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Social modulation of adult brain cell proliferation: Influence of sex and gonadal hormones

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Social modulation of adult brain cell proliferation: Influence of sex and gonadal hormones

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Social modulation of adult brain cell proliferation: Influence of sex and gonadal hormones

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Environmental factors are known to have far-reaching effects on nervous system function, and in the adult brain, it is clear that a wide range of environmental stimuli modulate cell proliferation and survival (e.g., neurogenesis). This project investigated whether social stimulation and concomitant changes in gonadal hormones can influence the proliferation of new cells in the adult brain. The adult green treefrog (*Hyla cinerea*) was used as the model system; studying the courtship behavior of the highly social treefrog affords a direct, quantifiable way to measure the effects of acoustic social cues and hormonal intervention on adult brain cell proliferation.

Using immunohistochemistry techiques, endocrinological manipulations, and socially-relevant acoustic stimulus presentations, I report that social cues modulate cell proliferation in the brains of adult male and female *H. cinerea*. I first mapped the distribution of proliferative areas in the adult treefrog brain using 5-bromo-2′-deoxyuridine (BrdU) labeling. I then exposed naturally-cycling male and female treefrogs to random tones or a recording of a natural *H. cinerea* chorus for ten days during the breeding season. I found that male and female treefrogs that heard their conspecific chorus exhibited increased brain cell proliferation compared to animals that heard random tones. Moreover, this modulation was region-specific and occurred in those

regions which reflected their presumed involvement in reproductive physiology and behavior: the preoptic area (POA) and the infundibular hypothalamus (IF). To determine the involvement of gonadal hormones in cell proliferation with and without social stimulation, I gonadectomized and implanted male and female *H. cinerea* with blank or steroid-filled implants. After exposing the treefrogs to the same acoustic conditions as above, I discovered that social modulation of adult cell proliferation can occur without the influence of gonadal hormones (i.e., androgens in the male and estrogen in the female). Furthermore, the results revealed that neither hormone was neurotrophic and in fact, chronically-elevated estrogen levels decreased cell proliferation in the female POA and IF. Together, these results indicate that the reception of acoustic social cues increases cell proliferation in brain regions mediating sexual behavior and endocrine regulation; furthermore, this modulation occurs in a sexually-differentiated fashion without gonadal hormone influence.

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Chapter 1: Introduction

A principle feature of brain development is producing the necessary neural architecture to allow the integration of information from the external environment with internal cues; these cues reflect important aspects of an animal's physiological state (Simerly, 2002). Thus, adaptive physiological responses are those that ensure successful reproduction and require the ability to coordinate behavior and physiology in response to sexually-relevant signals (Simerly, 2002). Consequently, the ability to recognize socio-sexual cues and respond accordingly is critical to survival in many species. Social cues that regulate the neural, physiological, and hormonal changes that accompany the expression of reproductive behavior in vertebrates may also regulate a form of neuroplasticity, specifically adult neurogenesis. The following contains an introduction to the model system for this dissertation research and a review of the current knowledge of the social modulation of adult neurogenesis in vertebrates.

Anuran amphibian socio-sexual system

Similar to other vertebrates, reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis via neural and hormonal feedback (e.g., Francis et al., 1993). The HPG axis is subject to both endogenous and exogenous cues, including photoperiod and social cues, and regulates gonadotropin releasing hormone (GnRH; reviewed in Wingfield & Kenagy, 1991). In turn, GnRH stimulates the gonads to release gonadal steroid hormones. As in other vertebrates, high levels of gonadal steroid hormones are necessary for reproductive behavior in amphibians (e.g., Licht et al., 1983; Mendonça et al., 1985). In seasonally breeding anuran amphibians, gonadal hormones fluctuate seasonally in concert with reproductive social behavior. This occurs with androgens in the male (Varriale et al., 1986; Itoh et al., 1990; Itoh and Ishii, 1990; Gobbetti et al., 1991; Polzonetti-Magni et al., 1998) and estrogen (among others) in the female (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986). Gonadal hormones

also fluctuate within the breeding season in some female anurans (Lea et al., 2000; Bosch and Boyero, 2004; Lynch and Wilczynski, 2005).

Courtship behavior and neural control in male anurans

In frogs, calling is the major socio-sexual behavior expressed by males; it is stereotyped and sexually-dimorphic, with most species having only one or two calls in their repertoire. Males aggregate in lek-type formations (Wells, 1977) and call to attract females; in fact, the most important determinant of mating success is the number of nights a male spends calling (e.g., Ryan, 1985; Gerhardt, 1988; Dyson et al., 1998). Calling is also androgen dependent (Wada et al., 1976; Wada and Gorbman, 1977b; Wetzel and Kelley, 1983; Solis and Penna, 1997; Burmeister and Wilczynski, 2001) and eliminated upon castration (Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976; Burmeister and Wilczynski, 2001). This is not to imply a causal relationship between androgens and calling behavior; in fact, a few studies have reported that androgens are lower in calling frogs compared to non-callers (e.g., Mendonça et al., 1985; Orchinik et al., 1988), where others have shown the opposite relationship (e.g., Townsend and Moger, 1987; Marler and Ryan, 1996) or alternatively, no correlation (e.g., Burmeister and Wilczynski, 2000). Thus, androgens may influence the motivation to call but do not determine the actual level of calling (Burmeister & Wilczynski, 2000).

It is clear that sufficient levels of androgens are necessary for calling behavior in the male anuran, but there may be other contributing factors, including corticosterone. Interestingly, corticosterone, like androgens, is socially-modulated (Burmeister and Wilczynski, 2000). Corticosterone levels are also seasonally-modulated (e.g., Licht et al., 1983) and have been suggested to regulate male reproductive behavior (Marler and Ryan, 1996). The effects of corticosterone may be species-dependent as in some species corticosterone inhibits male calling behavior (Marler and Ryan, 1996) and is associated with low androgen levels (Licht et al., 1983; Moore and Zoeller, 1985; Marler and Ryan, 1996); whereas in other species, corticosterone is higher in calling males (Mendonça et al., 1985; Orchinik et al., 1988; Harvey et al., 1997; Leary et al., 2004) and has no effect on androgen levels (Leary et al., 2004; Orchinik, 1988). A model for the complex

relationship between calling behavior and corticosterone in male anurans has been proposed by Emerson (2001).

The anuran amphibian central nervous system has unique qualities related to processing social signals. For instance, the anuran auditory system is specialized to detect and recognize conspecific calls (reviewed in Capranica & Moffat, 1983; Walkowiak, 1988). Central auditory pathways are summarized in Wilczynski and Capranica (1984) and Feng et al. (1990). In brief, the torus semicircularis and thalamic nuclei transmit auditory information to the preoptic area (POA) and ventral hypothalamus (VH), which are the reproductive centers of the basal forebrain. The POA and VH concentrate androgens (Kelley et al., 1975; Kelley et al., 1978; di Meglio et al., 1987) and respond to acoustic stimulation as shown by single cell recordings (Wilczynski and Allison, 1989; Allison, 1992). The POA also plays an important role in male social behavior, i.e., calling behavior, as shown by lesion and stimulation studies (Knorr, 1976; Schmidt, 1968; Wada and Gorbman, 1977a); the sensory system thus links social cues to endocrine control regions of the anuran brain (Wilczynski et al., 1993). In addition, socially- or hormonally-induced changes have been shown to occur in the socio-sexual system of male anurans; this includes GnRH (e.g., Iela et al. 1994; Burmeister & Wilczynski, 2005), arginine vasotocin (AVT; the nonmammalian analog to arginine vasopressin; Boyd, 1994; Emerson and Boyd, 1999), and tyrosine hydroxylase (Chu and Wilczynski, 2002). These studies highlight the plasticity in the adult anuran brain as it relates to socio-sexual behavior.

Receptive behavior and neural control in female anurans

In female frogs, socio-sexual behavior is displayed when the female is hormonally-receptive to mating. This receptive behavior can be considered an approach toward advertising males, i.e., phonotaxis (Schmidt, 1984, 1985a, b; Gordon and Gerhardt, 2009), emitting a vocalization (e.g., Tobias et al., 1998; Shen et al., 2008), or the inhibition of release calls or leg extensions (e.g., Diakow and Nemiroff, 1981; Kelley, 1982). Receptive behavior tends to occur when gonadal hormones, i.e., estrogen and progesterone, are highest (Lynch and Wilczynski, 2005). A recent study, however,

suggested that estradiol alone could promote phonotaxis (Chakraborty and Burmeister, 2009). These authors conclude that changes in female sexual behavior during a reproductive cycle is controlled by fluctuations in estradiol concentrations (Chakraborty and Burmeister, 2009). Nonetheless, it seems apparent that other hormones, including progesterone and prostaglandin, may also be involved in mediating receptive behaviors in the female anuran amphibian (Schmidt, 1984, 1985a, b).

The neural control of socio-sexual behavior in females is not as well-documented as calling behavior in male anurans; however, there have been studies on the involvement of various brain regions in acoustically-guided behavior in female anurans. For example, an early report using lesion studies (Schmidt, 1988) suggests that neither the dorsal thalamus nor the torus semicircularis (the homologue of the mammalian inferior colliculus; Wilczynski and Endepols, 2007) are necessary for female phonotaxis. However, a more recent study demonstrated that the torus semicircularis is in fact crucial for phonotaxis in reproductively active females (Endepols et al., 2003). Further, another study reports that lesions of the POA abolish phonotaxic behavior in female anurans (Walkowiak et al., 1999). Although the neural control of phonotaxis may be unresolved, we do know that these putative brain regions concentrate estrogen (Morrell et al., 1975; Kelley at al., 1978). Similar to male anuran amphibians, females exhibit socially-induced changes in gonadal morphology (e.g., Lea et al., 2001), gonadal hormone production (Lynch and Wilczynski, 2006), and immediate early gene expression (i.e., egr-1 in the torus semicircularis; Lynch and Wilczynski, 2008). Thus, both male and female anuran amphibian exhibit plasticity as adults in response to socio-sexual stimuli.

Adult neurogenesis: emphasis on social and gonadal hormone modulation in vertebrates

Adult neurogenesis consists of several processes including cell proliferation, differentiation, migration and cell survival (see review in Ming and Song, 2005). The number of new cells in the brain can be increased either by enhancing cell proliferation or survival of the new cells, or both. Studies of adult neurogenesis commonly use 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog that is incorporated into DNA during cell synthesis (Taupin, 2007), to observe proliferative activity in the brain.

Bromodeoxyuridine is typically injected into the animal, and differing lengths of time before sacrifice determine whether cell proliferation or cell survival is observed. In addition, BrdU labeling in brain tissue can be co-localized with phenotypic markers to determine whether proliferating cells are neurons or glia. An elaboration on the detection of adult neurogenesis can be found in Kuhn and Peterson (2008).

A recent review has highlighted the importance of social behavior in regulating adult neurogenesis in the vertebrate central nervous system (Gheusi et al., 2009). For example, mating activity enhances cell proliferation and/or survival in female prairie voles (Fowler et al., 2002). In males, on the other hand, mating activity failed to increase cell proliferation in hamsters (Antzoulatos et al., 2008). Pheromonal signals have been a focus in these mammalian studies as they tend to positively regulate adult neurogenesis; specifically, chemosensory socio-sexual cues influence adult neurogenesis primarily in regions related to the olfactory system (e.g., Smith et al., 2001; Fowler et al., 2002; Baudoin et al., 2005). Social stress is another regulating factor of adult neurogenesis, however, it typically is a negative one (reviewed in Gheusi et al., 2009). Further, social behavior is usually reliant on gonadal steroid hormones, thus their influence on adult neurogenesis may be linked together. In fact, Pawluski et al. (2009) suggest that the seasonal fluctuation of steroid hormones may have evolved to modulate neurogenesis in order to cope with the changing environment. Thus, the social modulation of adult neurogenesis and concomitant gonadal hormone mediation may be an adaptive response in many vertebrates. The following includes a review of the current knowledge of the social and hormonal modulation of cell proliferation in vertebrates.

Songbirds

Research in songbirds has been very influential on the field of adult neurogenesis, because it was the first system that provided evidence that neurons generated in the adult brain can be recruited into functional circuits (Paton and Nottebohm, 1984). This study used electrophysiological studies to demonstrate that new neurons in the adult songbird brain respond to acoustic stimuli (Paton and Nottebohm 1984). In addition, the social modulation of adult neurogenesis has been reported in the song control nuclei of

songbirds via acoustic social cues (e.g., Lipkind et al., 2002). In regards to gonadal hormone modulation of adult neurogenesis, studies report that estrogen increases the recruitment and/or survival of new neurons (e.g., Hidalgo et al., 1995). However, Gahr et al. (2002) suggest that estrogens are unlikely to act directly in songbirds since new neurons do not express estrogen receptors (e.g., Hidalgo et al., 1995). Testosterone also appears to increase the survival (Goldman and Nottebohm, 1983; Rasika et al., 1994; for an exception, see Brown et al., 1993) and proliferation (Absil et al., 2003) of new HVC neurons. In songbirds, gonadal hormones have also been suggested to affect neurogenesis indirectly through the regional induction of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF). Brain-derived neurotrophic factor infusion increases neuronal survival within the HVC of canaries (Alvarez-Borda et al., 2004), and the neurotrophic effect of testosterone has been demonstrated to be mediated by BDNF (Rasika et al., 1999). Brain-derived neurotrophic factor is also regulated by social behavior; for example, singing upregulates BDNF in songbirds (Li et al., 2000; Alvarez-Borda et al., 2004).

Mammals

In mammals, the modulation of adult neurogenesis by gonadal hormones is dependent on the species, region, and dose/administration of the hormone (see reviews in Fowler et al., 2008; Galea, 2008; Pawluski et al., 2009). Few studies have investigated the effects of androgens on neurogenesis in mammals. However, reproductively active male meadow voles have been shown to exhibit increased cell survival in the hippocampus compared to reproductively inactive males (Ormerod and Galea, 2003). Using exogenous manipulations of testosterone, there is evidence of a direct effect of testosterone on increased cell proliferation and/or survival in the mammalian hippocampus (Spritzer and Galea, 2007). This effect may be mediated by dihydrotestosterone (DHT; Spritzer and Galea, 2007) or an estrogen-dependent pathway (e.g., Fowler et al., 2003). From research on gonadal hormone modulation of adult neurogenesis in mammals, a potential mechanism of action has been suggested by Spritzer and Galea (2007) among others: gonadal hormones may influence cell

proliferation or survival directly by binding to steroid receptors on newly proliferated cells. Thus, androgens (or estrogen) may act as transcription factors and induce changes in gene transcription (Spritzer and Galea, 2007).

Naturally occurring fluctuations in gonadal hormones across the estrous cycle influence hippocampal neurogenesis in adult females (Tanapat et al., 1999; Galea et al., 2006; reviewed in Pawluski et al., 2009). Natural estrogen levels can enhance cell proliferation and decrease cell death, but this effect is transient (Tanapat et al., 1999). These effects are also species-dependent as seen in conflicting reports of the effects of estradiol in different species of rodents (Galea and McEwen, 1999; Banasr et al., 2001) Lagace et al., 2007; Ormerod and Galea, 2001). There are also important differences in the effects of estrogen when dose and administration time are considered (reviewed in Pawluski et al., 2009). For example, short-term exposure (30 minutes to 4 hours) to an acute high level of estradiol enhances hippocampal cell proliferation (Barha et al., 2009; Ormerod et al., 2003). Chronic administration of estradiol resulting in elevated estrogen levels, on the other hand, inhibits or decreases the process of adult neurogenesis (Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003; Tanapat et al., 2005; Barker and Galea, 2008). Interestingly, chronically-elevated estrogen also impairs hippocampal-dependent learning and memory, which suggests an optimal level of estrogen for both neurogenesis and behavioral tasks (Barha et al., 2009). Pawluski et al. (2009) suggest that estradiol influences neurogenesis and cell death in the adult female rodent via interactions with estrogen receptors (ERs) expressed on neural progenitor cells. In fact, both ERα and ERβ mRNA and/or protein have been located on progenitor cells and immature neurons in the dentate gyrus (Isgor and Watson, 2005; Mazzucco et al., 2006). Lastly, in regards to social behavior and associated gonadal hormones, reproductively active female voles have been shown to exhibit lower levels of cell proliferation than reproductively inactive females (Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003).

Fish

Adult neurogenesis is more widespread and occurs at a higher level in non-mammals than in mammals (García-Verdugo et al., 2002). As such, fish have been considered the 'champions' of adult neurogenesis (Mouriec et al., 2008) due to the high degree of cell proliferation in most major subdivisions of the adult brain (Zupanc and Horschke, 1995). The social and hormonal modulation of adult neurogenesis in fishes is not well-documented; however, social interactions and cortisol treatment increase cell addition in the prepacemaker nucleus of the electric fish (Dunlap et al., 2006). Further, Mouriec et al. (2008) suggest that estrogens are involved in the high proliferative activity of the adult fish brain; however, this has not been tested experimentally.

Amphibians

Adult cell proliferation has been previously demonstrated in anuran amphibians including *Rana temporaria* (using 3H-thymidine; Bernocchi et al., 1990, Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), *Rana esculenta* [using proliferating cell nuclear antigen (PCNA); Raucci et al., 2006], and *Rana catesbeiana* (using BrdU; Simmons et al., 2008). Although these studies use different methods to label proliferating cells and investigate different time points, the primary result is that proliferative activity in the anuran amphibian brain is extensive. Previous studies have suggested that the high proliferative activity in anuran amphibians may be related to the continual growth of these animals throughout adulthood (e.g., Bernocchi et al. 1990), which is similar to that suggested in adult fishes (Zupanc, 2001) and reptiles (Font et al., 2001). At any rate, this constitutive cell proliferation occurs primarily in the ventricular zone of the lateral ventricles, similar to that seen in other vertebrates (Doetsch and Scharff, 2001; Lindsey and Tropepe, 2006). Fewer proliferating cells are observed in the fourth ventricle than around the lateral, third, and tectal ventricles (Bernocchi et al., 1990; Simmons et al., 2008; Raucci et al., 2006). Interestingly, many studies report high proliferative activity in the POA and hypothalamus (Bernocchi et al., 1990, Chetverukhin and Polenov, 1993; Raucci et al., 2006; Simmons et al., 2008; Polenov and Chetverukhin, 1993). Discrepancies do exist, however, in the extent of proliferative activity in other brain regions. For example, Simmons et al. (2008) found higher levels of labeling in the

optic tectum and torus semicircularis compared to other anuran amphibian studies (e.g., Bernocchi et al., 1990). Also, there were mixed reports regarding whether the habenula contained proliferating cells: cells were observed in the study by Margotta et al. (2002) but not by others (e.g., Raucci et al., 2006). Lastly, the hindbrain regions (e.g., cerebellum and medulla) also had fewer (if any) proliferating cells in most anuran studies; however, Bernocchi et al. (1990) noted moderately high labeling in the medulla and Raucci et al (2006) noted many labeled cells in the granular layer of the cerebellum.

The time line and seasonality of adult neurogenesis in anuran amphibians is unresolved at this time, but we can speculate a few parameters from the available studies in anurans. For example, cells born in the ventricular zone may migrate into parenchymal regions after two (Simmons et al., 2008) or three days (Polenov and Chetverukhin, 1993). However, Bernocchi et al. (1990) did find proliferating cells in the parenchyma within two hours, but those were likely born *in situ*. Further, labeled cells can survive for at least one month (Polenov and Chetverukhin, 1993; Simmons et al., 2008), although studies did not investigate possible longer life-spans. Using 3H-thymidine labeling, Bernocchi et al. (1990) and Chetverukhin and Polenov (1993) intimate seasonal differences in proliferative activity in the anuran brain. In summary, amphibians and fish are the only vertebrates as yet to exhibit neurogenesis in all major subdivisions of the adult brain (Raucci et al., 2006). However, modulating factors, such as the role of social behavior or gonadal hormones, in adult neurogenesis have not been previously investigated in anuran amphibians.

Overview of Chapters

The objective of the research detailed in this dissertation was to understand the link among social cues, gonadal steroid hormones, and cell proliferation in adult *H. cinerea*. Using immunohistochemistry techiques, endocrinological manipulations, and socially-relevant acoustic stimulus presentations, I investigate the distribution of proliferative areas in the adult treefrog brain using BrdU labeling, the influence and region-specificity of socially-relevant cues on cell proliferation in the brains of males and females, and the influence of gonadal hormones on cell proliferation with and without

social stimulation. In addition, I investigate the involvement of evoked calling behavior in the modulation of cell proliferation in male treefrogs.

The research detailed in Chapter 2[1] is designed to determine regions of proliferative activity in the adult *H. cinerea* brain. Adult green treefrogs received injections of BrdU and were sacrificed 2 hours, 2 days, 2 weeks, or 30 days later. Bromodeoxyuridine immunoreactive (BrdU+) cells were counted in 12 brain regions and notations were made on whether the cells were located in ventricular or parenchymal areas. The percentage of parenchymal BrdU+ cells to the total number of new cells was used as an indicator of potential cell migration. The results of this study indicated that the POA and infundibular hypothalamus (IF), a brain region which includes the VH, have extensive proliferative activity. Interestingly, these regions are involved in socio-sexual behavior in anuran amphibians.

In Chapter 3, I investigate the influence of acoustic social signals on adult male and female green treefrogs. In acoustically-isolated chambers, I presented males and females with nightly acoustic stimulation: a conspecific mating chorus ('Chorus') or random tones ('Tone'), which were matched in duration and approximate amplitude to the calls in the mating chorus. The treefrogs received acoustic stimulation for 10 days (i.e., 9 nites) as per a time course conducted in a previous study (Burmeister and Wilcynski, 2000). In the evening on days 1, 5, and 9, each treefrog received an intraperitoneal injection of BrdU ca. 30 minutes prior to acoustic stimulation. I counted BrdU+ cells in the POA and IF, two regions involved in acoustic communication and endocrine regulation, in addition to a control region containing the olfactory bulb. These regions were selected to determine whether social stimulation modulated cell proliferation in general or had specific effects on brain regions mediating social behavior. I qualifed the resulting number of BrdU+ cells as indicative of cell proliferation; thus, this terminology is used throughout the dissertation research. Using the number of BrdU+ cells in the POA and IF, I investigated the influence of sex, time during the breeding season, and acoustic stimulus type on cell proliferation. I also monitored daily calling

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[1]L.M. Almli and W. Wilczynski. 2007. Brain Research 1159: 112-118.

behavior in male treefrogs in order to determine the effect of calling on cell proliferation. I categorized calling during the acoustic stimulation (Chorus or Tone) as 'evoked calling behavior.' In addition, I measured plasma steroids (androgens and estradiol in males and females, respectively) and using those levels, I statistically determined the importance of gonadal hormones in socially-modulated cell proliferation. In order to further characterize the factors modulating cell proliferation in males, male treefrogs were divided into two groups depending on their evoked calling behavior: those that called back to the stimulus ('Caller') or those that did not call ('Non-caller'). The contribution of both calling behavior and androgen levels on cell proliferation in males was then investigated statistically.

Due to the implication of hormone involvement, I investigate the influence of gonadal hormones on cell proliferation in male and female *H. cinerea*, with and without social modulation in Chapter 4. Seven days prior to the acoustic stimulation procedure, I gonadectomized and implanted male and female *H. cinerea* with blank or steroid-filled implants (testosterone for males and estradiol for females). For 10 days, I exposed the treefrogs to the same acoustic conditions as in Ch. 3, monitored calling behavior of males, collected plasma samples, and counted BrdU+ cells in the POA and IF. I analyzed the effect of acoustic stimulation type (Chorus or Tone) and hormone implant on cell proliferation in males and females. In males, I determined the influence of evoked calling behavior on cell proliferation in the POA and IF.

Chapter 5 is a comparison of staining methodologies for cell proliferation. Although BrdU is a common marker to label proliferating cells in the brain, BrdU immunohistochemical staining may be problematic (see Taupin, 2007). In this chapter, I briefly review some common issues with BrdU staining. Using the subjects from Ch. 3, I quantified cell proliferation in the POA and IF using two staining procedures: immunohistochemistry using fluorescein (i.e., immunofluorescence) or 3,3′-diaminobenzidine tetrahydrocloride (DAB) as the chromagen to label proliferating cells. I then compared whether proliferative activity in those regions were influenced by the time during the breeding season, acoustic stimulus type (Chorus or Tone) and sex. Because the subjects were the same in both staining methods, I used the number of BrdU+ cells with

each staining method as a repeated factor in a statistical analysis; this allowed me to isolate trends across both staining methods. Correlations between the number of fluorescein-labeled and DAB-labeled BrdU+ cells in the POA and IF were also performed. In addition, I calculated a ratio consisting of the number of fluorescein-labeled BrdU+ cells to DAB-labeled BrdU+ cells. The ratio data depicted the differences in cell proliferation due to the staining method in regards to the time during the breeding season, acoustic stimulus type, and sex.

Chapter 6 consists of concluding remarks, including a synthesis of the experimental data from the dissertation and additional comments.

Chapter 2: Regional distribution and migration of proliferating cell populations in the adult brain of Hyla cinerea (Anura, Amphibia)[1]

Abstract

We examined the distribution of adult cell proliferation throughout the brain of an anuran amphibian using 5-bromo-2′-deoxyuridine (BrdU). BrdU, a thymidine analog, is a commonly used cellular marker that is incorporated into actively dividing progenitor cells. Adult green treefrogs, *Hyla cinerea*, received injections of BrdU and were sacrificed 2 h, 2 days, 2 weeks, or 30 days later. Immunohistochemistry revealed BrdU-immunopositive (BrdU+) cells to be distributed in ventricular zones throughout the brain. The heaviest concentrations of cells were located in the telencephalon, primarily in the ventrolateral region of the lateral ventricles, and the ventricles of olfactory bulbs. Numerous BrdU+ cells were located around the preoptic and hypothalamic recesses and few around the third ventricle in the diencephalon. Proceeding caudally towards the midbrain, there was a marked decrease in BrdU labeling and few BrdU+ cells were found in the hindbrain. Consistent with previous studies in ectothermic vertebrates, BrdU+ cells were found predominantly in the ventricular zone (VZ) and immediately adjacent to the VZ; at later time points (i.e., 30 days), the cells appeared to have migrated into parenchymal regions. The extent of cellular proliferation in anurans is similar to that of fishes and reptiles and thus is more widespread compared to mammals.

Introduction

Documentation of postnatal neurogenesis has generated interest in patterns of cell proliferation in brains of adult vertebrates. Adult neurogenesis is defined as the birth and maturation of new neurons that add to or replace neurons in existing circuitry (Lindsey and Tropepe, 2006), encompassing four processes: proliferation, migration, differentiation and survival. Patterns of cellular proliferation have been mapped in the

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[1]L.M. Almli and W. Wilczynski. 2007. Brain Research 1159: 112-118.

developing anuran brain (Schmidt and Roth, 1993; Chapman et al., 2006; Simmons et al., 2008), but comprehensive studies of cell proliferation in adults are not well-documented. Adult cell proliferation has been demonstrated in anurans (Raucci et al., 2006 and references therein); specifically, in *Rana temporaria* via tritiated thymidine (i.e., radioactive thymidine; Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), in *R. esculenta* via proliferating cell nuclear antigen (PCNA; Raucci et al., 2006), and in *R. catesbeiana* via BrdU labeling (Saijo et al., 2006).

Although there are a few studies detailing some aspect (i.e., proliferation or differentiation) of adult neurogenesis in anuran amphibians, the story is not yet complete. With the exception of the work of Saijo et al. (2006), the ranid studies focus on specific brain regions (e.g., the preoptic area in Chetverukhin and Polenov, 1993), include only one time point of cell proliferation (Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), or use alternative cell proliferation markers (tritiated thymidine in Bernocchi et al., 1990; PCNA in Raucci et al., 2006). The amount and potential sites of cell proliferation should be interpreted cautiously as cell proliferation markers differ in their method of labeling (such as labeling molecules only during specific parts of the cell cycle; e.g., PCNA, Wullimann and Puelles, 1999). BrdU is currently the most utilized technique to assess cell proliferation (due to the ease of double-labeling with cell phenotypic markers; Taupin, 2007); hence, a description of the distribution of BrdU labeling is necessary in anurans if the results are to be compared to the current literature on the distribution of cell proliferation across vertebrates. To our knowledge, this report is the first comprehensive study of the regional distribution of cell proliferation via BrdU labeling in adult *Hyla cinerea* incorporating the time course and migration of new cells. A preliminary report of this work has been presented (Almli and Wilczynski, 2004).

Materials and Methods

Animals

Adult male *H. cinerea* were purchased from a commercial supplier (Charles Sullivan, Inc., Nashville, TN) and group-housed in glass aquaria containing water bowls,

plastic plants, and rocks. The frogs had free access to water and were fed crickets (*Acheta domestica*, ca. 12 mm long) twice/week. The animal room was maintained at ca. 24 °C on a 14:10 L:D cycle. Animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines.

Frogs (N = 12; mean snout-vent length = 50 mm, range = 46–52 mm) received two consecutive injections intraperitoneally at 12:00 h (Day 1) with BrdU (100 mg/kg; Sigma, St. Louis, MO) and sacrificed at 2 h, 48 h, 14, or 30 days later (N = 3 per group). Brains were immersion-fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose. Using a cryostat (Leica CM 1900), brains were sectioned coronally at a thickness of 20 μm. Sections were collected on gelatinized subbed slides and four series of adjacent sections were created per animal. Sections were stored at − 20 °C until immunohistochemical staining was carried out.

Immunohistochemistry

One series of sections for each animal was processed for BrdU using 3,3′-diaminobenzidine tetrahydrocloride (DAB) as the chromogen; the other three series were reserved for future studies. Slide edges were marked with a PAP pen (Research Products International Corp, Mount Prospect, IL). For detection of BrdU-labeled nuclei, the DNA was denatured with 2 N HCl at 37 °C for 30 min and neutralized with 0.1 M boric acid buffer (pH 8.5) for 15 min. Endogenous peroxidases were quenched with 1% (v/v) H_2O_2 in 0.1 M PBS with 1% Triton X-100 (v/v; Sigma) for 10 min at room temperature. Nonspecific antigen binding sites were blocked by preincubation with 10% (v/v) normal rabbit serum in blocking solution [0.1 M PBS, 0.3% (v/v) Triton X-100, 0.2% (w/v) bovine serum albumin (Sigma)] that included avidin (1 drop/ml; Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Slides were then incubated with anti-BrdU antibody (1:100; Accurate Chemicals, Westbury, NY) in blocking solution containing biotin (2 drops/ml; Vector Laboratories) for 48 h at 4 °C. Avidin–biotin blocking was used in previous steps to reduce nonspecific biotin binding. After washing four times with PBS-T [0.3% (v/v) Triton X-100], sections were incubated in biotinylated

secondary antibody (rabbit anti-rat, 1:200; Vector Laboratories) for 60 min. Signal amplification was achieved by exposing the tissue to horseradish peroxidase conjugated avidin–biotin complex (Vector Laboratories) for 60 min at room temperature. After washing, sections were immersed in a DAB solution [0.25% (w/v) DAB with 0.006% (v/v) H_2O_2] for 15 min at room temperature to yield brown nuclei, and the cell bodies were counterstained with toluidine blue [0.25% (w/v)] to visualize regional boundaries. (The photomicrographs shown in Figs. 2.2B, C, E, F were taken of alternate sections that were not counterstained by toluidine blue.) Slides were coverslipped using Permount (Fisher Scientific, Pittsburgh, PA) after dehydration with graded ethanols and clearance in xylenes. Omission of the primary antibody eliminated all staining and served as a negative control. Control slides from an animal that did not receive a BrdU injection revealed no staining.

Quantification

Every section in the stained series (i.e., first series) was counted to determine the numbers of labeled cells in each ROI, the boundaries of which were determined by toluidine blue counterstaining. Slides were coded so that cell counts were obtained blind to time point condition, and sections were observed with a light microscope (Nikon Eclipse 80i, Melville, NY) attached to a digital camera (Nikon Digital Sight, DS-2Mv). DAB-positive cells (i.e., BrdU+ cells) were visualized and counted using a 20× objective (unless multiple cells overlapped and then a 40× objective was used to confirm the number of labeled cells). All BrdU+ cells were counted within the ROI. Because anuran cells in the ROI are only ca. 10 μm in diameter and sections counted were 60 μm apart, there is little possibility that labeled nuclei would be split between sections and be counted twice, causing an overestimation of proliferating cells. Furthermore, a positive DAB-labeled cell was counted when the cell appeared of the same general size/shape of a Nissl-labeled cell (with the exception of elongated cells in the ependymal layer, see below), thereby excluding partial cells (which were rarely identifiable). In addition, the BrdU label becomes diluted with subsequent divisions and may appear punctate (though not apoptotic; Miller and Nowakowski, 1988); punctate deposits were not counted.

Therefore, we might have underestimated the number of proliferating cells at latter time points. For this reason, we only reported qualitative indications of BrdU cell density and no statistical comparisons were made between time points.

We counted the total number of BrdU+ cells in the VZ in addition to the BrdU+ cells outside the VZ (i.e., parenchyma) using the same criteria as above. To assess cellular migration, we calculated the percentage of parenchymal cells to the total number of BrdU+ cells and compared this ratio for early and late time points (e.g., 2 h and 30 days). An increase in the ratio after the 2 h time point suggests migration into the parenchymal layer.

Results

Distribution

Proliferating cells were regionally distributed in the adult amphibian brain as seen in Fig. 2.1. Within 48 h, proliferating cells (i.e., labeled with BrdU) mainly occurred in the VZ (i.e., ependymal layer or first layer of cells) especially in forebrain regions and often appeared in clusters or pairs of labeled cells. Cells within the first layer of cells may be ependymal in nature as some were elongated, but the type of cell is undetermined as of yet. The BrdU+ cells in the parenchyma were not elongated and resembled the surrounding Nissl-labeled cells. The heaviest concentrations of BrdU+ cells were located in the telencephalon, primarily around the lateral ventricles and the ventricles of olfactory bulbs (Table 2.1). Proliferating cells were scattered throughout the olfactory bulbs, including the accessory olfactory bulb, and were uniformly distributed (Fig. 2.1A). In the middle to caudal telencephalon, a greater number of progenitor cells were distributed in the ventrolateral region of the lateral ventricles (adjacent to the striatum laterally and septal nuclei medially) as compared to the dorsal half of the ventricle (Figs. 2.1B, C). Furthermore, the lateral and ventral regions, specifically the lateral pallium and subpallial regions, contained more BrdU+ cells than the medial pallium (Table 2.1). An exception to the mainly ventricular location of proliferating cells in the forebrain was the amygdala, in which there were occasional labeled cells not immediately adjacent to a ventricle (Fig. 2.1D). There were many proliferating cells in the preoptic recess, especially in the ventral

extent of the preoptic area (POA; Fig. 2.1D). Proceeding caudally in the POA, the proliferating cells extended to more dorsal regions (in addition to the ventral region), though never reached the dorsal extent of the POA. Proliferating cells in the thalamic regions were lesser in number considering the greater regional area included in this sample (Fig. 2.1E). BrdU+ cells appeared more abundant in the dorsal extent of the anterior thalamus near the habenula. Occasional proliferating cells were located in the habenula (not shown) and suprachiasmatic nucleus (Fig. 2.1E). Moving ventrally, numerous BrdU+ cells were located around the hypothalamic recess (mostly on the ventral edge) and became more abundant caudally, while a few appeared around the third ventricle in the diencephalon throughout its rostral–caudal extent (Fig. 2.1F).

Proceeding caudally into the brainstem, there was a significant decrease in BrdU+ cells. Proliferating cells were distributed with lesser density along the cerebral aquaduct (Fig. 2.1G), with cells predominantly in the dorsal extent of the aquaduct. The VZ of the tectal ventricles contained few cells and appeared to lack a rostral–caudal gradient (Figs. 2.1F, G). Of note were occasional BrdU+ cells in the nucleus isthmi (Fig. 2.1G). Few BrdU+ cells were found in the hindbrain; these included rare non-ventricular cells in the cerebellum and reticular formation (Fig. 2.1H).

Survival and migration

Although the number of BrdU+ cells varied in the adult amphibian brain depending on the region sampled, there was a relative stability in the numbers of labeled cells per region of interest (ROI) over time (2 h–30 days; Table 2.1). However, when the location of the BrdU+ cells was noted (Table 2.1), the percentage of parenchymal cells was different depending on the time point sampled (specifically in the forebrain at 2 h compared with 30 days). BrdU+ cells in forebrain regions (excluding the amygdala) were mostly located in the VZ at time points up to 2 weeks, but at 30 days ca. 25% of cells appeared in the parenchyma (Table 2.1). This change in cell location is depicted in Fig. 2.2, which shows most BrdU+ cells inside the VZ of the POA and ventral hypothalamus (VH) at 48 h compared to outside the VZ at 30 days. Midbrain and hindbrain regions also showed an increase in the percentage of cells in the parenchyma over time, although not

as dramatically because parenchymal cells were also seen at the 2 h time point in these regions.

Discussion

Amphibians have been central to studies of proliferation and regeneration for decades (reviewed in Raucci et al., 2006), yet the lack of consistency or completeness in these studies has made taxonomic comparisons with other vertebrates difficult. We viewed proliferating cells using BrdU staining, the most commonly used procedure in other vertebrates. Consistent with previous research in vertebrates, we found BrdU+ cells in adult amphibians in the VZ and immediately adjacent to the VZ after short-term survival, primarily in the ventrolateral extent of the telencephalic ventricles. There were regional differences in the numbers of proliferating cells with forebrain regions having the highest numbers of BrdU+ cells (pallial, subpallial regions, and the VH being the most noteworthy). This is not to imply that all of the newly formed cells migrate to and/or remain in those regions, but they are born or proliferate there. Despite regional differences in the number of BrdU+ cells, there is a temporal stability in all sampled brain regions between 2 h and 30 days; however, in most brain regions, more notably in the forebrain, cells appear to migrate away from the VZ and into the parenchyma.

Proliferation

Different techniques make comparing between species and treatments very challenging (Rakic, 2002; Lindsey and Tropepe, 2006). Nevertheless, results in anurans using different cell proliferation markers were largely comparable to those described in this study, although we find some differences in the amount and location of proliferating brain cells. The medial pallium in our study had much lower numbers of proliferating cells than the lateral pallium; the opposite is reported in other amphibian studies using different markers of cell proliferation (Bernocchi et al., 1990; Raucci et al., 2006). Compared to the forebrain, fewer proliferating cells were found in the midbrain (e.g., tectum) and hindbrain regions (e.g., cerebellum) in all anuran studies. However, the medulla was noted to have a moderately high labeling index (percent of labeled cells with

respect to the total number of cells per brain region) in Bernocchi et al. (1990) but had very few labeled cells in our study. Future work using density counts instead of total number of labeled cells may reconcile this discrepancy.

The taxonomic variation in adult cell proliferation is significant. However, in general, the occurrence of adult cell proliferation in amniotic vertebrates (reptiles, birds, and mammals) is mostly restricted to the forebrain (García-Verdugo et al., 2002; Nottebohm, 2002) unlike that seen in anamniotes (e.g., fishes and anurans). In reptiles (see Font et al., 2001) and birds (Alvarez-Buylla et al., 1990), adult cell proliferation has been described in many forebrain regions, but proliferating cells are rarely seen in the walls of the third, tectal, and fourth ventricles. Mammals have the most limited distribution of adult generated neurons: the hippocampus, the olfactory bulb (reviewed in Ming and Song, 2005), and perhaps the cerebellum and neocortex (e.g., Magavi et al., 2000). In fish, 75% of proliferating cells are generated in the cerebellum, but many other areas exhibit adult cell proliferation (reviewed in Zupanc, 2006). Anuran amphibians exhibit a cell proliferation pattern intermediate between fish and reptiles.

Although differences exist in the destination of proliferating cells in vertebrates, there appears to be a common neurogenic region in the telencephalic ventricles (usually the lateral ventricles; Doetsch and Scharff, 2001; Lindsey and Tropepe, 2006) that can be considered a "hot spot" or concentrated region of proliferating cells (Alvarez-Buylla et al., 1990). Teleost fishes exhibit the greatest number of proliferation zones/neurogenic regions and mammals have only two, the subventricular zone and the subgranular zone of the dentate gyrus (reviewed in Lindsey and Tropepe, 2006). In frogs, the ventrolateral wall of the lateral ventricles is a proliferation hot spot as it is in birds (Alvarez-Buylla et al., 1990). However, many regions of the anuran telencephalic VZ appear to be proliferation zones, a pattern similar to that generally seen in reptiles (for exceptions cf. Font et al., 2001).

Survival and migration

In the adult anuran, the number of BrdU+ cells in each ROI between 2 h and 30 days remained constant. This suggests that newly proliferating cells survived for at least

30 days. Alternatively, there was a balance between dividing and dying cells; e.g., newly proliferating cells died before 30 days and the population of labeled cells at 30 days consisted of daughter cells from initially labeled cells. BrdU studies such as ours cannot distinguish this.

The temporal stability of proliferating cells in anurans is similar to that seen in fish (specifically the fish cerebellum) and reptiles, in contrast to birds and mammals. In birds, there seems to be a significant amount of turnover in the number of surviving cells (Alvarez-Buylla and Nottebohm, 1988). There are regional differences reported in the stability of proliferating cells in mammals as well: most of the new cells die upon reaching the olfactory bulb (Biebl et al., 2000; Kempermann and Gage, 2002), though most are integrated into the neuronal circuitry in the hippocampus (Kempermann et al., 2003). Because little or no degenerating cells have been detected, the majority of cells produced in the adult fish and reptile brains seem to complete their development and become part of existing neuronal populations (Zupanc et al., 1996; Font et al., 2001). The present study suggests that frogs are similar due to comparable numbers of BrdU+ cells labeled over time.

Migration of newly proliferated cells (ca. 25%) away from the ventricle was evident at the 30 day time point in most brain regions sampled. The forebrain regions had few if any parenchymal cells at early time points (less than 2 weeks) and a greater percentage after 30 days. Most forebrain areas (with the exception of the amygdala) largely surround the ventricles, whereas brainstem regions have more of their cellular areas distant from ventricles. The greater amount of non-ventricular area in the brainstem could be the reason why many BrdU+ cells were found in the parenchyma at 2 h. These cells were most likely born *in situ*.

The time course of cellular migration in anurans is consistent with that of fishes, reptiles, and birds, where most new cells take ca. 30 days to reach their final destination (Perez-Canellas and Garcia-Verdugo, 1996; Zupanc et al., 1996; Nottebohm, 2002). The phenomenon in mammals is unique with cells migrating in under 2 weeks (for review see Ming and Song, 2005). Furthermore, the spatial restriction of neuronal migration in mammals is unlike that seen in other vertebrates that exhibit widespread migration into

parenchymal regions (Goldman, 1998). Equally important to the migration of proliferating cells in the adult brain is the differentiation of cells into neurons and glia. It would be informative to address, in future studies, whether the new cells in amphibians are of neuronal or glial origin.

Mechanism and functional significance

Ectothermic vertebrates, in general, tend to generate new neurons in many brain regions as compared to the limited distribution in mammals (Font et al., 2001; Zupanc, 2001; Raucci et al., 2006), a phenomenon which may be incidental to the continual brain growth of non-mammalian vertebrates (Kaslin et al., 2007). One mechanism that may suppress adult neurogenesis in mammals appears to be the absence of permissive signals for proliferation and further development of neuronal precursors (Emsley et al., 2005; Zupanc, 2006; Kaslin et al., 2008). By comparison, such neurogenesis-permissive factors are present in fish and reptilian brains (Zupanc, 2006) and are likely to be present in amphibians in light of their ability for organ and limb regeneration (Brockes and Kumar, 2002).

Although the phenomenon of adult neurogenesis is now widely accepted, it is difficult to determine the functional significance of the new cells. The degree to which new cells are incorporated into existing circuitry or are able to replace injured or dying cells varies widely in non-mammalian vertebrates (Doetsch and Scharff, 2001; Font et al., 2001; Zupanc, 2001) and is quite limited in mammals (reviewed in Cayre et al., 2002; Emsley et al., 2005; Lindsey and Tropepe, 2006). Undoubtedly, more work needs to be conducted on adult neurogenesis in frogs; anurans have such widespread and high rates of cell proliferation as adults and thus are excellent models in which to pursue questions surrounding the interplay between behavior, changing environment, and cell proliferation.

Timepoint	MP	LP	St/Ac	Septum	Amyg	POA	Thal	VH	Tectum	Torus	Cb	RTF
2 hours	++ (0.00)	+++ (0.00)	+++ (0.00)	+++ (0.01)	+ (0.36)	+ (0.00)	++ (0.04)	+++ (0.00)	+ (0.44)	+ (0.38)	+ (0.25)	+ (0.56)
48 hours	++ (0.02)	+++ (0.00)	+++ (0.00)	+++ (0.01)	+ (0.50)	+ (0.00)	++ (0.04)	+++ (0.00)	+ (0.25)	+ (0.00)	+ (0.17)	+ (0.85)
14 days	++ (0.03)	+++ (0.00)	+++ (0.00)	+++ (0.10)	+ (0.18)	++ (0.04)	++ (0.21)	+++ (0.15)	+ (0.70)	+ (0.53)	+ (0.63)	+ (0.64)
30 days	++ (0.16)	+++ (0.17)	+++ (0.31)	+++ (0.27)	+ (0.82)	++ (0.33)	+ (0.35)	+++ (0.27)	+ (0.59)	+ (0.54)	+ (0.41)	+ (0.74)

Table 2.1. BrdU-labeled cells in each region of interest (N=3/time point) in the *Hyla cinerea* brain. Numbers in parentheses correspond to the ratio of the number of parenchymal cells to the total number of cells (cells in the VZ plus cells in the parenchyma). [Note: + = 0-50 cells, ++ = 51-100 cells, +++ > 101 cells. Abbreviations: MP, *medial pallium*; LP, *lateral pallium*; St/Ac, *striatum/nucleus accumbens*; Amyg, *amygdala*; POA, *preoptic area*; Thal, *thalamus*; VH, *ventral hypothalamus*; Torus, *torus semicircularis*; Cb, *cerebellum*; RTF, *reticular formation*.]

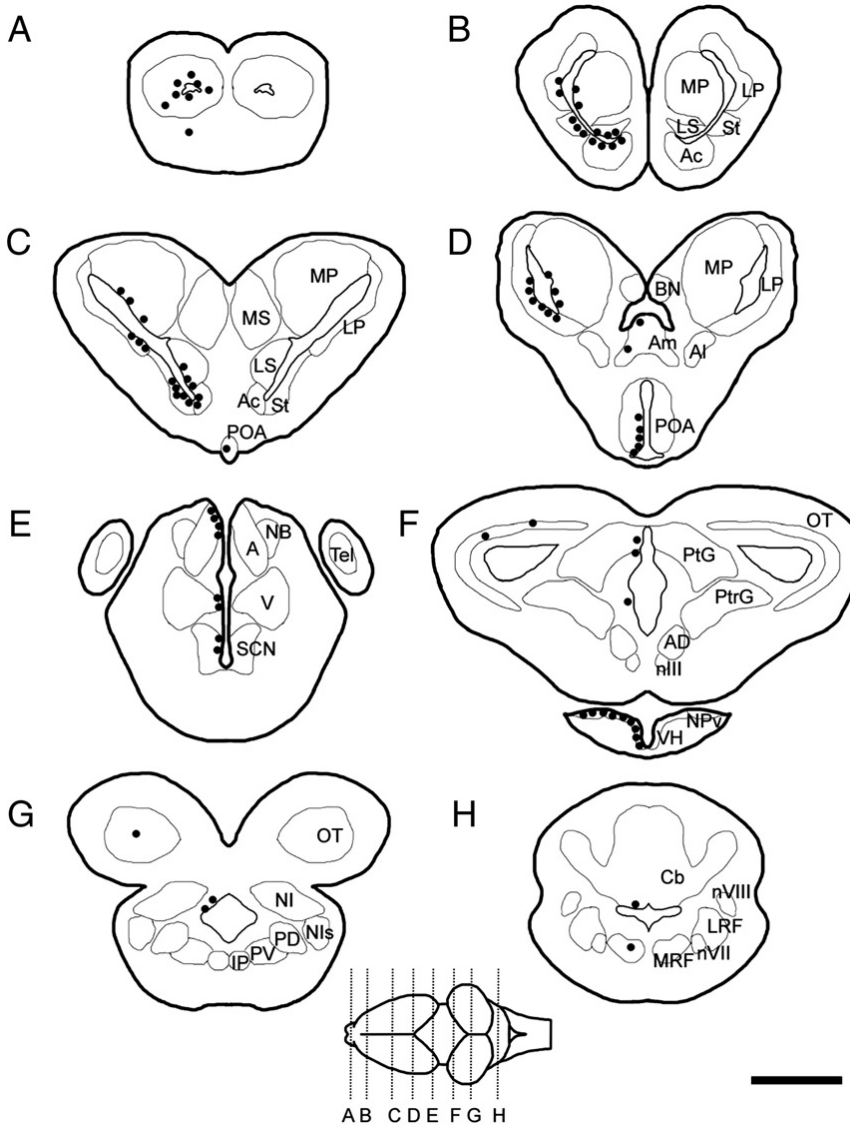

Figure 2.1. Schematics of brain sections showing the distribution of BrdU+ cells (black circles) at 2-48 hours in the *Hyla cinerea* brain. Scale bar = 1 mm. Abbreviations: A, *anterior thalamic nucleus*; Ac, *nucleus accumbens*; AD, *anterodorsal tegmentum*; Al, *lateral amygdala*; Am, *medial amygdala*; BN, *bed nucleus of the pallial commissure*; Cb, *cerebellum*; Ip, *interpeduncular nucleus*; LP, *lateral pallium*; LRF, *lateral reticular formation*; LS, *lateral septal nucleus*; MP, *medial pallium*; MRF, *medial reticular formation*; MS, *medial septal nucleus*; NB, *nucleus of Bellonci*; NI, *nucleus isthmi*; NIs, *secondary isthmal nucleus*; NPv, *nucleus of the periventricular organ*; nIII, *oculomotor nucleus*, nVII, *vestibular nucleus*; nVIII, *facial motor nucleus*; OT, *optic tectum*; PD, *posterodorsal tegmentum*; PV, *posteroventral tegmentum*; POA, *preoptic area*; PtG, *pretectal gray*; PtrG, *pretoral gray*; SCN, *suprachiasmatic nucleus*; St, *striatum*; V, *ventral thalamus*; VH, *ventral hypothalamus.*

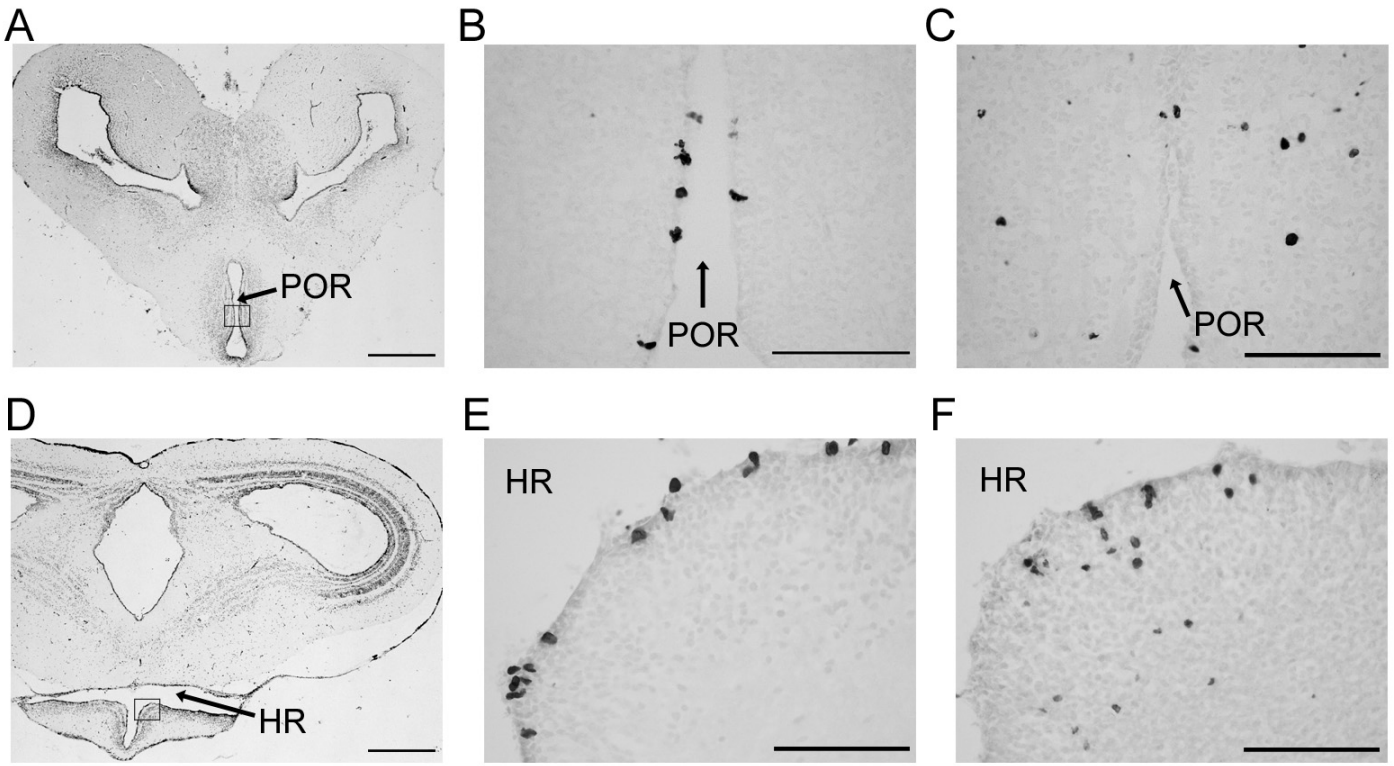

Figure 2.2. Photomicrographs of the anuran amphibian telencephalon showing BrdU+ cells in ventricular and parenchymal regions. Nissl-stained sections (A,D) include squared regions containing BrdU-labeled cells (B,C,E,F). Preoptic area depicted in top row and ventral hypothalamus in bottom row. BrdU+ cells are in the ependymal layer at 48 hrs (B,E) and in the parenchyma at 30 days (C,F). Scale bar in A,D = 500 µm; scale bar in B,C,E,F = 100 µm. Abbreviations: POR, *preoptic recess*; HR, *hypothalamic recess*.

Chapter 3: Sex-specific modulation of cell proliferation by socially-relevant stimuli in the adult green treefrog brain (*Hyla cinerea*)

Abstract

Social experience plays an important role in regulating the neural, physiological, and hormonal changes that accompany the expression of reproductive behavior in vertebrates. This suite of functions is sexually-dimorphic, with different neural control areas preeminent in males and females. In anuran amphibians, social experience comes in the form of acoustic communication, which is central to their reproductive behavior. We sought to determine whether acoustic cues regulate cell proliferation in the brain of adult green treefrogs (*Hyla cinerea)*. Our results show that both male and female treefrogs that heard a conspecific chorus during the breeding season exhibited increased brain cell proliferation compared to animals that heard random tones. Increased cell proliferation, as assessed by the number of 5-bromo-2'-deoxyuridine-immunopositive (BrdU+) cells, were found near the ventricles of acoustically-sensitive brain regions such as the preoptic area (POA) and the infundibular hypothalamus (IF). Sex differences emerged in the location of this socially-modulated cell proliferation: increases occurred primarily in the male POA and the female IF. In addition, gonadal hormones may have played a role in the social modulation of cell proliferation: by statistically controlling for hormone level, we revealed that androgens may influence socially-induced increases in BrdU+ cells in the male POA, but estrogen did not contribute to socially-induced increases in the female IF. These results indicate that the reception of social cues increases cell proliferation in brain regions mediating sexual behavior and endocrine regulation, and moreover that social modulation of cell proliferation occurs in a sexually-differentiated fashion.

Introduction

The idea of a "social brain" has received considerable attention (Insel and Fernald, 2004), which is not surprising considering the importance of social information to individual animals. In many vertebrates, the ability to recognize sensory stimuli from conspecifics and respond accordingly is vital to successful reproduction and survival

(Insel and Fernald, 2004). Social interactions, which naturally include both the reception of social signals and the responses to them, have sexually-dimorphic patterns. These patterns coincide with sex differences in hormonal and physiological regulation as well as sex differences in the neural systems that ultimately control both behavioral and physiological social responses. Furthermore, social interactions often result in dramatic and persistent changes in behavior and physiology, as well as in key neural systems underlying both.

Brain plasticity, defined by changes in structure and/or function, is an essential feature of the adult brain. Historically, experiential changes have been studied on the synaptic level, but they can be also be reflected in the addition of new cells, a phenomenon termed adult neurogenesis. This is a multi-step process that includes cell proliferation, neuronal fate specification of progenitor cells, and the maturation and functional integration of neuronal progeny into neuronal circuits (Ming and Song, 2005). Cell proliferation consists of the division of precursor or progenitor cells. Progenitor cells may continuously divide or subsequently differentiate into neurons or glial cells. 5-Bromo-2'-deoxyuridine (BrdU), a thymidine analogue, is commonly used as a marker of cell proliferation (Taupin, 2007). Brain areas (or zones) with high proliferative activity are limited in adult endothermic vertebrates, although recent studies have suggested that the adult mammalian brain contains progenitor cells which may be induced to proliferate into neurons under certain conditions (Pencea et al., 2001). In contrast to this, ectotherms such as fish (Zupanc and Horschke, 1995; Byrd and Brunjes, 1998; Zikopoulos et al., 2000; Ekstrom et al., 2001; Adolf et al., 2006; Grandel et al., 2006; Zupanc, 2006) and frogs (Almli, Ch. 2; Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993; Raucci et al., 2006; Simmons et al., 2008) demonstrate extensive brain cell proliferation as adults.

The reception of social stimuli has been shown to modulate adult neurogenesis (or cell proliferation) in specialized nuclei related to signal production, such as the prepacemaker nucleus of electric fish (Dunlap et al., 2006) and song control nuclei of songbirds (e.g., Lipkind et al., 2002; Barnea et al., 2006), and in the olfactory system of mammals (e.g., Smith et al., 2001; Fowler et al., 2002; Baudoin et al., 2005), for which

pheromone signals are important. It remains to be seen whether social stimulation influences cell proliferation in more integrative areas of the social brain that are less strictly tied to motor pattern generation or sensory reception, such as the preoptic area (POA) and caudal/ventral hypothalamus. These areas mediate the integration of behavioral, physiological, endocrinological, and motivational processes. Moreover, the POA and caudal hypothalamus are sexually-specialized in their function (Wade et al., 1993), although they and presumably their functions are present in both sexes. The POA's role as a central integrative site for the regulation of male sexual behavior in mammals has been well-established (Hull et al., 2002) and can be inferred in male amphibians (Wada and Gorbman, 1977a). On the other hand, the ventromedial hypothalamus (VMH) has long been known as the center for reproductive control in female mammals (Pfaff and Schwartz-Giblin, 1988) and due to studies in lizards (Wade and Crews, 1991; Kendrick et al., 1995) may be generalized to ectotherms.

We investigated socially-stimulated plasticity in an anuran amphibian in these integrative areas in the green treefrog (*Hyla cinerea*). The main social signal used by anurans during socio-sexual behavior is acoustic, the advertisement (mating) call. Males aggregate in groups and call to attract females (Wells, 1977; Rand, 1988; Kelley, 2004). Only males produce an advertisement call; both males and females respond to calling, but their behaviors are different. Females use it as a mate attractant and it triggers phonotaxis; males, on the other hand, call back in response to the same calls and use them as spacing cues. The POA and infundibular hypothalamus (IF), site of the ventral hypothalamic nucleus, are parts of a well-defined neural circuit controlling the expression of these socio-sexual behaviors as well as associated endocrine responses (Wilczynski et al., 1993; Emerson and Boyd, 1999; Wilczynski et al., 2005; Wilczynski and Endepols, 2007). Both receive auditory input and have neurons that are acoustically-sensitive (Wilczynski and Allison, 1989; Allison, 1992). Additionally, the POA has been linked to male calling behavior (Schmidt, 1968; Knorr, 1976; Wada and Gorbman, 1977a). Although the neural substrate of female phonotaxis is unresolved, activity in caudal areas of the hypothalamus has been shown to be associated with an interaction of hearing

conspecific calls and locomotion (Hoke et al., 2007), which implies a closer relationship to phonotaxis than evoked calling.

In this study, we examined three competing hypotheses about the effects of social stimulation on cell proliferation in more integrative areas of the vertebrate brain: (1) social stimulation causes a general increase in cell proliferation across brain areas; (2) social stimulation causes an increase in cell proliferation targeted to integrative brain areas involved in reproductive physiology and behavior; (3) social stimulation causes a sexually different pattern of cell proliferation in integrative areas reflecting their differential involvement in reproductive physiology and behavior. Our results support the third hypothesis, showing sexually-dimorphic patterns in socially-induced cell proliferation and sexual differences in the influence of sex steroid hormones on this effect.

Materials and Methods

Experimental Animals and Acoustic Stimulation Procedure

Adult male and female *Hyla cinerea* (snout-vent length range: 48-55 mm) were purchased from a commercial supplier (Charles Sullivan, Inc., Nashville, TN USA) and group-housed in glass aquaria containing water bowls, plastic plants, and rocks. The treefrogs had free access to water and were fed crickets twice/week. The animal room was maintained at ca. 24°C on a 14:10 L:D cycle. Animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee and conformed to NIH guidelines.

The acoustic stimulation procedure was modified from methods detailed previously (Burmeister and Wilczynski, 2000; Burmeister and Wilczynski, 2005). Each subject was housed individually in an acoustically-isolated test chamber containing water, plastic plants, rocks, and a light on a timer. Each acoustic chamber (14 x 14 x 20 cm) contained a speaker (Radioshack 277-1008C) connected to a tape deck for presenting acoustic stimuli, and a microphone connected to a custom-designed circuit board. The output of the board connected to a computer with custom-designed software that counted each male subject's calls.

Hyla cinerea (N=42, 21 males and 21 females) were exposed to either a recording of natural chorus ('Chorus') or random pure tones ('Tone') between 21:00 and 2:00 (typical of a breeding chorus) on 9 consecutive nights. The Tone stimulus was generated using SoundEdit 16, (Macromedia, Inc.) to replace each treefrog call in the Chorus stimulus with a single pure tone that matched the call in duration and approximate amplitude. Subjects were divided equally by sex and then randomly assigned to receive the acoustic stimulation procedure at one of three times during the breeding season, July through early September ('acoustic stimulus group').

BrdU Immunohistochemistry

Treefrogs were injected with BrdU (100 mg/kg in saline; Sigma, St. Louis, MO USA) at 20:30 on Days 1, 5, and 9, and sacrificed between 10:00-12:00 on Day 10 (Fig. 3.1). Brains were removed, immersion-fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose. Brain tissue was sectioned on a cryostat into four series; sections (20 μm thick) were collected on gelatinized subbed slides and stored at -20°C until immunohistochemical staining was carried out. One series of brain sections (every fourth section in total) was processed for BrdU using 3,3′-diaminobenzidine tetrahydrocloride (DAB) as the chromogen as per the immunohistochemistry procedure published previously (Almli and Wilczynski, 2007). Briefly, DNA was denatured with 2 N HCl and neutralized with 0.1 M boric acid buffer (pH 8.5). Endogenous peroxidases were quenched with 1% H_2O_2 solution, and nonspecific antigen binding sites were blocked by preincubation buffer. The slides were incubated in buffer plus anti-BrdU antibody (Accurate Chemical, Westbury, NY USA). Signal amplification was achieved by exposing the tissue to horseradish peroxidase conjugated avidin–biotin complex (Vector Laboratories, Burlingame, CA USA). To yield brown nuclei, sections were immersed in a DAB solution (0.25% DAB with 0.006% H_2O_2), and the cell bodies were counterstained with toluidine blue (0.25%) to visualize regional boundaries. Immunohistochemistry for BrdU, as above, was carried out on sections from a control animal that did not receive BrdU injections and revealed no staining. Omission of the primary antibody also eliminated staining.

Analysis of Cell Proliferation

Slides were coded so that cell counts were obtained blind to experimental conditions. 3,3′-diaminobenzidine tetrahydrocloride-positive cells (i.e., BrdU+ cells) were visualized and counted using a 40× objective (Olympus BX60 microscope). Every section in the stained series (i.e., every fourth section in total) was counted to determine the numbers of labeled cells in each region of interest (ROI), the boundaries of which were determined by toluidine blue counterstaining (for published boundaries, see Northcutt and Kicliter, 1980; Neary and Northcutt, 1983). A section containing the olfactory bulb (OB) was chosen as a control region not involved in acoustic communication. This was standardized by selecting the section that included the beginning of the accessory olfactory bulb. BrdU+ cells were counted in this control region, which contained caudal olfactory bulb in addition to pallial structures and the accessory olfactory bulb. Cells were not counted if they were more than 50 μm from the ventricle or outside of the cell dense area of the ROI, whichever came first. Further, a positive DAB-labeled cell was counted when the cell appeared of the same general size of a nissl-labeled cell, thereby excluding partial cells (which were rarely identifiable). Corrections for overcounting due to cell splitting were not incorporated in this study due to the distance between the sampled sections (60 μm), and the unlikely occurrence that cell splitting would differ between experimental conditions.

To control for variability due to sampling error, the volume of the region used for cell counts (i.e, approximately one quarter of the total volume of the brain region) was calculated for each animal. The perimeter of the ROI in each section was traced with SPOT software (Diagnostics Instruments, Sterling Heights, MI); three measurements per section were averaged to obtain the section area. Each section area was multiplied by the thickness (20 μm) and then summed to get the volume of each sampled ROI per animal. This volume measurement was then used as a covariate in a statistical analysis where noted in the text. To evaluate the precision of our sampling scheme, we calculated coefficients of error (CE) for the measured volumes of the ROIs of each animal using the formula in Gundersen and Jensen (Gundersen and Jensen, 1987): $CE=\sqrt{[(3A+C-$

4B)/12]/∑P, where P=area per section; $A=\sum P_i^2$; $B=\sum P_{i+1}$; $C=\sum P_{i+2}$. The relative variance of the individual volume measurements for the POA (mean: 0.06, range: 0.03-0.09) and IF (mean: 0.03, range: 0.02-0.06) were under 0.1, supporting the reliability of our measurements and sampling scheme.

Density measurements were calculated by dividing the number of BrdU+ cells counted by the volume of the region sampled. Additionally, the total number of cells per ROI were quantified by multiplying the cell counts by four to obtain an estimate of the total number of labeled cells per ROI (because every fourth section was sampled). This fractionator method (e.g., West et al., 1991) was chosen as opposed to more traditional stereological procedures (see Kuhn and Peterson, 2008), e.g., optical disector, due to the heterogeneous distribution of new cells.

Gonadal Hormone Analysis

Hormone assays were conducted using enzyme immunoassays (EIA; Cayman Chemicals, Ann Arbor, MI) to determine circulating estrogen in the females and androgens in the males; this procedure was modified from methods published previously (Lynch and Wilczynski, 2006). The volume of plasma used in each assay was 40 µL for estrogen and ranged from 3 to 5 µL for androgen. Plasma samples were spiked with 20 µL of tritiated estrogen or testosterone and extracted using 3 mL of diethyl ether. The extraction procedure resulted in a mean recovery (± SEM) of 26.7± 0.6% for estrogen and 87.8 ± 1.3% for androgen. Recovery values were used to correct the concentration of hormone estimated in each sample. We assayed each sample in triplicate and used between one and three dilutions per sample. Inter-assay variation was 5.9% and 3.7% in the estrogen and androgen assays, respectively. Intra-assay variation was 10.8% and 3.0% in the estrogen and androgen assays, respectively. Cross-reactivity in the estrogen kit was 0.1% for testosterone and 5α-dihydrotestosterone, 0.07% for 17α-estradiol, and 0.03% for progesterone. The testosterone kit had 27.4% cross-reactivity with 5α-dihydrotestosterone and 18.9% for 5β-dihydrotestosterone. The detection limits were 8 pg/mL and 6 pg/mL for the estrogen and testosterone EIA kits, respectively.

The EIA kits were previously validated by extracting hormone from a pooled plasma sample, and comparing that serially-diluted sample (with three dilutions) to the standard curve for that hormone. Hormone concentrations were natural log transformed to reveal a straight line. The slope of the line was −0.14 for both the serially-diluted estrogen sample and the standard curve. The slope of the line was −0.09 and −0.07 for the serially-diluted androgen sample and the standard curve, respectively. Although the slopes of the lines produced from our serially-diluted sample and the standard curve were not compared statistically (due to small sample size), they were clearly parallel.

Statistical Analyses

All statistical analyses were conducted with SPSS 15.0 (Chicago, 2006), and *p*-values were labeled as significant at $p < 0.05$. Post-hoc testing, where appropriate, utilized Bonferroni correction. Independent variables include: acoustic stimulus group (1, 2, or 3), sex (male or female), and acoustic stimulus type (Chorus or Tone). Dependent variables include: number of BrdU+ cells in the POA, IF, and OB section, density of BrdU+ cells, volume of the POA and IF, hormone level (androgens in males and estrogen in females), and number of evoked calls in males. The total number of BrdU+ cells in the POA, IF, and OB section were analyzed with separate three-way MANOVA. The sampling scheme was validated by using the volume of the sections sampled as a covariate. Separate three-way ANOVAs were also used to examine the effects of sex, acoustic stimulus type, and acoustic stimulus group on the density of BrdU+ cells in the POA and IF and the volumes of the POA and IF. Additionally, cell proliferation in males and females were analyzed separately using a two-way MANOVA. Correlations between hormone level and number of BrdU+ cells in the POA and IF were performed and if significant, an ANCOVA was computed with hormone level as a covariate. Males were divided into two groups depending on their evoked calling behavior: those that called back to the stimulus (‘Caller’) or those that did not call (‘Non-caller’). Evoked calling behavior and androgen level were then analyzed with a two-way MANOVA. Correlation analyses between the number of evoked calls and cell proliferation in the POA and IF were conducted and if significant, an ANCOVA was used with the number of evoked

calls as a covariate. Subjects that were missing consecutive sections within the POA or IF or had sections with aberrant staining were excluded from analyses. The resultant number of animals per group is listed under each analysis in the Results section.

Results

Proliferating cell numbers increase in frogs hearing their conspecific chorus

Proliferating cells in the POA, IF (containing the ventral and dorsal hypothalamus, the posterior tuberculum, and the nucleus of the periventricular organ; Fig. 3.2), and rostral telencephalon including the caudal olfactory bulb were labeled with BrdU. Because BrdU is incorporated by cells synthesizing DNA in preparation for division (Taupin, 2007), we use the term "cell proliferation" to describe our results due to our injection scheme and length of time before sacrifice. In studies of adult neurogenesis in birds, the term "recruitment" is used to describe a similar condition including the contributions of both cell proliferation and survival (Alvarez-Borda and Nottebohm, 2002). We note that we may be observing several components of adult neurogenesis with our procedure; resolving this would necessitate colocalization with other cellular markers in future studies. 5-bromo-2'-deoxyuridine-immunopositive (BrdU+) cells were found mainly in or near the ventricular zone of the POA, IF, and OB section, though this is not to imply that all of the newly formed cells migrate to and/or remain in those regions. Labeled cells were often oval-shaped and found in clusters. BrdU+ cells were also found in the parenchymal areas of the ROIs; these cells tended to be smaller and round in shape.

A MANOVA was conducted with the number of BrdU+ cells in the POA, IF, and OB section as dependent variables and acoustic stimulus group (1, N = 12; 2, N = 11, or 3, N = 10), sex (male, N=14; or female, N=19), and acoustic stimulus type (Chorus, N=15; or Tone, N=18) as independent variables. Because the independent variable for acoustic stimulus group was not significant, it was collapsed and the MANOVA was recalculated. Overall, there was a main effect of acoustic stimulus type ($\lambda = 0.671$, $F_{3,27} = 4.420$, $p = 0.012$) and a sex by acoustic stimulus type interaction ($\lambda = 0.747$, $F_{3,27} = 3.040$, $p = 0.046$) on the number of BrdU+ cells. Between-subject analyses on the number of BrdU+ cells in the POA ($F_{1,29} = 8.519$, $p = 0.007$) and IF ($F_{1,29}=4.451$, $p = 0.044$)

found each affected by the acoustic stimulus treatment: the Chorus condition exhibited increased numbers of proliferating cells compared to Tone controls (Fig. 3.3). Proliferation in the OB section, the control region, was unaffected by acoustic stimulus group, sex, or acoustic stimulus type (mean ± SEM: Chorus: 425.2 ± 46.0; Tone: 407.6 ± 36.8).

To correct for sampling error, we used the total volume of the sections sampled to determine whether that could cause differences between the independent variables. Separate ANCOVAs, with volume of the sampled ROI as a covariate, were conducted on the numbers of BrdU+ cells in the POA and IF. The analyses remained similar to the MANOVA (except for a loss of power): treefrogs hearing the Chorus condition exhibited increased numbers of BrdU+ cells compared to Tone controls in both the POA ($F_{1,32}$=4.234, p = 0.048; covariate: $F_{1,32}$ = 0.305, p = 0.585) and IF ($F_{1,33}$ = 5.283, p = 0.028; covariate: $F_{1,33}$ = 0.175, p = 0.679).

Separate three-way ANOVAs, using acoustic stimulus group (1, N = 11; 2, N = 12; or 3, N = 8), acoustic stimulus type (Chorus N = 14, Tone N = 17), and sex (male N = 12, female N = 19) as independent variables, were also conducted on the density of BrdU+ cells in the POA and IF and the volume of the POA and IF. In the ANOVAs using the densities of proliferating cells in the POA and IF, the 'acoustic stimulus group' variable was not significant, thus it was collapsed and the analysis was recalculated. There were main effects of acoustic stimulus type in the POA ($F_{1,24}$ = 5.528, p = 0.027) and IF ($F_{1,24}$ = 7.600, p = 0.011). Similar to that reported with the total number of BrdU+ cells, these results yielded increases in proliferating cell density in the POA and IF of Chorus-stimulated frogs compared to Tone controls. There was also a sex by acoustic stimulus type interaction in the POA ($F_{2,24}$ = 3.628, p = 0.042), but not in the IF (p > 0.05); this revealed a significant increase in BrdU+ cell number in the male POA with Chorus- stimulation compared to Tone controls (Table 3.1). There were no significant main effects or interactions in the MANOVA using volume as the dependent variable (Table 3.2).

Sex differences occur in regions that exhibit socially-modulated cell proliferation

In order to investigate the significant interaction between sex and acoustic stimulus type on the number of proliferating cells in the ROIs, separate MANOVAs in males (Chorus N=5, Tone N=9) and females (Chorus N=10, Tone N=9) were performed. There was an overall effect of acoustic stimulus type on BrdU+ cells in males ($\lambda = 0.362$, $F_{3,10} = 5.877$, $p = 0.014$) and females ($\lambda = 0.541$, $F_{3,15} = 4.234$, $p = 0.023$). In males, the number of BrdU+ cells in the POA, but not the IF, was significantly different (Fig. 3.4A) such that proliferating cell numbers were increased in the Chorus-stimulated group compared to Tone controls (POA: $F_{1,12} = 9.720$, $p = 0.009$; IF: $p > 0.1$; OB: $p > 0.1$). In females, the number of BrdU+ cells in the IF, but not the POA, increased due to the Chorus stimulation (Fig. 3.4B; IF: $F_{1,17} = 10.885$, $p = 0.004$; POA: $p > 0.1$; OB: $p > 0.1$). Correcting for sampling error (i.e., using section volume as a covariate) did not affect the significant results (data not shown). In summary, hearing the Chorus condition increased the number of BrdU+ cells in the POA of male treefrogs and the IF of female treefrogs compared to controls in the Tone condition.

Sex differences occur in the effects of gonadal steroids on cell proliferation

To determine the relationship between gonadal hormone level and cell proliferation in the POA and IF, we used estrogen (Chorus: 4.0 ± 0.4 ng/mL; Tone: 4.5 ± 0.5 ng/mL) and androgen (Chorus: 21.3 ± 3.6 ng/mL; Tone: 13.6 ± 2.3 ng/mL) levels in a correlation analysis with the number of BrdU+ cells in the ROI and as a covariate in an ANCOVA. Using hormone level as a covariate in an ANCOVA allowed us to determine whether hormones were driving the socially-modulated cell proliferation in anurans. In males, androgen level was significantly correlated with the number of BrdU+ cells in the POA ($r = 0.659$, $p = 0.005$; Fig. 3.5A) but not the IF ($p > 0.1$). When statistically controlling for androgen level as a covariate in an ANCOVA, there was no main effect of acoustic treatment ($F_{1,13} = 3.816$, $p = 0.073$; covariate: $F_{1,13} = 4.702$, $p = 0.049$). In females, estrogen levels were significantly correlated with cell proliferation in the IF ($r = -0.466$, $p = 0.033$; Fig. 3.5B) but not the POA ($p > 0.1$). As a covariate, estrogen level did not influence socially-modulated increases in cell proliferation in the IF (acoustic stimulus type: $F_{1,18} = 5.617$, $p = 0.029$; covariate: $F_{1,18} = 3.900$, $p = 0.064$). Hence,

androgens may influence socially-induced increases in cell proliferation in the male POA, but estrogen did not modulate socially-induced increases in the female IF, although it may have an influence there.

Male frogs that called back to chorus stimulation have elevated numbers of BrdU+ cells in the POA

To determine whether calling behavior was driving socially-modulated increases in cell proliferation, male treefrogs were divided into two groups based on their evoked calling behavior: Caller (N=10) and Non-caller (N=5). A MANOVA was conducted with androgen level and number of BrdU+ cells in the POA and IF as dependent variables, and acoustic stimulus (Chorus or Tone) and calling behavior (Caller or Non-caller) as independent variables. There was a significant main effect of calling behavior ($\lambda = 0.245$, $F_{3,9} = 9.232$, $p = 0.004$) and an interaction between calling behavior and acoustic stimulus type ($\lambda = 0.378$, $F_{3,9} = 4.942$, $p = 0.027$), suggesting that the influence of calling on cell proliferation depended upon whether the male was calling back to the Chorus or Tone stimulus. A significant between-subjects analysis demonstrated that the number of BrdU+ cells in the POA of males that called back to their conspecific chorus ($F_{1,11} = 6.468$, $p = 0.027$) increased compared to the number of BrdU+ cells in the POA of males calling during the Tone stimulus or compared to non-calling males in general (Fig. 3.6A). Further, a significant between-subjects analysis within calling behavior revealed higher androgen levels for callers, i.e., males that engaged in evoked calling ($F_{1,11} = 8.968$, $p = 0.012$). To assess whether behavioral output, as measured by the amount of evoked calling, influences cell proliferation in the POA, a correlation analysis was conducted. This analysis revealed a positive correlation between evoked calling behavior and cell proliferation in the POA of all males ($r = 0.726$, $p = 0.017$) but not in the IF ($p > 0.1$). Including only males that engaged in evoked calling, there was also significant positive correlation between evoked calling levels and BrdU+ cell number in the POA ($r = 0.659$, $p = 0.003$; Fig 3.6B) but not in the IF ($p > 0.1$). Subsequently, an ANCOVA was conducted with the number of calls as a covariate. A significant effect of acoustic stimulation after statistically controlling for the number of evoked calls ($F_{1,7} = 7.616$, $p =$

0.028; covariate: $F_{1,7}$=0.510, p = 0.498) confirmed that calling back to acoustic stimuli was not driving the social modulation of cell proliferation. Overall, this analysis suggests that in males, hearing calls alone can increase cell proliferation, but calling back to the Chorus (and not calling in general) enhances this effect.

Discussion

Our results demonstrate that the number of proliferating cells, as labeled by BrdU incorporation, can be modulated in adult animals via acoustic social stimuli in a region- and sex-specific manner. This modulation occurs in brain regions involved in processing social information, the POA and IF, and does not upregulate cell proliferation in general, as evidenced by similar levels of cell proliferation in a control region containing the olfactory bulb. Acoustic stimulation affected male and female *H. cinerea* differently. In males, social stimulation increased proliferating cell numbers in the POA, while in females this increase occurred in the IF. There were sex differences in the effect of gonadal steroids on cell proliferation: androgens may have contributed to the social modulation of cell proliferation in the male, but estrogens did not seem to modulate socially-induced cell proliferation in the female.

The sex-specific manner in which social signals influenced the differential cell proliferation in the POA and IF may be a result of the sexually-dimorphic functions of the POA and IF. Chorus-stimulation increased BrdU+ cell numbers in the POA of male *H. cinerea*, but female *H. cinerea* exhibited Chorus-stimulated increases in BrdU+ cell numbers in the IF. We can infer the importance of the POA in male social behavior from previous studies: lesions of the POA abolish male sexual behavior in anurans (Schmidt, 1968; Urano, 1988) and stimulating the POA elicits evoked calling behavior (Schmidt, 1968; Knorr, 1976; Wada and Gorbman, 1977a). Because the neural correlates of phonotaxis, the key reproductive behavior in female anurans, are not resolved, it is difficult to determine the contribution of the IF in females.

The mechanisms underlying the sex-specific cell proliferation are unclear; however, we speculate that gonadal hormones and their receptors may play a role in the social modulation of cell proliferation in males. Although gonadal hormone receptors

have not been localized on new brain cells in the anuran amphibian as yet, the POA does concentrate androgens (Kelley et al., 1975; Kelley et al., 1978; di Meglio et al., 1987) and contains androgen receptor immunoreactive cells (Guerriero et al., 2005), and the IF concentrates estrogen (Morrell et al., 1975; Kelley et al., 1978). In the current study, Chorus-stimulated males exhibited higher numbers of proliferating cells than Tone controls. The increased cell proliferation in Chorus-stimulated males may be due in part to socially-induced increases in androgens (Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001) in the Chorus group as evidenced by the loss of a significant stimulus effect overall (albeit marginal) after controlling for the hormonal covariate in the statistical analysis. The females in this study did not exhibit modulation in estrogen levels due to social stimulation (c.f., Lynch and Wilczynski, 2006), nor did estrogen levels seem to influence cell proliferation in the brain regions studied here. However, the reproductive state of the females was not controlled, and it may be that in other conditions they would demonstrate both an estrogen elevation and an estrogenic mediation of cell proliferation.

Gonadal hormones, both estrogen in females and androgens in males, have been shown to modulate adult neurogenesis (cell proliferation and/or survival) in vertebrates. In songbirds, estrogen and testosterone can promote the survival of new neurons, though there is little evidence that these hormones directly affect cell proliferation (Brown et al., 1993; Burek et al., 1994; Rasika et al., 1994; Burek et al., 1995; Hidalgo et al., 1995; for an exception, see Absil et al., 2003). In mammals, the role of hormones in modulating adult neurogenesis is less clear and seems to be region- and species-dependent. In general, acute estradiol initially enhances then suppresses cell proliferation in the female rodent hippocampus whereas androgens increase cell survival, not proliferation, in the male (Galea, 2008). Further, both estrogen and testosterone have been shown to affect cell proliferation in the amygdala but not the hypothalamus of the vole (Fowler et al., 2003). As cell survival or other aspects of hormonal modulation were not directly measured in our study, we can only report that gonadal hormones are not the sole mediators in socially-modulated cell proliferation in the anuran brain.

It is possible that both sensory experience and behavioral performance can affect cell proliferation, as suggested by research on song-control nuclei volume in songbirds

(Sartor and Ball, 2005; c.f., Brenowitz et al., 2007). By analyzing male call-back data in this study, we can assess the relative roles of sensory experience via acoustic stimulation and behavioral performance via evoked calling. Because the POA plays a central role in the activation of calling (Schmidt, 1968; Knorr, 1976; Wada and Gorbman, 1977b), we hypothesized a relationship between calling behavior and cell proliferation in that region. Male treefrogs who called back to the conspecific chorus exhibited increases in BrdU+ cells in the POA compared to Tone controls (Callers and Non-callers), but calling behavior was not directly driving this increase (as evidenced by a significant effect of Chorus-stimulation after controlling for the degree of calling behavior). Thus, these results indicate that hearing a conspecific chorus induces cell proliferation and/or survival in the POA regardless of the behavioral output, and that calling in response to the chorus may enhance this effect on cell proliferation. In songbirds, singing has been shown to increase neuron proliferation and survival in the high vocal center (HVC; Li et al., 2000; Alvarez-Borda and Nottebohm, 2002) but removing auditory feedback by deafening caused zebra finches to have decreased new HVC cells in one study (Wang et al., 1999) but increased cell addition in another study (Hurley et al., 2008). Because deafened birds engage in singing behavior, albeit altered, it is difficult to separate the factors affecting cell proliferation and/or survival in that system. At any rate, in the anuran model system, a parallel experiment with females on the contribution of sensory experience and behavioral performance on cell proliferation could be determined by measuring movement during phonotaxic behavior and correlating directed movement with cell proliferation.

It is well established that socio-sexual signals can influence the physiology and neurobiology of the receiver in vertebrates and specifically in anuran amphibians (Moore et al., 2005; Wilczynski et al., 2005). The increased neuronal activity in response to socio-sexual cues in the POA and ventral hypothalamus (Wilczynski and Allison, 1989; Allison, 1992) may be related to the increased cell proliferation observed in our study. This socially-induced cell proliferation in the POA and IF is noteworthy in light of the fact that neither region is a primary sensory or motor region (although the POA does have an important role in evoked calling); rather, these regions serve global regulatory and

motivational roles. In contrast, a recent study testing this activity-dependent hypothesis in rodents demonstrated that new cells in the mating circuit, specifically the medial POA and medial amygdala, were not enhanced with mating activity (Antzoulatos et al., 2008). It should be noted, however, that the mammalian brain has considerably less constitutive cell proliferation compared to the anuran brain, and thus may not be as plastic; furthermore, our experimental design provided nightly stimulation with socio-sexual cues compared to weekly stimulation in the rodent study. In addition to our findings, few studies have shown modulations in cell proliferation in a social context outside of the chemosensory system (e.g., hypothalamus: Fowler et al., 2002).

One possible explanation for our results, the region- and sex-specific increase in BrdU+ cells, is that the microenvironments (or "niche"; Alvarez-Buylla and Lim, 2004) of the POA and IF may be suitable for enhancing cell proliferation. Candidate mechanisms for the observed enhancement may include the upregulation of growth factors such as brain-derived neurotrophic factor (BDNF), as well as morphological changes such as increased glial support. The relationship between hormones, behavior, neurotrophins, and neurogenesis has been investigated in the highly specialized birdsong vocal learning system (Li et al., 2000; Brenowitz, 2004). Studies have shown that BDNF mRNA in the canary HVC increased due to singing (Li et al., 2000), and BDNF mediated the effects of testosterone on the survival of new neurons in the adult canary brain (Rasika et al., 1999). Enhancement of cell proliferation may also be a result of increases in glial support, specifically radial glial cells which are known to assist in the process of neurogenesis (both developmental and adult), as they are often correlated with increases in neurogenesis (e.g. Alonso, 2001). Although glia were not specifically labeled in this study, research in electric fish (Dunlap et al., 2006) has shown that radial glial fibers, proposed to be the progenitor cells of non-mammalian vertebrates (Garcia-Verdugo et al., 2002), increase during social interactions.

Our results indicate that hearing social signals does increase cell proliferation outside of specialized sensory and motor centers. Furthermore, male and female brains respond differently to these effects. In males, changes occur in the POA and are influenced by androgen levels and the calling behavior of males displayed specifically in

response to hearing those calls. In females, however, changes occur in the IF, and although cell proliferation there is correlated with estrogen levels, the effects of social stimulation are independent of this gonadal steroid. This is not to say that these new cells survive and become incorporated into existing circuitry, but just that hearing social signals increases cell proliferation outside of traditional sensory and motor nuclei, and it does so in a sex-specific manner with effects targeted toward the brain region most associated with regulation of behavior and physiological responses of that sex. Future studies may elucidate the functional significance of this socially-modulated cell proliferation.

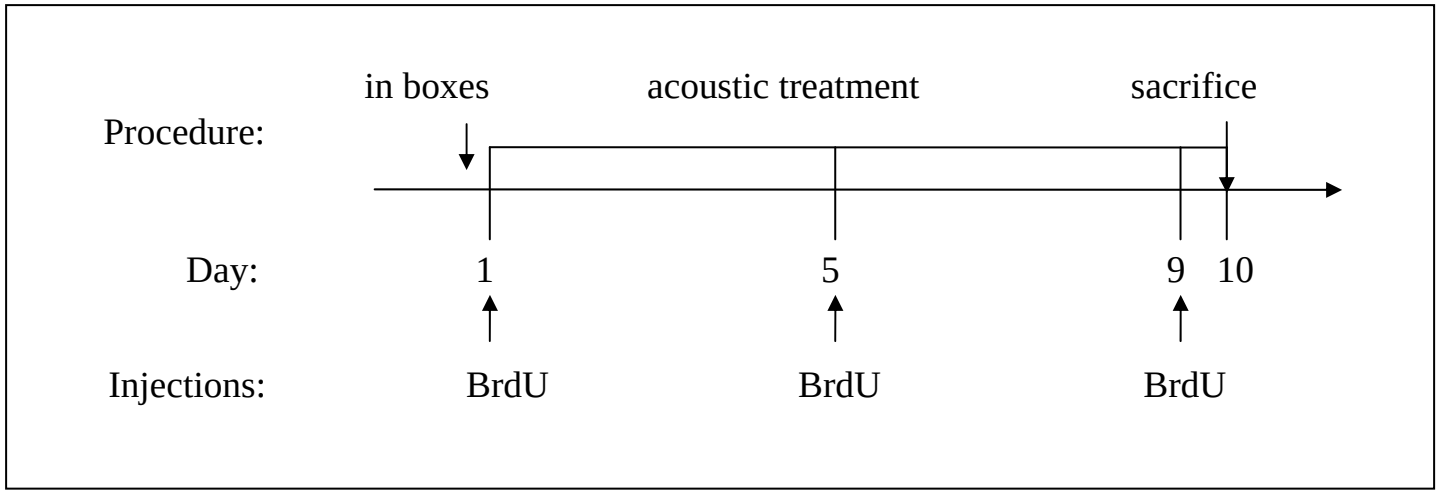

Figure 3.1. Timeline of acoustic treatment and BrdU injections for male and female *Hyla cinerea*.

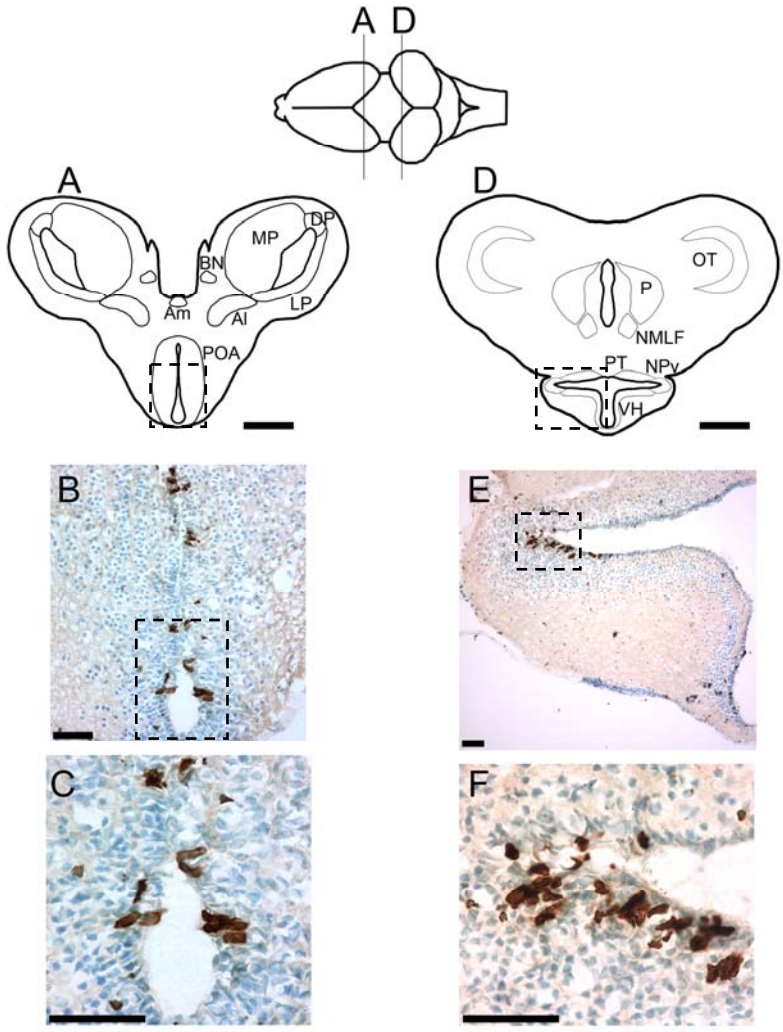

Figure 3.2. Schematics (top rows) and photomicrographs (bottom rows) of brain sections showing adult cell proliferation in *Hyla cinerea*. Sections (B,C) are from a representative male in the Tone condition and (E,F) from a representative female in the Tone condition. A top-view drawing of the *H. cinerea* brain is oriented with anterior to the left. The lines indicate the level of the sections through the POA (A) and IF (D). Photomicrographs of transverse sections through the brain show the distribution of proliferating cells (i.e., dark brown cells) labeled with BrdU in the POA (B,C) and the IF (E,F); sections are nissl-stained with toluidine blue, which labels non-proliferating cells blue. Photomicrographs are shown in increasing magnification down the left and right panels. The dotted line boxes represent the location of the subsequent photomicrograph. Scale bar = 500 µm for (A,D) and 50 µm for (B,C,E,F). Abbreviations: Al, *lateral amygdala*; Am, *medial amygdala*; BN, *bed nucleus of the pallial commissure*; DP, *dorsal pallium*, LP, *lateral pallium*; MP, *medial pallium*; NPv, *nucleus of the periventricular organ*; OT, *optic tectum*; P, *posterior thalamic nucleus*; POA, *preoptic area*; PTd, *dorsal posterior tuberculum*; PTv, *ventral posterior tuberculum*; VH, *ventral hypothalamus*.

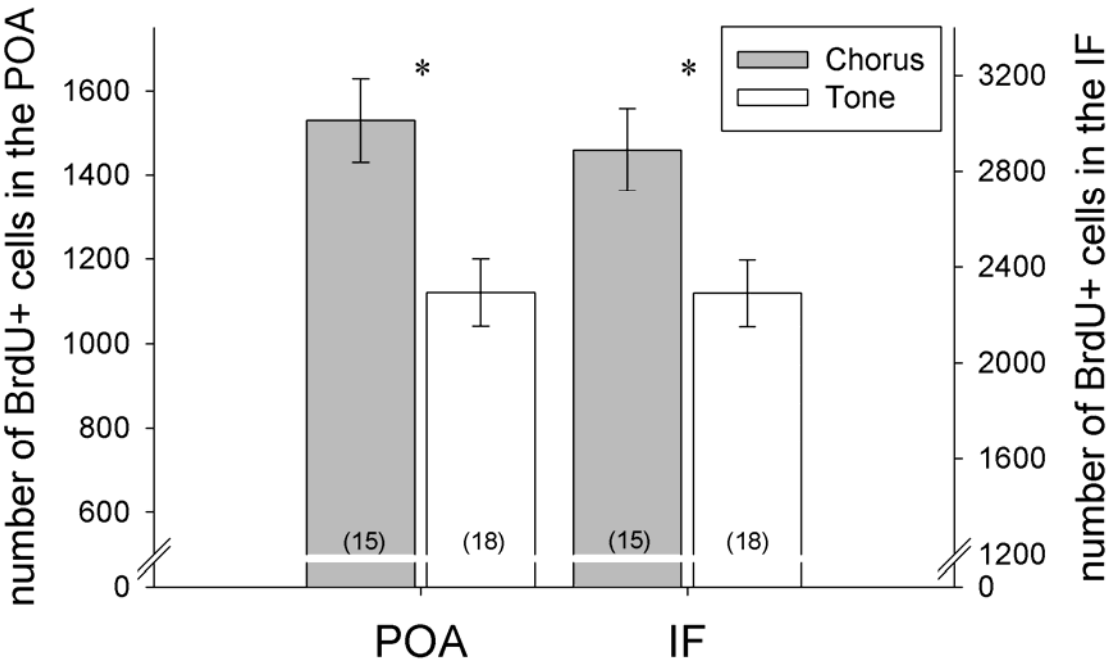

Figure 3.3. The number of BrdU+ cells in the POA (left side) and IF (right side) of Chorus- and Tone-stimulated *Hyla cinerea*. Chorus-stimulated males and females exhibited increased cell proliferation in acoustically-sensitive brains regions, the POA and IF, compared to Tone controls. Shown are the mean number of cells counted [± the standard error of the mean (SEM)]. Asterisks (*) represent significant between-subjects tests at $p < 0.05$.

ROI	Acoustic stimulus	Sex	N	Density (BrdU+ cells/mm^3) Mean ± SEM
POA	Chorus	female	11	10437.5 ± 988.4
		male	5	10860.0 ± 1003.8
		total	16	10569.5 ± 730.9
	Tone	female	10	8968.4 ± 1077.3
		male	9	8510.9 ± 571.2
		total	19	8751.7 ± 614.0
IF	Chorus	female	11	12782.2 ± 856.6
		male	5	11567.6 ± 2181.1
		total	16	12402.6 ± 868.3
	Tone	female	10	9932.3 ± 773.0
		male	10	11411.8 ± 611.3
		total	20	10672.0 ± 508.8

Table 3.1. The density of BrdU+ cells (per mm^3) in the regions of interest (ROI) of male and female *Hyla cinerea* as a function of acoustic stimulus type. The densities in the preoptic area (POA) and infundibular hypothalamus (IF) are listed as means ± the standard error of the mean (SEM).

ROI	Acoustic stimulus	Sex	N	Volume (mm^3) Mean ± SEM
POA	Chorus	female	11	0.1405 ± 0.007
		male	5	0.1545 ± 0.001
		total	16	0.1448 ± 0.007
	Tone	female	10	0.1341 ± 0.004
		male	9	0.1449 ± 0.007
		total	19	0.1392 ± 0.004
IF	Chorus	female	11	0.2271 ± 0.005
		male	5	0.2421 ± 0.001
		total	16	0.2318 ± 0.005
	Tone	female	10	0.2152 ± 0.007
		male	10	0.2067 ± 0.009
		total	20	0.2109 ± 0.006

Table 3.2. The volume of the regions of interest (ROI) of male and female *Hyla cinerea* as a function of acoustic stimulus type. The volumes of the preoptic area (POA) and infundibular hypothalamus (IF) are listed as means ± the standard error of the mean (SEM).

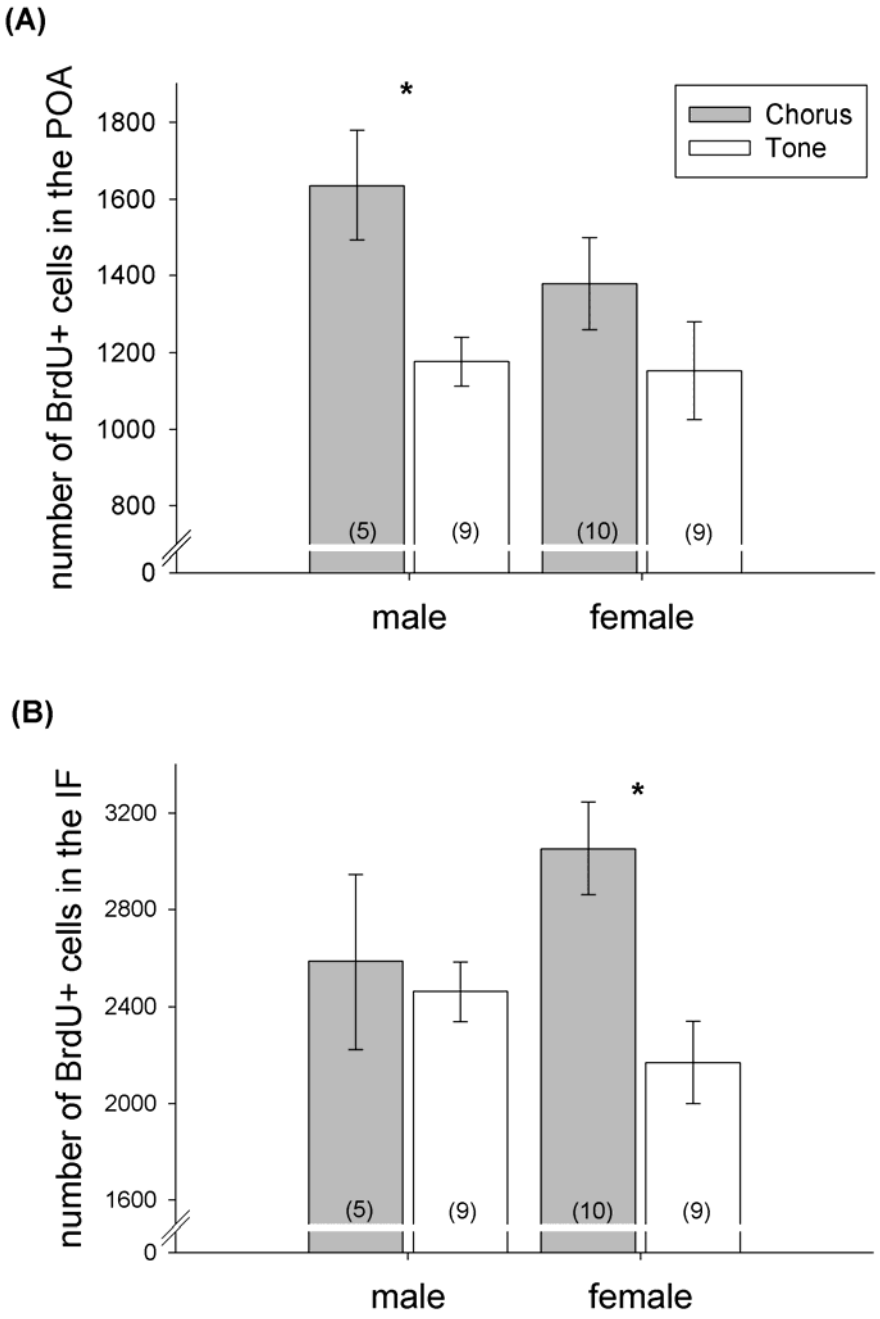

Figure 3.4. Proliferating cells, as labeled by BrdU, were socially-modulated in a sex- and region-specific manner. (A) In male *H. cinerea,* hearing their conspecific chorus increased BrdU+ cell numbers in the POA. (B) In females, hearing their conspecific chorus increased BrdU+ cell numbers in the IF. Shown are means ± SEM; asterisks (*) represent significant post-hoc tests at $p < 0.05$.

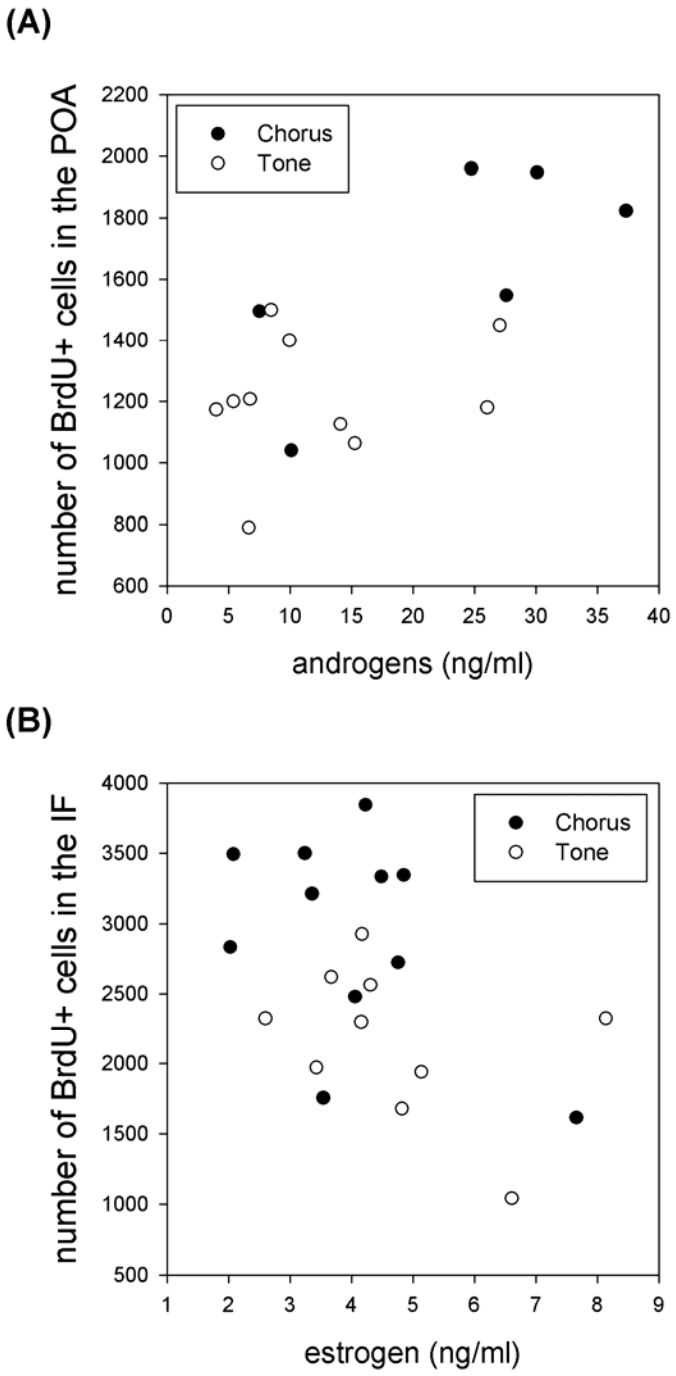

Figure 3.5. The relationship between hormone level and BrdU+ cells in male and female *Hyla cinerea*. (A) There is a significant positive correlation between androgen level and BrdU+ cells in the POA of males ($r = 0.659$, $p = 0.005$). (B) In females, there is a significant negative correlation between BrdU+ cells in the IF and estrogen level ($r = -0.466$, $p = 0.033$).

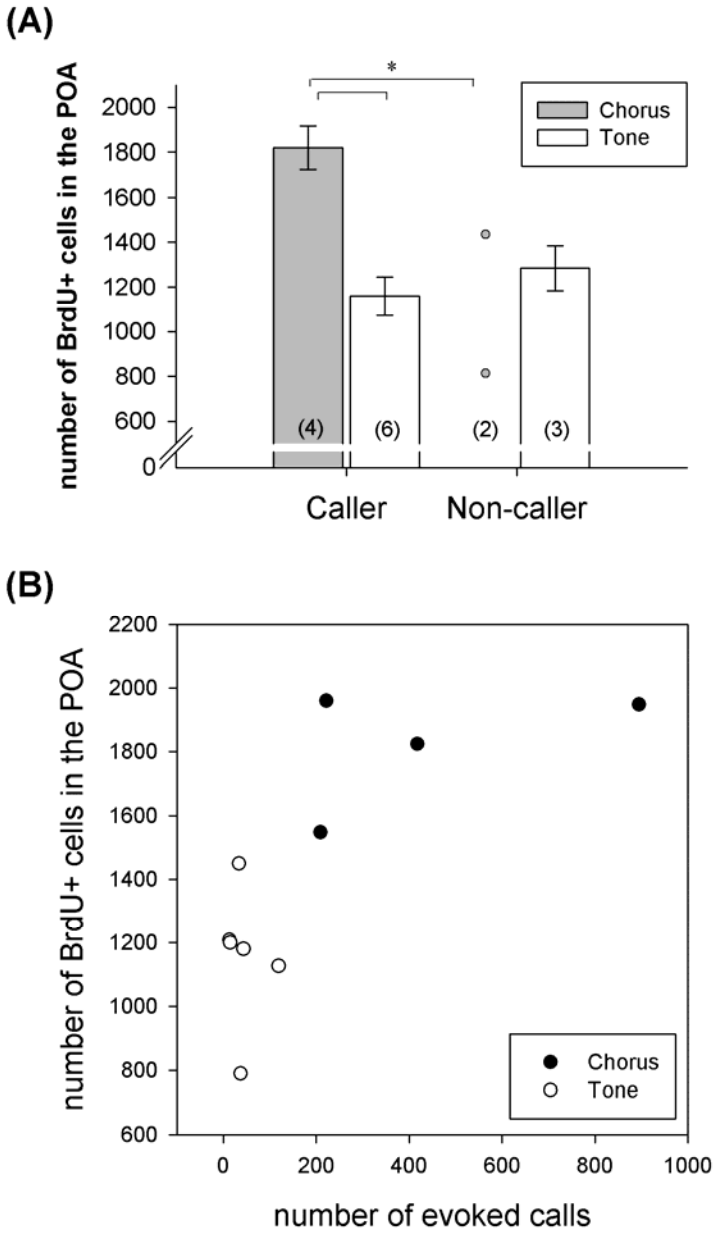

Figure 3.6. The influence of acoustic stimulation and calling behavior on cell proliferation in the POA of male *Hyla cinerea*. (A) Callers (i.e., those that called back to stimulation) in response to Chorus-stimulation exhibited increased cell proliferation in the POA compared to Non-callers. Shown are means ± SEM. Because only two males in the Chorus condition did not call back to stimulation (i.e., Non-caller), they are represented as dots with no SEM. Asterisks (*) represent significant post-hoc tests at $p < 0.05$. (B) The number of evoked calls was positively correlated with cell proliferation in the male POA ($r = 0.659$, $p = 0.003$).

Chapter 4: Cell proliferation in the adult green treefrog brain (*Hyla cinerea*): Influence of gonadal hormones and social cues

Abstract

Gonadal steroid hormones have been shown to influence adult neurogenesis in addition to their well-defined role in regulating social behavior. Adult neurogenesis consists of several processes including cell proliferation, which can be studied via 5-bromo-2'-deoxyuridine (BrdU) labeling. In this study, we examined whether BrdU-labeling is modulated by gonadal hormones and influenced by social behavior. We investigated this using a gonadectomy-implant paradigm and by exposing male and female green treefrogs (*Hyla cinerea*) to their conspecific chorus or control stimuli (i.e., random tones). Our results indicate that neither androgens in the male or estrogen in the female were neurotrophic in the preoptic area (POA) and infundibular hypothalamus (IF), brain regions involved in endocrine regulation and acoustic communication. In fact, elevated estrogen levels decreased cell proliferation in those brain regions in the implanted female. Further, androgens in the male and estrogen in the female did not mediate socially-induced cell proliferation in the POA and IF in this study. These results suggest that the social modulation of cell proliferation can occur without gonadal hormone involvement in the adult anuran amphibian.

Introduction

Despite early discoveries relating social interactions to the plasticity of the adult brain (Rosenzweig and Bennett, 1977), the relation between social behavior or social interactions and adult neurogenesis is an emerging field, recently reviewed by Gheusi et al. (2009). Neurogenesis consists of several processes including cell proliferation, differentiation, migration, and survival. These processes have been shown to be modulated by the reception of social stimuli in specialized nuclei related to signal production in birds (e.g., Lipkind et al., 2002; Barnea et al., 2006), mammals (e.g., Smith et al., 2001; Fowler et al., 2002; Baudoin et al., 2005), and fish (Dunlap et al., 2006). Often separately from social behavior, gonadal steroid hormones have been shown to

affect the process of adult neurogenesis but their effects seem to be dependent on the species, region, and dose of the hormone (reviewed in Fowler et al., 2008 and Galea, 2008).

Gonadal steroid hormones regulate social behaviors and ensure the appropriate timing of reproduction. In seasonally breeding anuran amphibians, gonadal hormones fluctuate seasonally in concert with reproductive social behavior. This occurs with androgens in the male (Varriale et al., 1986; Itoh et al., 1990; Itoh and Ishii, 1990; Gobbetti et al., 1991; Polzonetti-Magni et al., 1998) and estrogen (among others) in the female (Licht et al., 1983; Pierantoni et al., 1984; Rastogi et al., 1986). Gonadal hormones and reproductive behavior also fluctuate within the breeding season of female anurans (Lea et al., 2000; Bosch and Boyero, 2004; Lynch and Wilczynski, 2005). The reciprocal case is also evident, or in other words, reproductive or social behavior can influence gonadal hormone levels. In anurans, social cues emanating from interactions with conspecifics can regulate gonadal hormone production (Burmeister and Wilczynski, 2000; Chu and Wilczynski, 2001; Lynch and Wilczynski, 2006).

Green treefrogs (*Hyla cinerea*) are highly social seasonal breeders (e.g., Gerhardt, 1987). During the breeding season, male treefrogs gather nightly and call to attract females, forming a mating chorus. Calling is the major socio-sexual behavior expressed by males, whereas phonotaxic behavior in response to male calls is the main socio-sexual behavior expressed by females. Thus, social signals are almost solely acoustic in anuran amphibians. Calling behavior in the male anuran is dependent on testosterone (Wada et al., 1976; Wada and Gorbman, 1977b; Wetzel and Kelley, 1983; Solis and Penna, 1997; Burmeister and Wilczynski, 2001) and eliminated upon castration (Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976; Burmeister and Wilczynski, 2001). The endocrine control of social behavior in females (i.e., phonotaxis), on the other hand, may involve multiple factors (for review see Burmeister and Wilczynski, 2005; Gordon and Gerhardt, 2009). However, studies suggest that estrogen (Chakraborty and Burmeister, 2009), estrogen and progesterone (e.g., Kelley, 1982), or progesterone and prostaglandin (Gordon and Gerhardt, 2009) are necessary to promote phonotaxis in females.

The anuran amphibian central nervous system has unique qualities that facilitate the study of the social modulation of adult neurogenesis. For example, adult anuran brains exhibit high levels of constitutive cell proliferation (e.g., Almli, Ch. 2; Simmons et al., 2008) and contain well-defined circuitries that control the expression of socio-sexual behavior (e.g., Wilczynski et al., 1993). Further, neuroplasticity in the anuran socio-sexual system has been demonstrated via the social modulation of gonadotropin-releasing hormone (GnRH) immunoreactive cell number (Burmeister and Wilczynski, 2005) and the proliferation of new brain cells (Almli, Ch. 3). The green treefrog is thus an excellent model for studying the link between social behavior, gonadal steroids, and adult neurogenesis, specifically cell proliferation.

We have previously demonstrated increased cell proliferation via 5-bromo-2'-deoxyuridine (BrdU) labeling in the preoptic area (POA) and infundibular hypothalamus (IF) of naturally cycling female and male *H. cinerea* with exposure to a conspecific chorus (Almli, Ch. 3). Further, we determined statistically that androgens in males may influence socially-modulated increases in cell proliferation (Almli, Ch. 3). In this study, we sought to determine experimentally the role of gonadal steroid hormones in cell proliferation in general and whether they are important for socially-modulated cell proliferation. To address this question, we gonadectomized male and female *H. cinerea*; in half of the frogs, we replaced gonadal steroid hormones (testosterone in males and estrogen in females). We then exposed the gonadectomized and implanted frogs to a conspecific mating chorus or a control stimulus (random tones) and assessed the number of proliferating cells via BrdU immunoreactivity.

Materials and Methods

Animal Care

We purchased adult male and female green treefrogs (*Hyla cinerea*) from Charles Sullivan Co. (Nashville, TN) and housed them in small groups (ca. 4-6) in an aquarium with water, plastic plants, and rocks. The treefrogs were fed crickets twice weekly and provided water *ad libitum*. Environmental conditions included an ambient temperature of 23ºC and a 14:10 light:dark cycle. All procedures were performed in accordance with a

protocol approved by The University of Texas at Austin Institutional Animal Care and Use Committee.

Hormone manipulation and surgical procedures

Blank and hormone implants were made in advance and soaked in 0.9% saline overnight before inserting into the frog. Silastic© capsules (1.47 mm i.d. x 1.96 mm o.d. x 6.5 mm length) were sealed empty (Blank; N=15) or filled with ca. 6 mg of crystalline testosterone propionate (Sigma, St. Louis, MO) and sealed (Testosterone; N=15). Due to extremely high estrogen levels produced from Silastic© implants containing estrogen (Almli, unpublished observation), female frogs received implants of RTV silicone elastomer (6382 Factor 11 Inc., Lakeside, AZ; see Salek et al, 2001; Sisneros et al., 2004). For estrogen containing pellets (Estrogen; N=17), 1 ml of elastomer was mixed with ca. 5 mg of 17-β-estradiol-3-benzoate (Fisher, Fair Lawn, NJ), allowed to cure, and then formed into disks (3 mm diameter x 2.5 mm total length). The remaining females received the same-sized pellets which contained silicone elastomer only (Blank; N=17).

For gonadectomy and the implantation of blank pellets or pellets containing sex steroid hormones, we first anesthetized animals by immersion in 0.2% tricaine methanosulfate (MS-222; Sigma-Aldrich, Milwaukee, WI). The males were castrated and the females ovariectomized by extracting the testes and ovaries, respectively, through a small ventrolateral incision. Each frog received a blank or hormone implant, inserted intra-abdominally, before the incision was sealed with a combination of nylon non-absorbable sutures (CP Medical; Portland, OR) and Vetbond (World Precision Instruments, Sarasota, FL). After several days of recovery in individual cages, the frogs were group housed, by hormone treatment, until the acoustic procedure was started seven days after surgery. Pilot studies determined that androgens and estrogen are depleted within seven days after gonadectomy (Almli, unpublished observation). Following the acoustic stimulation procedure, we euthanized the animals and collected blood samples for hormone analysis. Gonadectomy and implantation did not seem to differentially affect the health of the animals as measured by the mass of the animal before and after the acoustic stimulation procedure (female Blank pre: 8.1 ± 0.3 g, post: 7.8 ± 0.4 g; Estrogen:

pre: 8.0 ± 0.3 g, post: 7.7 ± 0.4 g; male Blank pre: 7.6 ± 0.3 g, post: 7.5 ± 0.2 g; Testosterone: pre: 6.7 ± 0.2 g, post: 7.0 ± 0.2 g).

Acoustic stimulation procedure

The acoustic stimulation procedure was modified from methods detailed previously (Almli, Ch. 3). Each treefrog was housed individually in an acoustically-isolated test chamber (14 x 14 x 20 cm) containing water, plastic plants, rocks, and a light on a timer. The acoustic chamber contained a speaker (Radioshack 277-1008C) connected to a tape deck for presenting acoustic stimuli and a microphone connected to a custom-designed circuit board. The output of the board connected to a computer with custom-designed software that counted each male subject's calls.

Gonadectomized and implanted *H. cinerea* were exposed to either a recording of a natural chorus ('Chorus') or random pure tones ('Tone') between 21:00 and 2:00 (typical of a breeding chorus) on nine consecutive nights. The Tone stimulus was generated using SoundEdit 16 (Macromedia, Inc.) by replacing each frog call in the Chorus stimulus with a single pure tone that matched the call in duration and approximate amplitude. Due to a limitation in the number of acoustic chambers available, male and female treefrogs were divided into two groups each ('cohort'), gonadectomized and implanted seven days prior to the acoustic stimulation procedure. Within each sex, the subjects were divided equally by hormone and acoustic treatment type (Chorus or Tone).

BrdU immunofluorescence and cell proliferation analysis

During the acoustic stimulation procedure, frogs were injected with BrdU (100 mg/kg in saline; Sigma, St. Louis, MO) at 20:30 on days 1, 5, and 9, and sacrificed between 10:00 - 12:00 on Day 10 (Fig. 4.1). Brains were removed, immersion-fixed in 4% paraformaldehyde, and cryoprotected in 20% sucrose. Brain tissue was sectioned on a cryostat into four series; sections (20 µm thick) were collected on gelatinized subbed slides and stored at -20°C until immunofluorescence staining was carried out. One series of brain sections (every fourth section in total) was processed for BrdU using the 5-bromo-2'-deoxyuridine Labeling and Detection Kit I (Roche Applied Science,

Indianapolis, IN). For detection of BrdU-labeled nuclei, the DNA was denatured with 2 N HCl at 37° C and neutralized with 0.1M boric acid buffer (pH 8.5). Nonspecific antigen binding sites were blocked by preincubation with blocking solution containing 10% normal sheep serum. Slides were incubated with anti-BrdU antibody (1:100) at 37° C followed by fluorescein-labeled anti-mouse secondary antibody (1:100) to visualize BrdU-positive cells. Slides were coverslipped using Fluoromount – G (Electron Microscopy Science, Hatfield, PA). Omission of the primary antibody eliminated all specific staining.

Slides were coded so that cell counts were obtained blind to experimental conditions. Fluorescein-positive cells (i.e., BrdU+ cells) were visualized and counted under a FITC filter cube using a 40× objective (Olympus BX60 microscope). Every section in the stained series was counted to determine the numbers of labeled cells in the POA and IF using published boundaries in (Northcutt and Kicliter, 1980; Neary and Northcutt, 1983). The quantification procedure, using the fractionator method (West et al., 1991), consisted of multiplying the cell counts by four to obtain an estimate of the total number of labeled cells per ROI (because every fourth section was sampled). Previous studies have determined that correcting the total number of cells by the volume of the sections sampled does not influence the results (Almli, Ch. 3). Subjects that were missing consecutive sections within the POA or IF or had sections with aberrant staining were excluded from analyses. The resultant number of animals per group is listed under each analysis in the Results section.

Enzyme Immunoassays

To verify the effectiveness of the hormone manipulation, we used an enzyme immunoassay kit (EIA; Cayman Chemical, Ann Arbor, MI) to measure levels of androgens and estrogen in males and females, respectively. General methods for the EIA procedure have been previously described (Almli, Ch. 3). The volume of plasma used in each estradiol assay was 8 μL and 50 μL (estrogen implants and blanks, respectively) and 5 to 8 μL for androgen (testosterone implants and blanks, respectively). Plasma samples were spiked with 20 μL of tritiated estradiol or testosterone and extracted using 3 mL of

diethyl ether. The extraction procedure resulted in a mean recovery ± the standard error of the mean (SEM) of 24.8 ± 0.5% for estrogen and 80.3 ± 1.4% for androgen. Recovery values were used to correct the concentration of hormone estimated in each sample. We assayed each sample in this study in triplicate and used between one and three dilutions per sample. In males, testosterone implants yielded a mean androgen level of 110.3 ± 5.9 ng/mL and blanks yielded 2.1 ± 0.2 ng/mL. In females, estrogen implants yielded a mean estradiol level of 17.1 ± 1.4 ng/mL; the estradiol levels of the female frogs which received blank implants were undetectable. For statistical purposes, the estradiol levels were assigned a value of 0.008 ng/mL (or 8 pg/mL, the detection limit of the kit) for blank implants. Inter-assay variation was 13.6% and 6.6% in the estradiol and androgen assays, respectively. Intra-assay variation was 6.4% and 7.1% in the estradiol and androgen assays, respectively. Cross-reactivity in the estrogen kit was 0.1% for testosterone and 5α-dihydrotestosterone, 0.07% for 17α-estradiol, and 0.03% for progesterone. The testosterone kit had 27.4% cross-reactivity with 5α-dihydrotestosterone and 18.9% for 5β-dihydrotestosterone. The detection limits were 8 pg/mL and 6 pg/mL for the estradiol and testosterone EIA kits, respectively. The EIA kits were previously validated by extracting hormone from a pooled plasma sample, and comparing that serially-diluted sample (with three dilutions) to the standard curve for that hormone (see Almli, Ch. 3).

Statistical analysis

All statistical analyses were conducted with SPSS 15.0 (Chicago, 2006), and *p*-values were considered significant at $p < 0.05$. For both male and female treefrogs, the number of BrdU+ cells in the POA and IF were analyzed with a three-way MANOVA with cohort (1 or 2), implant (in males: Testosterone or Blank; in females: Estrogen or Blank), and acoustic stimulus type (Chorus or Tone) as independent variables. Post-hoc analyses were computed using the Tukey procedure. Correlation analyses were conducted with evoked calling behavior in males and the number of BrdU+ cells in the POA and IF; and if significant, an ANCOVA was conducted on the number of proliferating cells using evoked calling as a covariate.

Results

We use the term cell proliferation to describe the component of neurogenesis observed in this study, but we note that we may be observing several processes of adult neurogenesis with our procedure (e.g., cell survival). Proliferating cells, labeled with BrdU, were found in or near the ventricular zone of the POA (Fig. 4.2) and IF (containing the ventral and dorsal hypothalamus, the posterior tuberculum, and the nucleus of the periventricular organ; Fig. 4.3). Labeled cells were also found in the parenchymal areas of the POA and IF, though they were lesser in number.

Proliferating cell numbers increase in male treefrogs hearing their conspecific chorus

A MANOVA was conducted with the number of BrdU+ cells in the POA and IF as dependent variables and cohort (1, N = 12 or 2, N = 14), acoustic stimulus type (Chorus, N = 11 or Tone, N = 15) and hormone implant (Testosterone, N = 13 or Blank, N = 13) as independent variables. Since the cohort variable did not significantly affect the dependent variables, it was collapsed and the analysis was recalculated. In males, there was a main effect of acoustic stimulus type ($\lambda = 0.644$, $F_{2,21} = 5.793$, $p = 0.010$) on the number of BrdU+ cells, but no main effect of hormone implant ($\lambda = 0.981$, $F_{2,21} = 0.198$, $p = 0.822$). Between-subject analyses on the number of BrdU+ cells in the POA ($F_{1,22} = 8.251$, $p = 0.009$) and IF ($F_{1,22}=8.065$, $p = 0.010$) indicate that each area was affected by the acoustic stimulus treatment: the Chorus-stimulated males exhibited increased numbers of proliferating cells compared to Tone controls (Fig. 4.4). Although the pattern between the effects of Chorus and Tone-stimulation on cell proliferation is the same in each hormone implant group, post hoc tests reached significance in the testosterone-implanted males in the POA ($p = 0.016$; Fig. 4.4A) and IF ($p = 0.023$; Fig. 4.4B).

Evoked calling behavior is not driving socially-modulated cell proliferation in male treefrogs

In order to determine the effects of calling behavior on cell proliferation, correlations between evoked calling and cell proliferation in the POA and IF were

conducted. Using only males that called back to acoustic stimulation, a significant positive correlation was observed between calling behavior and cell proliferation in the POA ($r = 0.697$, $p = 0.037$; Fig. 4.5), but not in the IF ($p > 0.1$). To statistically control for calling behavior, an ANCOVA was conducted on the number of BrdU+ cells in the POA with the number of evoked calls as a covariate. This analysis included only males that engaged in evoked calling behavior, which was a portion of the testosterone-implanted males as sufficient levels of testosterone are necessary to engage in calling behavior. A significant effect of acoustic stimulation after statistically controlling for the number of evoked calls ($F_{1,7} = 7.245$, $p = 0.031$; covariate: $F_{1,7}=0.035$, $p = 0.857$) confirmed that calling back to acoustic stimuli was not driving the social modulation of cell proliferation in the POA. Overall, this analysis suggests that in male treefrogs, hearing their conspecific chorus was sufficient to exhibit increases in cell proliferation in the POA without regard to calling behavior.

Estradiol decreased proliferating cell numbers in female treefrogs

A MANOVA was conducted with the number of BrdU+ cells in the POA and IF as dependent variables and cohort (1, N = 13 or 2, N = 15), acoustic stimulus type (Chorus, N = 14 or Tone, N = 14) and hormone implant (Estrogen, N = 14 or Blank, N = 14) as independent variables. Again, the cohort variable did not significantly affect the dependent variables; thus, the variable was collapsed and the analysis recalculated. Overall, in females, there was no main effect of acoustic stimulus type ($\lambda = 0.860$, $F_{2,23} = 1.879$, $p = 0.175$) on the number of BrdU+ cells, but there was a significant main effect of hormone implant ($\lambda = 0.323$, $F_{2,23} = 24.149$, $p < 0.001$). Estrogen implants significantly decreased BrdU+ cell numbers in the POA ($F_{1,24} = 39.820$, $p < 0.001$; Fig. 4.6A) and IF ($F_{1,24}=21.800$, $p < 0.001$; Fig. 4.6B). Significant post-hoc tests revealed that the females with undetectable levels of estrogen (i.e., blank implants) exhibited socially-induced increases in cell proliferation in the IF ($p = 0.016$; Fig 4.6B) but not the POA ($p > 0.1$).

Discussion

We examined the influence of gonadal hormones on proliferative activity in the brains of gonadectomized and implanted male and female *H. cinerea* and in addition, whether these hormones were necessary for the social modulation of cell proliferation. Our results demonstrated that androgens in males did not modulate the number of proliferating cells as labeled by BrdU incorporation in adult treefrogs; however, elevated estrogen levels in females suppressed cell proliferation. Using acoustic social cues, we demonstrated that cell proliferation was socially-modulated in the POA and IF, brain regions involved in the expression of socio-sexual behaviors as well as associated endocrine responses. Overall, gonadal steroids did not modulate socially-induced increases in cell proliferation in the male or female green treefrog in this study.

Both the testosterone and estrogen implants yielded hormone levels within the physiological range; however, the levels are significantly higher than those reported for unmanipulated frogs (testosterone implants: 110.3 ± 5.9 ng/mL versus 12-14 ng/mL for unmanipulated males; estrogen implants: 17.1 ± 1.4 ng/mL versus 4-5 ng/mL for unmanipulated females). In the two regions studied here, it is clear that gonadal hormones affect cell proliferation in the sexes differently. Testosterone alone does not modulate cell proliferation in the POA and IF as evidenced by comparable numbers of BrdU+ cells in the males that heard Tones with and without a testosterone implant. In female treefrogs, high estrogen levels decreased cell proliferation in the POA and IF compared to control levels in females with undetectable estrogen levels (i.e., blank-implanted females).

In other vertebrates, testosterone appears to increase the survival, but not the proliferation, of new cells (reviewed in Galea, 2008). In songbirds, testosterone enhances the survival of HVC neurons (Goldman and Nottebohm, 1983; Rasika et al., 1994; for an exception, see Brown et al., 1993). In mammals, Ormerod and Galea (2003) found increased cell survival in the hippocampus when testosterone levels were naturally high during the breeding season compared to outside the breeding season. However, cell proliferation was not similarly enhanced with high testosterone levels (Galea and McEwen, 1999; Ormerod and Galea (2003). Further, exogenous testosterone has been

shown to increase cell survival in the rat hippocampus (Spritzer and Galea, 2007) and amygdala (voles: Fowler et al., 2003; hamsters: Antzoulatos et al., 2008). In the medial POA, however, testosterone did not increase cell proliferation or survival (Antzoulatos et al., 2008). Since we did not measure cell survival directly in this study, we can only conclude a similar result to that found in the study of cell proliferation in the POA by Antzoulatos et al. (2008).

Estradiol has been traditionally considered neuroprotective (Brann et al., 2007) with acute estradiol administration enhancing cell proliferation (e.g., Tanapat et al., 1999; Banasr et al., 2001). However, high estrogen levels have been shown to inhibit or decrease the process of adult neurogenesis (Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003; Tanapat et al., 2005; Barker and Galea, 2008). A possible explanation for the suppression of cell proliferation by estrogen could be a result of the different patterns of hormonal administration reported in these mammalian studies (Perez-Martin et al., 2005). For example, chronically-elevated estrogen via estrogen implants or repeated injections affects neurogenesis differently than acute estrogen administration, which mimics the pulsatile release of hormones during the estrous cycle. Perez-Martin et al., (2005) suggest that acute effects of estradiol on neurogenesis may be lost after a sustained increase in hormonal levels. Indeed, chronically-elevated estradiol levels during the breeding season reduce cell proliferation in the hippocampus (Galea and McEwen, 1999; Ormerod and Galea, 2001; Ormerod et al., 2003). In our study, plasma estradiol levels were ca. four times higher than those of naturally-cycling females (Almli, Ch. 3). These levels may still be much higher than reported plasma estradiol in amplexing ovulatory females, which yield the highest levels compared to other reproductive stages in anurans (Gobbetti and Zerani, 1999; Lynch and Wilczynski, 2005). Thus, the estrogen in our study could be considered similar to the chronic estrogen paradigm described above.

Barker and Galea (2008) have suggested a mechanism for the inhibition of cell proliferation and/or survival with high estrogen levels: chronic estrogen "may alter the responsiveness of cells to estradiol itself (via regulating estrogen receptors), potentially reversing the survival-enhancing effects of acute estradiol exposure." Likewise, there is a

dose-response relationship for estrogen, such that low doses facilitate but high doses may inhibit responses (Scharfman and MacLusky, 2006). Indeed, Cheng (1977) reported that high estrogen levels can suppress sexual behavior in female ring doves: low doses of estradiol promote sexual behavior, but high doses inhibit sexual behavior through the inhibition of GnRH. This negative feedback regulation could be due to the down-regulation of estrogen receptors (ER; Weiland et al., 1997) as evidenced by decreased ERα gene expression in ovariectomized rodents receiving chronic estradiol treatment (Jin et al., 2005; Iivonen et al., 2006). Because the POA and IF concentrate estrogen (Morrell et al., 1975; Kelley et al., 1978), it would be interesting to determine the levels of ER expression in the POA and IF in *H. cinerea* with and without circulating estrogen. Furthermore, chronic estradiol treatment has been reported to significantly increase plasma levels of corticosterone in mammals (Ormerod et al., 2003; Perez-Martin et al., 2005). Corticosterone traditionally negatively affects neurogenesis in mammals (e.g., (Gould et al., 1992); however, its effects on proliferative activity in anuran amphibians is unknown at this time.

Previously, we reported that in naturally-cycling adult males and females, hearing acoustic social signals increased cell proliferation in the POA and IF but did not upregulate cell proliferation in general (Almli, Ch. 3). In this study, chorus-stimulation increased BrdU+ cell numbers in the POA and IF of male *H. cinerea*, but female *H. cinerea* exhibited Chorus-stimulated increases in BrdU+ cell numbers only in the IF. This region- and sex-specific difference in the social modulation of cell proliferation may be consistent with the sexually-dimorphic functions of the POA and IF. For example, male sexual behavior is linked to the POA: lesions of the POA abolish male sexual behavior in anurans (Schmidt, 1968; Urano, 1988) and stimulating the POA elicits evoked calling behavior (Schmidt, 1968; Knorr, 1976; Wada and Gorbman, 1977a). On the other hand, female sexual behavior is linked to the ventromedial hypothalamus (VH), a component of the IF, in other vertebrate groups (in mammals: Pfaff and Schwartz-Giblin, 1988; in reptiles: Wade and Crews, 1991; Kendrick et al., 1995). However, the neural correlates of female anuran reproductive behavior (i.e., phonotaxis) are unresolved at this time. In addition to being the reproductive centers of the basal forebrain, the POA and VH (part of

the IF) receive auditory information from thalamic and midbrain nuclei (Wilczynski et al., 1993; Emerson and Boyd, 1999; Wilczynski and Endepols, 2007) and contain neurons that respond to a conspecific chorus (Wilczynski and Allison, 1989; Allison, 1992).

In contrast to our previous study, the IF also exhibited socially-induced cell proliferation in males with testosterone implants. Because androgen-concentrating cells have been localized in the POA and VH of anuran amphibians (Kelley et al., 1975; Kelley et al., 1978; di Meglio et al., 1987), it is possible that chronically high levels of androgens from testosterone implants upregulated androgen receptor expression in the IF causing that region to be more sensitive to testosterone levels. Future studies are necessary to reconcile this discrepancy in region-specific cell proliferation and address the involvement of androgen receptors in socially-induced cell proliferation.

We observed that gonadal hormones were not necessary for the social modulation of cell proliferation in *H. cinerea*. Our results are in contrast to that found in male mammals but in concert to that found in female mammals. Specifically, mating activity did not enhance cell proliferation in the medial POA of male hamsters (Antzoulatos et al., 2008). In female prairie voles, male exposure and mating activity did enhance cell proliferation/survival in the VH (Fowler et al., 2002), but this was effect was not mediated by estrogen (Fowler et al., 2005). What may be driving these socially-induced changes in cell proliferation or survival is beyond the scope of this study; however, the regional induction of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), may be a candidate mechanism. In other vertebrates, for example, BDNF infusion increases neuronal survival within the HVC of canaries (Alvarez-Borda et al., 2004) and the number of new neurons in the rat hypothalamus (Pencea et al., 2001). Brain-derived neurotrophic factor is also regulated by social behavior; for example, both testosterone and singing upregulate BDNF in songbirds (Li et al., 2000; Alvarez-Borda et al., 2004). Future research should investigate the influence of BDNF as a mechanism for the social modulation of cell proliferation in anuran amphibians.

Behavioral responses to social stimuli have been known to affect adult neurogenesis (e.g., Sartor and Ball, 2005). As reported in other studies, calling depends

on the presence of androgens (Wada et al., 1976; Wada and Gorbman, 1977b; Wetzel and Kelley, 1983; Solis and Penna, 1997; Burmeister and Wilczynski, 2001), as castrated treefrogs in our study did not call. Evoked calling behavior was positively correlated with cell proliferation in the POA and not in the IF, which is interesting considering the involvement of the POA in calling behavior. However, the behavioral response of calling was not driving the socially-induced increases in BrdU-labeling in the POA, as demonstrated statistically by controlling for the amount of evoked calling behavior. Thus, unlike that observed in the song control nuclei of songbirds (Wang et al., 1999; Sartor and Ball, 2005), the behavioral output of the male treefrogs in this study did not influence cell proliferation in the brain regions studied here.

In conclusion, our results indicate that gonadal hormones differentially influence cell proliferation: testosterone in the male treefrog did not affect cell proliferation, whereas elevated estrogen levels in the female suppressed cell proliferation. Further, gonadal steroids did not mediate the social modulation of cell proliferation in males and females. There were sex differences in the location of socially-modulated cell proliferation: in the male, socially-induced cell proliferation occurred in the POA and IF, where as in the female, socially-induced increases occurred in the IF. Additionally, in males, evoked calling behavior was positively correlated with BrdU-labeling in the POA (and not the IF); however, this behavior did not modulate socially-induced cell proliferation. Even though gonadal steroids may be correlative with cell proliferation (Almli, Ch. 4), it appears that they are not interdependent with the effects on cell proliferation from social modulation. Further studies are necessary to examine potential neurotrophin involvement in mediating the observed social modulation of cell proliferation. Moreover, it is not known at this time whether these new cells survive and become incorporated into existing circuitry within the POA and/or IF or whether they mature into neurons or glia. Future research could elucidate the impact of these new cells on social behavior; such studies will provide insight into the importance of cell proliferation (or neurogenesis in general) in mediating seasonal changes in socio-sexual behavior.

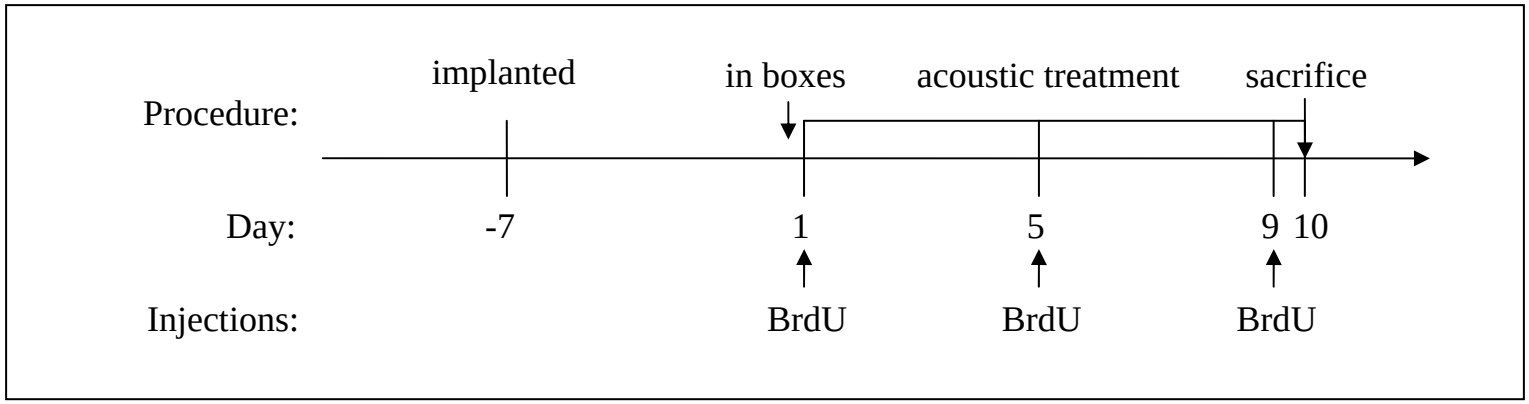

Figure 4.1. Timeline of gonadectomy/implantation, BrdU injections, and acoustic treatment for male and female *Hyla cinerea*.

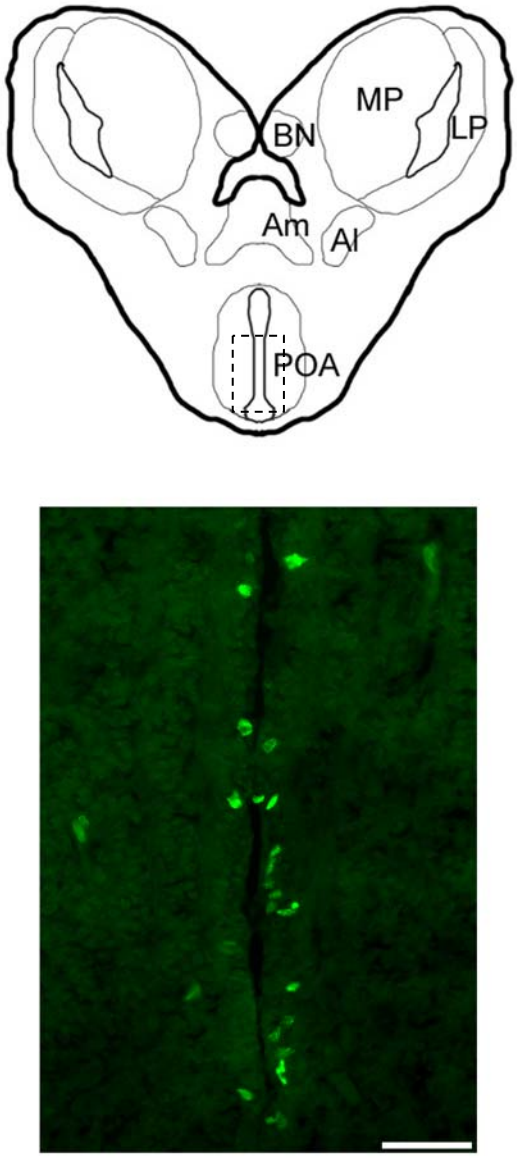

Figure 4.2. Schematic and photomicrograph of a horizontal section through the telencephalon of a representative *Hyla cinerea* brain at the level of the POA. Dotted rectangle in upper image represents the area including the preoptic recess photographed in the lower image. BrdU-labeled cells (i.e., green cells) are located mainly near the ventricle edge in the center of the image. Scale bar = 50 μm. Abbreviations: Al, *lateral amygdala*; Am, *medial amygdala*; BN, *bed nucleus of the pallial commissure*; LP, *lateral pallium*; MP, *medial pallium*; POA, *preoptic area*.

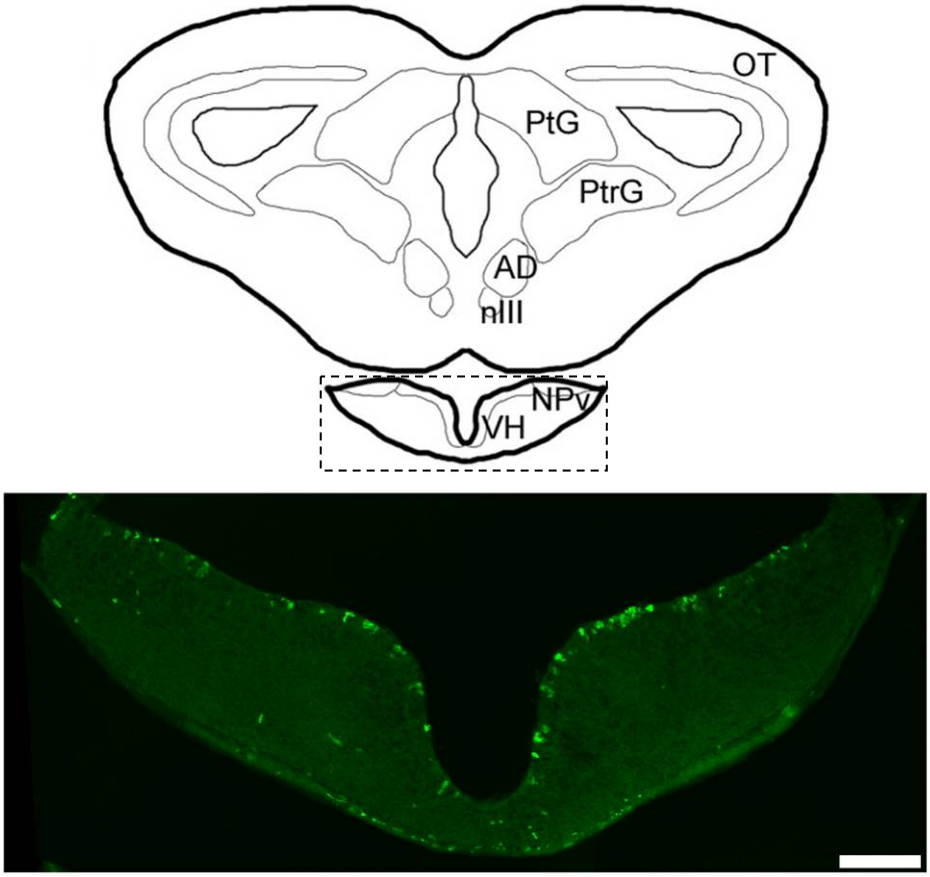

Figure 4.3. Schematic and photomicrograph of a horizontal section through the telencephalon of a representative *Hyla cinerea* brain at the level of the IF. Dotted rectangle in upper image represents the area including the hypothalamic recess photographed in the lower image. BrdU-labeled cells (i.e., green cells) are located mainly near the ventricle edge in the center of the image. Scale bar = 100 μm. Abbreviations: AD, *anterodorsal tegmentum*; IF, *infundibular hypothalamus*; NPv, *nucleus of the periventricular organ*; nIII, *oculomotor nucleus*; OT, *optic tectum*; PtG, *pretectal gray*; PtrG, *pretoral gray*; VH, *ventral hypothalamus*.

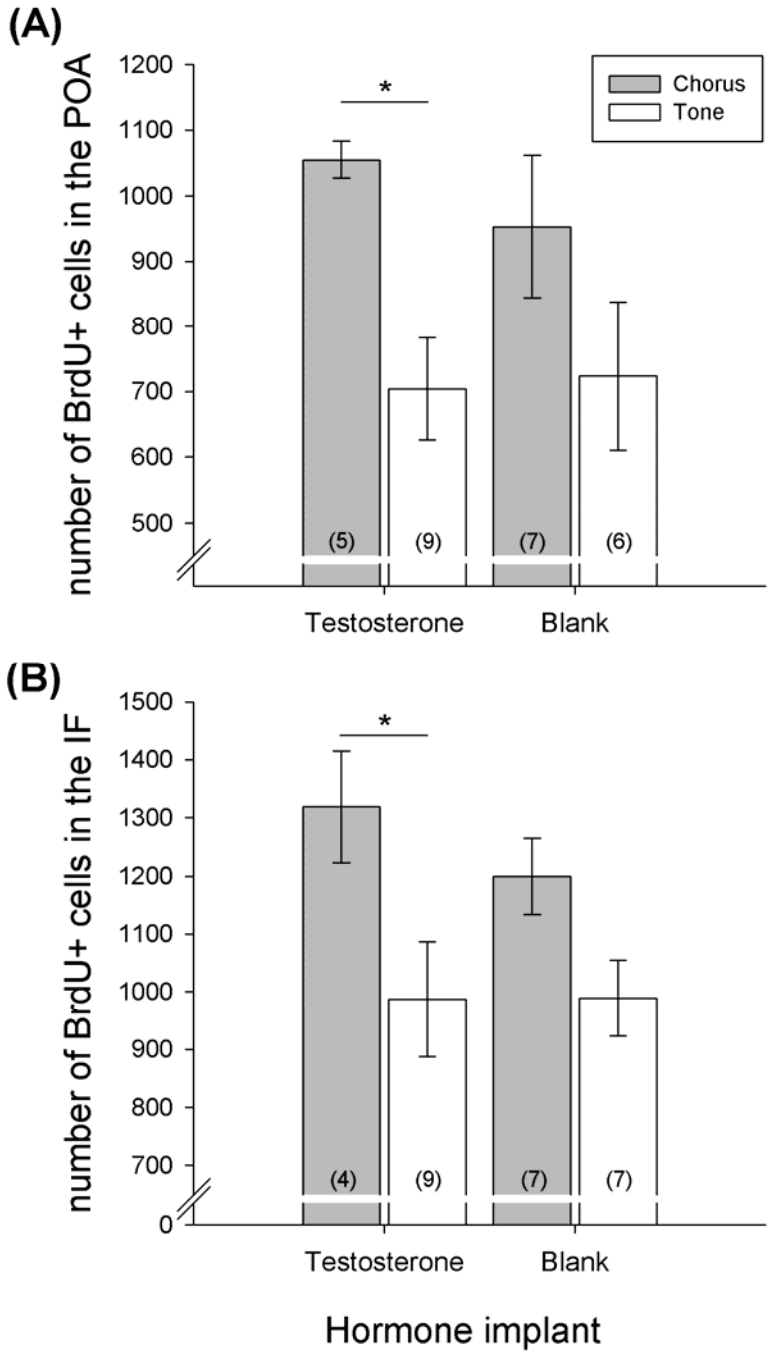

Figure 4.4. The influence of testosterone implants and acoustic stimulation on the number of BrdU+ cells in the POA and IF of male *Hyla cinerea*. Males exhibited increased cell proliferation in the POA (A) and IF (B) in response to hearing their conspecific Chorus compared to Tone controls. Shown are means ± SEM. Significant post-hoc tests are represented by asterisks, *.

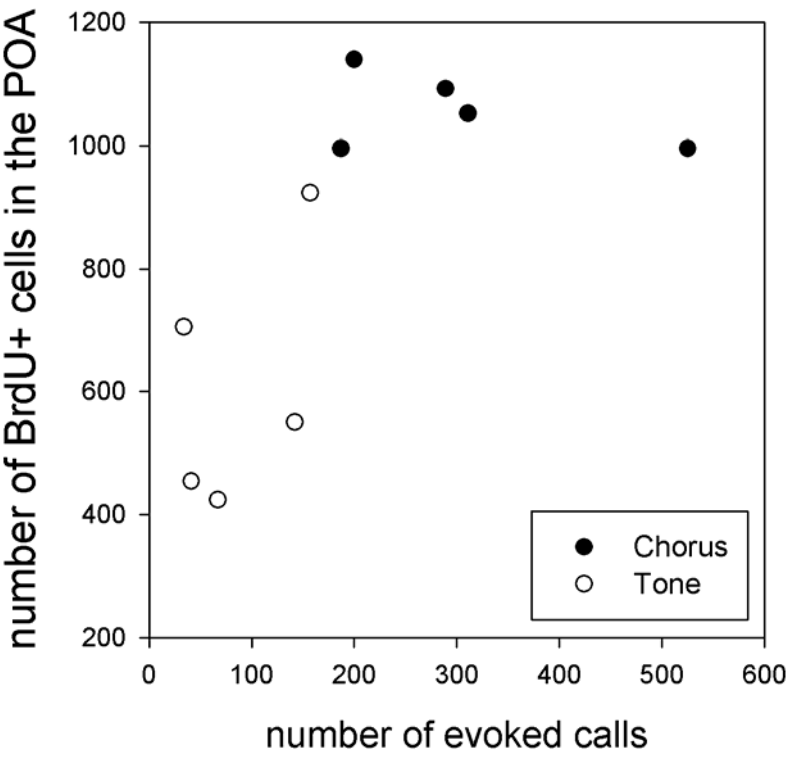

Figure 4.5. The relationship between the number of evoked calls and the number of BrdU+ cells in the POA of male *Hyla cinerea*. There was a significant positive correlation between calling behavior and cell proliferation in the male POA ($r = 0.697$, $p = 0.035$).

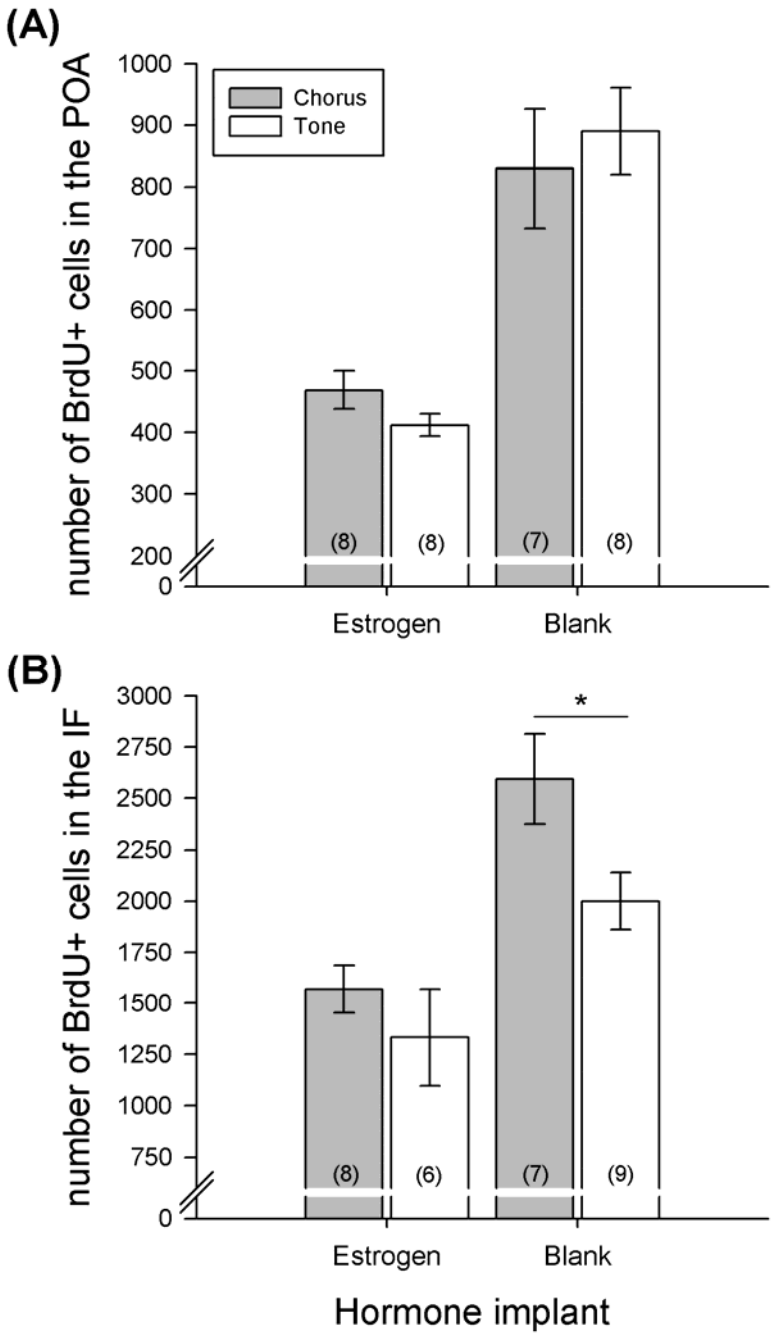

Figure 4.6. The influence of estrogen implants and acoustic stimulation on the number of BrdU+ cells in the POA and IF of female *Hyla cinerea*. Estrogen implants significantly decreased cell proliferation in the POA (A) and IF (B). Females with undetectable levels of estrogen (< 8 pg/mL) exhibited socially-modulated cell proliferation in the IF as revealed by a significant post-hoc test (represented by an asterisk, *). Shown are means ± SEM.

Chapter 5: Methodological considerations in the measurement of the proliferative activity in the adult green treefrog brain (*Hyla cinerea*)

Abstract

Bromodeoxyuridine (BrdU) is a common marker to label proliferating cells in the brain, but BrdU immunohistochemical staining is not without problems. In this chapter, We briefly review some common issues with BrdU staining and describe our own issue with BrdU-labeling when comparing immunohistochemical methodologies. The take-home message from this chapter is that studies using BrdU-labeling should be evaluated with caution and careful interpretation and that the results from different staining methods cannot be compared.

Introduction

With the advent of molecular markers to label proliferating cells and determine their phenotypes, there has been an explosion of research on adult neurogenesis in recent years. Still, the most common marker to label proliferating cells in the adult brain is 5-bromo-2'-deoxyuridine (BrdU; Taupin, 2007). Immunohistochemical (IHC) methods to detect BrdU require pretreatment of the tissue by DNA denaturation/digestion/antigen retrieval; this pretreatment includes acid hydrolysis, thermal denaturation, enzymatic degradation with nuclease and proteinase, and/or any combination of these methods (Dolbeare, 1995). The harsh treatment, in some cases, is necessary to render BrdU available for antibody binding (e.g., to break double-stranded DNA into single-strands), due to the situation that antibodies are directed against BrdU on single-stranded DNA, (Ngwenya et al., 2005; Taupin, 2007) or to remove histones and reduce tertiary DNA structures (Kuhn and Cooper-Kuhn, 2007). In some cases, it has been reported that the denaturation or digestion steps of some protocols do not confer reproducible results (Dover and Patel, 1994). However, in others, it has been reported that standard BrdU IHC methods can accurately and reliably detect labeling of cells in tissue that is well preserved (Ngwenya et al., 2005).

Historically, 3, 3'-diaminobenzidine tetrahydrocloride (DAB) was a commonly used chromagen for IHC staining, but that methodology does not allow for the facilitation of double and triple-labeling in studies of adult neurogenesis. Thus, many studies use immunofluorescence to visualize newly proliferating cells which colocalize with a cell phenotypic marker; however, this method is not without problems either (see Kuhn and Cooper-Kuhn, 2007). We used both methods to assess cell proliferation in our study, described in Chapter 3, and found differences in BrdU-labeling depending upon the IHC protocol used; i.e., immunofluorescence using fluorescein as the chromagen to visualize BrdU-immunoreactive cells (BrdU+) or IHC using DAB as the chromagen.

Materials and Methods

The methodology detailing the experimental animals, acoustic stimulation procedure, and IHC procedures can be referenced in the Materials and Methods in Ch. 3. One series of brain sections was processed for BrdU using DAB as the chromagen (see Almli, Ch. 3) and another processed with immunofluorescence (see Almli, Ch. 4).

Analysis of cell proliferation

Slides were coded so that cell counts were obtained blind to experimental conditions. Bromodeoxyuridine-immunoreactive cells (i.e., fluorescein-positive cells or DAB-positive cells) were visualized and counted using a 40× objective (Olympus BX60 microscope, Optronics camera). For IHC using DAB, every section in the each stained series (i.e., every fourth section in total) was counted to determine the numbers of labeled cells in the preoptic area (POA) and the infundibular hypothalamus (IF), the boundaries of which were determined by toluidine blue counterstaining (published boundaries: Northcutt and Kicliter, 1980; Neary and Northcutt, 1983). Similarly with immunofluorescent staining, every section in the each stained series (again, every fourth section in total) was examined and counted using a FITC filter cube, and faint autofluorescent background staining was used to determine the boundaries of the POA and IF. As per the fractionator method (e.g., West et al., 1991), the cell counts were multiplied by four to obtain an estimate of the total number of BrdU+ cells in the whole

ROI (because every fourth section was sampled). Previous studies have determined that correcting the total number of cells by the volume of the sections sampled does not influence the results (see Almi, Ch. 3).

Statistical analysis

All statistical analyses were conducted with SPSS 15.0 (Chicago, 2006), and *p*-values were labeled as significant at $p < 0.05$. Post-hoc analysis, where appropriate, was tested with Tukey's procedure. The number of BrdU+ cells with immunofluorescence staining and IHC staining with DAB as the chromagen in the POA and IF were analyzed separately with a three-way ANOVA. Additionally, BrdU+ cells in the POA and IF were analyzed separately using a repeated measures ANOVA, with the staining method using DAB or fluorescein as the chromagen as the repeated factor. Correlations between the number of fluorescein-labeled and DAB-labeled BrdU+ cells in the POA and IF were performed. Lastly, we calculated a ratio consisting of the number of fluorescein-labeled BrdU+ cells to DAB-labeled BrdU+ cells and used that ratio as the dependent variable in separate ANOVAs for the POA and IF. Subjects that were missing consecutive sections within the POA or IF or had sections with aberrant staining were excluded from analyses. The resultant number of animals per group is listed under each analysis in the Results section.

Results

Proliferating cells in the IF (containing the ventral and dorsal hypothalamus, the posterior tuberculum, and the nucleus of the periventricular organ) as labeled by BrdU using both staining methodologies can be seen in Figure 5.1. Labeled cells often appeared in clusters, including some with up to 10 cells stacked near or on top of each other. These clusters included cells that were oval-shaped and tended to be observed in the ventricular zones of the POA and IF. Smaller, round-shaped cells were often found singly in the parenchyma.

Immunofluorescence and IHC using DAB show different seasonal results

Fluorescein-labeled BrdU+ data

Separate three-way ANOVAs for the POA and IF were calculated using the data collected from fluorescein-labeled BrdU+ cells. In the POA, sex (male, N=20; or female, N=22), time during breeding season (middle, N=15; middle to late, N=14; and late, N=13), and acoustic stimulus type (Chorus, N=21; or Tone, N=21) were the independent variables. The 'sex' variable did not yield any significant results, thus the variable was collapsed and the analysis was recalculated. Between subjects tests revealed a significant acoustic stimulus type by time during breeding season interaction ($F_{2,36} = 4.445$, $p = 0.019$) and main effects of acoustic stimulus type ($F_{1,36} = 4.471$, $p = 0.041$) and time during breeding season ($F_{2,36} = 21.466$, $p < 0.001$). Significant post-hoc tests ($p < 0.05$) using Tukey's procedure are shown on Figure 5.2A.

Similar results occurred in the IF using sex (male, N=21; or female, N=22), time during breeding season (middle, N=15; middle to late, N=15; and late, N=13), and acoustic stimulus type (Chorus, N=19; or Tone, N=24) as the independent variables. An ANOVA on the number of fluorescein-labeled BrdU+ cells in the IF was calculated, but the effect of sex was not significant. After removing the variable sex, the ANOVA was recalculated and between subjects tests yielded significant main effects of acoustic stimulus type ($F_{1,37} = 5.524$, $p = 0.024$) and time during breeding season ($F_{2,37} = 17.943$, $p < 0.001$). Significant post-hoc tests ($p < 0.05$) using Tukey's procedure are shown in Figure 5.2B. In summary, significant differences between acoustic stimulus type occurred in the middle of the season only; in other words, cell proliferation in the POA and IF due to Chorus stimulation returned to Tone control levels at the end of the breeding season.

DAB-labeled BrdU+ data

Separate ANOVAs were calculated with the data collected from DAB-labeled BrdU+ cells in the POA and IF: sex (male or female), time during breeding season (middle, middle to late, or late), and acoustic stimulus type (Chorus or Tone) were the independent variables. The 'sex' variable did not yield any significant results in the POA or IF, thus the variable was collapsed and the analysis recalculated. (See Ch. 3 for detailed results.) The time during breeding season did not significantly affect the number

of BrdU+ cells in the POA or IF. Significant post-hoc tests ($p < 0.05$) using Tukey's procedure are shown in Figure 5.2C, D. Using DAB as the chromagen to label BrdU+ cells, a significant difference between acoustic stimulus type only occurred late in the season; however, there was no overall seasonal trend in cell proliferation at the time points sampled in this study.

Different staining methods revealed increased cell proliferation due to Chorus stimulation

To ensure that our differing methodologies were comparable in regards to the social modulation of cell proliferation, we calculated repeated measures ANOVAs using the number of fluorescein-labeled cells and DAB-labeled cells as the repeated factor ('chromagen'). Additionally, we ran correlation analyses between the measurements of cell proliferation using different stains and found they were correlated: POA ($r = 0.416$, p=0.012) and IF ($r = 0.418$, p=0.010). A three-way repeated measures ANOVA on the number of BrdU+ cells in the POA was calculated with sex (male, N=15; or female, N=21), time during breeding season (middle, N = 13; middle to late, N = 13, or late, N = 10), and acoustic stimulus (Chorus, N=17; or Tone, N=19) as independent factors. The 'sex' variable did not yield significant results, thus the variable was collapsed and the analysis was recalculated. Within subjects tests revealed that all variables were significantly different: there was a three-way interaction, chromagen by time during breeding season by acoustic stimulus type ($F_{2,30} = 7.032$, $p = 0.003$), and a main effect of chromagen ($F_{1,30} = 236.805$, $p < 0.001$). Between subjects tests yielded main effects of time during breeding season ($F_{2,30} = 3.592$, $p = 0.040$) and acoustic stimulus type ($F_{1,30} = 8.882$, $p = 0.006$). Tukey's post-hoc tests revealed a significant difference between Chorus and Tone stimulation at the end of the breeding season using the estimated marginal means of the BrdU+ cells in the POA (Fig. 5.3A).

Similarly, another three-way repeated measures ANOVA on the number of BrdU+ cells in the IF was calculated with sex (male, N=17; or female, N=20), time during breeding season (middle, N = 13, middle to late, N = 13, or late, N = 11), and acoustic stimulus type (Chorus, N=17; or Tone, N=20) as independent factors; the

number of fluorescent-labeled cells and DAB-labeled cells were the repeated factor. Again, the 'sex' variable did not yield significant results, thus the variable was collapsed and the analysis was recalculated. Within subjects tests revealed that all were significant: there was a three-way interaction, chromagen by time during breeding season by acoustic stimulus type ($F_{2,31} = 7.523$, $p = 0.002$), and a main effect of chromagen ($F_{1,31} = 327.352$, $p < 0.001$). Between subjects tests yielded main effects of time during breeding season ($F_{2,31} = 3.414$, $p = 0.046$) and acoustic stimulus type ($F_{1,30} = 10.977$, $p = 0.002$). Tukey's post-hoc tests revealed a significant difference between Chorus and Tone stimulation at the end of the breeding season using the estimated marginal means of BrdU+ cells in the IF (Fig. 5.3B). The repeated measures ANOVAs in the POA and IF demonstrated that although there was a significant difference between 'chromagen' or in other words, the different staining methodologies, there maintained a significant main effect of acoustic stimulus type regardless of the staining methodology. However, statistically controlling for the variation between staining methods (i.e., the repeated factor) yielded a main effect of time during the breeding season with a slight decreasing trend in BrdU+ in the Tone stimulation group compared to the Chorus; this was supported by a significant Tukey's post-hoc test at the late breeding season time point.

Ratio of BrdU+ cells changed across the breeding season

To further characterize the relationship between the time during the breeding season and the different methodologies to measure cell proliferation, we calculated a ratio consisting of the number of fluorescein-labeled BrdU+ cells to DAB-labeled BrdU+ cells. Using the ratio of the number of BrdU+ cells, separate ANOVAs (for the POA and IF) were calculated using sex (male or female), time during breeding season (middle, middle to late, and late), and acoustic stimulus (Chorus or Tone) as independent factors. Again, as in the analyses above, 'sex' did not contribute to any significant results, thus it was collapsed and the analysis was recalculated. In regards to the POA (Fig. 5.A), between subjects tests yielded an acoustic stimulus type by time during the breeding season interaction ($F_{2,30} = 7.583$, $p = 0.002$) and a main effect of time during breeding season ($F_{2,30} = 12.500$, $p < 0.001$; acoustic stimulus type: $p > 0.05$). Similar results

occurred in the IF (Fig. 5.4B): between subjects tests yielded an acoustic stimulus type by time during the breeding season interaction ($F_{2,31} = 9.434$, $p = 0.001$) and a main effect of time during breeding season ($F_{2,31} = 16.578$, $p < 0.001$; acoustic stimulus type: $p > 0.05$). Significant post-hoc test results are presented in Figure 5.4, however, of note was the differences between the methodologies occurred mainly at the end of the breeding season (i.e., Chorus: between middle and late time points; Tone: between mid-late and late time points). Although there was a significant interaction between the independent variables, the ratio data revealed that the methodology differences were mainly affected by the time during the breeding season, because the main effect of acoustic stimulus type was lost.

Discussion

Our results demonstrate that cell proliferation, as measured by BrdU-labeling, using different visualization methodologies can yield different results; this was revealed by a significant effect of staining method in a repeated measures ANOVA. In our data using immunofluorescence methodology, we saw a seasonal decline in cell proliferation in the POA and IF towards the end of the breeding season, with the main differences in cell proliferation between acoustic stimulus type (Chorus or Tone) observed at the first time point measured, i.e, in the middle of the breeding season. In regards to the data using DAB as the chromagen to label proliferating cells, we saw no seasonal trend; however, it was only late in the season where we observed a significant difference in cell proliferation between acoustic stimulus type. When a ratio between the numbers of fluorescein-labeled BrdU+ cells to DAB-labeled BrdU+ cells was used in the statistical analyses, we were able to compare the methodologies directly. Late in the breeding season, the differences between the methodologies were more apparent: specifically, the methodology using DAB as the chromagen was labeling more proliferating cells than immunofluorescence. With the exception of the ratio analysis (which would essentially factor out components of the staining methods that exhibited similar trends; i.e., social modulation in BrdU+ cell number), significant differences between acoustic stimulus type remained regardless of the methodology used. The similarities between the analyses suggest that certain manipulations (i.e., acoustic stimulation) are resistant to

methodological differences in staining, but that subtle manipulations such as seasonality may be affected in more intricate ways.

Using statistical methods that compared the methodologies directly (i.e., repeated measures ANOVA and the ANOVA on the ratio data), we observed that differences in methodology became more apparent late in the breeding season but consistently exhibited a significant interaction between time during the breeding season and acoustic stimulus type. In the methodological analysis with the data from each staining method analyzed separately, we observed that immunofluorescence was more sensitive to differences between acoustic stimulus type early in the breeding season whereas IHC using DAB was more sensitive to differences between acoustic stimulus types late in the breeding season. In other studies of cell proliferation, Taupin (2007) explains that the intensity or extent of BrdU-labeling is highly dependent on the method used for detection; thus the discrepancy in our results may not be surprising. In fact, discrepancies in cell numbers with differing methodologies have been seen in other ectothermic vertebrates (Enrique Font, personal communication). In regards to seasonal differences in BrdU labeling, however, we are aware that many factors are known to affect neurogenesis (reviewed in Ming and Song, 2005). These factors, which may be environmental, may have consequences such as changes in blood flow which may change the rate of BrdU uptake into the brain (Taupin, 2007). Hence, this may explain the observations of seasonality in BrdU labeling, but does not necessarily explain the differences in BrdU+ due to methodology.

In light of the unexpected differences in BrdU+ depending upon IHC methodology, slight differences between the staining methods warranted further consideration. The main difference between the staining protocols is the composition of the anti-BrdU antibody: in the immunofluorescence protocol, the primary antibody has added nucleases. This difference is critical because other studies have shown that protocols using a denaturation step with nucleases yield the best results (e.g., Gonchoroff et al., 1986). Thus, it is possible that the added nucleases allowed the anti-BrdU antibody to have more access to antigen sites on labeled DNA and hence the extent of staining should be greater. However, our results demonstrate less labeling, on average, using this antibody with immunofluorescence; this could be due to technical limitations with the

traditional fluorescent microscopy. Because proliferating cells often appear in clusters (e.g., Cameron and McKay, 2001), the actual number of BrdU+ cells becomes obscured when multiple cells were proliferating within the same z-plane, thus BrdU+ numbers may be underrepresented in our study using immunofluorescence. At any rate, these observations are difficult to reconcile with reference to other studies as, to our knowledge, there are no published comparisons of BrdU IHC using DAB versus immunofluorescence to consult.

Although BrdU staining is not stoichiometric (Taupin, 2007), there are some observations that can be made about the general appearance of the BrdU-labeling within the cells. For example, cells that have divided multiple times may incur cumulative labeling depending upon the administration times of BrdU (Taupin, 2007), which would yield more intense labeling. Alternatively, labeling becomes diluted with every new cell division (Cooper-Kuhn and Kuhn, 2002) causing less intense labeling or no labeling if multiple divisions have diluted the label below the detection level of the staining method (Kuhn and Cooper-Kuhn, 2007). It is unknown whether there is a difference between the detection limits of our immunofluorescence method and IHC method using DAB. It is possible that depending upon the time during the breeding season (and hence the physiological state of the animal), the IHC method is labeling more or less cells that have divided multiple times. Again, this situation may favor IHC methods using DAB as the chromagen due to the ease of counting faintly-labeled BrdU+ cells as compared to immunofluorescence in which autofluorescence of the background cells may mask faintly-labeled BrdU+ cells (Kuhn and Cooper-Kuhn, 2007). Without further experiments, it is difficult to speculate on the cause of the discrepancy between staining methods in our study, which is clearly modulated by acoustic stimulation and the time during the breeding season. However, it should be noted that consistency in IHC staining methodology is of utmost importance to obtain an accurate depiction of adult cell proliferation or neurogenesis.

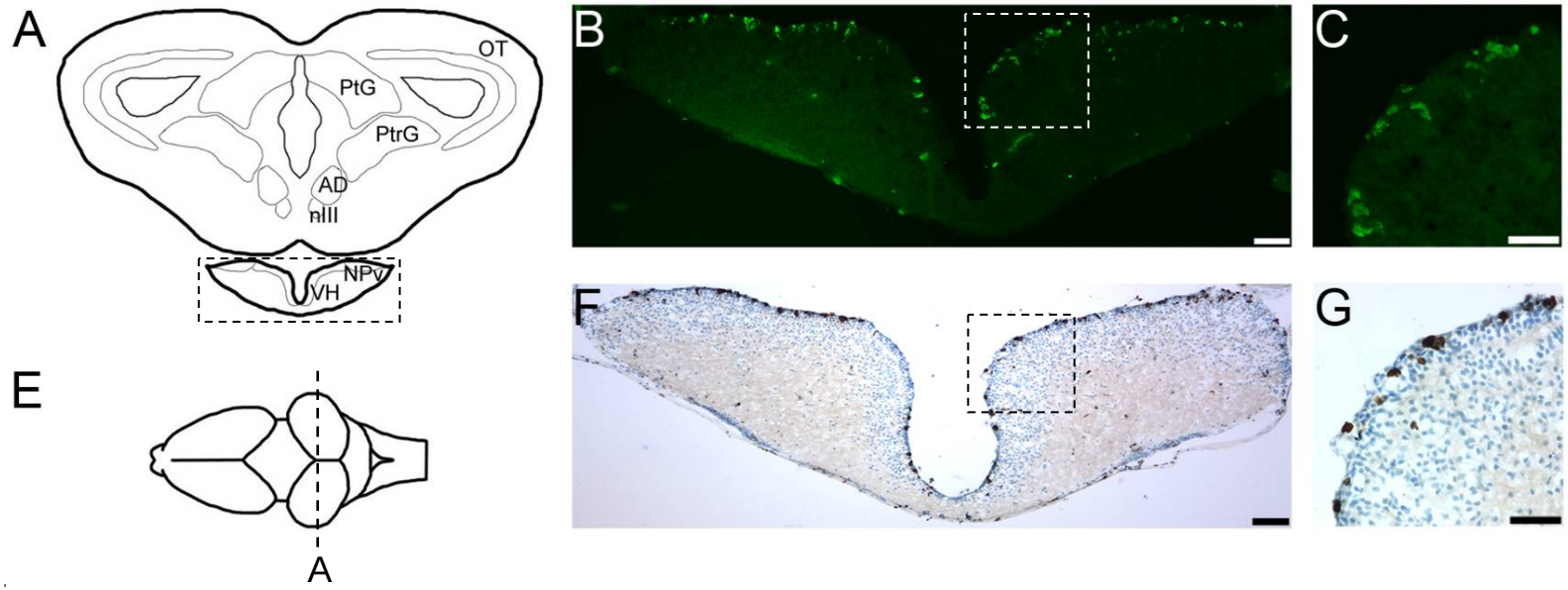

Figure 5.1. Comparison of BrdU-labeling in the IF with two staining methods. Dotted rectangle in (A) represents the region photographed with the two staining methods using fluorescein (B,C) or DAB (F,G) as the chromagen. Dotted rectangles in B and F are shown at higher magnification in C and G, respectively. (E) Top view of *Hyla cinerea* brain showing the location of the horizontal sections in A, B, C, F, and G. Scale bars = 50 µm. Abbreviations: AD, *anterodorsal tegmentum*; DAB, *3, 3'-diaminobenzidine tetrahydrocloride*; IF, *infundibular hypothalamus*; NPv, *nucleus of the periventricular organ*; nIII, *oculomotor nucleus*; OT, *optic tectum*; PtG, *pretectal gray*; PtrG, *pretoral gray*; VH, *ventral hypothalamus*.

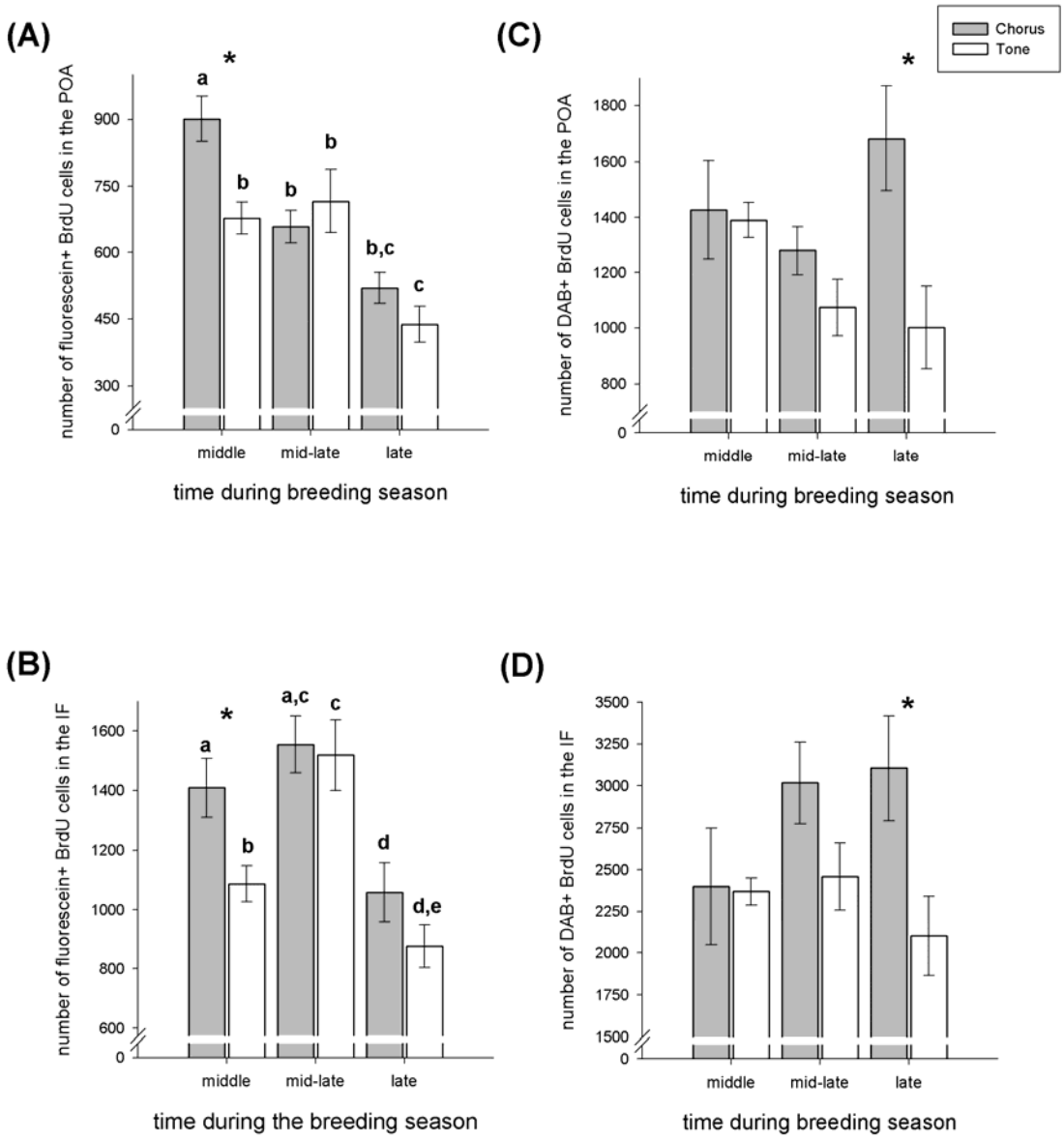

Figure 5.2. The influence of season and acoustic stimulation on the number of BrdU+ cells in *Hyla cinerea* using two staining methods. Cell proliferation was quantified in the POA (A,B) and IF (C,D). Fluorescent staining (A,C) revealed differences in BrdU+ cell number between acoustic stimulation groups in the middle of the breeding season whereas DAB staining (B,D) picked up differences between acoustic stimulation groups in the late breeding season. Asterisks (*) represent the only significant post-hoc tests between acoustic stimulation groups ($p < 0.05$). The letters represent significant differences between the time during the breeding season within each acoustic stimulation group ($p < 0.05$).

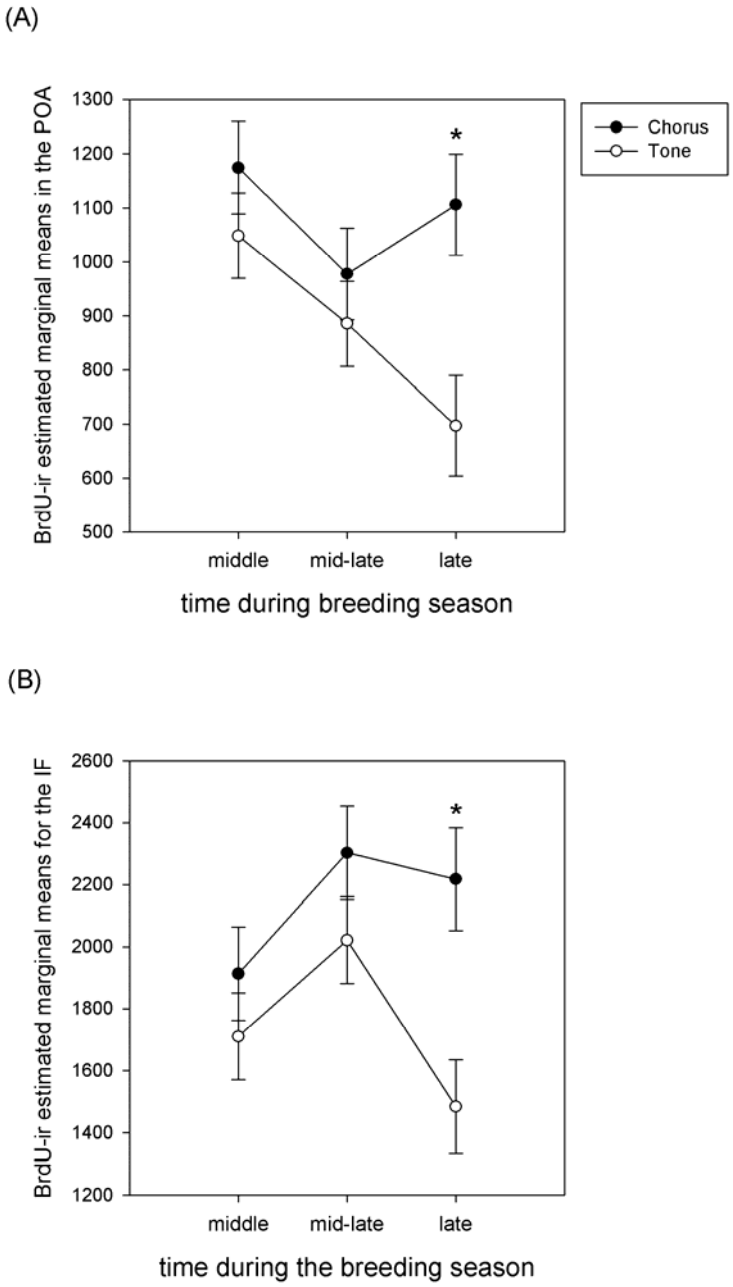

Figure 5.3. The influence of acoustic stimulation and time during the breeding season on the estimated marginal means calculated from the number of BrdU+ cells from each staining method. Proliferating cells from immunofluorescence with fluorescein as the chromagen and immunohistochemical staining with DAB as the chromagen in the POA (A) and IF (B) are shown. Asterisks (*) represent the only significant post-hoc tests between acoustic stimulation groups ($p < 0.05$). There were no interactions between acoustic stimulus type and time during the breeding season in either the POA or IF.

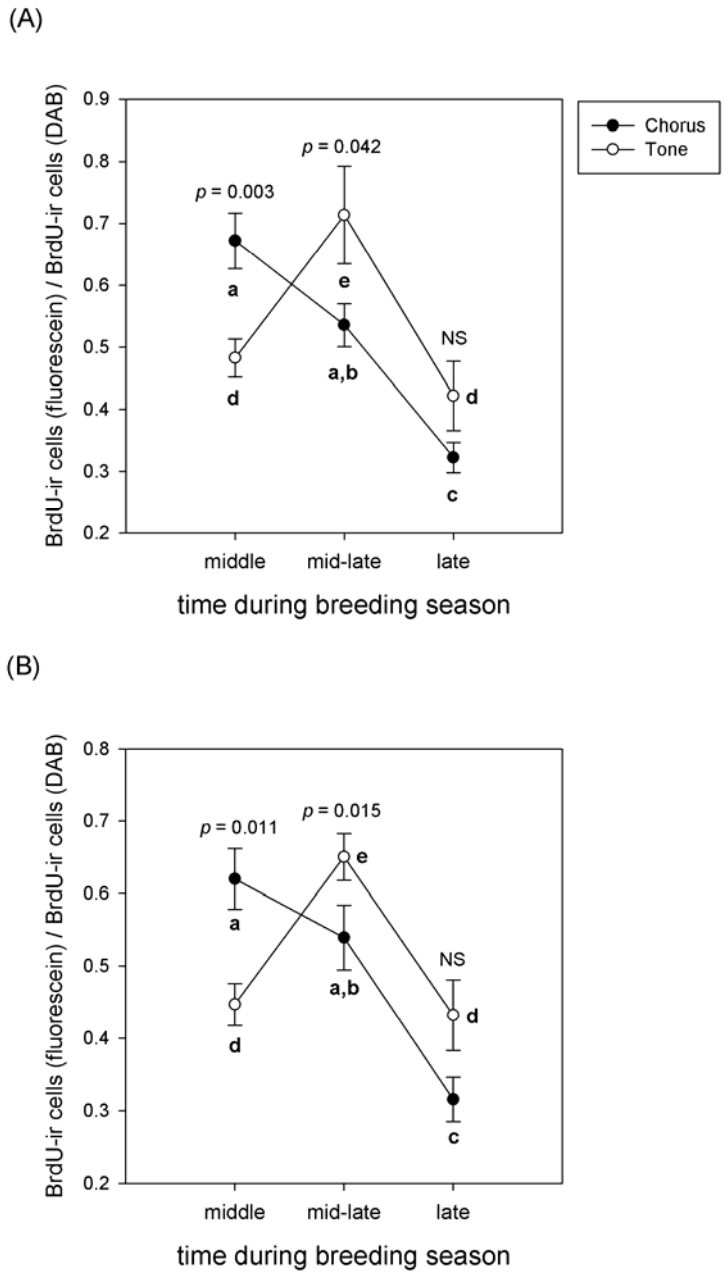

Figure 5.4. The ratio of the number of BrdU+ cells (fluorescein/DAB) changed across the breeding season depending upon the type of acoustic stimulus presented. In the POA (A) and IF (B), the ratio of cells in the Chorus group declined towards the end of the breeding season, whereas there was no trend in Tone controls. Significant post-hoc tests between acoustic stimulus groups are listed with *p*-values above the data points and within acoustic stimulus groups are listed with lower-case letters under or to the side of the data points.

Chapter 6: Concluding remarks

The objective of this research was to understand the relationship between social behavior, concomitant gonadal steroid hormones, and adult neurogenesis using the green treefrog, *Hyla cinerea*, as a model system. Similar to other vertebrates, treefrogs use social interactions as regulators of socio-sexual behavior; however, the treefrog's main social cue, the mating call, comprises only one modality unlike the multisensory cues used by many vertebrates (although other signals may influence behavioral responses to the call). Thus, in the green treefrog model, it is possible to link potential socially-induced changes in adult neurogenesis to exposure to their sole acoustic cue, i.e., the conspecific mating call. In this dissertation research, several hypotheses regarding the social and hormonal modulation of adult neurogenesis, specifically cell proliferation, were tested and the results synthesized below (Fig. 6.1).

Synthesis of results

To begin the assessment of the social modulation of cell proliferation, I needed to understand the extent of endogenous cell proliferation in the adult treefrog brain. Thus, in Chapter 2, I detailed the distribution of proliferative areas in the adult *Hyla cinerea* brain using 5-bromo-2′-deoxyuridine (BrdU) labeling (Almli and Wilczynski, 2007). I discovered that BrdU immunoreactive (BrdU+) cells were mainly distributed in ventricular zones throughout the brain at 2 hrs, 2 days, 2 weeks, and 30 days. The heaviest concentrations of cells were located in the telencephalon, primarily in the ventral regions of the lateral ventricles. Numerous BrdU+ cells were also located around the preoptic and hypothalamic recesses. Proceeding caudally towards the midbrain, there was a marked decrease in BrdU labeling and few BrdU+ cells were found in the hindbrain. At earlier time points, cells were primarily ventricular; however, at later time points (i.e., 30 days), the cells appeared to have migrated into parenchymal regions. Findings from this study were essential to locate areas of the brain to investigate in the experimental chapters of this dissertation.

Results in anuran amphibians using different cell proliferation markers were largely comparable to those described in this study, although there were some differences in the amount and location of proliferating brain cells. Compared to the forebrain, fewer proliferating cells were found in the midbrain (e.g., tectum) regions in most anuran amphibian studies, with the exception of Simmons et al. (2008) who found higher levels of labeling in the optic tectum and torus semicircularis. Further, the hindbrain regions (e.g., cerebellum and medulla) also had fewer (if any) proliferating cells in most anuran studies; however, Bernocchi et al. (1990) noted moderately high labeling in the medulla and Raucci et al. (2006) noted many labeled cells in the granular layer of the cerebellum. It is unclear at this point whether differences in proliferative activity reported in these studies are species-dependent or dependent on the type of proliferative marker used. Further, widespread neurogenesis in adult fishes (Zupanc, 2001) and reptiles (Font et al., 2001) has been related to the continual growth of these animals throughout adulthood. It has been suggested this may also be the case in anuran amphibians (Bernocchi et al., 1990).

I report here that socially-relevant cues modulate cell proliferation in the brains of male and female *Hyla* cinerea; this was accomplished using immunohistochemistry techiques, endocrinological manipulations, and socially-relevant acoustic stimulus presentations. In Chapters 3 and 4, I found that BrdU+ cell number was increased in the preoptic area (POA) and infundibular hypothalamus (IF), two regions involved in acoustic communication and endocrine regulation, of males and females that heard their conspecific chorus. However, BrdU+ cell number was not socially-modulated in a control region containing the olfactory bulb. I discovered that proliferating cells were socially-modulated in a sex by region-specific manner; specifically, males exhibited socially-modulated increases in BrdU+ cells in the POA whereas females exhibited socially-modulated increases in BrdU+ cells in the IF. Statistically, I was able to factor out the influence of gonadal steroid hormones, i.e., androgens in the male and estrogen in the female, on cell proliferation in naturally cycling males and females in Chapter 3. Those results demonstrated that androgens may mediate the social modulation of cell

proliferation in the male, and estrogen did not mediate the social modulation of cell proliferation in the female.

In Chaper 4, I used gonadectomized and implanted (blank- or steroid-filled implants) male and female *H. cinerea* to further uncover the involvement of gonadal steroids in cell proliferation with and without social stimulation. I found that gonadal steroid hormones, i.e., androgens in the male and estrogen in the female, were not required for the social modulation of cell proliferation nor were the hormones neurotrophic in anuran amphibians. In fact, elevated estrogen levels suppressed cell proliferation in the POA and IF of female *H. cinerea*. This is not to imply that gonadal steroids do not exert some type of an effect on cell proliferation (e.g., see correlations with androgens and cell proliferation, Ch. 3), but those effects (unknown at this time), are independent of those mediating cell proliferation with social stimulation. In this study, the social modulation of cell proliferation occurred in both the POA and IF of male treefrogs (in contrast to only the POA, Ch. 3) and only the IF of females (same in Ch. 3). It is possible that the exogenous elevated testosterone from the implants, as opposed to naturally cycling testosterone, influenced the region-specificity of social modulation in the male. However, further investigation with more subjects and the incorporation of testosterone implants producing physiological levels of androgens would be necessary to reconcile this discrepancy between Chapters 3 and 4. Also, using females in one reproductive state or categorizing the female into a reproductive state prior to the acoustic stimulation procedure would be an interesting addition to this study.

Lastly, I assessed whether behavioral output, i.e., evoked calling behavior, modulated cell proliferation in male treefrogs. Interestingly, I found that calling behavior was not driving the increased cell proliferation due to social modulation in either naturally-cylcing males (Ch. 3) or testosterone-implanted males (Ch. 4). However, evoked calling levels were positively correlated with cell proliferation in the POA in both studies, a notable observation considering the involvement of the POA in calling behavior (Schmidt, 1968; Knorr, 1976; Wada and Gorbman, 1977b). Although female behavior was not quantified in this study, future studies could correlate movement toward the “signaler”, for example, with cell proliferation. In fact, activity in caudal areas of the

hypothalamus has been shown to be associated with an interaction of hearing conspecific calls and locomotion (Hoke et al., 2007).

Because calling behavior did not induce cell proliferation in these studies, it may also be unlikely that the reciprocal is true; in other words, that cell proliferation influences calling behavior. This idea is supported by the time course data presented in Ch. 2 in which significant migration away from the ventricular zone does not seem to occur until after at least two weeks after BrdU injection. Thus, the majority of the cells remained in the ventricular zone during the 10 day period during which I tracked calling behavior in Chapters 3 and 4. Further, studies in mammals (reviewed in Abrous et al., 2005) suggest that newly born cells do not become active until ca. 1-2 weeks after birth. In fact, experimental studies report that new cells participate in olfactory mediated behaviors after three weeks in mammals (Huang and Bittman, 2002; Rochefort et al., 2002), and in song control nuclei of songbirds after 30 days (Paton and Nottebohm, 1984). Thus, it would seem unlikely that the cell proliferation observed in this study is behaviorally meaningful over the 10 day period measured in this dissertation research.

Chapter 5 was a comparison of staining methodologies for cell proliferation. When attempting the co-localization of phenotypic markers with proliferating cells using fluorescent immunohistochemical methods, I noticed differences between the amount of labeling between cells stained with traditional methods [using 3,3′-diaminobenzidine tetrahydrocloride (DAB) as the chromagen] and immunofluorescence (with fluorescein as the chromagen). Using the subjects from Ch. 3, I quantified cell proliferation in the POA and IF using the two staining procedures and compared whether proliferative activity in those regions were influenced by the time during the breeding season, acoustic stimulus type (Chorus or Tone) and sex. In the data using immunofluorescence methodology, I observed a seasonal decline in cell proliferation in the POA and IF towards the end of the breeding season. In regards to the data using DAB as the chromagen to label proliferating cells, I observed no seasonal trend. Further, differences in methodology in regards to proliferating cell number became more apparent late in the breeding season, but the results consistently exhibited a significant effect of acoustic stimulus type with Chorus-stimulated treefrogs having increased cell proliferation compared to Tone controls. The

results of Chapter 5 suggest that BrdU-labeling should be evaluated with caution as differences between IHC staining methodology can obscure an accurate depiction of adult cell proliferation or neurogenesis.

This dissertation research provides the groundwork for future studies detailing the social modulation of adult neurogenesis in *Hyla cinerea*. It seems that many aspects of socio-sexual behavior in the adult green treefrog have independent modulators. For example, social cues modulate gonadal hormones (Almli, Ch. 3; Burmeister and Wilczynski, 2000; Lynch and Wilczynski, 2006), behavior (evoked calling in the male; Burmeister and Wilczynski, 2000, 2001), and now cell proliferation (Almli, Ch. 3 and 4). Interestingly in these studies, cell proliferation was not increased by gonadal steroid hormones or evoked calling behavior. This "independence" is similar to that seen in Burmeister and Wilczynski (2000) in which social cues increased androgen levels equally in male treefrogs whether or not they engaged in calling behavior. In summary, social cues can have multiple effects on physiology and behavior, but one could argue that these effects are not inter-dependent. This is not to imply that gonadal hormones or behavior are not important for cell proliferation in general, but just that they did not modulate cell proliferation in the conditions studied here.

Further Considerations

The studies presented in this dissertation were not designed to elucidate which component(s) of neurogenesis is/are primarily affected by social stimulation. In these studies, I have qualified my observations as cell proliferation, although multiple injection paradigms cause uncertainty in which process of neurogenesis is being observed (Taupin, 2007). Typically, it is thought that a process which affects cell proliferation either suppresses or induces mitosis in precursor cells (Galea, 2008). On the other hand, factors that affect cell survival either promote or prevent the differentiation and/or maturation of new cells (Galea, 2008). In order to address this dichotomy in our model system, I would make these suggestions for future studies: to assess cell proliferation, treefrogs could receive a BrdU injection immediately prior to the acoustic stimulation procedure (on the last night) and then sacrificed after the acoustic treatment and to assess cell survival,

treefrogs could receive a BrdU injection two hours prior to acoustic stimulation on the first day of the experiment. At any rate, because in other vertebrates, over 50% of proliferating cells die within the first month (mammals: e.g., Cameron et al., 1993; birds: e.g., Kirn et al., 1999), it would be interesting to determine whether the socially-induced increases in cell proliferation would translate into increased survival and potential incorporation into neuronal circuitry.

Future studies could also investigate the phenotypes of the proliferating cells and whether they were differentially modulated by social stimulation. The relative percentage of neurons and glial cells produced in the adult amphibian brain is unclear at this time, but studies suggest between 10 (Simmons et al., 2008) to 15% (Polenov and Chetverukhin, 1993) become neurons and 20 (Simmons et al., 2008) to 65% become glia (Polenov and Chetverukhin, 1993); the fate of the rest of the cells is uncertain. More detailed analysis of proliferating cells within the ventricular zone of the POA (using electron microscopy) suggest the cells to be proliferating neuroepithelial germinative stem cells, which may serve as the progenitors of new neuronal cells (Chetverukhin and Polenov, 1993). In fact, Polenov and Chetverukhin (1993) characterize one third of these proliferating cells in the POA to be peptidergic neurosecretory cells and conventional neurons. They suggest that labeled cells found in the POA are the result of the proliferation, migration, and subsequent differentitation of ventricular zone precursor cells (Polenov and Chetverukhin, 1993). Because more detailed analyses were not conducted in this study, I can only report that both immature neurons (Fig. 6.2) and glia (Figs. 6.3 - 6.4) are found in the adult anuran amphibian brain within 10 days. This finding is supported by Western blot analysis (Almli, unpublished data).

In regards to the social modulation of adult neurogenesis, the mechanisms causing the increases in cell proliferation were not explicitly tested in this dissertation research and thus the following hypotheses are only speculation. The region- and sex-specific increase in BrdU+ cells reported here may be due to permissive microenvironments (or "niche"; Alvarez-Buylla and Lim, 2004) in the POA and IF, which may be suitable for enhancing cell proliferation. Newly proliferated cells are thought to be easily excitable (e.g., Cameron et al., 1993) and preferentially incorporated into existing circuitry (e.g.,

Kee et al., 2007). This is important in light of the sexually-dimorphic functions of the POA and ventral hypothalamus (VH), part of the IF: the POA is mainly involved in male socio-sexual behavior whereas the VH is primarily involved in female socio-sexual behavior. Under certain conditions (e.g., social stimulation), the constitutive proliferative activity in the adult anuran brain may initiate a chain-reaction by releasing neurotrophic factors or neurotransmitters which may enhance proliferative activity in neighboring cells. This mechanism has been suggested due to the clustering of BrdU labeled cells found in different phases of neurogenesis (e.g., Palmer et al., 2000). From studies in other vertebrates, we know that growth factors, such as brain-derived neurotrophic factor (BDNF), are upregulated by socio-sexual cues and behavior in the songbird system which directly or indirectly enhances adult neurogenesis (e.g., Li et al., 2000).

Studies also suggest that morphological changes such as increased glial support may create a permissive microenvironment and enhance cell proliferation (e.g., Dunlap et al., 2006). Specifically, Dunlap et al., 2006) have shown that radial glial fibers increase during social interactions. This study is extremely relevant to the studies presented here as many of the proliferating cells express glial fibrillary acidic protein (GFAP) and have radial fibers (Fig. 6.4). Thus, it seems that the BrdU labeled cells in this study are proliferating radial glial cells (Enrique Font, personal communication). We know that radial glial cells, or a subset of them, are multipotent stem cells for adult neurogenesis in reptiles and birds (e.g., Garcia-Verdugo et al., 2002) and more recently, in mammals *in vitro* (Merkle et al., 2004). Hence in our study, radial glial cell division may be giving rise to neurons and perhaps other cell types (e.g. more radial glial cells).

Adult neurogenesis represents not only a constitutive replacement of dying neurons but also an adaptive process allowing experience-dependent plasticity throughout life (Gheusi et al., 2009). Polenov and Chetverukhin (1993) have suggested that there is a physiological regeneration of the hypothalamic neurosecretory centers in the adult anuran amphibian (Polenov and Chetverukhin, 1993), which is supported by seasonal differences in cell proliferation (Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993). Why this region (or any others) may regenerate seasonally is unresolved, but it may represent an adaptive response in seasonally breeding vertebrates

to limit expensive metabolic processes not necessary for immediate survival (Pawluski et al., 2009). At any rate, the ability of the adult brain to respond to social experiences by modulating neural or glial development is unquestionable (reviewed in Gheusi et al., 2009). The fact that it does so in the POA and IF (this study), which are integrative areas involved in reproductive physiology and behavior, may suggest an adaptive response to the seasonal demands of breeding. It is not clear at this time whether the production of new cells in the anuran amphibian brain are integrated into existing circuitry and/or are directly involved in mediating socio-sexual behavior. It is clear, however, that showing this modulation is the first step in elucidating these answers.

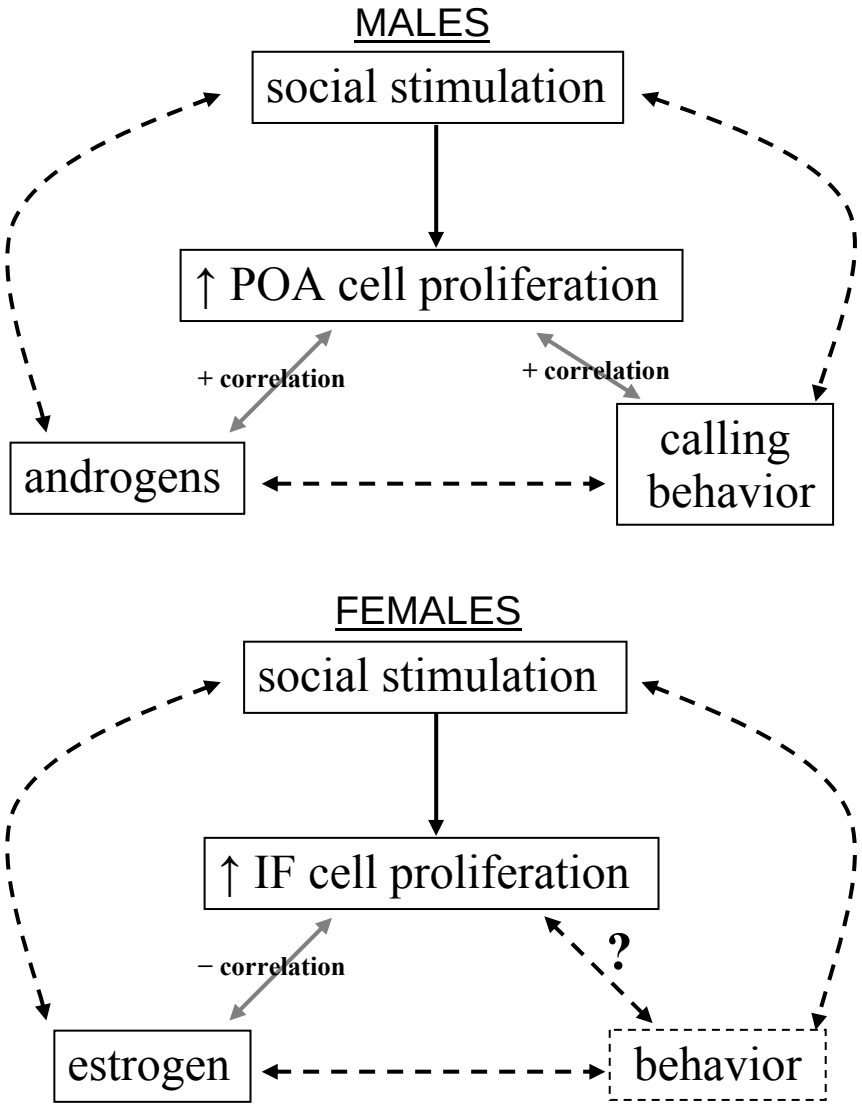

Figure 6.1. Summary of independent modulating factors of cell proliferation in *Hyla cinerea*. The solid lines and boxes indicate novel conclusions from this dissertation research. For example, in males, social stimulation increased cell proliferation in the preoptic area (POA). Further, there were positive correlations between cell proliferation in the POA and androgens and calling behavior, but gonadal steroids and behavior did not modulate the social modulation of cell proliferation in this study. In females, social stimulation increased cell proliferation in the infundibular hypothalamus (IF), but this was not modulated by gonadal steroids. In fact, estrogen and cell proliferation in the IF were negatively correlated. The dotted lines and boxes indicate either relationships that are known but were not explicitly tested in these studies or are areas for further investigation. For example, future studies may look at the effect of female behavior on cell proliferation.

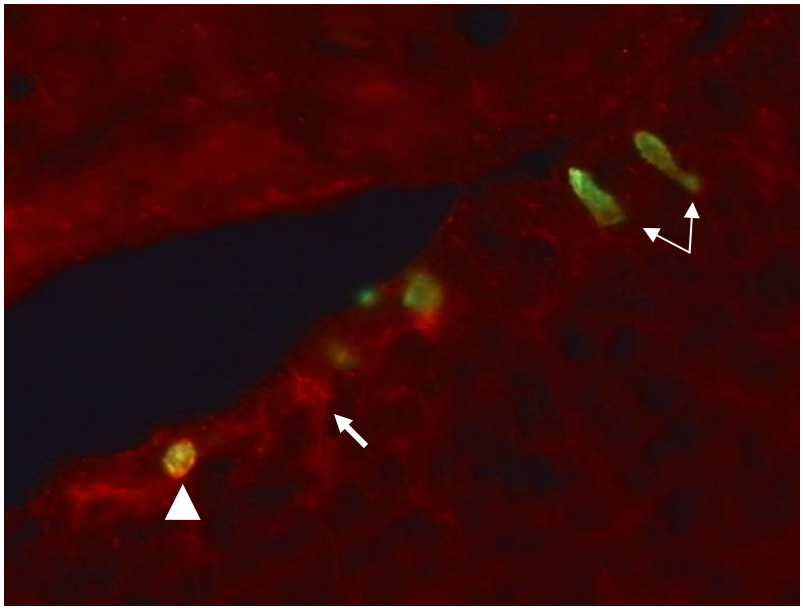

Figure 6.2. Different colored cells represent different stages of neurogenesis in the adult *Hyla cinerea* telecephalon. Double arrows show elongated ependymal cells positive for BrdU (green), but undifferentiated (i.e., not neuronal at this point). The single arrow points at a neuron positive for doublecortin (DCX; a marker for immature neurons) but negative for BrdU. Arrowhead shows a proliferating neuron labeled with BrdU and DCX (yellow).

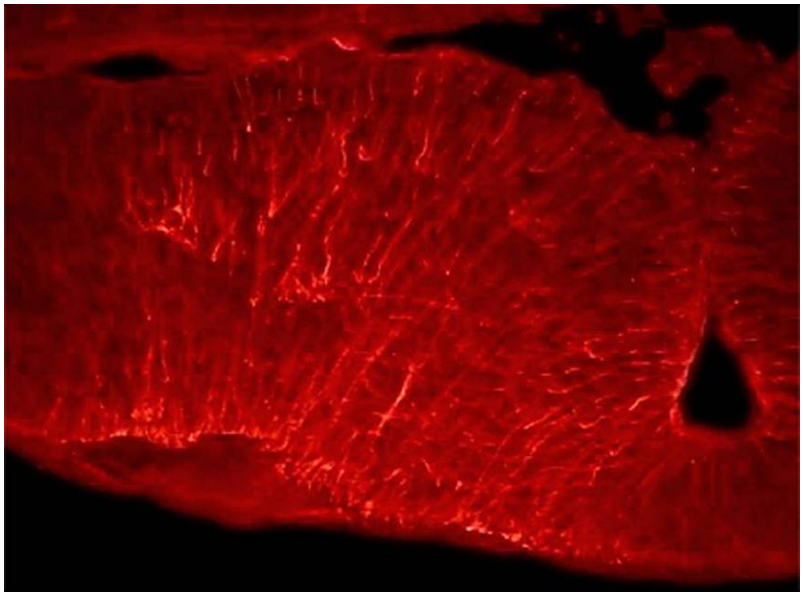

Figure 6.3. Radial glial fibers, stained with glial fibrillary acidic protein (GFAP), in the infundibular hypothalamus of the adult *Hyla cinerea* brain**.**

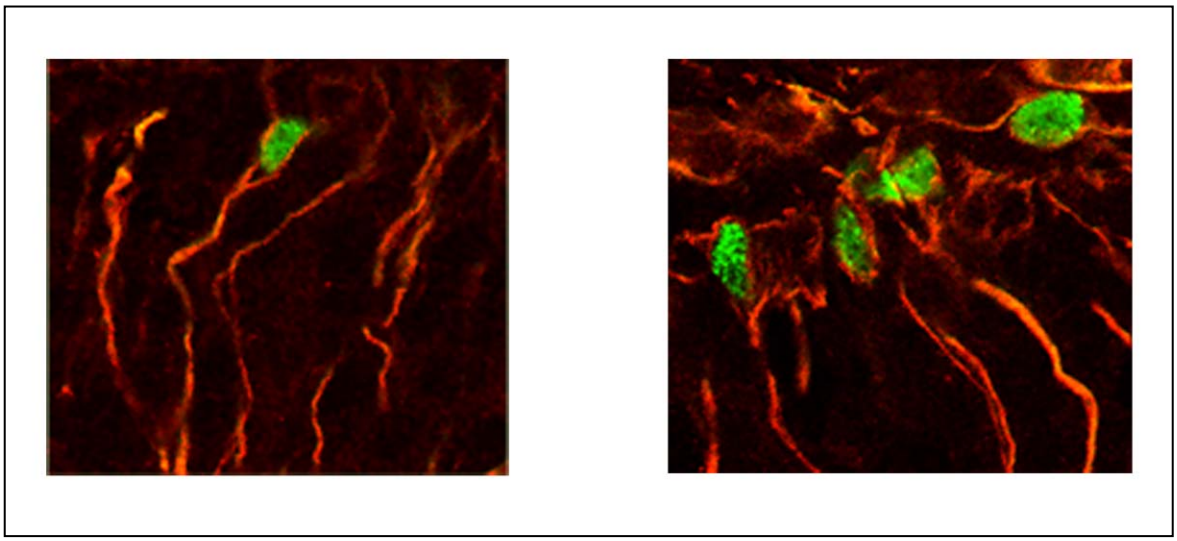

Figure 6.4. Confocal images of the lateral ventricles in the *Hyla cinerea* telecephalon. Radial glial fibers (GFAP+ red fibers) in the ependymal layer are associated with BrdU-labeled nuclei (green cells). All but one BrdU-labeled nuclei has a corresponding radial fiber in these images.

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