27	-
cVH	PAREN	6.50	1.59	7.13	1.77	2.33	2.05	13.00	3.55	-
DST	EPEN	14.50	2.60	8.50	2.91	9.50	3.36	17.50	5.81	-
DST	PAREN	3.30	0.71	1.63	0.79	2.83	0.92	1.00	1.59	-
IGL	EPEN	8.00	2.96	4.75	3.31	16.83	3.83	7.00	6.63	-
IGL	PAREN	7.80	1.66	4.38	1.86	10.17	2.14	3.00	3.71	-
LS	EPEN	29.00	4.37	16.75	4.89	15.17	5.65	23.50	9.78	-
LS	PAREN	12.00	1.62	10.00	1.81	6.17	2.09	8.00	3.62	-
NA	EPEN	30.10	5.72	19.50	6.39	28.00	7.38	45.00	12.78	*
NA	PAREN	3.70	0.76	3.63	0.84	2.17	0.97	7.00	1.69	-
POA	EPEN	5.50	2.00	4.00	2.23	5.83	2.58	22.50	4.47	-
POA	PAREN	6.00	2.64	9.75	2.95	4.50	3.41	20.50	5.90	-
rMP	EPEN	1.60	0.45	0.75	0.50	2.17	0.58	2.50	1.01	-
rMP	PAREN	18.10	1.72	9.00	1.92	6.17	2.22	20.50	3.85	-

Table 1. Number of BrdU-labeled Cells at the 2d Time Point. Asterisks indicate significant seasonal difference (p-value <0.05) in post-hoc test.

		Number of BrdU-labeled Cells per Section @ 2d								
Region	Cell Layer	PRE	SEM	EARLY	SEM	LATE	SEM	POST	SEM	Sig.
cMP	EPEN	3.50	0.98	2.63	1.10	2.00	1.26	15.00	2.19	-
cMP	PAREN	11.70	2.92	10.88	3.26	11.33	3.76	10.50	6.52	-
cNPV	EPEN	2.40	1.93	10.00	2.16	27.50	2.49	8.50	4.31	*
cNPV	PAREN	5.40	2.06	9.50	2.30	3.00	2.66	4.50	4.61	-
cVH	EPEN	2.60	2.36	3.25	2.63	19.00	3.04	4.50	5.27	-
cVH	PAREN	6.50	1.59	7.13	1.77	2.33	2.05	13.00	3.55	-
DST	EPEN	14.50	2.60	8.50	2.91	9.50	3.36	17.50	5.81	-
DST	PAREN	3.30	0.71	1.63	0.79	2.83	0.92	1.00	1.59	-
IGL	EPEN	8.00	2.96	4.75	3.31	16.83	3.83	7.00	6.63	-
IGL	PAREN	7.80	1.66	4.38	1.86	10.17	2.14	3.00	3.71	-
LS	EPEN	29.00	4.37	16.75	4.89	15.17	5.65	23.50	9.78	-
LS	PAREN	12.00	1.62	10.00	1.81	6.17	2.09	8.00	3.62	-
NA	EPEN	30.10	5.72	19.50	6.39	28.00	7.38	45.00	12.78	*
NA	PAREN	3.70	0.76	3.63	0.84	2.17	0.97	7.00	1.69	-
POA	EPEN	5.50	2.00	4.00	2.23	5.83	2.58	22.50	4.47	-
POA	PAREN	6.00	2.64	9.75	2.95	4.50	3.41	20.50	5.90	-
rMP	EPEN	1.60	0.45	0.75	0.50	2.17	0.58	2.50	1.01	-
rMP	PAREN	18.10	1.72	9.00	1.92	6.17	2.22	20.50	3.85	-

Table 2. Number of BrdU-labeled Cells at the 6wk Time Point. Asterisks indicate significant seasonal difference (p-value <0.05) in post-hoc test.