Effects of estradiol on neural responses to social signals in female túngara frogs

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SUMMARY

Estradiol plays an important role in mediating changes in female sexual behavior across reproductive cycles. In the túngara frog (*Engystomops=Physalaemus pustulosus*), the relationship between gonadal activity and female sexual behavior, as expressed by phonotaxis, is mediated primarily by estradiol. Estradiol receptors are expressed in auditory and motivational brain areas and the hormone could serve as an important modulator of neural responses to conspecific calls. To better understand how estradiol modifies neural responses to conspecific social signals, we manipulated estradiol levels and measured expression of the immediate early gene *egr-1* in the auditory midbrain, thalamus, and limbic forebrain in response to conspecific or heterospecific calls. We found that estradiol and conspecific calls increased *egr-1* expression in the auditory midbrain and limbic forebrain, but in the thalamus, only conspecific calls were effective. In the preoptic area, estradiol enhanced the effect of the conspecific call on *egr-1* expression, suggesting that the preoptic area could act as a hormonal gatekeeper to phonotaxis. Overall, the results suggest that estradiol has broad influences on the neural circuit involved in female reproduction, particularly those implicated in phonotaxis.

INTRODUCTION

In many female vertebrates, estradiol modulates sexual behavior by influencing sensory, motor, and motivational brain regions such that sexual behavior occurs when gonadal activity is high. A principal way that estradiol affects sexual behavior is by regulating neural responses in the basal forebrain, which serves to integrate hormonal and sensory information and, in turn, influences motor circuits underlying sexual behavior. For example, the preoptic area in female rats is estradiol sensitive and enhances lordosis by reducing the excitatory neural impulses that project from the preoptic area to the ventral tegmental area (Sakuma, 2015). Estradiol can also impact sensory systems directly. For example, estradiol shapes auditory tuning in midshipmen fish so that, during the breeding season, females are more sensitive to the harmonics in the calls of courting males which enhances the adaptive coupling of the sender and the receiver for reproductive success (Sisneros, 2009a; Sisneros, 2009b; Sisneros, 2009c; Sisneros et al., 2004). Similar mechanisms of estradiol-dependent modulation of acoustic communication circuits are present in birds in response to plasma estradiol (Maney et al., 2006; Maney et al., 2008; Maney and Pinaud, 2011; Sanford et al., 2010), and locally-produced neuroestrogens (Krentzel and Remage-Healey, 2015; Pawlisch and Remage-Healey, 2015; Remage-Healey, 2012; Remage-Healey, 2014; Remage-Healey et al., 2010; Remage-Healey and Joshi, 2012; Remage-Healey et al., 2008).

Anurans serve as excellent models to explore hormonal effects on auditory plasticity due to a wealth of data available on their courtship behaviors and their associated changing hormone levels (see reviews by Arch and Narins, 2009; Caras, 2013). In túngara frogs (*Physalaemus=Engystomops pustulosus*), a primary component of female sexual behavior is phonotaxis, or the approach toward a calling male for the purpose of initiating a mating clasp (amplexus). The expression of selective phonotaxis depends on estradiol (Chakraborty and Burmeister, 2009) and estradiol receptors are expressed widely in auditory, motor, and motivational brain regions (Chakraborty and Burmeister, 2010). Furthermore, in the auditory midbrain and parts of the limbic forebrain, estradiol receptor expression is sexually dimorphic (Chakraborty and Burmeister, 2010), suggesting that estradiol is an important modulator of neural circuits underlying female-typical behavior. In addition, injections of human chorionic gonadotropins (hCG), which stimulate production of gonadal steroids (Lynch et al., 2006; Wu et al., 2001), increase neural responses to conspecific social signals in the auditory midbrain (i.e., torus semicircularis) (Lynch and Wilczynski, 2008), suggesting that one way that estradiol modulates behavioral responses to conspecific calls is through

modulation of auditory responses (Lynch and Wilczynski, 2008), or through auditory-motor integration (see Hoke & Pitts). In fact, Hoke and Pitts (2012) propose that a portion of the auditory midbrain (laminar nucleus of the torus semicircularis) is a critical node in initiating behavioral responses to socially relevant sounds in a reproductive context, a finding also supported by another study (Mangiamele and Burmeister, 2011). Additionally, in female green treefrogs (*Hyla cinerea*), auditory-evoked multi-unit responses are stronger in unmated females compared to females that have recently mated, suggesting that periods of sexual receptivity are accompanied by enhanced sensitivity of the anuran auditory midbrain to facilitate processing of male advertisement calls (Miranda and Wilczynski, 2009).

To better understand how estradiol promotes phonotaxis in túngara frogs -- whether through effects on auditory, motor, and/or motivational brain regions -- we manipulated estradiol levels by injecting estradiol or an estradiol synthesis blocker (fadrozole) and measured neural responses to conspecific or heterospecific calls throughout the brain. We measured neural responses by measuring expression of the transcription factor *egr-1* (*early growth response 1*; synonymous with *zif268*, *NGFI-A, ZENK*). Expression of *egr-1* is linked to changes in membrane depolarization (via MAP kinase pathways) and it controls expression of many plasticity-related genes (Clayton, 2000). Because of its links to membrane depolarization and synaptic plasticity, the expression of *egr-1* is a useful tool for investigating neural responses to behaviorally relevant cues. It also allows measurement of multiple brain regions simultaneously.

MATERIALS AND METHODS

Hormone treatment and tissue collection

We used adult female túngara frogs from a laboratory stock maintained at the University of North Carolina that was originally derived from natural populations near Rio Píro on the Osa Peninsula in Costa Rica. Photoperiod (12:12) and temperature (28 °C) approximated natural conditions. Because túngara frogs are small, we manipulated hormones through injection rather than surgery, which meant that females had intact gonads that could produce endogenous steroids. Therefore, we began by inducing breeding so that our subjects would start the experiment with low levels of gonadal activity. To do this, we injected females with 500 IU per g body mass of hCG and paired them with a male and water dish to stimulate breeding. We collected 48 females that made nests over a period of 7 days for further hormone manipulations. Ten days after the females had made nests, a time when endogenous gonadal steroids are low (Lynch and Wilczynski, 2005), we raised estradiol levels in half of the females and blocked estradiol synthesis in the other half (Fig. 1), as follows. To block estradiol synthesis, we injected females twice with the estradiol synthesis blocker fadrozole (50 μg each injection in 50 μl), 24 hours apart. To raise estradiol, we injected females with saline (50 μl of 0.9% sodium chloride) followed 24 hours later by estradiol (0.07 μg per g body mass in 50 μl); the purpose of the initial saline injection was to match the number of injections in the fadrozole group. We gave injections in the afternoons.

We were not able to collect blood plasma from the females in this experiment in order to document the effects of our hormone manipulations because blood collection is incompatible with brain collection in this species due to small blood volume (i.e., they do not bleed enough following decapitation). However, these hormone manipulations have been validated previously for túngara frogs. Specifically, two injections of fadrozole are effective at inhibiting estradiol synthesis in hCG-treated females (Chakraborty and Burmeister, 2009; Lynch and Wilczynski, 2005). In addition, when measured 24 hours later, a single injection of estradiol at this dose is sufficient to raise estradiol levels above fadrozole treated females and increases phonotaxis behavior (Chakraborty and Burmeister, 2009). It is important to note that fadrozole would affect both neural and gonadal synthesis of estradiol. Likewise, our estradiol injections likely influence pituitary gonadotropins through negative feedback.

After the final injection, we placed pairs of females, one from each hormone treatment into acoustic isolation chambers; individual females in a pair were maintained separately in individual mesh containers. After an 18-hr acclimation period in the dark, we

presented the female pairs with a single exemplar of a conspecific (n = 24) or a heterospecific call (n = 24) for 30 minutes (see below for stimulus details). We interspersed females assigned to each acoustic treatment across days and chambers. We rapidly decapitated females 1 h after onset of stimuli, which corresponds to peak accumulation of acoustically induced *egr-1* mRNA expression (Burmeister et al., 2008) but occurs before habituation of the *egr-1* response is evident (RM Glaeser, LA Mangiamele, & SS Burmeister, unpublished). After decapitation, we opened the skull in order to fix the brains (10 min in 4% paraformaldehyde) before removing them. We froze the brains in liquid nitrogen in 2 ml tubes containing Tissue-Tek OCT Compound (Sakura, Finetek, Torrance, CA), and stored them at − 80° C until sectioning. The University of North Carolina Institutional Animal Care and Use Committee (IACUC) approved our experimental procedures.

Acoustic stimuli

We presented hormonally manipulated females with a conspecific call from the Osa Peninsula or a heterospecific (*P. enesefae)* call from Venezuela (Fig. 2). The túngara frog produces a two-note advertisement call (whine-chuck) (Ryan, 1985) while *P. enesefae* only produces a single-note call (whine). The whines of both species are characterized by a downward frequency sweep that spans similar frequencies but they do so with very different time signatures (Fig. 2). Female túngara frogs do not recognize the *P. enesefae* whine as an acceptable sexual signal (e.g., Ryan et al., 2003). In both cases, the call was repeated every 2 seconds to reflect the calling rate of *P. pustulosus* and stimulus amplitude was 82 dB SPL at a distance of 20 cm from the speaker. The speaker was a midlevel woofer (Misco JC46W-4A, Minneapolis Speaker Company, Minneapolis, MN) that was connected to an AMP Five series monoblock amplifier (Audiosource, Portland, OR) and a Macintosh computer. We controlled playback using ProTools audio software (V. 7.3; Digidesign, Daly City, CA). Using these exemplars, we have previously demonstrated that the conspecific call, but not the heterospecific call, induces significant expression of *egr-1* compared to no sound in the auditory midbrain, thalamus, and limbic forebrain of túngara frog females (Chakraborty et al., 2010).

Quantification of egr-1 expression

We sectioned brains in the transverse plane at 16 μm in 3 series on a cryostat. To localize *egr-1* mRNA, we used radioactive *in situ* procedures previously described in (Burmeister et al., 2008). Briefly, we generated radioactively labeled sense and antisense

probes from reverse transcription of a 309-nucleotide subclone of *P. pustulosus egr-1*. Before hybridization, we fixed the tissue for 10 sec in 4% paraformaldehyde and washed in phosphate-buffered saline, triethanolamine, acetic anhydride, 2× SSC, and a series of ethanols. We hybridized the tissue with 90 μl of 3.0×10^5 cpm/ml of hybridization buffer at 65° C overnight. We removed unbound probe with a series of 65° C washes, first in 50% formamide and 2× SSC (1.25 h) followed by two washes in 0.1× SSC (30 min each). We visualized the bound riboprobe as silver grains by exposing the slides to NTB emulsion diluted 1:1 in distilled water for 14 days at 4° C, and we visualized the cell bodies by staining the tissue with thionin. Tissue incubated with the sense probe showed no significant binding above background.

We followed the nomenclature of O'Connell et al. (O'Connell et al., 2011; O'Connell et al., 2010). We measured *egr-1* in brain regions that are known to receive significant auditory input as well as those that are part of the anuran social behavior network (Newman, 1999; O'Connell et al., 2010): the auditory midbrain (principal and laminar nuclei of the torus semicircularis), parts of the thalamus that receive ascending auditory input (posterior, central, and anterior thalamic nuclei), and parts of the limbic forebrain that either receive auditory input and/or are directly implicated in modulating phonotaxis (preoptic area, striatum, lateral septum, dorsal part of the medial pallium, and nucleus accumbens). Of these brain regions, all express estradiol receptors at detectable levels except the posterior thalamus (Chakraborty and Burmeister, 2010).

Our quantification method follows previously described methods (Burmeister et al., 2008; Chakraborty et al., 2010) and was derived from Mize et al. (1994), Masseroli et al. (1993), and Lucas et al. (1994). Briefly, for each brain region, we calculated an individual's mean from three brain sections. For each section, we took three images at 630× magnification: a color image of Nissl-stained tissue in the region of interest, a blue-filtered image of only the silver grains in the same field of view (grains image) and a blue-filtered image of an area of the slide adjacent to the tissue to represent local background silver grain density (background image). We measured local background silver grain density to account for emulsion thickness, which varies across the slide and influences silver grain density. In the blue-filtered images that we used to measure silver grain density, exposure, brightness and contrast settings were the same for each picture of a given section. We used ImageJ (http:// rsbweb.nih.gov/ij/) to convert the grains and background images to binary and to count the silver grains in each image using the analyze particles feature. We subtracted the number of background silver grains from the number of silver grains in the region of interest

to get the number of silver grains above background per image. We then used the point selection tool in ImageJ to mark and count all visible cells in the color image of the region of interest. We identified cells visually by their clearly stained soma and the cell counting was performed by experienced individuals who were blind to treatment. Our final measure of *egr-1* mRNA expression for each section was the number of silver grains above background per cell. The quality of the brain sections influenced the sample sizes reported for each brain region, as we were unable to obtain data from all individuals in the study for all brain regions sampled.

Statistical analyses

To test whether hormone treatment, call type, or their interaction influenced *egr-1* expression we conducted separate two-way analysis of variances (ANOVAs) for each brain region with call type, hormone treatment, and their interaction as between-subjects factors. Although brain regions are not independent of one another, we could not account for covariation among brain regions with a multivariate analysis (e.g., repeated-measures ANOVA) because missing values for individual brain regions would result in the exclusion of most subjects. For the same reason, missing samples prevented us from including pair as a variable in the analyses.

RESULTS

The conspecific call elicited higher *egr*-1 expression than the heterospecific call in the auditory midbrain, thalamus, and limbic forebrain (main effects for call type, see Table 1, Fig. 3, Fig. 4), demonstrating that conspecific social signals are a highly salient signal that elicits robust responses throughout the brain, largely independent of hormone status. When compared to fadrozole, injections of estradiol increased *egr-1* expression in the auditory midbrain and the limbic forebrain, but not in the thalamus, (main effects of hormone treatment, see Table 1, Fig. 3, Fig. 4). That is, estradiol increases *egr-1* expression in the midbrain and forebrain independently of the acoustic environment, suggesting that estradiol increases the basal activity of these brain regions.

For most brain regions, the main effects of call type and hormone were independent of one another, and both conspecific calls and estradiol increased *egr-1* expression, creating an additive effect. In the preoptic area, however, the effect of call type depended on the hormonal status of the animal ($F_{1,32} = 4.1$, $p = 0.05$). That is, estradiol enhanced the effect of the conspecific call, suggesting that the preoptic area may have a particularly important role in integrating hormonal and social cues. We observed a similar pattern for the septum, but with less robust statistical support ($F_{1,29} = 3.5$, $p = 0.07$).

DISCUSSION

We have previously shown that estradiol is necessary and sufficient for the expression of phonotaxis in the túngara frog (Chakraborty and Burmeister, 2009) and that estradiol receptors show sexually dimorphic expression in the auditory midbrain and parts of the limbic forebrain (Chakraborty and Burmeister, 2010), indicating that estradiol could mediate changes in phonotaxis through direct effects on these brain regions. Consistent with this, we now show that estradiol and conspecific calls both increase *egr-1* in the auditory midbrain and parts of the limbic forebrain, including motor (striatum) and motivational (preoptic area, septum, and nucleus accumbens) brain regions. In the preoptic area, the effect of conspecific calls depended on the hormonal status of the animal. Such an effect is consistent with behavioral responses to conspecific calls, which only occur in the presence of estradiol (Chakraborty and Burmeister, 2009) raising the possibility that the preoptic area is acting as a hormonal gatekeeper to reproductive behavior in the túngara frog.

Our results for the auditory midbrain parallel a number of studies from other taxa that describe an important role for estradiol in sensory processing of social signals and of audition, in particular (Arch and Narins, 2009; Caras, 2013; Earp and Maney, 2012; Maney, 2013; Maney et al., 2006; Maney et al., 2008; Maney and Pinaud, 2011; Petrulis, 2013; Remage-Healey, 2012; Remage-Healey, 2014; Sanford et al., 2010; Sisneros, 2009c; Tremere et al., 2009; Walpurger et al., 2004; Yovanof and Feng, 1983). In the túngara frog, a previous study showed that hCG, which stimulates production of gonadal steroids (Lynch et al., 2006; Wu et al., 2001), increases *egr-1* expression in the laminar nucleus of the torus (Lynch and Wilczynski, 2008). Our findings suggest that the effect observed by Lynch & Wilczynski (2008) was mediated by estradiol and not by direct effects of gonadotropins (Yang et al., 2007) or through changes in other steroids, such as progesterone or androgens. The increased expression of *egr-1* in response to estradiol may reflect a lower behavioral threshold for approaching calls: when females are nearing oviposition, a time when gonadal hormones are high, they are willing to approach calls that are typically unattractive, although their ability to discriminate among calls remains intact (Lynch et al., 2006). The limited electrophysiological data support this conclusion. That is, both reproductive activity (Miranda and Wilczynski, 2009) and estradiol treatment (Yovanof and Feng, 1983) increase auditory responses to sounds in frogs, including biologically irrelevant sounds (noise burst, tones) as well as conspecific calls. There are also broad correspondences between egr-1 (also known as ZENK) expression and electrophysiological activity in songbirds: ZENK expression is selective for conspecific social signals (song) over tones only in the presence of estradiol in

female white-throated sparrows (*Zonotrichia albicolis*) (Maney et al., 2006; Maney et al., 2008; Maney and Pinaud, 2011). Estradiol also enhances auditory encoding of song, sometimes through rapid effects of brain-derived estradiol (Krentzel and Remage-Healey, 2015; Remage-Healey, 2012; Remage-Healey, 2014; Remage-Healey et al., 2010; Tremere and Pinaud, 2011). In zebra finches, the impact of estradiol extends further to the song control system, as estradiol enhances the neural selectivity for the bird's own song (BOS) but not to conspecific songs, and fadrozole injections reduces this selectivity (Remage-Healey and Joshi, 2012). Thus, effects of estradiol on auditory processing of social signals are manifest at multiple levels (i.e., MAPK-dependent gene expression and electrophysiological activity) and across vertebrate classes.

An interesting pattern that we observed was that estradiol affected *egr-1* expression in the midbrain and limbic forebrain, but not the thalamus. This, in spite of the fact that estradiol receptors are expressed in parts of the thalamus (central and anterior nuclei; Chakraborty and Burmeister, 2010) and that the primary auditory afferent to the thalamus (i.e., the torus) was strongly affected by estradiol. Although intriguing, the implications of these results will remain unclear until we have a more complete understanding of the effects of estradiol on electrophysiological responses to conspecific calls. At this time, however, we can conclude that the thalamus appears to lack estradiol-induced auditory plasticity.

We found that estradiol had potent effects on *egr-1* expression in the preoptic area and other parts of the limbic forebrain that are thought to modulate socially relevant behavioral responses. While the preoptic area is central to sexual behavior in all vertebrates, a role of the preoptic area in anuran sexual behavior has been less well studied. It has long been known that the anuran preoptic area receives auditory input and that it is sensitive to conspecific calls (Allison, 1992). We recently demonstrated that the preoptic area expresses estradiol receptors (Chakraborty and Burmeister, 2010) and we now show that estradiol modifies its response to conspecific calls (present study). Together, these data indicate that, like other vertebrates, the anuran preoptic area influences reproductive behaviors by integrating sensory and hormonal information. Further, the fact that estradiol's effect on the preoptic area mirrors the effect of estradiol on female phonotaxis suggests that the preoptic area may play a particularly important role in female phonotaxis.

In summary, we found that both conspecific calls and estradiol increased *egr-1* expression in the auditory midbrain and limbic forebrain. Together with previous behavioral (Chakraborty and Burmeister, 2009) and electrophysiological studies (Yovanof and Feng, 1983), our results suggest that estradiol could promote selective phonotaxis by lowering the

threshold for responses in both the midbrain and limbic forebrain, while highlighting a role for the preoptic area. The effect of estradiol at multiple points in the neural circuit for sexual behavior in túngara frogs is part of a common theme in vertebrates for the influence of steroids on social behavior.

Abbreviations: A, anterior nucleus of the thalamus; AA, anterior amygdala; Acc, nucleus accumbens; C, central nucleus of the thalamus; CeA, central amygdala; Dp, dorsal pallium; DP, dorsal pallidum; dMp, dorsal part of medial pallium; egr-1, early growth response 1; LA, lateral amygdala; Lp, lateral pallium; LP, lateral pallidum; Ls, lateral septum; Ltor, laminar nucleus of the torus semicircularis; MeA, medial amygdala; Mp, medial pallium; Ms, medial septum; OT, optic tectum; P, posterior nucleus of the thalamus; POA, preoptic area; Ptor, principal nucleus of the torus semicircularis; Teg, tegmentum; VP, ventral pallidum; Str, striatum.

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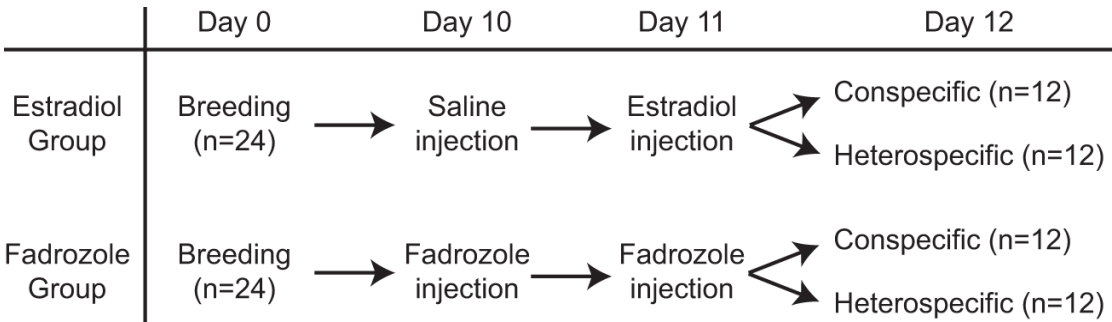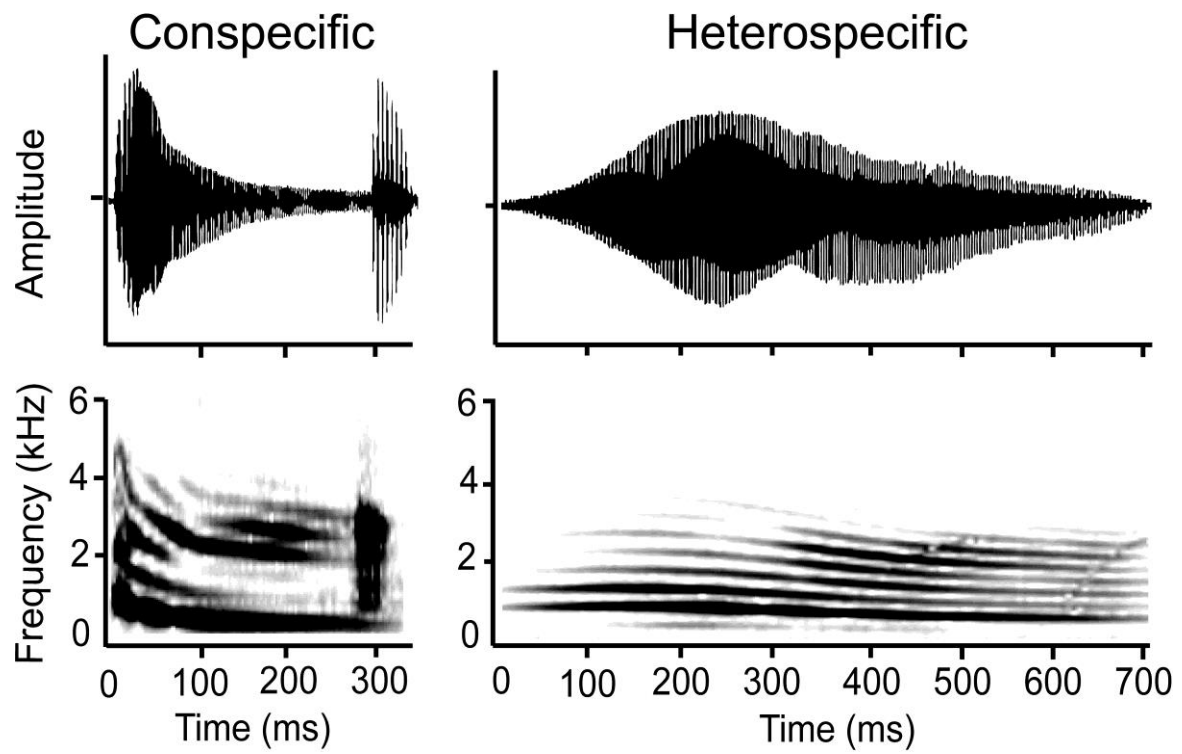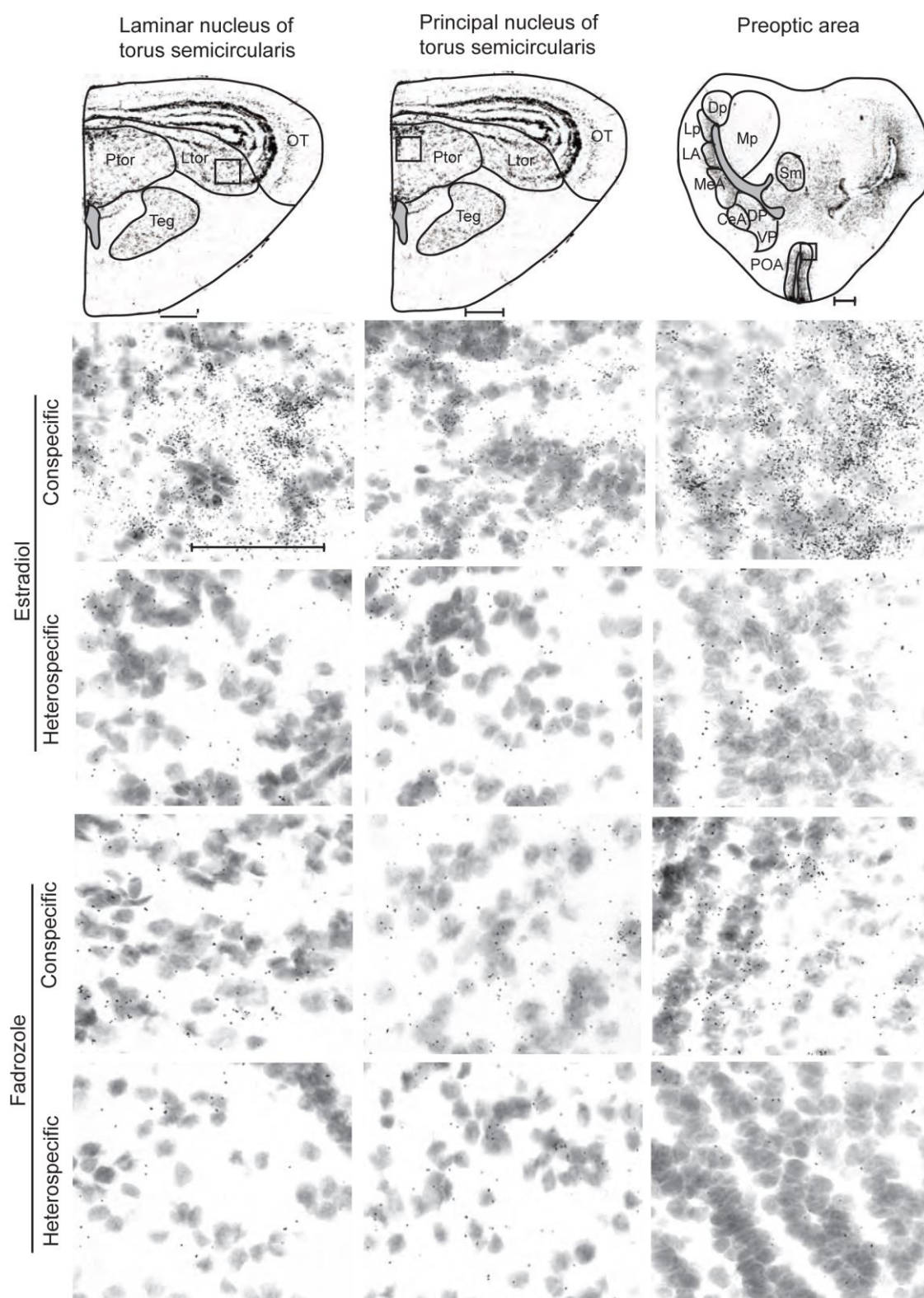
Figures

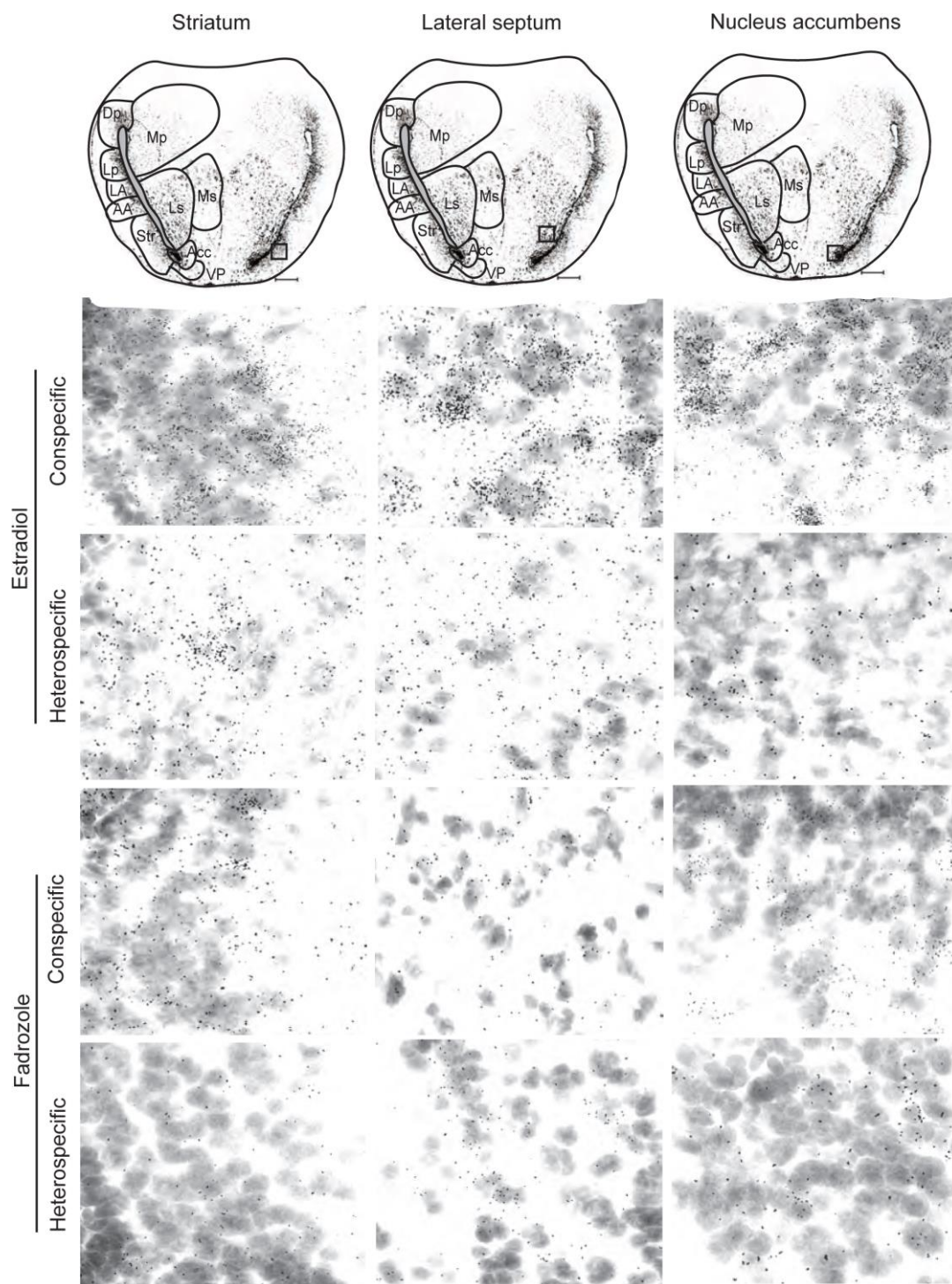|	Day 0	Day 10	Day 11	Day 12
Estradiol Group	Breeding (n=24) →	Saline injection →	Estradiol injection	Conspecific (n=12) / Heterospecific (n=12)
Fadrozole Group	Breeding (n=24) →	Fadrozole injection →	Fadrozole injection	Conspecific (n=12) / Heterospecific (n=12)

**Figure 1**. Timeline of experimental treatments.

Figure 2. Waveforms and spectrograms of the call exemplars that we used to represent conspecific (*Physalaemus pustulosus*) and heterospecific (*Physalaemus enesefae*) calls.

Laminar nucleus of torus semicircularis

Principal nucleus of torus semicircularis

Preoptic area

Estradiol — Conspecific

Estradiol — Heterospecific

Fadrozole — Conspecific

Fadrozole — Heterospecific

Figure 3. Schematic drawings superimposed on photomicrographs of Nissl-stained sections (top row) and photomicrographs (remaining rows) showing variation in silver grain density in response to advertisement calls and hormone treatment in the auditory midbrain (Ptor, Ltor), preoptic area (POA), striatum (Str), lateral septum (Ls), and nucleus accumbens (Acc). The sampling windows (rectangular box) are shown in the schematic drawings. Scale bars represent 400 μm (schematic drawings) and 100 μm (photomicrographs). See list for abbreviations.
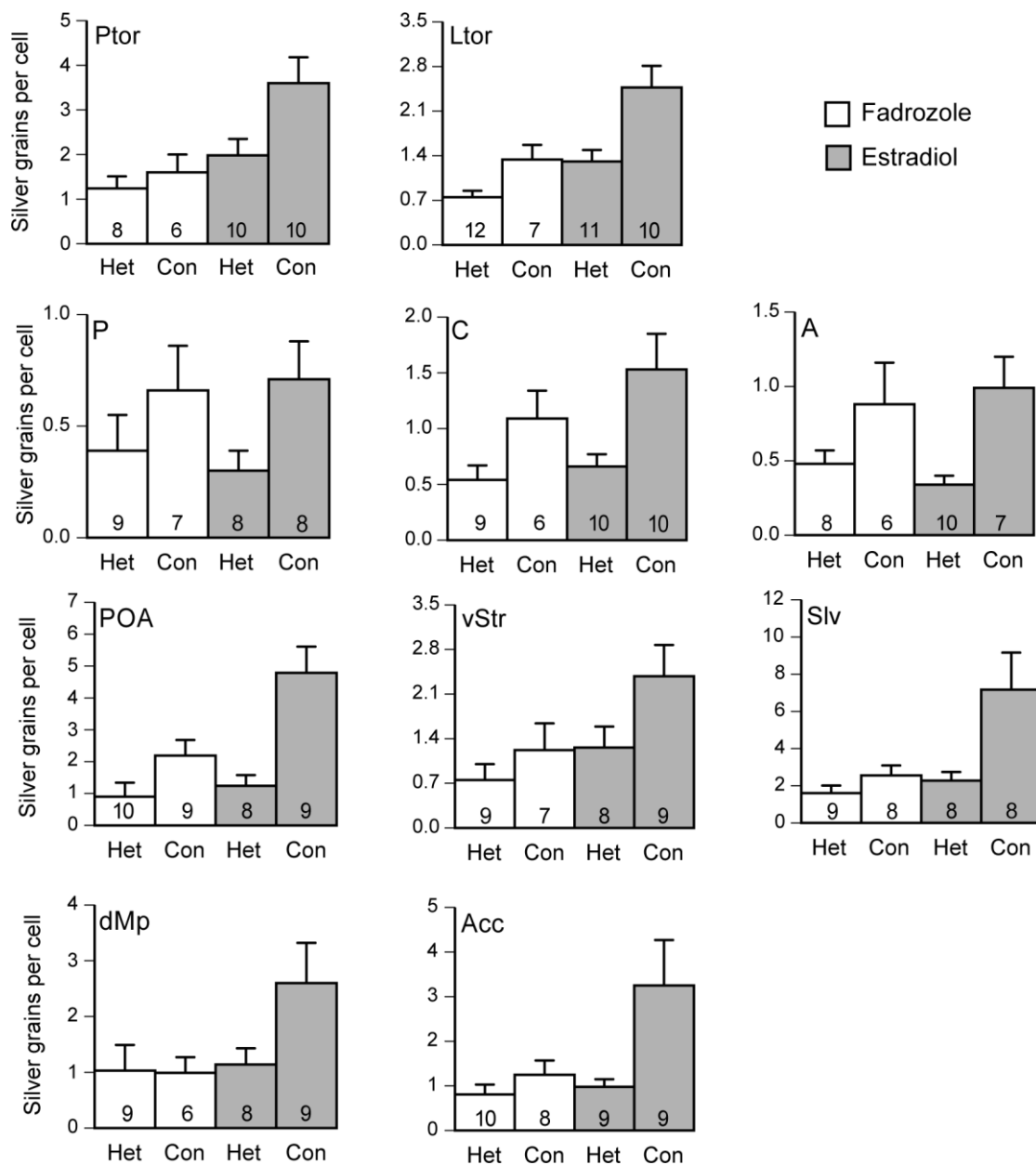
Figure 4. Effects of call type and hormone treatment on *egr-1* gene expression in the auditory midbrain (Ptor, Ltor), thalamus (P, C, A), and forebrain (POA, Str, Ls, dMp, Acc). Data are shown as mean (± SE) silver grains per cell above background. Sample sizes for each group are indicated within each column. Het = heterospecific call; Con = conspecific call. See list for abbreviations.

TABLES

Table 1. Statistical results from two-way ANOVAs testing for effects of call type, hormone treatment, and their interaction on expression of *egr-1* in the auditory midbrain (Ptor, Ltor), thalamus (P, C, A), and forebrain (POA, Str, Ls, dMp, Acc) of female túngara frogs; *p* values of 0.1 and less are indicated with bold text.

| | df | Call type | | | Hormone | | | Call x Hormone | |
		F	*p*	*F*	*p*	*F*	*p*
Ptor	1,30	4.7	**0.04**	8.9	**0.006**	1.9	0.18
Ltor	1,36	15.0	**0.0004**	13.9	**0.0007**	1.6	0.22
P	1,28	4.7	**0.04**	0.01	0.92	0.21	0.65
C	1,31	10.1	**0.003**	1.6	0.22	0.5	0.48
A	1,27	11.4	**0.002**	0.01	0.94	0.63	0.43
POA	1,32	18.7	**0.0001**	6.9	**0.01**	4.1	**0.05**
Str	1,29	4.2	**0.049**	4.6	**0.04**	0.71	0.41
Ls	1,29	7.8	**0.009**	6.4	**0.02**	3.5	**0.07**
dMp	1,28	1.9	0.18	2.7	0.11	2.1	0.16
Acc	1,32	6.0	**0.02**	3.8	0.059	2.8	0.11