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RESEARCH ARTICLE

Prior experience with conspecific signals enhances auditory midbrain responsiveness to conspecific vocalizations

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ABSTRACT

There is a long history in neuroethology of investigating how communication signals influence the brain and behavior. It has become increasingly clear that brain areas associated with sensory processing are plastic in adults and that this plasticity is related to reproductive condition. However, the role of communication signal reception in adult auditory plasticity has received relatively little attention. Here, we investigated whether the reception of communication signals (a frog chorus) could enhance the responsiveness of the auditory system to future reception of communication signals (a single male call). We found that animals that had been exposed to 10 days of a male chorus had stronger auditory midbrain immediate early gene expression than animals that had been exposed to 10 days of random tones when tested with 30 min of male calls or 30 min of tones. Our results suggest that exposure to dynamic social stimuli, like frog choruses, may play an important role in shaping the neural and behavioral responses to communication signals.

KEY WORDS: Plasticity, *Hyla cinerea*, Immediate early genes, Lek, Social signals

INTRODUCTION

Communication signals play an important role in mate selection and reproduction (Bradbury and Vehrenkamp, 2011). Males use conspecific signals to evaluate rivals, modulate aggression and determine spacing patterns, while females use male communication signals to locate, sample and select mates. In vertebrates, female mate sampling typically takes one of two forms: sequential sampling [e.g. territorial birds (Dale and Slagsvold, 1996)] or simultaneous sampling [e.g. leks, such as frog choruses (Murphy, 2012); note: individuals in leks may also sequentially sample]. Both males and females may be exposed to mate attraction or courtship signals of conspecifics over a period of days or weeks before, during and after females are actively sampling and selecting mates (Catchpole and Slater, 2008; Elliott et al., 2009; Höglund and Alatalo, 1995; Wells, 1977).

Males defending territory boundaries and females sequentially sampling mates experience chronic exposure to vocal signals. Much of the previous work on the significance of repeated exposure to social signals has focused on neural habituation and diminished behavioral responses to individual signals (Bee and Gerhart, 2001a; Dong and Clayton, 2009; Mello et al., 1995) (but see Sockman et al., 2002; Sockman et al., 2005). Diminished neural and behavioral responses to a signal have important consequences for territory defense, mate choice and reproductive success of both the sender and the receiver, as they can alter the perception of both signal and sender quality (Catchpole and Slater, 2008; Peeke, 1984). Neural habituation has been invoked as a potential neural mechanism underlying the 'dear enemy' effect (Bee and Gerhardt, 2001b; Peeke, 1984). It has been hypothesized that male songbirds with a greater diversity in their song repertoires may be more successful because song-type switching results in dishabituation and increased arousal in females (Catchpole and Slater, 2008).

In all vertebrate taxa there are species that form leks or communal breeding assemblies in which individuals perform communication displays for a long period of time (Höglund and Alatalo, 1995). This results in individuals – both males and females – being exposed to a variety of conspecific advertisement calls in varying patterns over a long period of time, rather than to a single call from a predictable source. Exposure to these conspecific calls changes hormone state and other aspects of physiological reproductive state in most vertebrate taxa (Adkins-Regan, 2005). Hormonal state, in turn, influences the behavioral response to communication signals (Adkins-Regan, 2005), as well as the reception and processing of acoustic signals (Arch and Narins, 2009; Yoder and Vicario, 2012). Although it is clear that prolonged short-term exposure to individual signals can diminish neural responses, it is less clear how longer term exposure (days or weeks) to dynamic assemblages of mate attraction signals encountered on a lek affects auditory processing.

Here, we examined whether prolonged exposure to dynamic conspecific signals, such as those encountered in a lek, could alter neural responses to a conspecific signal in the green treefrog, *Hyla cinerea* (Schneider 1799), a well-studied anuran model for acoustic communication. Green treefrogs are an ideal model system in which to address this question because they have an extended breeding period that lasts for several months (Elliott et al., 2009) and females may lay multiple clutches of eggs (Perrill and Daniel, 1983). During this time both males and females are exposed to choruses composed of the signals of multiple males, which vary in temporal patterns and amplitude as males begin to call, cease to call, or overlap the calls of their neighbors. In *H. cinerea* and other anurans, the importance of communication signals is reflected in specializations in the neural systems processing them. Communication signals – particularly mate-attraction signals – and synthetic stimuli with similar spectral–temporal features are processed selectively or with enhanced sensitivity throughout the auditory system, and particularly in the midbrain (Feng et al., 1990; Leary et al., 2008; Rose and Gooler, 2007; Wilczynski and Ryan, 2010). There is good evidence to suggest that exposure to conspecific chorus sounds can influence the hormonal state of the green treefrog and other frogs (Burmeister and Wilczynski, 2000; Burmeister and Wilczynski, 2001; Lynch and Wilczynski, 2006; Wilczynski and Lynch, 2011); however, it is not clear how this exposure to conspecific choruses might alter the neural processing of male calls in the auditory system.

We focused our investigation on three areas of the torus semicircularis (hereafter torus): the principal nucleus, the laminar

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nucleus and the midline nucleus. The torus is a midbrain auditory processing area that is equivalent to the inferior colliculus of mammals and has previously been shown to respond selectively to stimulation with conspecific calls in other frog species (Burmeister et al., 2008; Hoke et al., 2004; Hoke et al., 2008; Leary et al., 2008). The torus is also a critical part of the auditory system for connecting sensory processing with both motor and endocrine areas of the forebrain (Hoke et al., 2008; Hoke et al., 2010; Wilczynski and Ryan, 2010). We hypothesized that ecologically relevant exposure to mate signals would increase expression of the activity-dependent immediate early gene *egr-1* in the torus in response to mate attraction signals. We explored this hypothesis by measuring the level of *egr-1* that was evoked by either a conspecific call or a random tone (test stimulus) in animals that had been exposed to either 10 days of conspecific chorus or random tones (sound exposure).

RESULTS

There was a significant main effect of sound exposure ($F_{1,18}$=13.9, P=0.002) whereby animals that were exposed to 10 days of chorus had a greater number of silver grains per cell (indicating *egr-1* expression) than animals that were exposed to 10 days of tones (Fig. 1). Similarly, there was a significant main effect of the 30 min test stimulus ($F_{1,18}$=30.5, P<0.001); individuals that received the call stimulus had a greater number of silver grains per cell than individuals that received the tone stimulus (Fig. 1). We did not find a significant sound exposure × test stimulus interaction on the number of silver grains per cell ($F_{1,18}$=0.76, P=0.41), suggesting that exposure to a chorus stimulus for 10 days had a general sensitizing effect on auditory processing, rather than differentially upregulating the response to conspecific vocalizations.

There was a significant main effect of brain area ($F_{2,20.5}$=11.9, P<0.001) and brain area × test stimulus interaction ($F_{2,20.5}$=4.6, P=0.02) on the number of silver grains per cell (Fig. 2). When animals were tested with the 30 min call stimulus, the midline area had a significantly greater silver grain density than the principal nucleus, but there were no other differences among brain areas. However, when animals were tested with the 30 min tone stimulus, the midline area had a significantly greater silver grain density than both the principal nucleus and the laminar nucleus. There was not a significant effect of sex ($F_{1,18}$=0.67, P=0.42) on the number of silver grains per cell.

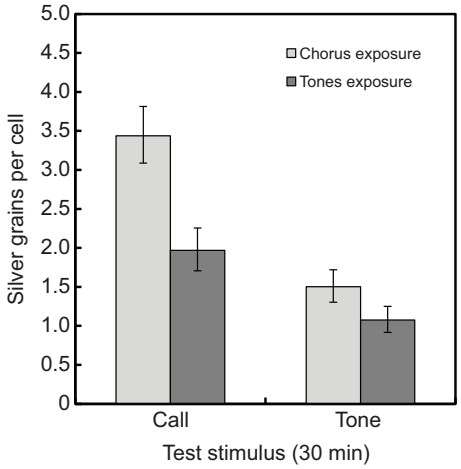

Fig. 1. The number of silver grains per cell (*egr-1* expression) as a function of both sound exposure and test stimulus. Animals exposed to 10 days of the green tree frog chorus and tested with the call had greater *egr-1* than any other group.

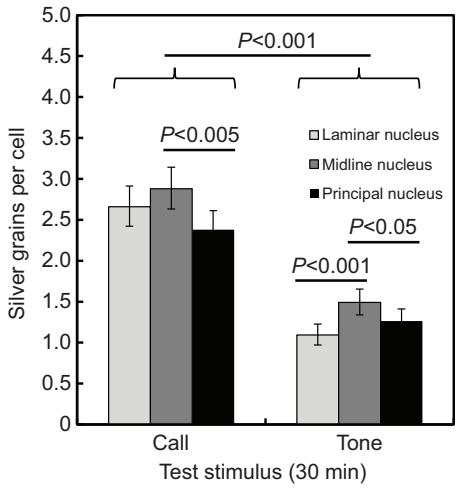

Fig. 2. Effects of nucleus and test stimulus on the number of silver grains per cell (*egr-1* expression). The midline nucleus had greater expression than the principal nucleus in both test conditions. The midline nucleus also had a greater expression level than the laminar nucleus in the animals tested with tones. *P*-values are from Bonferroni corrected *post hoc* *t*-tests.

DISCUSSION

We found that all three of the nuclei we investigated (laminar nucleus, principal nucleus, midline nucleus) showed increased *egr-1* expression when exposed, both in the long and short term, to conspecific signals, consistent with previous findings in frogs (Arch et al., 2011; Burmeister et al., 2008; Charkraborty et al., 2010; Hoke et al., 2004; Hoke et al., 2007; Mangiamele and Burmeister, 2011).We also found that daily exposure to an acoustic social stimulus can influence the future immediate early gene expression in response to acoustic stimulation. Specifically, we found a main effect of sound exposure on immediate early gene expression, which suggests that hearing a chorus for 10 days increased the responsiveness of the midbrain auditory center to acoustic stimuli. Our findings are similar to those in songbirds that have shown that exposure to specific types of conspecific signals can elevate immediate early gene expression to those signals in forebrain areas (Sockman et al., 2002; Sockman et al., 2005). Although we found no interaction effect between the sound exposure and test stimuli, our examination of the data suggests that the effect of sound exposure may be slightly greater when the test stimulus is a socially relevant call (1.7-fold increase for calls, 1.3-fold increase for tones). This issue of specific enhancement deserves greater attention in the future, either with a greater sample size or with control stimuli that have a random assortment of acoustic features. Our call test stimulus and our tone stimulus were designed to be similar in many acoustic features, which may make the control stimuli more likely to evoke ‘call-like’ auditory responses than control stimuli with randomized acoustic features. We believe this is the first documented instance of a mate attraction signal modulating the strength of future neural responses to that signal in anurans.

In frogs (and most other vertebrate taxa) (Bradbury and Vehrenkamp, 2011), these communication signals uniquely evoke courtship and intra-sexual aggressive behaviors [e.g. female phonotaxis and male antiphonal calling behavior (Diekamp and Gerhardt, 1995; Gerhardt, 1974; Gerhardt, 1981a; Gerhardt, 1981b; Gerhardt, 1991; Simmons et al., 1993)]. The importance of communication signals is reflected in specializations in the neural systems processing them. Communication signals – particularly

mate attraction signals – and synthetic stimuli with similar spectral–temporal features are processed selectively or with enhanced responsiveness by neurons in many brain regions (Wilczynski and Ryan, 2010) [as revealed in electrophysiology studies (Diekamp and Gerhardt, 1995; Eggermont and Epping, 1986; Elliott et al., 2011; Fuzessery and Feng, 1983; Miranda and Wilczynski, 2009a; Miranda and Wilczynski, 2009b; Rose et al., 1985) and immediate early gene studies (Chakraborty et al., 2010; Hoke et al., 2004; Hoke et al., 2008; Lynch and Wilczynski, 2008; Mangiamele and Burmeister, 2011)]. In anuran amphibians, this is particularly apparent in the midbrain (reviewed in Rose and Gooler, 2007; Wilczynski and Ryan, 2010).

The auditory midbrain (torus) plays a particularly important role in processing acoustic communication signals in anuran amphibians (Wilczynski and Ryan, 2010), serving as an important sensory–motor interface linking sensory input to behavioral responses (Emerson and Boyd, 1999; Endepols et al., 2003; Walkowiak and Luksch, 1994; Wilczynski and Endepols, 2007). Neurons in the laminar nucleus, a subdivision of the torus, are a major source of auditory midbrain output to both forebrain and brainstem areas (Endepols and Walkowiak, 2001; Wilczynski and Endepols, 2007). It also has a heavy concentration of receptors for numerous behaviorally relevant neuropeptides and steroid hormones (reviewed in Wilczynski and Endepols, 2007). The torus is sensitive not only to conspecific signals (Hoke et al., 2004) (for review, see Rose and Gooler, 2007) but also to artificial stimuli that contain a frequency step necessary and sufficient for species identification (Mangiamele and Burmeister, 2011), suggesting it plays an important role in acoustically based species recognition. It has been hypothesized that the activation level of the torus may be directly related to the level of acoustically evoked reproductive behaviors, such as phonotaxis (Lynch and Wilczynski, 2008). Therefore, factors that modulate the responsiveness of the torus are expected to have major consequences for reproductive behaviors.

In fact, both midbrain neural processing (Goense and Feng, 2005; Lynch and Wilczynski, 2008; Miranda and Wilczynski, 2009a; Penna et al., 1992; Yovanof and Feng, 1983) and behavioral responses (Burmeister and Wilczynski, 2001; Chakraborty and Burmeister, 2009; Lynch and Wilczynski, 2006) to communication signals are plastic in anurans, with variation most often being attributed to reproductive or hormonal state. This temporal variation in the neural processing of communication signals presumably reflects variation in the value of communication signals across time (e.g. within and across the breeding season) and the consequences of behavioral responses to communication signals. Modulating the responsiveness of the auditory systems through exposure to communication signals may serve to functionally couple the behaviors of senders and receivers, be it for reproductive or aggressive interactions.

In many taxa there are reciprocal interactions between communication and hormonal state (Adkins-Regan, 2005). It has been known for some time that the reception of mating signals can alter hormone levels and enhance gonadal development [e.g. mammals (Gudermuth et al., 1992; McComb, 1987), birds (Bentley et al., 2000; Cheng et al., 1998; Lehrman, 1959; Moore, 1983), reptiles (Crews, 1975), fish (Francis et al., 1993) and anurans (Brzoska and Obert, 1980; Burmeister and Wilczynski, 2000; Burmeister and Wilczynski, 2001; Lea et al., 2001; Lynch and Wilczynski, 2006)]. In turn, hormone levels affect behavioral responses to communication signals in frogs, fish, birds, reptiles and mammals (Adkins-Regen, 2005). In the case of acoustic communication, the reception of signals themselves can be influenced by hormones, as hormones have been shown to increase immediate early gene and electrophysiological responses in the auditory system (Arch and Narins, 2009; Lynch and Wilczynski, 2008; Maney and Pinaud, 2011; Penna et al., 1992; Yoder and Vicario, 2012). Here, we found that the responsiveness of the auditory midbrain to acoustic communication signals can be similarly enhanced by chronic exposure to dynamic conspecific signals. However, it is unclear at present whether enhanced neural responsiveness is a direct result of acoustic stimulation, an indirect result of acoustic stimulation elevating systemic or brain-derived hormones levels, or some combination of the above.

In this study we used expression of the immediate early gene *egr-1* as a surrogate for neural excitation, an approach that has frequently been used to explore the reception of conspecific mating signals (Hoke et al., 2004; Sockman et al., 2005; Maney et al., 2006; Wong et al., 2012). It is important to note, however, that immediate early genes are themselves important regulators of further genomic action. *Egr-1* is an inducible transcription factor that is linked to membrane depolarization by a second messenger cascade. EGR-1 binds to a short consensus sequence found in many genes and can act both as an activator and as a repressor of transcription. Downstream targets of immediate early genes include trophic factors, constituents of axons and presynaptic terminals, such as neurofilament and synapsin, and enzymes involved in neurotransmitter synthesis (reviewed in Clayton, 2000). As such, EGR-1 could be involved in both short-term experience-dependent synaptic change and long-term consolidation through synapse building. This action is believed to provide enhanced selectivity for the behaviorally relevant stimuli that can induce learning and memory formation. This enhanced *egr-1* expression may feed-forward to enhance the selectivity of the system for activation in response to future stimulation with behaviorally relevant stimuli, thereby continually reinforcing the greater activation of auditory pathways to socially relevant signals.

Conclusions

In summary, we found that experience with a social communication signal modulated the responsiveness, as measured with early immediate gene expression, of the auditory midbrain to conspecific communication signals. We believe this is the first report of stimulation with an assemblage of mate attraction signals enhancing future sensory processing in anurans. Modulating the sensory processing of communication signals through previous reception may be particularly important for coordinating reproductive efforts in species with dynamic mating assemblages. Previous work has shown that communication signals can influence hormone levels, and hormone levels can influence the reception of communication signals. We now show that the same type of social stimulation that elevates gonadal steroid levels also increases the responses of midbrain neurons to conspecific communication signals. This work raises a number of questions that should be explored in future work including: what mechanisms are responsible for acoustically modulating auditory responses, and, in particular, are endocrine and sensory changes functionally linked?; where in the auditory system do these effects emerge?; what is the functional significance of acoustically modulating auditory responses?

MATERIALS AND METHODS

Auditory experiment

All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee (protocol A12036). The animals used in this experiment were acquired in November of 2012 through a commercial vendor (Big Apple Herp) that collected them in Florida. We tested a total of

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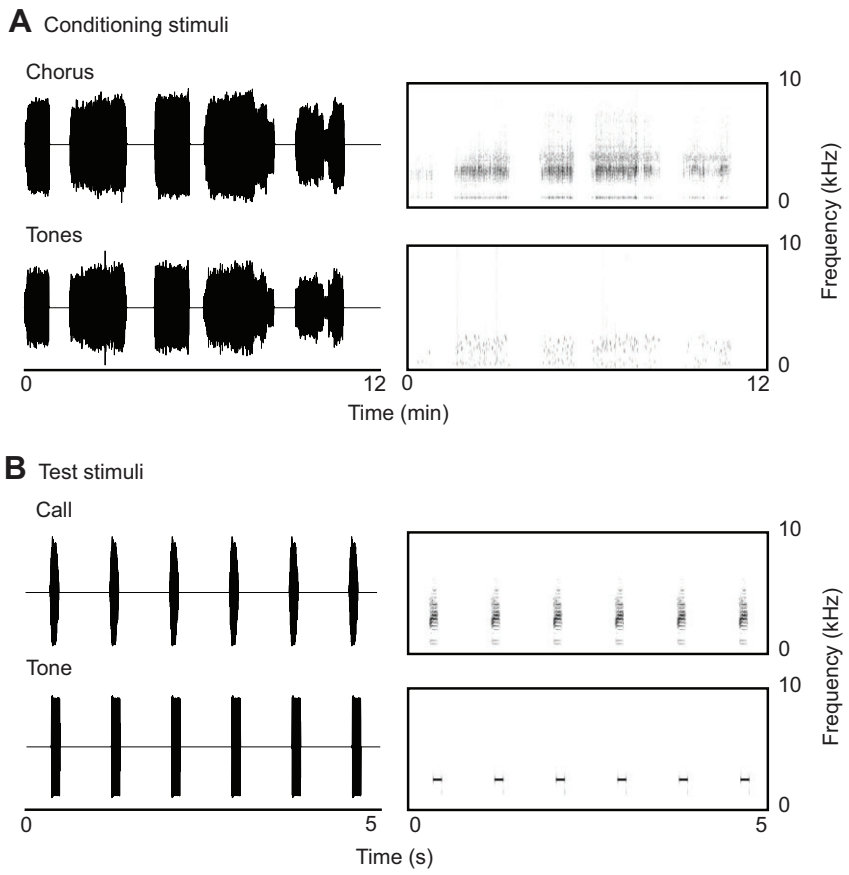

Fig. 3. Examples of stimuli used for the 10 day sound exposure period and the 30 min test period. The left-hand panel shows the waveforms of the stimuli and the right-hand panel shows the spectrograms of the stimuli. (A) Animals were exposed for 6 h to either a green tree frog chorus (including ambient environmental noise) or a series of tones with the same repetition rate, duration and amplitude envelope as the frog chorus for each of 10 days. (B) Animals were tested with either a single green tree frog call or a single tone repeated every 0.85 s. Tones and calls were matched for duration, amplitude and repetition rate. Each individual was tested with a different call or tone. Spectrograms were created in Praat ver. 5.3.23 with a 0.01 ms Gaussian window and a 40 dB dynamic range.

24 (12 males, 12 females) green tree frogs in late February and early March of 2013. Animals were maintained on a 14 h:10 h light:dark cycle. On the day prior to the start of the experiment, animals were placed in custom-built acoustically isolated chambers that contained a water dish, rock and artificial vegetation. Each sound chamber was also equipped with a speaker. Animals were randomly divided into two sound exposure groups: chorus and tones. The animals in the chorus group were exposed to 6 h of green tree frog chorus recordings and the animals in the tone groups were exposed to 6 h of random tone recordings for 10 consecutive days (Fig. 3A). The tone and chorus recordings were matched for call length, calling bout length and amplitude. Both were presented with a peak amplitude of 82 dB SPL, A weighting. The playback began 1 h after lights out. On day 11, all of the animals were kept in silence to minimize both habituation effects and system-wide *egr-1* expression. On day 12, animals were exposed to 30 min of the test stimulus, which consisted of either a single call or a single tone (~100 ms in length, repeated every 0.85 s) followed by 30 min of silence (Fig. 3A). A unique call or tone was used for each animal to avoid pseudoreplication. This resulted in four sound exposure × test stimulus groups (chorus–call, tones–call, chorus–tone, tones–tone). Animals were killed immediately after the silent period following the test stimulus (5 min in 0.2% MS-222). The brains were removed, embedded in tissue mounting media (Shandon M1 Embedding Matrix; Thermo Fisher Scientific) and frozen on dry ice. Embedded brains were stored at −80°C until sectioning (no more than 21 days). Brains were sectioned into four series (16 µm sections) on a Leica cryostat and mounted on FrostPlus slides. We stored the slides at −80°C until *in situ* hybridization.

Primer and probe design

We developed *in situ* hybridization procedures for the immediate early gene *egr-1* (also known as *zenk*) in *Hyla cinerea*. We isolated mRNA from the green tree frog brain with TRIzol (Invitrogen). We then created a cDNA library using a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). We designed species-specific primers (forward: TTC CAC AGC AAC AGG GAG ATG TCA; reverse: ATT CCT CAT GCA AAT GCG GCA CTG) using consensus sequences from several species of frogs and a single avian outgroup. Our target gene was amplified using a touch-down PCR and Platinum PCR SuperMix High Fidelity (Invitrogen). Amplification success was determined by gel electrophoresis. The band was extracted with a QIAquick gel extraction kit (Qiagen) and the PCR product was sequenced. Our product had >85% homology with genes from other frog species including *Physalaemus pustulosus*, *Xenopus tropicalis* and *Xenopus laevis*.

We cloned fresh PCR product into pCRII-TOPO TA vector (Invitrogen) and transformed chemically competent *E. coli*. Successful clones were identified by sequencing. Plasmids were harvested with a Plasmid Plus Maxi Kit (Qiagen) and linearized with *Eco*RV (anti-sense; Invitrogen) or *Hin*dIII (sense, Invitrogen) restriction enzymes. Radiolabeled probes (^{35}S UTP; Perkin Elmer) were generated by transcription with SP6 (anti-sense) or T7 (sense) promoters using a MAXIscript kit (Invitrogen). Unincorporated nucleotides were removed with NucAway spin columns (Invitrogen).

In situ hybridization

Slides were retrieved from the −80°C freezer and allowed to air dry at room temperature for ~30 min. Once the slides were dry they were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline (PBS; Fisher Scientific) for 2.5 min. Slides were then quickly rinsed in DEPC-treated water and tissue charge was neutralized in 0.05% acetic anhydride (Sigma) in 0.1 mol l^{-1} triethanolamine (TEA; Sigma) for 10 min. Slides were then dehydrated in an alcohol series (50, 75, 95, 100% in DEPC water) for 3 min each. Slides were then allowed to air dry for ~15 min prior to hybridization. Slides were rehydrated with 75 µl of 1× hybridization buffer containing the radiolabeled riboprobe (3×10^5 CMP per slide) and 0.01 mol l^{-1} dithiothreitol (Sigma). Slides were placed in humidified chambers and allowed to hybridize at 55°C overnight. The following day, we removed the coverslips and washed the slides in three washes of 2× SSC (saline sodium citrate) and one wash of 1× SSC (10 min each). The slides were then denatured in 50% formamide in 2× SSC at 52°C for 1.25 h. The formamide was removed in two washes of 0.1× SSC (30 min each) and then slides were dehydrated in an alcohol series. In a light-proof room the slides were dipped in NTB

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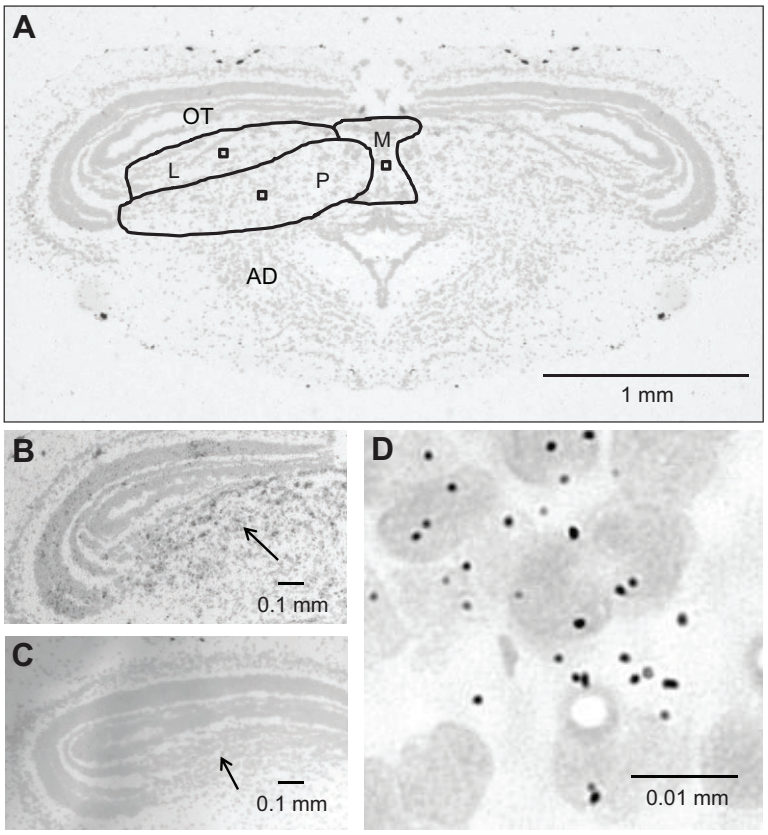

Fig. 4. Photomicrographs of green tree frog brains showing *egr-1* expression and typical cytoarchitecture. (A) Cresyl Violet-stained sections showing three subdivisions of the torus semicircularis: the laminar nucleus (L), principal nucleus (P) and midline nucleus (M). The torus lies between the optic tectum (OT) and the anterodorsal tegmentum (AD). To enhance clarity, the left-hand side of the image is a mirror image of the right hemisphere. (B) Sections hybridized with antisense probe showed the highest expression of *egr-1* in the toral subdivisions. (C) Sections hybridized with sense probes showed no *egr-1* expression. (D) Example of an image used for silver grain quantification. Images were taken with a 40× objective and subsequently divided into 100-image subsections. We counted the number of cells and the number of silver grains overlapping cell bodies.

autoradiography emulsion (Carestream Molecular Imaging) diluted 1:1 with distilled water and warmed to 42°C. The slides were allowed to dry overnight at room temperature. The slides were then placed in slide boxes with desiccant packs, wrapped with foil and stored at 4°C for 10 days. The slides were processed in D-19 developer (diluted 1:1 with distilled water; 4 min), rinsed with distilled water (10 s), fixed in Kodak fixer (5 min), and rinsed again in distilled water (5 min). All developing and fixing solutions were kept at 15°C. Fixed slides were counterstained with Cresyl Violet and coverslipped with Eukitt mounting medium (Sigma).

Imaging and statistics

Brain areas were imaged on an Olympus BX-41 microscope with a SPOT digital camera and software. We focused on three subdivisions of the torus that can be identified by their cytoarchitecture and have previously been shown to respond selectively to conspecific stimuli in other frog species: the laminar nucleus, the principal nucleus and the midline area (Hoke et al., 2004; Hoke et al., 2008; Mangiamele and Burmeister, 2011). We randomly selected a hemisphere and took a single 40× image of each of the three brain areas (Fig. 4). The image was centered in the brain area of interest. The number of sections we sampled varied across animals (four to eight sections) with an average of five sections per animal. One individual was excluded from the analysis because the tissue sections containing the torus were damaged. In ImageJ we place a 10×10 grid on the image, which divided the image into 100 equal parts. Using a random number table we randomly selected the *x*- and *y*-coordinates of 10 sub-areas to sample. We counted the number of cells and the number of silver grains in each cell using the ImageJ counter plugin. We then calculated the average number of silver grains per cell in each brain area for each animal.

We analyzed our data with a repeated measures linear mixed model in SPSS 19. The dependent variable in the model was average silver grains per cell. The independent variables were sex (male or female), brain area (laminar nucleus, midline nucleus, principal nucleus), sound exposure (chorus or tones), the 30 min test stimulus (call or tone) and their interactions. Brain area was a within-subject factor, modeled with an unstructured covariance structure (the model was not qualitatively different using compound symmetry or autoregressive covariance structures). Non-significant higher order interactions were removed from the model according to *P*-value and the resulting AIC value for the new model. The only significant interaction term that remained in the final model was brain area × test stimulus. The test stimulus × condition stimulus interaction was an important test of our hypothesis, so we left it in the model, although it was not significant (this did not qualitatively affect the other model terms). Significant main effects and interaction terms were explored *post hoc* with tests of simple effects or pairwise comparisons. Statistics were Bonferroni corrected for multiple comparisons as appropriate. We cube-root transformed the dependent variable (silver grains per cell) to achieve normality and homogeneity of variance. We therefore report back-transformed marginal means (±s.e.) throughout.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.D.G. and W.W. jointly contributed to the conception and design of the experiments, interpretation of the findings being published and the drafting and revising of the article. M.D.G. was principally responsible for the execution of the experiments.

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