The Journal of Experimental Biology 213, 3463-3472

doi:10.1242/jeb.043992

Sexually dimorphic sensory gating drives behavioral differences in túngara frogs

Kim L. Hoke[1,*], Michael J. Ryan[1,2] and Walter Wilczynski[3]

[1]Section of Integrative Biology, University of Texas at Austin, 1 University Station C0930, Austin, TX 78712, USA, [2]Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama and [3]Neuroscience Institute and Center for Behavioral Neuroscience, Georgia State University, PO Box 5030, Atlanta, GA 30302, USA

*Author for correspondence at present address: Department of Biology, Colorado State University, 1878 Campus Delivery, Fort Collins, CO 80523-1878, USA (kim.hoke@colostate.edu)

Accepted 11 July 2010

SUMMARY

Males and females can differ both in the social behaviors they perform and in the contexts in which they engage in these behaviors. One possible mechanism of sex differences in behavior is a sexual dimorphism in the relay of sensory information to motor areas, but no studies have examined the role of such a relay in vertebrate sexually dimorphic behaviors. We used *egr-1* expression as a marker of neural activation in frogs exposed to conspecific and heterospecific acoustic signals to compare activation patterns throughout the brains of males and females. We determined how the sexes differ in the transformation of social signals into motor responses in the context of social communication. We examined the relationships between *egr-1* mRNA levels in the auditory midbrain and forebrain areas, as well as how forebrain expression related to the behavioral responses of the animals. Forebrain network activation patterns and forebrain–behavior relationships were similar in males and females. By contrast, we found a sex difference in the relationship between midbrain and forebrain activation; midbrain auditory responses predicted forebrain responses in females but not in males. This sex difference suggests that sensory inputs differentially regulate motor systems underlying social behaviors in males and females. This sensorimotor transformation may be a common locus for generating sex differences in behavior.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/20/3463/DC1

Key words: immediate-early genes, *Physalaemus pustulosus*, túngara frog, sensorimotor transformation, sex differences, acoustic communication.

INTRODUCTION

Sex differences in social behaviors are widespread. A common behavioral domain in which males and females differ is in the response to conspecific social signals. Even when encountering the same signal, such as a male advertisement call or a territorial pheromone, males and females often react very differently. Olfactory cues that trigger a lordosis response in female rodents elicit no response at all in males (Harlan et al., 1984); similarly, ultrasonic cries from rat pups trigger stereotyped retrieval behavior in mother, but not in father, rats (Allin and Banks, 1972). In response to a male's vocal or visual display, female birds and frogs often approach, produce solicitation displays, or otherwise express affiliative or courtship behaviors. To the same signal, males may increase their own display behavior or become aggressive toward the signaler (Berglund et al., 1996). When both sexes respond to social cues, the range of cues triggering a response can differ in males and females. As a general rule, females are predicted to respond to a more restricted range of potential male signals than do males to potential female signals, or to male signals. This difference in selectivity is predicted by the different costs to the sexes in responding inappropriately to sexual solicitiation signals (higher in females than males) compared with the costs of not responding to a potential sexual signal (higher in males than females) (Searcy and Brenowitz, 1988).

The evolution of sexually dimorphic behaviors in response to the distinct selective pressures on males and females is dependent on the evolution of the neural systems underlying those behaviors. The degree to which the neural mechanisms of social behavior overlap in males and females constrains the opportunity for sex-specific responses to selection and directs the sites at which selection can modify neural systems. Sexually dimorphic behaviors may be generated by either dedicated or multifunctional neural systems. A dedicated neural system is one in which the neurons involved subserve a specific sensory or motor task, such as motor circuits involved in controlling the vocal apparatus or the sensory neurons that respond to pheromones, both of which have specific roles in social communication. A multifunctional neural system is one in which the neurons contribute to a number of sensory or motor tasks, such as the motor systems controlling limb movements (walking, jumping, swimming and turning) or the visual system (social communication, protection from predators and localizing food). We have recently proposed that the sensory–motor relay may be a particularly flexible site for modification when sensory and motor systems subserve multiple functions (Hoke and Wilczynski, 2010). Modifying the sensory responses or motor pathways could have widespread effects on other behaviors utilizing the same neural systems, thus limiting the range of alterations possible for any one behavioral task. The links between the sensory and motor systems are probably restricted to a small number of contexts, so varying those links could influence one behavior while leaving others intact. Sexually dimorphic behaviors are a special case of the distinction between dedicated and multifunctional neural systems. Sexually dimorphic behaviors could depend on sex-specific neural systems dedicated to the male or female behavioral response, or could depend on neural systems that are common to both sexes and potentially regulate a number of behaviors. For behaviors that are not regulated by dedicated neural systems in males or females, the existence of sex-specific sensory motor relays within shared neural systems

would offer a flexible site for evolution in response to sex-specific selective pressures. In this paper we ask if sex differences in the influence of sensory relays on motor circuits contribute to sex differences in neural systems underlying social behavior.

To address this, we examined sex differences in neural and behavioral responses to reproductive signals in *Physalaemus (=Engystomops) pustulosus*, the túngara frog. Túngara frogs differ in both sex-typical motor responses and behavioral selectivity for signals (Baugh and Ryan, 2010; Bernal et al., 2007). Male túngara frogs vocalize both to attract mates and to engage in male–male competition. Sexually receptive females respond to mating calls by phonotaxis, i.e. by approaching an attractive stimulus. Reproductive males typically respond to the same suite of conspecific signals by evoked calling, i.e. vocalizing alternately with the competitor. Laboratory assays of these sex-typical behaviors (phonotaxis assays for females and evoked playback assays for males) demonstrate sex differences in selectivity for signals, with males responding to a broader range of signals than females (Bernal et al., 2007). When not given the opportunity to vocalize, males will perform phonotaxis – a behavioral output in which they show similar selectivity for stimuli as females (Bernal et al., 2009). Despite similar signal selectivity, male and female túngara frogs differ in the motor patterns that comprise a phonotaxis response. Both males and females readily approach speakers playing conspecific signals as adults but only reproductively active females continue to engage in extensive locomotor activity near the source of a conspecific call (Baugh and Ryan, 2010). Male and female túngara frogs therefore demonstrate a number of sex differences in behavioral responses to social signals depending on the experimental and reproductive context, including sex-typical behavioral response, selectivity for signals and pattern of locomotive activity.

Neural activation at a proposed sensory–motor interface in the midbrain (Walkowiak and Luksch, 1994), the laminar nucleus of the torus semicircularis (homolog of the inferior colliculus), shows sexually dimorphic selectivity for signals using *egr-1* mRNA levels as a measure of neural response; a conspecific mating call elicits responses in both males and females whereas a heterospecific call elicits a significantly elevated response only in males (Hoke et al., 2008). The laminar nucleus receives extensive auditory inputs from midbrain and hindbrain auditory nuclei and acts as the primary output region of the torus with widespread connections with forebrain regions (Wilczynski and Endepols, 2007). The laminar nucleus probably acts as a sensory–motor gateway, relaying auditory information both to brainstem motor centers in the tegmentum and to telencephalic areas that modulate motor output *via* multiple pathways through the thalamus and hypothalamus (Wilczynski and Endepols, 2007). Lesioning the torus semicircularis blocks phonotaxis responses of females, although effects of lesioning specific toral nuclei have not been described (Endepols et al., 2003). The sexual dimorphism in the signal specificity of laminar responses suggests that sex differences in responsiveness at this putative sensory–motor gateway could regulate behavioral selectivity for signals. However, it does not explain the sex differences in which behaviors are typically evoked by the signals, nor in the different motor patterns that comprise behavioral responses in males and females. Two explanations for this aspect of sex differences in social behavior are that (1) the midbrain's relay of information to forebrain areas controlling behavior differs in the sexes, and (2) forebrain areas control behavior differently in the sexes. If the former is true, we would predict that the relationship between activity in the midbrain and forebrain would be different in males and females. If the latter is true, we would predict that the relationship between functional patterns in the forebrain and patterns of behavior would differ in the sexes. The two possibilities are not mutually exclusive.

In this study, we extended our previous analysis of sex differences in the auditory system to the forebrain to test the hypothesis that sex differences in sensory–motor relays underlie sexually dimorphic locomotive responses to social signals. We used the immediate-early gene *egr-1* as an indicator of neural activation throughout the brain. We compared both mean *egr-1* responses and auditory–forebrain relationships in males and females, and we related activation patterns to sex differences in locomotive responses to signals. Through our analyses, we linked activation of sensory systems to behavior through intervening motor systems, and we identified the most likely point in the system responsible for sex differences in distinct aspects of phonotaxis behavior.

MATERIALS AND METHODS

Animals

Animal procedures were approved by both University of Texas IACUC and Autoridad Nacional del Ambiente del República de Panamá. The animals included in this experiments are the same individuals whose auditory system we analyzed previously (Hoke et al., 2008). We collected amplexed pairs of male and female túngara frogs (*Physalaemus pustulosus*, Cope 1864) at mating ponds near Gamboa, Panama, to ensure reproductive condition. Frogs were separated and placed in dark, sound isolation chambers for two hours to decrease baseline levels of *egr-1* mRNA. After the waiting period, frogs were assigned to one of three acoustic conditions: no acoustic stimulation (silence), calls from heterospecific *Physalaemus petersi* (Jiménez de la Espada 1872) males, or calls from conspecific *P. pustulosus* males (Fig. 1). Sample sizes ranged from 9 to 11 frogs of each sex in each stimulus condition. Acoustic stimuli consisted of a single exemplar of each call repeatedly broadcast every second from speakers on alternate sides of the chamber (SME-AFS, Saul Mineroff Electronics, Elmont, NY, USA). Amplitudes were adjusted to be 82 dB SPL (re. 20 μP) in the center of the chamber, which measured 0.7 m in length.

We videotaped behavioral responses of frogs during the 30-minute stimulus presentation using infrared cameras (PC-6EX-2 IR video camera, Supercircuits, Liberty Hill, TX, USA) mounted on the chamber ceiling and captured images using a ZR60 miniDV digital camcorder (Canon, Lake Success, NY, USA). For behavioral analysis of the videotapes, we recorded the amount of time each frog was in motion using a stopwatch and estimated association with the speaker by noting every 30 s the location of the frog on a grid overlaying the monitor. We calculated the association time by determining the proportion of time the frog was located in the parts of the chamber abutting the speaker rather than the central portion.

Measuring *egr-1* expression

We assessed neural responses in the brain using mRNA expression of *egr-1*, an immediate-early gene with mRNA or protein levels that increase upon activation of neurons. *Egr-1* levels are related nonlinearly to a neuron's electrical activity, as *egr-1* induction in a neuron requires depolarization but also relies on the complement of co-factors present in the cell (Clayton, 2000; Jarvis, 2004; Knapska and Kaczmarek, 2004). Immediately following the 30-minute acoustic treatment, we decapitated animals and froze their heads embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) in liquid nitrogen. We later processed brains for radioactive *in situ* hybridization for *P. pustulosus egr-1* and quantified silver grain density as previously described (Hoke et al., 2004; Hoke et al., 2008). Briefly, our quantification entailed

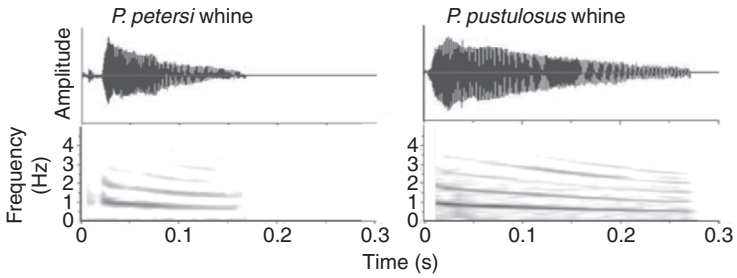


Fig. 1. Acoustic stimuli used in the experiment. Waveforms (top) and spectrograms (bottom) of heterospecific *P. petersi* and conspecific *P. pustulosus* whines used as stimuli in the experiment.

capturing high magnification images sampled at specific sites throughout the brain (3–15 per brain region) and using automated image processing methods to save separate images of Nissl-stained cell bodies and the silver grains over cells. We then calculated silver grain density as the proportion of the area of cells covered in silver grains. We reported *egr-1* mRNA levels in the auditory system (Hoke et al., 2008), then extended our analyses of the same brains to encompass 25 forebrain regions in the same individuals using unbiased sampling methods detailed previously (Hoke et al., 2005; Hoke et al., 2007) (supplementary material Fig. S1).

Statistical analysis

Analysis of the data was done in a set of sequential steps to investigate potential sex differences at several levels. First, we used univariate analyses of covariance (ANCOVAs) to identify parts of the brain that function differently in males and females. We also used analyses of variance (ANOVAs) to test for sex differences in locomotive responses to stimuli. We then used resampling approaches to ask whether the relationships between activity measurements in subsets of brain regions differ in males and females. We complemented resampling approaches with structural equation modeling (SEM) to test hypotheses about sex differences in information flow in the brain.

ANOVAs

We examined sex differences in the *egr-1* responses of each brain region separately using univariate ANCOVAs with SPSS 11 (SPSS, Inc., Chicago, IL, USA). The dependent variables were the natural logarithms of the mean *egr-1* levels in each brain region for each individual. None of the log-transformed variables significantly deviated from a normal distribution, as assessed by the Shapiro–Wilks test. We tested for main effects of sex, stimulus and the sex × stimulus interaction. We included as covariates a variable representing overall brain activation for the individual (Hoke et al., 2007; Hoke et al., 2008) and the proportion of time in motion. Because the relationship between the movement covariate and dependent variable might vary with sex or stimulus, we included three additional interaction terms: stimulus × movement, sex × movement, and sex × stimulus × movement. We retained these three interactions of main effects terms and movement covariate in the analyses if the interaction explained variation in the dependent variable with $P<0.2$. For those cases in which we retained the three-way interaction (sex × stimulus × movement), we also retained both two-way interactions. If interaction terms were not retained ($P>0.2$) we then re-ran ANCOVA analyses without the interactions and present the latter results here.

To determine how males and females differed in their behavioral responses to acoustic stimuli, we used ANOVAs with either proportion time in motion or proportion time associated with the speaker as dependent variables and main effects of sex and stimulus as well as the sex × stimulus interaction term. For analyses in which the effect of stimulus condition was significant, we used *post-hoc* pairwise analyses and corrected for multiple comparisons using Tukey's HSD to isolate which stimuli evoked differences in behavior. We further assessed significant sex × stimulus interactions using planned comparisons to assess sex differences within each stimulus condition.

Resampling

We used resampling approaches in R in an extension of our previously described methods (Hoke et al., 2007) to ask where sex differences in the brain arise, i.e. whether auditory–forebrain relationships, within forebrain networks, and/or forebrain–behavior associations differed between males and females. Resampling is a flexible method of comparing group differences in user-defined measurements using random permutation of the dataset to create the null distribution against which to test statistical significance of a comparison. We calculated an index of association, R_{SqDiff}, to summarize the sex differences in the individual pairwise correlation coefficients comprising each comparison as detailed below. We then compared the actual association index with the range of indices found by random permutation of the sex of each individual in the data set 2000 times without replacement, reassigning each individual as either male or female to reflect the overall sex ratio in the original data set on every round. We determined how often the measured sex differences would occur by chance, with the *P*-value calculated as the proportion of group differences in the original data set plus permutations that were as large as the calculated sex difference.

We first tested the hypothesis that the set of midbrain auditory–forebrain correlations differed in the sexes, specifically focusing on the laminar nucleus as the major output area of the midbrain auditory system (Wilczynski and Endepols, 2007). To do so, we calculated R_{SqDiff} as the sum over all forebrain regions of the squared difference between the Pearson correlation coefficient of laminar *egr-1* levels with *egr-1* levels in that forebrain region in females and the corresponding coefficient in males.

We next addressed whether there were sex differences in the functional associations within forebrain networks to test the hypothesis that forebrain systems operate differently in males and females. We tested the hypothesis that correlations among all pairs of forebrain regions differed significantly in males and females. We calculated the full Pearson correlation matrix describing relationships among *egr-1* mRNA levels in each pair of forebrain regions in males and females. We then calculated R_{SqDiff} as the sum over all off-diagonal elements in the correlation matrix of the squared difference between the corresponding correlation coefficients in males and females.

We finally asked whether the relationship between forebrain activation and behavior was sexually dimorphic. We tested the hypothesis that males and females differed significantly in how *egr-1*

mRNA levels in the group of forebrain regions correlated with time in motion. We calculated R_{SqDiff} as the sum over all brain regions of the squared difference in the Pearson correlation coefficient between *egr-1* levels in that forebrain region and the proportion of time in motion for males and females.

Structural equation modeling

The resampling methods detailed above allowed us to test for sex differences in networks of interacting brain regions, but did not enable us to consider simultaneously co-activation patterns across the brain and isolate specific pathways that functioned differently in males and females. To determine the sexually dimorphic functional pathways responsible for the sex differences in behavior, we compared connectivity of neural systems using SEM. SEM is an extension of linear regression in which the researcher tests and compares different models of the relationships among variables. Models proposed relationships between variables based on anatomical connections, including both direct causal effects of one variable on another and covariation between variables that were not detailed explicitly in the model (correlated error terms). Direct relationships are termed paths, and the strengths of those relationships, akin to regression coefficients, are termed path coefficients.

SEM enables simultaneous tests of sex differences in auditory–forebrain, forebrain or forebrain–behavior relationships but precludes modeling each brain region individually given our sample sizes. To reduce the number of variables, we summarized *egr-1* levels for each forebrain division using principal component analysis. We calculated principal components representing activation in the three forebrain divisions separately (thalamus, hypothalamus and telencephalon) using SPSS 11 (SPSS, Inc.). We prioritized by including all subjects over including all brain regions, and thus excluded the following brain regions that had several missing values: rostral striatum (rST), dorsal pallium (DP), dorsal hypothalamus (DH) and central thalamus (Cthal). We ran three separate principal components analyses for the three forebrain divisions, each based on the logarithm of the mean *egr-1* levels in the brain regions in that division. A single component for each division had an eigenvalue greater than 1, thus we represented each brain division by a single component. We calculated components by regression with cases excluded pairwise. Principal components calculated with and without the above excluded variables were highly correlated (correlation coefficient 0.980–0.998), thus we concluded that omitting these variables did not alter the generality of these components. A single component in each division explained more than 65% of the variance in *egr-1* levels in the telencephalon, hypothalamus and thalamus. We used those three components in SEM analyses along with the *egr-1* levels in the laminar nucleus and the percentage of time each frog moved.

We tested two types of SEM models – structured and unstructured. Both model types assumed a basic flow of information from the laminar nucleus to the forebrain areas to regulate locomotive behavior. The unstructured model did not postulate any particular relationship among forebrain areas whereas the structured model further assumed that the thalamus and hypothalamus received laminar inputs and relayed them to the telencephalon, which directly influenced locomotion. We based the structured model on the general view of the sensory–motor pathways underlying acoustic communication in frogs (Wilczynski and Endepols, 2007), and included the unstructured model due to the many anatomical connections among brain regions that are not detailed in the structured model. Due to mathematical limitations that require path diagrams to be non-recursive, we did not include any of the feedback pathways from the forebrain to the auditory system.

All SEM model estimation was run with Mplus using the MLM estimator (Muthén & Muthén, Los Angeles, CA, USA). After estimation of all of the model parameters, we tested each model for model fit based on how well the implied covariance matrix of that model matched the actual matrix, thus allowing us to reject models in which the proposed relationships among variables did not match the measured relationships. The overall model fit was tested by a χ^2 statistic as well as by close fit indices. We report the Chi-square test, with *P*-values less than 0.05 indicating poor model fit. Two close fit indices were assessed: Bentler's Comparative Fit Index (CFI), which compares model fit with baseline model positing zero relationship among variables, and the standardized Root Mean Square Residual (SRMR), a measure of the total amount of covariance explained. Commonly used criteria for good model fit include CFI>0.96 and SRMR<0.1 (Hu and Bentler, 1999). In addition, we report the Akaike Information Criteria (AIC) for comparisons of non-nested models. We tested unconstrained models in which all paths were estimated separately in males and females, and compared those unconstrained models with nested ones in which some or all paths were constrained to be equal in the sexes. If a constrained model had poor fit while the unconstrained model did not, then we concluded that one or more of the constrained paths were significantly different in males and females.

RESULTS

Sex differences in *egr-1* expression in forebrain regions

We found sex differences in neural activation in a number of forebrain regions that we selected based upon their proposed roles in motivation or behavior. In a subset of forebrain regions (supplementary material Table S1) *egr-1* mRNA levels consistently covaried with the locomotive responses of the individual and the acoustic stimulus to which it was subjected. Seven brain regions had significant main effects of sex [lateral pallium (LP), caudal striatum (cST), medial septum (MS), nucleus accumbens (NA), medial amygdala (MA), anterior preoptic area (aPOA), suprachiasmatic nucleus (SC); Fig. 2]. The lateral septum (LS), central septum (CS) and MA had significant interactions of sex and stimulus in predicting *egr-1* levels (Fig. 2). In addition, the relationships between *egr-1* level and locomotive behaviors differed in males and females in five brain regions (LP, rST, CS, MA, aPOA; sex × movement or sex × stimulus × movement interactions; not shown). Males and females did not consistently differ in the range or variability in *egr-1* levels across the brain (supplementary material Fig. S2). Together, these results demonstrate widespread sex differences in *egr-1* induction in forebrain regions, either on overall levels, or on the patterns of stimulus- or motor-related activation.

Sex differences in auditory–forebrain relationships

We found evidence that males and females differ in the relaying of auditory information to the forebrain. We examined the correlations between forebrain *egr-1* levels and the *egr-1* levels in the major auditory input to the forebrain, the laminar nucleus of the torus semicircularis (Fig. 3). Resampling demonstrated that the measured index of sex differences, R_{SqDiff}, was significantly higher than expected from the null distribution based on randomly assigning individuals to male and female groups (P=0.017); thus, the associations between *egr-1* levels in the laminar nucleus and each forebrain region were significantly higher in females than in males.

This laminar–forebrain association was unique. If we selected any of the 25 forebrain regions and compared its correlations with all of the other forebrain regions, none showed significant sex differences (all $P>0.05$). Thus, there was a sex difference in the correlation between *egr-1* levels in a midbrain auditory relay, the laminar nucleus and the responses in the forebrain.

Sex differences in forebrain–forebrain relationships

Sex differences in auditory–forebrain relationships do not coincide with sex differences in the pairwise associations between different forebrain regions. Using resampling to compare all pairwise correlations among forebrain areas, we found that intra-forebrain correlations did not differ consistently in males and females ($R_{SqDiff}=21.08$, $P=0.516$; Fig. 4). The higher auditory–forebrain covariation in females was therefore not indicative of widespread differences in correlations across forebrain networks. Instead, once activated, the functional networks within the forebrain operate similarly in males and females.

Sex differences in association between forebrain *egr-1* expression and locomotion

Males and females do not fundamentally differ in the relationship between forebrain *egr-1* mRNA levels and behavioral responses. We found no significant difference between males and females in the correlations between forebrain activation patterns and the proportion of time the frog spent moving using resampling ($R_{SqDiff}=2.47$, $P=0.097$; Fig. 5). Despite several forebrain regions with significant interactions between sex and movement in an ANCOVA, we did not find evidence for a widespread difference in males and females in how forebrain activation is translated into motor output.

Sex differences in locomotive responses to advertisement signals

The differential stimulus specificity of the laminar nucleus in males and females (Hoke et al., 2008), along with the sexually dimorphic associations between the laminar nucleus and forebrain *egr-1* levels, is reflected in sex differences in locomotive responses to

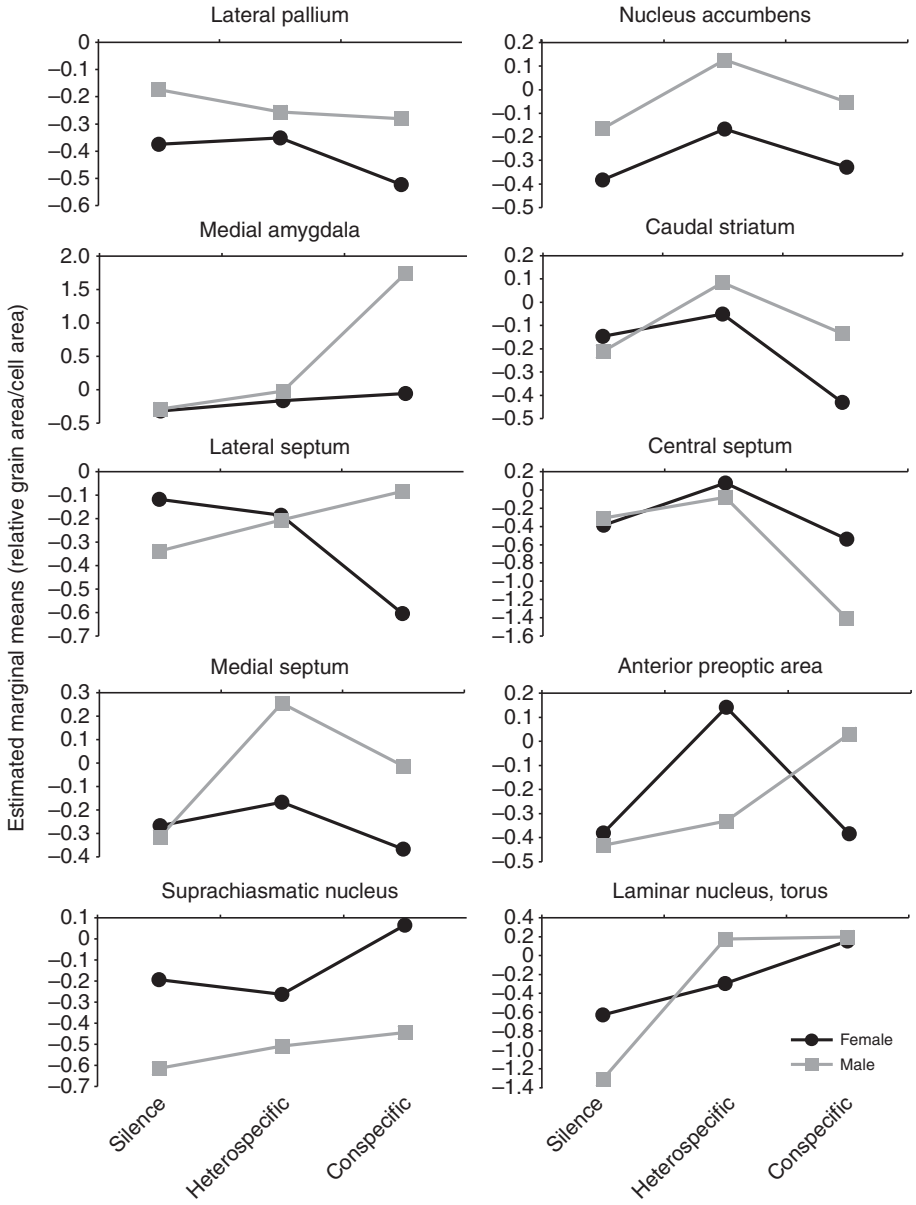


Fig. 2. Sex differences in the stimulus-induced *egr-1* mRNA levels were widespread in the forebrain. Plotted are the estimated marginal means for each stimulus category in males (gray) and females (black) based on ANCOVA analyses. We show only brain regions in which the main effects of sex or sex × stimulus interactions were statistically significant (supplementary material Table S1), and include the laminar nucleus results (modified from Hoke et al., 2008) for reference.

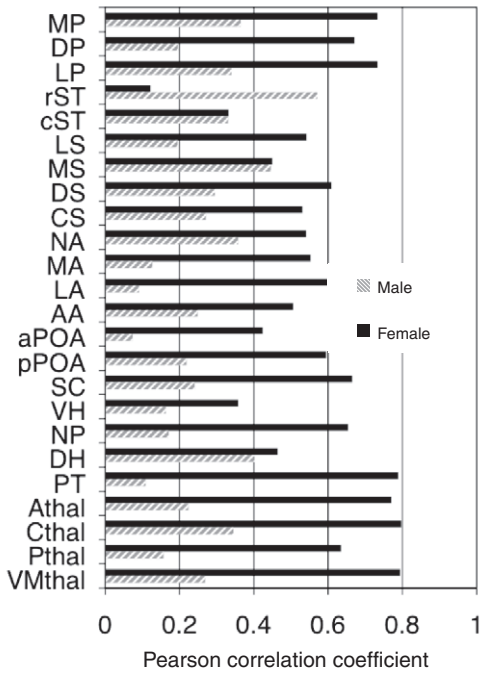


Fig. 3. The relationships between *egr-1* mRNA levels in the laminar nucleus and forebrain areas are stronger for females than males. Pearson correlation coefficients between *egr-1* levels in the laminar nucleus and in each forebrain region are shown for males (gray) and females (black). We used resampling approaches to demonstrate statistical significance of this sex difference. MP, medial pallium; DP, dorsal pallium; LP, lateral pallium; rST, rostral striatum; cST, caudal striatum; LS, lateral septum; MS, medial septum; DS, dorsal septum; CS, central septum; NA, nucleus accumbens; MA, medial amygdala; LA, lateral amygdala; AA, anterior amygdala; aPOA, anterior preoptic area; pPOA, posterior preoptic area; SC, suprachiasmatic nucleus; VH, ventral hypothalamus; NP, periventricular nucleus; DH, dorsal hypothalamus; PT, posterior tuberculum; Athal, anterior thalamus; Cthal, central thalamus; Pthal, posterior thalamus; VMthal, ventromedial thalamus.

advertisement calls. We found a significant difference in the amount males and females moved in response to the three stimulus conditions using ANOVA (Fig. 6A, sex × stimulus interaction: $F_{2,52}$=6.605, P=0.003; main effect of sex: $F_{1,52}$=7.491, P=0.008; main effect of stimulus: $F_{2,52}$=0.595, P=0.555). Planned comparisons of estimated marginal means determined that the sexes differed specifically in their locomotive responses to conspecific signals ($F_{1,52}$=18.946, P<0.001 for sex differences in behavioral responses to conspecific calls; $F_{1,52}$=0.618, P=0.435 for locomotion without acoustic stimulation; $F_{1,52}$=1.525, P=0.222 for locomotion in response to heterospecific calls). By contrast, we did not find sex differences in the time frogs spent near the speaker using ANOVA (Fig. 6B, main effect of sex: $F_{1,52}$=0.525, P=0.472; sex × stimulus interaction: $F_{2,52}$=0.285, P=0.753). Males and females spent more time in proximity to the speakers in response to the conspecific calls than in either the heterospecific or silence conditions (main effect of stimulus: $F_{2,52}$=5.815, P=0.005; *post-hoc* pairwise comparisons: silence *vs* heterospecific P=0.974; silence *vs* conspecific P=0.011; heterospecific *vs* conspecific P=0.021). Thus, we found sex differences in locomotive responses to conspecific signals (but not other signals) but not in association time with the sound source.

Linking auditory activation to behavior

We used SEM to relate auditory system activation to behavioral output through forebrain networks as a complement to the resampling approach. Both structured and unstructured models fit the data well (Table 1), suggesting that these simplified models (e.g. lacking feedback pathways) are able to approximate well the measured covariance matrix and thus are amenable to testing the hypothesis about sex differences in particular pathways in the brain.

To localize sex differences in SEM models, we tested a series of models in which different subsets of paths were constrained to be equal across sexes. Models in which all paths are constrained equally were rejected based on poor fit (U2 and S2, Table 1). Constraining any one of the laminar–forebrain relationships to be equal in males and females also resulted in poor model fit (U4, U5, U6, S4 and S5; Table 1). By contrast, models U3 and S3, in which direct paths between the laminar nucleus and forebrain were freely estimated in males and females while all other paths were constrained to be equal, adequately described relationships among variables (Table 1). Taken together, these results suggest that, if the models accurately describe information processing, males and females differ in their auditory–forebrain relationships but not in forebrain–forebrain networks or in forebrain influences on locomotive behaviors (Fig. 7B,C). This is consistent with the resampling results detailed above (summarized in Fig. 7A).

DISCUSSION

Our results support the hypothesis that sex differences in behavioral responses to social stimuli stemmed from sex differences in the relay of auditory information from the midbrain to forebrain

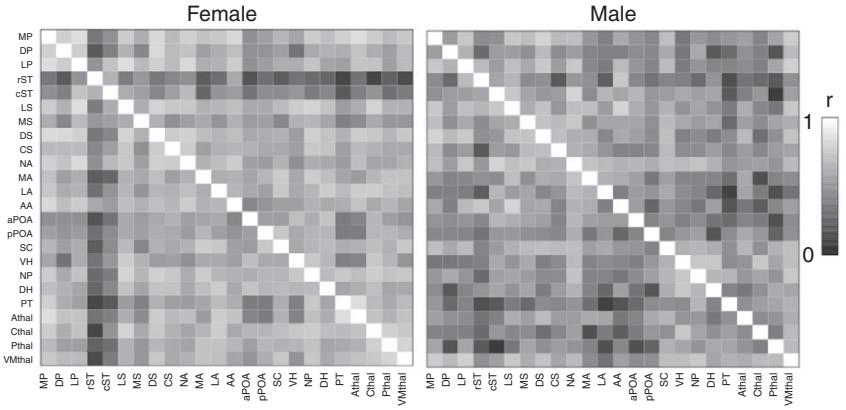


Fig. 4. Correlation structure in forebrain networks did not differ in males and females. Each small square represents a correlation coefficient between *egr-1* levels in two brain regions (arrayed rostrocaudally in the same order on each axis). White and light gray squares indicates positive correlation coefficient (diagonal indicates Pearson correlation coefficient of 1), dark squares indicates correlation coefficients near zero. No negative correlations were found in this dataset. See Fig. 3 legend for definitions.

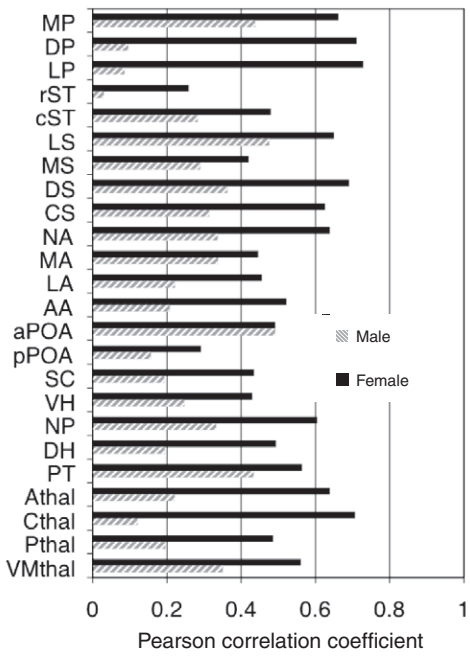


Fig. 5. The relationships between time in motion and the *egr-1* mRNA levels in forebrain areas are not signficantly different in females than males. Pearson correlation coefficients between movement levels and *egr-1* abundance in each forebrain region are shown for males (gray) and females (black). See Fig. 3 legend for definitions.

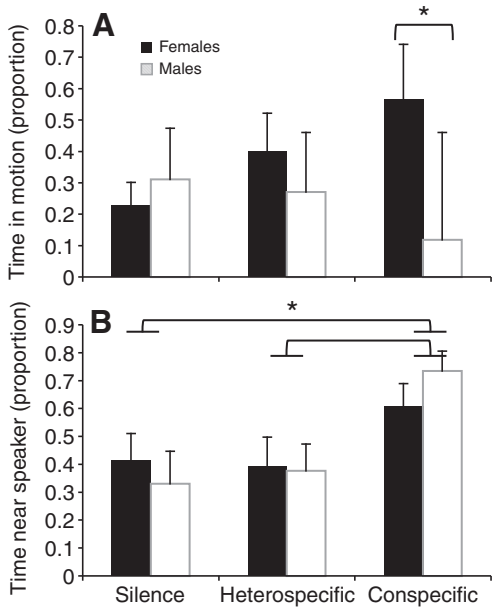


Fig. 6. The sexes differed in the amounts of stimulus-induced locomotion but not in their association with the speakers. Mean proportions of time in motion (A) and time in the quadrants near the speakers (bottom) for males (white) and females (black) are shown for each stimulus condition, with error bars representing standard error of the mean. Asterisks indicate statistically significant comparisons.

circuits. There was a linear relationship between *egr-1* levels in the laminar nucleus and in its forebrain targets in females but not in males. These functional networks within the forebrain otherwise operated similarly in males and females as assessed by correlated activation patterns, despite sex differences in mean *egr-1* levels in many forebrain regions. Moreover, we did not find evidence for sex differences in the relationship between *egr-1* induction in these forebrain networks and locomotive behavior, although the sexes differed in locomotive responses to signals. We propose that sex differences both in the selectivity of a midbrain sensory–motor gateway (Hoke et al., 2008) and in the influence of this gateway on forebrain targets together produce sex differences in neural activation within forebrain regions that are associated with sexually dimorphic behavioral responses to social signals.

Relating sex differences in brain and behavior

The experimental design we used offered a unique opportunity to relate variation in sensory responses to variation in behavioral output through a complex network of brain regions using two types of multivariate analyses. Resampling statistics considered the activation measure of each forebrain region individually, and few assumptions were made as to the specific roles of each region. This was followed by an SEM approach that allowed a global analysis that spanned activation in sensory regions, forebrain network patterns and

Table 1. Structural equation modelling (SEM) results comparing models that relate auditory responses to behavior

Model	Model type	Constrained	χ^2	d.f.	*P*-value	CFI	SRMR	AIC
U1	Unstructured	None	5.311	2	0.0688	0.985	0.043	382.5
U2	Unstructured	All	33.686	11	0.0004	0.897	0.300	383.4
U3	Unstructured	d, e, f, g, h, i	12.188	8	0.1434	0.981	0.066	374.3
U4	Unstructured	a	20.750	3	0.0001	0.920	0.379	394.0
U5	Unstructured	b	16.220	3	0.0010	0.940	0.245	390.0
U6	Unstructured	c	12.075	3	0.0071	0.959	0.190	385.6
S1	Structured	None	12.039	8	0.1494	0.982	0.050	374.2
S2	Structured	All	34.167	14	0.0019	0.909	0.302	377.6
S3	Structured	3, 4, 5, 6	14.948	12	0.2443	0.987	0.076	367.9
S4	Structured	1	30.357	9	0.0004	0.903	0.362	385.7
S5	Structured	2	24.136	9	0.0041	0.932	0.232	381.6

Models are described by model type (structured or unstructured) and which paths are constrained to be equal in males and females. Numbers and letters in the constrained column indicate which paths (labeled on Fig. 7) are constrained to be equal. d.f.=degrees of freedom, CFI=Bentler's comparative fit index, SRMR=standardized root mean square residual, AIC=Akaike information criteria. Values that indicate poor model fit are highlighted by a gray background.

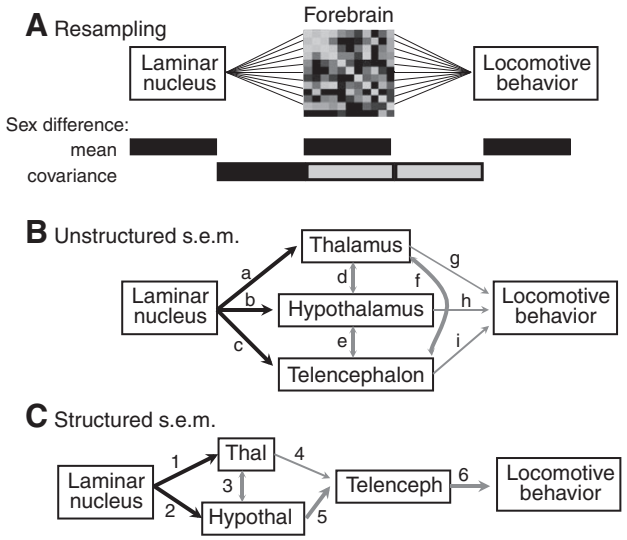


Fig. 7. Summary depicting consistent sex differences isolated to the laminar-forebrain associations using both resampling and structural equation modeling (SEM) analyses. Panel (A) summarizes the results from ANCOVAs and resampling, indicating a sex difference with black bars and the lack of difference with gray bars. The sexes differed in mean *egr-1* levels in the laminar nucleus, *egr-1* induction in some forebrain areas, and locomotive behaviors. The auditory-forebrain relationships were sexually dimorphic, but not the intra-forebrain associations or the dependence of locomotive responses on forebrain activation. Both classes of SEM models, the unstructured (B) and structured (C) ones, had concordant results. Black arrows indicate those that are significantly different in males and females, whereas gray arrows are not different. The models with good fit (U1, U3, S1, S3, Table 1) explained 15–25% of the variance in locomotive behavior in males, and 42–47% in females.

consequent behavioral outputs to isolate components of the functional network with sex differences (after summarizing average activity across each brain division due to sample size requirements). Despite the distinctly different statistical requirements of each approach, we found entirely consistent results; network covariation patterns did not differ significantly in males and females within the forebrain and between forebrain areas and behavior whereas the sexes differed in auditory–forebrain relationships.

We have focused on sex differences in correlations as a means to argue that neural systems function differently in the two sexes, and to propose specific locations and pathways that warrant more detailed physiological characterization. Network covariation patterns, or connectivity, are typically estimated using physiological measures such as electrophysiology or functional magnetic resonance imaging in which the researcher measures responses to multiple stimuli within each individual and determines how tightly correlated activity levels are in two different cells or different brain regions across stimulus presentations. Given that in our experiments each animal is subject to only one stimulus condition, our measure of connectivity is intrinsically different, as we compare correlations across individuals. Patterns of correlated *egr-1* expression could arise because of direct synaptic coupling between brain regions, because of shared common modulatory inputs or because of some generalized mechanisms that influence *egr-1* induction across the brain (e.g. hormone levels or neuromodulators). Moreover, given the long time scale of *egr-1* induction, we cannot demonstrate that auditory activation preceded forebrain activation, thus we cannot determine whether ascending synaptic connections underlie this pattern of covariation. Due to the nonlinearities in *egr-1* expression, we additionally cannot rule out the possibility that the lack of auditory–forebrain covariation in males could reflect differences in cofactors regulating *egr-1* induction in some brain region rather than differences in correlated electrical activities, as electrical activation does not always induce *egr-1* expression (Clayton, 2000; Jarvis, 2004; Mello and Riberio, 1998). The lack of widespread differences in the overall means or variances of *egr-1* levels in males and females (supplementary material Fig. S2) does suggest that a lack of covariation in males is not the trivial consequence of a lack of *egr-1* induction. We suggest that follow-up anatomical and physiological studies focus on laminar–forebrain pathways to detail cellular mechanisms for the sex differences we found in this study.

How do the sex differences we find in auditory–forebrain relationships correspond to the sex differences in social behavior? Sex differences in social behavior encompass (1) differences in the propensity to perform a particular behavioral response; (2) differences in the signal specificity in eliciting a response; and (3) differences in the motor patterns that comprise a given behavioral response. The difference in sex-typical behavioral responses in frogs is well established; in túngara frogs, as in most frog species, the males are the only ones that produce advertisement calls in response to calls, and females typically respond by phonotaxis (Ryan, 1985). The sexes also differ in signal specificity in túngara frogs, with males less selective in vocal responses than are females in phonotaxis responses (Bernal et al., 2007). Note, however, that this specificity difference depends on behavioral output. Bernal et al. (Bernal et al., 2009) found that male and female túngara frogs display similar signal specificity when performing phonotaxis responses, suggesting that the greater selectivity of females in their previous study (Bernal et al., 2007) is, in fact, due to a task difference (vocalization *vs* phonotaxis) rather than a sex difference *per se*. In their work demonstrating the lack of sex difference in phonotaxis responses, Bernal et al. used the dichotomous outcome of phonotaxis as an indicator of behavioral response (Bernal et al., 2009). A choice was defined as approaching within 10 cm of a speaker. A subsequent paper found sex differences in a quantitative measure of phonotaxis behavior, locomotive perseverance or the amount of locomotor activity performed by males and females in the region adjacent to the speakers as measured by path length, despite similar overall percentages of individuals that approached speakers playing conspecific signals (Baugh and Ryan, 2010). We found concordant sex differences in the motor responses to a conspecific signal; males approached the speaker and ceased moving whereas females approached the speaker and engaged in searching behavior.

The *egr-1* expression patterns we described reflect these different aspects of sex differences in social behavior. *Egr-1* induction within the laminar nucleus (Hoke et al., 2008) parallels the sex difference in behavioral selectivity when the animals are performing sex-typical responses (Bernal et al., 2007); *egr-1* levels are high only in females that heard conspecific signals whereas both conspecific and heterospecific *P. petersi* calls induce *egr-1* mRNA in males. Laminar nucleus expression does not predict the sex difference in motor output during phonotaxis – this difference emerged within laminar targets in the forebrain. Our data suggest that in females but not in males, the forebrain regions regulating phonotaxis depend on laminar inputs to forebrain motor areas in a straightforward fashion. The sex difference in signal selectivity within the laminar nucleus would be transformed into a distinct sex difference in the patterns of *egr-1* responses in forebrain targets based on the suggested sexually dimorphic influence of laminar inputs on forebrain regions. Because the pattern of activation in forebrain areas is strongly related to motor output in both males and females, the

sex differences in forebrain regions mirror the sex differences in behavior. We propose that the difference in signal selectivity in the laminar nucleus combined with differences in laminar–forebrain associations may fully account for sex differences in the motor output performed.

Sexually dimorphic sensory gating: a flexible solution

Our results extend our understanding of how sex differences in social behaviors can arise by differential activation of motor control circuits that are present in both sexes. The well-established mechanisms of sex differences in vocalizations of fish, frogs and birds depend on sex differences in the anatomy and function of motor circuits controlling vocalization (reviewed in Ball and MacDougall-Shackleton, 2001; Forlano et al., 2007; Zornik and Yamaguchi, 2008). In each of these cases, however, the motor neurons involved in the behavioral output are dedicated to the production of vocalizations. By contrast, sexually dimorphic gating of common motor circuits has been proposed to distinguish male-typical and female-typical behaviors in three systems in which the motor neurons generating the social behavior also contribute to other behavioral outputs: mounting behavior in mice, courtship behaviors in flies, and phonotactic locomotion in frogs. In mice, eliminating a pheromonal input in females results in the expression of male-typical mounting behavior (Kimchi et al., 2007), suggesting that motor circuits regulating male-typical behaviors are normally inhibited in female mice, although these experimental findings were not replicated in a subsequent study (Martel and Baum, 2009). In flies, males and females differ in their behavioral responses to a sex pheromone (Kurtovic et al., 2007) as a consequence of differences in the projection pattern of the olfactory neurons (Datta et al., 2008). Experimentally activating song motor commands in females produces song by patterning wing vibrations (Cline and Miesenbock, 2008), suggesting that female flies lack stimulatory inputs to drive the functional motor circuit that regulates male-typical singing behavior. Our results expand on the previous work in other systems by examining the functional activation of the neural systems that regulate female-typical behavioral responses. We found evidence that sensory-motor gating differed in males and females in evoking female-typical responses to acoustic signals. We detailed how the activation of this sensory–motor gateway differed both in its overall selectivity for conspecific signals (Hoke et al., 2008), and in the relationship between sensory inputs and forebrain motor-control circuits (this study). We speculate that sex differences in social behaviors that originate *via* sexually dimorphic sensory gating rather than dimorphic motor systems may be the consequence of multifunctionality of motor centers; perhaps the sensory–motor connections are dedicated to the particular behavioral response despite linking multifunctional auditory and locomotive circuits, and thus this sensory–motor gateway may be a malleable site for modulation by sex, reproductive condition, learning or evolution (Hoke and Wilczynski, 2010).

LIST OF ABBREVIATIONS

Telencephalon (Telenceph):

AA	anterior amygdala
CS	central septum
cST	caudal striatum
DP	dorsal pallium
DS	dorsal septum
LA	lateral amygdala
LP	lateral pallium
LS	lateral septum
MA	medial amygdala
MP	medial pallium
MS	medial septum
NA	nucleus accumbens
rST	rostral striatum

Hypothalamus (Hypothal):

aPOA	anterior preoptic area
DH	dorsal hypothalamus
NP	periventricular nucleus
pPOA	posterior preoptic area
PT	posterior tuberculum
SC	suprachiasmatic nucleus
VH	ventral hypothalamus

Thalamus (Thal):

Athal	anterior thalamus
Cthal	central thalamus
Pthal	posterior thalamus
VMthal	ventromedial thalamus

ACKNOWLEDGEMENTS

We thank Ryan Taylor and the Smithsonian Tropical Research Institute for logistical support in Panama, and the American Association of University Women and the National Science Foundation (IRCEB 0078150 and IOS 0940466) for funding. W.W. is an NIH-funded researcher. Deposited in PMC for release after 12 months.

REFERENCES

Allin, J. T. and Banks, E. M. (1972). Functional aspects of ultrasound production by infant albino rats (*Rattus norvegicus*). *Anim. Behav.* **20**, 175-185.

Ball, G. F. and MacDougall-Shackleton, E. A. (2001). Sex differences in songbirds 25 years later: what have we learned and where do we go? *Microsc. Res. Tech.* **54**, 327-334.

Baugh, A. T. and Ryan, M. J. (2010). The development of sexual behaviour in túngara frogs. *J. Comp. Psychol.* **124**, 66-80.

Berglund, A., Bisazza, A. and Pilastro, A. (1996). Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biol. J. Linn. Soc.* **58**, 385-399.

Bernal, X. E., Rand, A. S. and Ryan, M. J. (2007). Sexual differences in receiver permissiveness to advertisement calls in túngara frogs, *Physalaemus pustulosus*. *Anim. Behav.* **73**, 955-964.

Bernal, X. E., Rand, A. S. and Ryan, M. J. (2009). Task differences confound sex differences in receiver permissiveness in túngara frogs. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 1323-1329.

Clayton, D. F. (2000). The genomic action potential. *Neurobiol. Learn. Mem.* **74**, 185-216.

Cline, J. D. and Miesenbock, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in *Drosophila*. *Cell* **133**, 354-363.

Datta, S. R., Vasconcelos, M. L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B. J. and Axel, R. (2008). The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. *Nature* **452**, 473-477.

Endepols, H., Feng, A. S., Gerhardt, H. C., Schul, J. and Walkowiak, W. (2003). Roles of the auditory midbrain and thalamus in selective phonotaxis in female gray treefrogs (*Hyla versicolor*). *Behav. Brain Res.* **145**, 63-77.

Forlano, P. M., Remage-Healy, L., Sisneros, J. A. and Bass, A. H. (2007). Steroid-induced plasticity in the auditory and vocal motor system: recent studies in a teleost fish. In *Evolutionary Molecular Strategies and Plasticity* (ed. M. Canonaco and R. M. Facciolo), pp. 25-50. Kerala, India: Research Signpost.

Harlan, R. E., Shivers, B. D. and Pfaff, D. W. (1984). Lordosis as a sexually dimorphic neural function. *Prog. Brain Res.* **61**, 239-255.

Hoke, K. L. and Wilczynski, W. (2010). Multifunctional neural systems and plasticity in their sensory-motor transformations. In *Reciprocal Interactions Among Primary Sensory and Motor Cortical Areas and Higher Cognitive Processes* (ed. L. Hermer). Kerala, India: Research Signpost Press. (in press).

Hoke, K. L., Burmeister, S. S., Fernald, R. D., Rand, A. S., Ryan, M. J. and Wilczynski, W. (2004). Functional mapping of the auditory midbrain during mate call reception. *J. Neurosci.* **24**, 11264-11272.

Hoke, K. L., Ryan, M. J. and Wilczynski, W. (2005). Social cues shift functional connectivity in the hypothalamus. *Proc. Natl. Acad. Sci. USA* **102**, 10712-10717.

Hoke, K. L., Ryan, M. J. and Wilczynski, W. (2007). Integration of sensory and motor processing underlying social behaviour in túngara frogs. *Proc. R. Soc. Lond. B. Biol. Sci.* **274**, 641-649.

Hoke, K. L., Ryan, M. J. and Wilczynski, W. (2008). Candidate neural locus for sex differences inreproductive decisions. *Biol. Lett.* **4**, 518-521.

Hu, L. and Bentler, P. M. (1999). Cutoff criteria for fit indexes in covariance structure analysis: conventional criteria *versus* new alternatives. *Struct. Equ. Modeling* **6**, 1-55.

Jarvis, E. D. (2004). Brains and birdsong. In *Nature's Music: The Science of Birdsong* (ed. P. Marler and H. Slabberkoorn), pp. 239-275. New York: Elsevier.

Kimchi, T., Xu, J. and Dulac, C. (2007). A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature* **448**, 1009-1014.

Knapska, E. and Kaczmarek, L. (2004). A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/Krox-24/TIS8/ZENK? *Prog. Neurobiol.* **74**, 183-211.

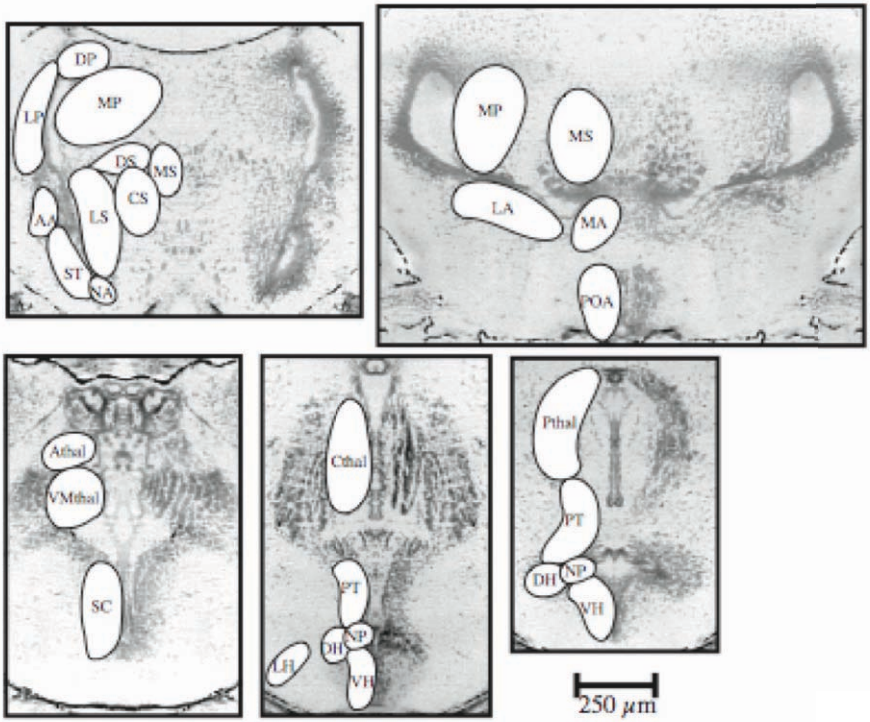
Kurtovic, A., Widmer, A. and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioral responses to a *Drosophila* sex pheromone. *Nature* **446**, 542-546.

Martel, K. L. and Baum, M. J. (2009). Adult testosterone treatment but not surgical disruption of vomeronasal function augments male-typical sexual behavior in female mice. *J. Neurosci.* **29**, 7658-7666.

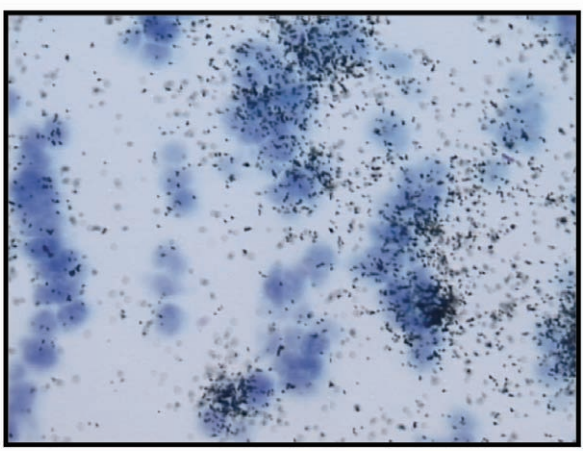
Mello, C. V. and Riberio, S. (1998). ZENK protein regulation by song in the brain of songbirds. *J. Comp. Neurol.* **393**, 426-438.

Ryan, M. J. (1985). *The Túngara Frog: a Study in Sexual Selection and Communication.* Chicago: University of Chicago Press.
Searcy, W. A. and Brenowitz, E. A. (1988). Sexual differences in species recognition of avian song. *Nature* **332**, 152-154.
Walkowiak, W. and Luksch, H. (1994). Sensory motor interfacing in acoustic behavior of aurans. *Am. Zool.* **34**, 685-695.
Wilczynski, W. and Endepols, H. (2007). Central auditory pathways in anuran amphibians: the anatomical basis of hearing and sound communication. In *Hearing and Sound Communication in Amphibians (*ed. P. N. Narins, A. S. Feng, R. R. Fay and A. N. Popper), pp. 221-249. New York: Springer-Verlag.
Zornik, E. and Yamaguchi, A. (2008). Sexually differentiated central pattern generators in *Xenopus laevis. Trends Neurosci.* **31**, 296-302.

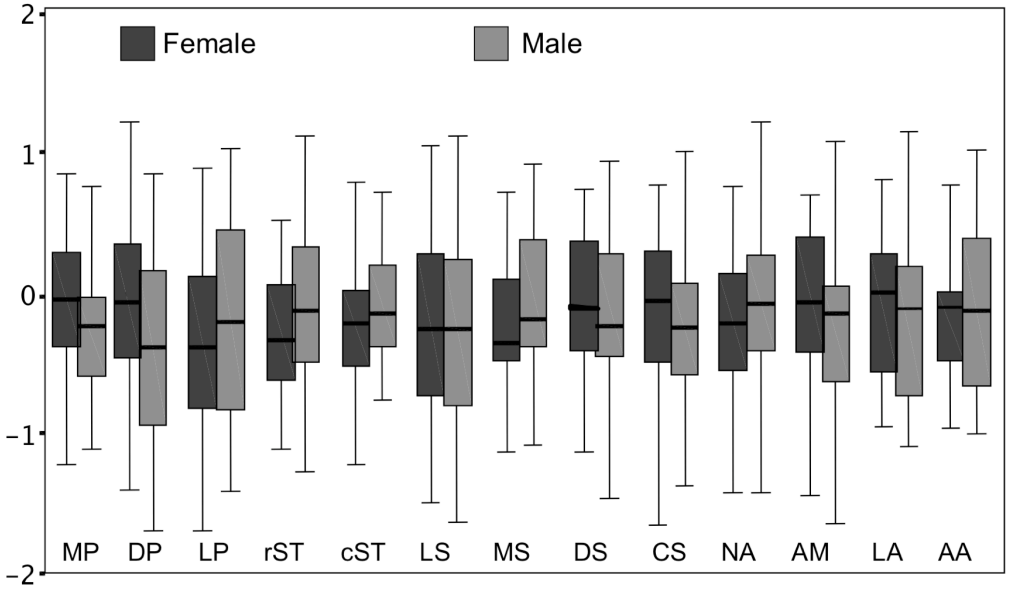
A


DP
MP
LP
DS
MS
CS
AA
LS
ST
NA
MP
MS
LA
MA
POA
Athal
VMthal
SC
Cthal
PT
NP
DH
LH
VH
Pthal
PT
DH
NP
VH
250 μm

B



Supplemental Fig. 2



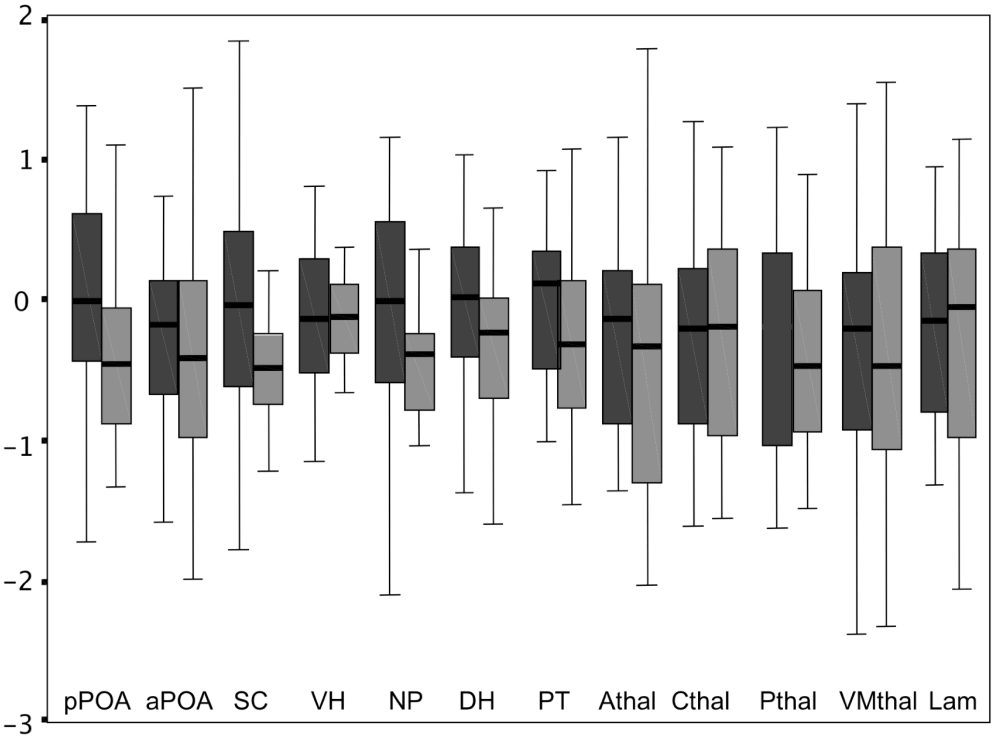


Table S1. ANCOVA results assessing effects of sex, stimulus and locomotion on mean *egr-1* levels in each forebrain region analyzed

Brain region	Sex	Stimulus	Sex × stimulus	Movement	Stimulus × movement	Sex × movement	Sex × stimulus × movement
MP	$F_{1,46}$=0.67	$F_{2,46}$=2.19	$F_{2,46}$=0.96	$F_{1,46}$=53.3	$F_{2,46}$=4.86		
	P=0.420	P=0.123	P=0.392	P<0.001	P=0.012		
DP	$F_{1;44}$=1.56	$F_{2;44}$=0.28	$F_{2;44}$=0.18	$F_{1;44}$=13.3	$F_{2;44}$=1.15	$F_{1;44=}$1.32	
	P=0.219	P=0.754	P=0.833	P=0.001	P=0.326	P=0.257	
LP	$F_{1,45}$=14.3	$F_{2,45}$=1.22	$F_{2,45}$=0.12	$F_{1,45}$=23.2	$F_{2,45}$=2.00	$F_{1,45}$=8.00	
	P<0.001	P=0.305	P=0.890	P<0.001	P=0.147	P=0.007	
rST	$F_{1,40}$=0.55	$F_{2,40}$=0.86	$F_{2,40}$=2.04	$F_{1,40}$=9.65	$F_{2,40}$=2.18	$F_{1,40}$=3.08	$F_{1,40}$=4.03
	P=0.461	P=0.429	P=0.143	P=0.003	P=0.127	P=0.087	P=0.025
cST	$F_{1,45}$=6.04	$F_{2,45}$=2.28	$F_{2,45}$=0.92	$F_{1,45}$=17.7	$F_{2,45}$=1.95	$F_{1,45}$=0.15	
	P=0.018	P=0.114	P=0.407	P<0.001	P=0.154	P=0.095	
LS	$F_{1,45}$=0.80	$F_{2,45}$=2.17	$F_{2,45}$=3.81	$F_{1,45}$=82.9	$F_{2,45}$=2.09		
	P=0.375	P=0.126	P=0.030	P<0.001	P=0.136		
MS	$F_{1,46}$=6.28	$F_{2,46}$=0.07	$F_{2,46}$=2.97	$F_{1,46}$=18.7	$F_{2,46}$=4.07		
	P=0.016	P=0.930	P=0.061	P<0.001	P=0.024		
DS	$F_{1,46}$=1.82	$F_{2,46}$=1.25	$F_{2,46}$=1.33	$F_{1,46}$=46.3	$F_{2,46}$=4.15		
	P=0.184	P=0.298	P=0.275	P<0.001	P=0.022		
CS	$F_{1,42}$=3.94	$F_{2,42}$=2.03	$F_{2,42}$=3.62	$F_{1,42}$=0.06	$F_{2,42}$=1.43	$F_{1,42}$=4.28	$F_{2,42}$=2.54
	P=0.054	P=0.145	P=0.035	P=0.813	P=0.250	P=0.045	P=0.091
NA	$F_{1,47}$=8.56	$F_{2,47}$=2.99	$F_{2,47}$=0.07	$F_{1,47}$=33.2			
	P=0.005	P=0.060	P=0.933	P<0.001			
MA	$F_{1,43}$=9.02	$F_{2,43}$=5.26	$F_{2,43}$=6.47	$F_{1,43}$=28.4	$F_{2,43}$=10.5	$F_{1,43}$=18.2	$F_{2,43}$=9.39
	P=0.004	P=0.009	P=0.004	P<0.001	P<0.001	P<0.001	P<0.001
LA	$F_{1,48}$=0.19	$F_{2,48}$=0.97	$F_{2,48}$=1.36	$F_{1,48}$=6.69			
	P=0.667	P=0.385	P=0.265	P=0.013			
AA	$F_{1,46}$=0.49	$F_{2,46}$=0.36	$F_{2,46}$=0.71	$F_{1,46}$=7.42	$F_{2,46}$=2.00		
	P=0.488	P=0.699	P=0.499	P=0.009	P=0.147		
aPOA	$F_{1,45}$=8.12	$F_{2,45}$=4.68	$F_{2,45}$=2.69	$F_{1,45}$=30.3	$F_{2,45}$=8.04	$F_{1,45}$=7.24	
	P=0.007	P=0.014	P=0.079	P<0.001	P=0.001	P=0.010	
pPOA	$F_{1,47}$=1.87	$F_{2,47}$=7.11	$F_{2,47}$=2.31	$F_{1,47}$=6.30		$F_{1,47}$=2.59	
	P=0.179	P=0.002	P=0.110	P=0.016		P=0.114	
SC	$F_{1,47}$=9.58	$F_{2,47}$=1.39	$F_{2,47}$=0.39	$F_{1,47}$=9.57			
	P=0.003	P=0.259	P=0.681	P=0.003			
VH	$F_{1,46}$=2.25	$F_{2,46}$=0.13	$F_{2,46}$=3.02	$F_{1,46}$=18.6			
	P=0.141	P=0.879	P=0.059	P<0.001			
NP	$F_{1,43}$=0.53	$F_{2,43}$=0.80	$F_{2,43}$=0.53	$F_{1,43}$=36.1	$F_{2,43}$=4.16		
	P=0.471	P=0.454	P=0.595	P<0.001	P=0.022		
DH	$F_{1,42}$=0.64	$F_{2,42}$=0.99	$F_{2,42}$=3.15	$F_{1,42}$=18.5		$F_{1,42}$=3.40	
	P=0.427	P=0.379	P=0.053	P<0.001		P=0.072	
PT	$F_{1,45}$=0.65	$F_{2,45}$=2.15	$F_{2,45}$=0.24	$F_{1,45}$=21.3			
	P=0.424	P=0.128	P=0.787	P<0.001			
Athal	$F_{1,41}$=0.08	$F_{2,41}$=0.21	$F_{2,41}$=0.16	$F_{1,41}$=15.9	$F_{2,41}$=1.57	$F_{1,41}$=1.49	$F_{2,41}$=2.24
	P=0.785	P=0.814	P=0.851	P<0.001	P=0.221	P=0.229	P=0.119
Cthal	$F_{1,42}$=0.15	$F_{2,42}$=7.44	$F_{2,42}$=1.75	$F_{1,42}$=21.1			
	P=0.705	P=0.002	P=0.187	P<0.001			
Pthal	$F_{1,44}$=0.04	$F_{2,44}$=1.96	$F_{2,44}$=1.54	$F_{1,44}$=6.87			
	P=0.838	P=0.153	P=0.226	P=0.012			
VMthal	$F_{1,43}$=3.26	$F_{2,43}$=5.30	$F_{2,43}$=0.10	$F_{1,43}$=30.7	$F_{2,43}$=2.80	$F_{1,43}$=2.49	
	P=0.078	P=0.009	P=0.902	P<0.001	P=0.072	P=0.122	

The ANCOVA for dorsal pallium includes both sex × movement and stimulus × movement interaction terms. Removing either interaction term causes the remaining term to be at or near significance, hence we removed neither.

The covariate representing global activity throughout the brain is included in all ANCOVA analyses and is a significant (P<0.001) covariate for every brain region (results not shown).

MP, medial pallium; DP, dorsal pallium; LP, lateral pallium; rST, rostral striatum; cST, caudal striatum; LS, lateral septum; MS, medial septum; DS, dorsal septum; CS, central septum; NA, nucleus accumbens; MA, medial amygdala; LA, lateral amygdala; AA, anterior amygdala; aPOA, anterior preoptic area; pPOA, posterior preoptic area; SC, suprachiasmatic nucleus; VH, ventral hypothalamus; NP, periventricular nucleus; DH, dorsal hypothalamus; PT, posterior tuberculum; Athal, anterior thalamus; Cthal, central thalamus; Pthal, posterior thalamus; VMthal, ventromedial thalamus.