Sex-Specific Modulation of Cell Proliferation by Socially Relevant Stimuli in the Adult Green Treefrog Brain (*Hyla cinerea*)

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Key Words

Amphibian • Cell proliferation • BrdU • Neurogenesis • Acoustic communication • Social behavior

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-immunoreactive (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 steroid hormones might have played a role in the social modulation of cell proliferation: by statistically control-

ling for hormone level, we revealed that androgens might 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.

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Abbreviations used in this paper

Al	lateral amygdala
Am	medial amygdala
BN	bed nucleus of the pallial commissure
DP	dorsal pallium
IF	infundibular hypothalamus
LP	lateral pallium
MP	medial pallium
NMLF	nucleus of the medial longitudinal fasciculus
NPv	nucleus of the periventricular organ
OB	olfactory bulb
OT	optic tectum
P	posterior thalamic nucleus
POA	preoptic area
PTd	dorsal posterior tuberculum
PTv	ventral posterior tuberculum
VH	ventral hypothalamus

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 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 might 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 [Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993; Raucci et al., 2006; Almlil and Wilczynski, 2007; 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, 1977]. On the other hand, the ventromedial hypothalamus has long been known as the center for reproductive control in female mammals [Pfaff and Schwartz-Giblin, 1988], and based on studies in lizards [Wade and Crews, 1991; Kendrick et al., 1995], this might be generalized to ectotherms.

We investigated socially stimulated plasticity in these integrative areas in an anuran amphibian, 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 [e.g., 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 the call 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, 2005; Emerson and Boyd, 1999; 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, 1977]. 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 *H. cinerea* (snout-vent length range: 48–55 mm) were purchased from a commercial supplier (Charles Sullivan, Inc., Nashville, Tenn., 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, 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 × 14 × 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.

H. 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 nine 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'). At the end of the acoustic stimulation procedure, trunk plasma samples, obtained at the time of sacrifice, were saved and stored at –20 °C until gonadal steroid analysis.

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. Brains were removed, immer-

sion-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 tetrahydrochloride (DAB) as chromogen for 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% H₂O₂ solution, and nonspecific antigen binding sites were blocked by preincubation buffer. The slides were incubated in buffer plus anti-BrdU antibody (Accurate Chemical, Westbury, N.Y., USA). Signal amplification was achieved by exposing the tissue to horseradish peroxidase conjugated avidin-biotin complex (Vector Laboratories, Burlingame, Calif., USA). To yield brown nuclei, sections were immersed in a DAB solution (0.25% DAB with 0.006% H₂O₂), 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. DAB-positive cells (i.e., BrdU+ cells) were visualized and counted using a 40× objective (Olympus BX60 microscope). Every section in each region of interest (ROI) in the stained series (i.e., every fourth section in total) was counted. The boundaries of the ROIs [Northcutt and Kicliter, 1980; Neary and Northcutt, 1983] were determined by toluidine blue counterstaining. 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 OB. BrdU+ cells were counted in this control region, which contained the caudal OB in addition to pallial structures and the accessory OB. 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 to be of the same general size as 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.

Proliferating cell numbers were quantified using the fractionator method [e.g., West et al., 1991]: the total numbers of cells in the POA and IF 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 procedure was chosen as opposed to more traditional stereological procedures [see Kuhn and Peterson, 2008], for example optical disector, due to the heterogeneous distribution of new cells. A measurement of proliferating cell density, calculated by dividing the original number of BrdU+ cells counted by the volume of the region sampled, rather than the estimated total number of BrdU+ cells per region, yielded the same results. This is consistent with the observation that the estimated volumes of the ROIs were compa-

rable between groups (acoustic stimulus type: $\lambda = 0.938$, $F_{2,19} = 0.629$, $p = 0.544$; sex: $\lambda = 0.760$, $F_{2,19} = 3.005$, $p = 0.073$; acoustic stimulus group: $\lambda = 0.689$, $F_{4,38} = 1.941$, $p = 0.123$), as well as with the results of an ANCOVA analysis using ROI volume as a covariate (see below).

To control for variability due to sampling error and ROI volume differences between groups, the volumes of the POA and IF were calculated for each animal. The perimeter of the ROI in each section was traced with SPOT software (Diagnostics Instruments, Sterling Heights, Mich., USA); 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 number was then multiplied by four to get the total volume of the ROI. 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 [1987]: $\text{CE} = \sqrt{[(3A + C - 4B)/12]/\Sigma P}$, where P = area per section; $A = \Sigma P_i^2$; $B = \Sigma P_{i+1}$; $C = \Sigma P_{i+2}$. 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.

Gonadal Steroid Hormone Analysis

Hormone assays, using plasma collected at the time of sacrifice, were conducted using enzyme immunoassays (EIA; Cayman Chemicals, Ann Arbor, Mich., USA) 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 ($\pm$ SEM) of $26.7 \pm 0.6\%$ for estrogen and $87.8 \pm 1.3\%$ 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 5 α -dihydrotestosterone, 0.07% for 17 α -estradiol, and 0.03% for progesterone. The testosterone kit had 27.4% cross-reactivity with 5 α -dihydrotestosterone and 18.9% for 5 β -dihydrotestosterone. The detection limits were 8 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 (SPSS Inc., Chicago, Ill., USA), 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, gonadal steroid 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 MANOVAs. The volume of the ROIs was then used as a covariate in separate ANCOVAs to evaluate potential differences in volume between treatment groups and to validate the sampling scheme. Additionally, cell proliferation in males and females was 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 gonadal steroid 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'). The effects of evoked calling behavior and androgen level on cell proliferation were then analyzed with a two-way MANOVA. Correlation analyses between the number of evoked calls and the number of BrdU+ cells in the POA and IF were conducted and if significant, an ANCOVA was calculated 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 Treefrogs 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. 1) and rostral telencephalon including the caudal OB 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 might be observing several components of adult neurogenesis with our procedure; resolving this would necessitate colocalization with other cellular markers in future studies. BrdU+ cells were found mainly in or near the ventricular zone of the POA, IF and OB section, although this is not to imply that all of the newly

Fig. 1. 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 μ m for **A**, **D** and 50 μ m for **B**, **C**, **E**, **F**. See list of abbreviations.

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. Over-

all, there was a main effect of acoustic stimulus type ($\lambda = 0.671$, $F_{3,27} = 4.420$, $p = 0.012$) and a sex/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: treefrogs receiving the Chorus condition exhibited increased numbers of proliferating cells compared to Tone controls (fig. 2). Proliferation in the OB section, the control region, was unaffected by acoustic stimulus group, sex, or acoustic stimulus type (mean $\pm$ SEM: Chorus: 425.2 ± 46.0 ; Tone: 407.6 ± 36.8).

Fig. 2. The total 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 brain regions, the POA and IF, compared to Tone controls. Shown are the mean number of cells counted $\pm$ the standard error of the mean (SEM). Asterisks (*) represent significant between-subjects tests at $p < 0.05$.

To correct for sampling error, we used the total volume of the ROIs to determine whether that could cause differences between the independent variables. Separate ANCOVAs, with volume of the 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: $F_{1,33} = 0.175$, $p = 0.679$).

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. 3A), such that proliferating cell numbers were increased in the Chorus-stimulated group compared to

Fig. 3. 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 compared to Tone controls. **(B)** In females, hearing their conspecific chorus increased BrdU+ cell numbers in the IF compared to Tone controls. Shown are means $\pm$ SEM; asterisks (*) represent significant post hoc tests at $p < 0.05$.

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. 3B; IF: $F_{1,17} = 10.885$, $p = 0.004$; POA: $p > 0.1$; OB: $p > 0.1$). Correcting for sampling error (i.e., using ROI 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 in the IF of female treefrogs compared to control frogs receiving the Tone condition.

Sex Differences Occur in the Effects of Gonadal Steroids on Cell Proliferation

To determine the relationship between gonadal steroid 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 gonadal steroid level as a covariate in an ANCOVA allowed us to determine whether hormones were mediating the socially induced 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. 4A) 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. 4B) 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 might influence socially-induced increases in cell proliferation in the male POA, but estrogen did not mediate socially induced increases in the female IF, although it could have an influence there.

Male Treefrogs that Called Back to Chorus-Stimulation have Elevated Numbers of BrdU+ Cells in the POA

To determine whether calling behavior mediates 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 type (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. Within the acoustic stimulus type by caller interaction, a significant between-subjects analysis demonstrated that the number of BrdU+ cells in the POA of males that called back to their conspecific chorus

Fig. 4. The relationship between gonadal steroid level and BrdU+ cells in male and female *Hyla cinerea*. **(A)** There was 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 was a significant negative correlation between BrdU+ cells in the IF and estrogen level ($r = -0.466$, $p = 0.033$).

($F_{1,11} = 6.468$, $p = 0.027$) 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. 5A). Further, a significant between-subjects analysis within calling behavior revealed higher androgen levels for callers, i.e., males that engaged in evoked

Fig. 5. 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 $\pm$ SEM. The asterisk (*) represents a significant post hoc test at $p < 0.05$. **(B)** The number of evoked calls was positively correlated with cell proliferation in the male POA ($r = 0.659$, $p = 0.003$).

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 there was no correlation in the IF ($p > 0.1$). Including only males that engaged in evoked calling, there was also a significant positive correlation between evoked calling levels and BrdU+ cell number in the POA ($r = 0.659$, $p = 0.003$; fig. 5B) 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 responsible for changes in cell proliferation due to social modulation. 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 OB. Acoustic stimulation affected male and female *H. cinerea* differently. In males, social stimulation increased proliferating cell numbers in the POA, whereas in females this increase occurred in the IF. There were sex differences in the effect of gonadal steroids on cell proliferation: androgens might 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 might 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, 1977]. 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 might 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, 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 might 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, on the other hand, did not exhibit modulation in estrogen levels due to social stimulation [but see Lynch and Wilczynski, 2006], nor did estrogen levels seem to influence cell proliferation in the brain regions studied here. The reproductive state of the females was not controlled, however, and it could 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, although there is little evidence that these hormones directly affect cell proliferation [Brown et al., 1993; Burek et al., 1994, 1995; Rasika et al., 1994; 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 voles [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 suggest-

ed 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 Gorman, 1977], we hypothesized a relationship between calling behavior and cell proliferation in that region. In fact, evoked calling levels were correlated with increased cell proliferation in the POA, but not the IF. Further, male treefrogs who called back to the conspecific chorus exhibited increases in BrdU+ cell number in the POA compared to Tone controls (Callers and Non-callers), but calling behavior was not directly mediating 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 might 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 phonotactic behavior and correlating directed movement with cell proliferation.

The correlation between behavior and gonadal steroid levels complicates the interpretation of the effects of these factors on socially modulated cell proliferation. In male *H. cinerea*, both evoked calling and androgen levels were positively correlated with cell proliferation in the POA (and were correlated with each other, $r = 0.506$, $p = 0.019$). It is therefore challenging to determine the roles of cause and effect. In other words, it is possible that the statistical effect of calling on cell proliferation is an artifact of calling males having higher levels of androgens. In fact, it seems that androgens might be more influential on cell proliferation. Our covariate analysis demonstrated that controlling for androgen levels negated the significant increase in cell proliferation from hearing a conspecific

chorus; in contrast, in the covariate analysis with calling behavior, a significant effect of Chorus stimulation remained. However, as the variables are tightly correlated it is impossible to disentangle their effects with certainty.

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] might 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 might 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 might be suitable for enhancing cell proliferation. Candidate mechanisms for the observed enhancement variously 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 among hormones, behavior, neurotrophins, and neurogenesis has been investigated in the highly specialized birdsong vocal learning system [e.g., 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 might 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 [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 in the conditions studied here. 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 could elucidate the functional significance of this socially modulated cell proliferation.

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