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RESEARCH ARTICLE

Auditory selectivity for acoustic features that confer species recognition in the túngara frog

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SUMMARY

In anurans, recognition of species-specific acoustic signals is essential to finding a mate. In many species, behavioral tests have elucidated which acoustic features contribute to species recognition, but the mechanisms by which the brain encodes these species-specific signal components are less well understood. The túngara frog produces a 'whine' mating call that is characterized by a descending frequency sweep. However, much of the signal is unnecessary for recognition, as recognition behavior can be triggered by a descending two-tone step that mimics the frequency change in a portion of the whine. To identify the brain regions that contribute to species recognition in the túngara frog, we exposed females to a full-spectrum whine, a descending two-tone step that elicits recognition, the reversed two-tone step that does not elicit recognition, or no sound, and we measured expression of the neural activity-dependent gene *egr-1* in the auditory brainstem and thalamus. We found that the behavioral relevance of the stimuli was the best predictor of *egr-1* expression in the laminar nucleus of the torus semicircularis but not elsewhere. That is, the laminar nucleus responded more to the whine and the two-tone step that elicits recognition than to the reversed two-tone step. In contrast, in other brainstem and thalamic nuclei, whines induced *egr-1* expression but tones did not. These data demonstrate that neural responses in the laminar nucleus correspond to behavioral responses of females and they suggest that the laminar nucleus may act as a feature detector for the descending frequencies characteristic of conspecific calls.

Key words: acoustic communication, call recognition, torus semicircularis, *Physalaemus* (=*Engystomops*) *pustulosus*.

INTRODUCTION

Sensory systems respond selectively to information in the environment, extracting some stimulus features while discarding others. Tinbergen elegantly demonstrated the role of stimulus features in eliciting behavior when he showed that sticklebacks attack a model fish if the model has an essential feature, a red belly (Tinbergen, 1951). The sensory mechanisms of species recognition can be thought of as a special case of this fundamental principle. Species-specific signals are often complex, even multimodal, but species recognition may depend only on a subset of features in natural signals. Thus, although we expect sensory systems to have general mechanisms that allow them to represent complex, species-specific signals, only some of these mechanisms are expected to contribute to species recognition *per se*. To better understand the sensory mechanisms of species recognition, therefore, it is necessary to focus on mechanisms that extract the salient components of species-specific signals. In order to identify these mechanisms, we must first identify the brain regions that respond selectively to the signal components that confer species recognition.

To identify brain regions that contribute to species recognition, we chose to examine auditory responses to species-specific signals and their salient components in the túngara frog, *Physalaemus* (=*Engystomops*) *pustulosus* (Cope 1864). Among species of *Physalaemus*, mating calls are characterized by a descending frequency-modulated (FM) sweep, or 'whine', that is necessary and sufficient for species recognition (Rand et al., 1992). The structure of the FM sweep varies among species (Ryan and Rand, 2001; Ryan et al., 2003) and behavioral studies indicate that specific features of the FM signal can account for patterns of mate recognition. Whereas the harmonics of the fundamental frequency of the whine are unnecessary (Rand et al., 1992), the direction of frequency modulation (high to low) is critical (Rose et al., 1988; Ryan, 1983). Stimulation anywhere in a high-frequency channel (900–560 Hz) followed by stimulation in a low-frequency channel (640–500 Hz) can elicit recognition, but no single frequency within each channel is critical for this response (Wilczynski et al., 1995). Thus, in túngara frogs, species recognition can be triggered by a simple descending frequency step that mimics a particular portion of the whine. Although we know something about how the anuran brain encodes amplitude-modulated signals (e.g. Feng et al., 1990; Leary et al., 2008; Rose and Capranica, 1994), we know little about how it encodes these types of changes in frequency (i.e. frequency steps) that are critical for species recognition in the túngara frog [although see Narins et al. (Narins et al., 1983)].

Studies that map auditory responses using activity-dependent gene expression have shown that, in the túngara frog, the auditory brainstem and its forebrain targets respond robustly to conspecific but not heterospecific calls (Chakraborty et al., 2010; Hoke et al., 2004; Hoke et al., 2008). However, these previous studies used natural calls and, because natural calls contain acoustic information that is unnecessary for species recognition, these prior studies may not have narrowly identified brain regions that contribute to species

recognition. To address this gap, we presented female túngara frogs with acoustic stimuli that vary in their ability to confer species recognition and characterized responses by measuring expression of the activity-dependent gene early growth response 1 (*egr-1*). Specifically, we presented females with a full-spectrum whine, a descending two-tone step that elicits recognition, the reversed two-tone step that does not elicit recognition, or no sound, and we measured *egr-1* expression in the auditory brainstem and thalamus. Based on prior studies showing that complex auditory responses emerge in the midbrain and thalamus (e.g. Edwards et al., 2002; Fuzessery and Feng, 1982; Fuzessery and Feng, 1983b; Hall and Feng, 1986; Hall and Feng, 1987), we predicted that the auditory midbrain and thalamus would respond to stimulus features that confer species recognition, but that the auditory hindbrain would respond to all acoustic stimuli. In contrast, we found that only one part of the auditory midbrain, the laminar nucleus of the torus semicircularis, responded to signal features that confer species recognition, suggesting that the laminar nucleus plays an especially important role in species recognition in the túngara frog.

MATERIALS AND METHODS

Acoustic stimuli

We created our whine stimuli by removing the chuck from three natural conspecific whine-chuck calls described in Ryan and Rand (Ryan and Rand, 2003) as 'Oc', 'M' and 'Sd'. All whines had a similar duration (mean ± s.d.=335.80±6.27 ms) and dominant frequency (mean ± s.d.=785.96±21.55Hz) and were at or close to the mean of the local population (Gamboa, Panama).

We based our tones stimuli on those used in the phonotaxis tests described in Wilczynski et al. (Wilczynski et al., 1995). Wilczynski et al. demonstrated that a two-tone, descending frequency step elicits recognition in female túngara frogs whereas the reversed two-tone step does not elicit recognition (Wilczynski et al., 1995). In that experiment, recognition was defined as a positive phonotactic response to the tone stimulus when it was paired against a noise stimulus. Tones stimuli consisted of a two-tone sequence with a total duration that approximates a natural whine and was partitioned as follows: 800 Hz for 100 ms followed by 500 Hz for 200 ms (800+500) or 500 Hz for 100 ms followed by 800 Hz for 200 ms (500+800). The frequencies and durations of the tones in the 800+500 two-tone step mimic the fundamental of a natural whine call, which begins at 900 Hz and sweeps to approximately 400 Hz in 300 ms, and the shorter duration of the 800 Hz tone (100 ms) approximates the time over which the fundamental sweeps to half its initial frequency. For the 500+800 stimulus, the sequence of the tones was reversed whereas the overall temporal structure of the two-tone step (a 100 ms tone followed by a 200 ms tone) was maintained. We shaped tones stimuli by the amplitude envelope of the whine exemplars described above so that each stimulus group had three exemplars. This resulted in whines and corresponding tones stimuli that had the same temporal characteristics (e.g. signal duration, rise time and fall time) and amplitude envelope. All stimuli were analyzed, synthesized and/or processed using Signal sound analysis software (Engineering Design, Berkeley, CA, USA).

Because we wanted to broadcast the frequencies in our acoustic stimuli without amplification or attenuation of a particular frequency band, we modified the stimuli to account for the frequency response characteristics of our amplified speaker system. Briefly, we used Vibrotoolbox (M. Gridi-Papp, University of the Pacific, Stockton, CA, USA) to create a transfer function that represented the frequency response of our speakers (PAL speaker, Tivoli Audio, Cambridge, MA, USA) between 100 and 6000 Hz. We then filtered each acoustic stimulus by the inverse of this transfer function, which effectively created a flat frequency response for the speaker during stimulus playback. Each playback consisted of a single call exemplar repeated every 2 s in order to approximate the average calling rate of túngara males. We set playback amplitude at 82 dB SPL (re. 20 µPa) at 25 cm from the speaker.

Animals

We captured female túngara frogs (*P. pustulosus*) in amplexus near Gamboa, Panama, from 29 October to 25 November 2007 between 20:00 and 22:45 h, transported them back to the laboratory of the Smithsonian Tropical Research Institute, and isolated them in individual dark acoustic chambers for 10 h. We lined the floors of the chambers with wet paper towels to prevent dehydration and enclosed the animals within circular mesh arenas (8 cm diameter). After the acclimation period, we exposed females to playbacks of descending tones (800+500; *N*=8), ascending tones (500+800; *N*=9) or whine calls (W; *N*=9) for 30 min followed by 30 min of silence. Animals were then killed by rapid decapitation. Other females (*N*=6) received no acoustic stimulation and were killed 1 h following the acclimation period. One hour after stimulus onset corresponds to peak accumulation of acoustically induced *egr-1* mRNA expression (Burmeister et al., 2008), and occurs before habituation of the *egr-1* response (R. M. Glaeser, L.A.M. and S.S.B., unpublished observation).

The government of Panama permitted tissue collection and export (permit nos SE/A-99-07, SEX/A-133-07), and the University of North Carolina Institutional Animal Care and Use Committee approved our experimental procedures (protocol no. 08-015).

Tissue preparation and *in situ* hybridization

We fixed females' brains in 4% paraformaldehyde for 10 min, embedded them in OCT embedding medium (Sakura Finetek, Torrance, CA, USA), and rapidly froze them in liquid nitrogen. We transported them to our University of North Carolina at Chapel Hill laboratory on dry ice and stored them at –80°C until sectioning. We sectioned brains on a cryostat at 16 µm thickness and mounted them onto slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA). We created radioactively labeled (^{35}S) sense and antisense probes by reverse transcription from plasmids containing *P. pustulosus egr-1* cDNA (GenBank accession no. AY562993) and performed *in situ* hybridization according to the protocol described in Burmeister et al. (Burmeister et al., 2008). All slides were processed simultaneously to eliminate variation between procedures. To visualize the bound riboprobe, we dipped slides in Kodak NTB emulsion, allowed them to dry, and stored them in lightproof boxes at 4°C for 14 days before developing with Kodak D-19 developer and Kodak fixer and counterstaining with thionin. We confirmed the binding specificity of our *egr-1* riboprobe by comparing antisense binding with sense binding under identical hybridization conditions. In tissue hybridized with the sense probe, no binding was evident (Fig. 1). Therefore, we can infer that the binding we observed in tissue hybridized with the antisense probe is a result of specific binding. In addition to probe specificity, our method identifies regionally specific *egr-1* expression in response to sound. For example, mating calls induce significantly elevated *egr-1* levels in the auditory midbrain but not in the optic tectum (see Hoke et al., 2004; Burmeister et al., 2008) (Fig. 1) or olfactory bulb (Mangiamele and Burmeister, 2008), indicating that species-specific sounds result in regionally specific *egr-1* expression and do not

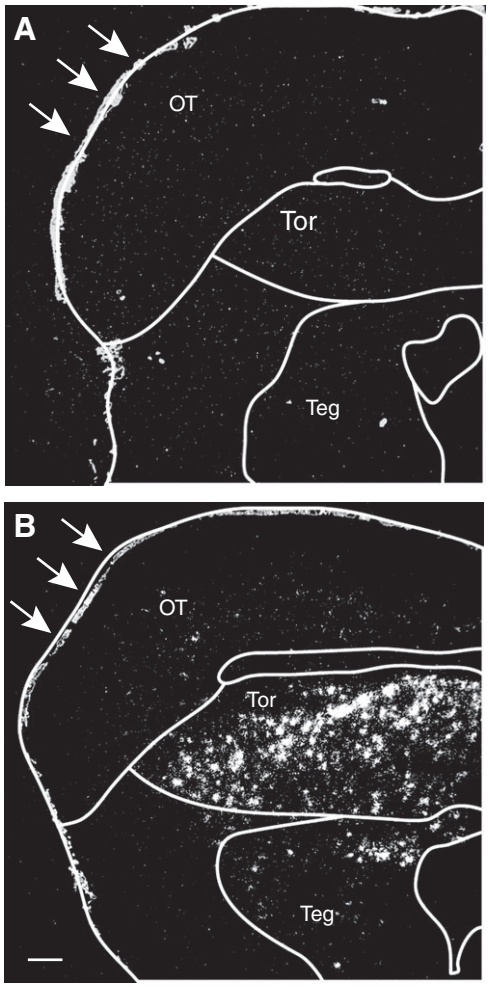

Fig. 1. Specificity of our *egr-1* riboprobe. Darkfield images of transverse sections of túngara frog midbrain hybridized with (A) sense or (B) antisense riboprobes. White spots (silver grains) represent radioactively labeled *egr-1* mRNA. The right edge of each photomicrograph is aligned with the midline of the midbrain. Arrows indicate edge of tissue section. Anatomical boundaries of the optic tectum (OT), torus semicircularis (Tor) and tegmentum (Teg) are outlined in white. Photomicrographs were taken with a 10× objective. Scale bar, 100 μm.

produce a generalized *egr-1* response because of heightened arousal, for example.

Quantification of *egr-1* expression

We measured *egr-1* expression in nuclei of the ascending auditory pathway. We sampled from the superior olivary nucleus (SON), the principal, magnocellular and laminar nuclei of the torus semicircularis (Ptor, MCtor and Ltor, respectively), and the posterior, central and anterior thalamus (Pthal, Cthal and Athal, respectively) (Fig. 2). We did not sample from the dorsal medullary nucleus (DMN; homolog of the cochlear nucleus) because *egr-1* expression in the DMN is not modulated by sound (Chakraborty et al., 2010). For all brain regions, we quantified *egr-1* mRNA expression using a 60× objective in one hemisphere of the brain chosen at random.

We quantified *egr-1* expression from digital photomicrographs taken with a SPOT FLEX camera attached to a Leica DM 5000B microscope. For each section, we took three images: a color image of Nissl-stained tissue in the brain region of interest, a blue-filtered image of only the silver grains in the same field of view (grains image) and a blue-filtered image of an area of the slide containing no tissue to represent local background silver grain density (background image). We measured local background silver grain density to account for emulsion thickness, which varies across the slide and influences silver grain density. In the blue-filtered images that we used to measure silver grain density, exposure, brightness and contrast settings were the same for each picture of a given section. We used ImageJ (National Institutes of Health, Bethesda, MD, USA) to convert the grains and background images to binary and to count the silver grains in each image using the analyze particles feature. This feature counts the number of discrete objects (silver grains or clusters of silver grains) in the image that have a minimum size of 1 pixel. We subtracted the number of background silver grains from the number of silver grains in the region of interest to get the number of silver grains above background per image. We then used the point selection tool in ImageJ to mark and count all visible cells in the color image of the region of interest. We identified cells visually by their clearly stained soma and cell counting was performed by experienced individuals who were blind to treatment. Our final measure of *egr-1* mRNA expression for each section was the number of silver grains above background per cell. We calculated mean *egr-1* expression from three consecutive sections per individual for each brain region. Although all statistical analyses were performed on silver grains per cell above background, we graphed *egr-1* levels in each brain region as fold change relative to the no-sound group by dividing each individual's value by the mean for the no-sound group. Using fold change to visualize our data provides ready comparison of the stimulus-induced *egr-1* expression patterns among brain regions by adjusting them all to the same *y*-axis. It is important to note that the fold-change data show the same pattern and magnitude of differences among stimulus groups and it does not affect the magnitude of the error bars relative to the means.

Statistical analysis

We were interested in determining whether signal components that confer species recognition elicit differential *egr-1* expression compared with stimuli that are not recognized and, if so, where in the auditory system this effect emerges. To address these questions, we used both null-hypothesis testing and information-theoretic approaches, as follows. To determine whether the stimuli influenced *egr-1* expression and whether this effect varied among brain regions, we used a hierarchical linear model (ANOVA) with brain region, acoustic stimulus and their interaction as fixed factors and individual as a random factor. We nested brain region within individual because *egr-1* measurements from different regions in the same brain are likely to be correlated. The inclusion of brain region in this model resulted in the exclusion of eight subjects that did not have data for every brain region. We then conducted separate one-way ANOVAs for each brain region to test whether *egr-1* levels varied with acoustic stimulus and we followed up the ANOVAs with *post hoc t*-tests to compare all pairs of groups. Because we did not have data from all subjects for all brain regions, sample sizes varied among brain regions, as shown in Fig. 3 and as reflected by the degrees of freedom shown in Table 1.

The ANOVAs and *t*-tests allowed us to determine whether our manipulations influenced *egr-1* expression, but they do not allow us to draw strong conclusions about which of several competing models best explain our data because a *P*-value for an ANOVA or *t*-test does not indicate anything about the underlying pattern of

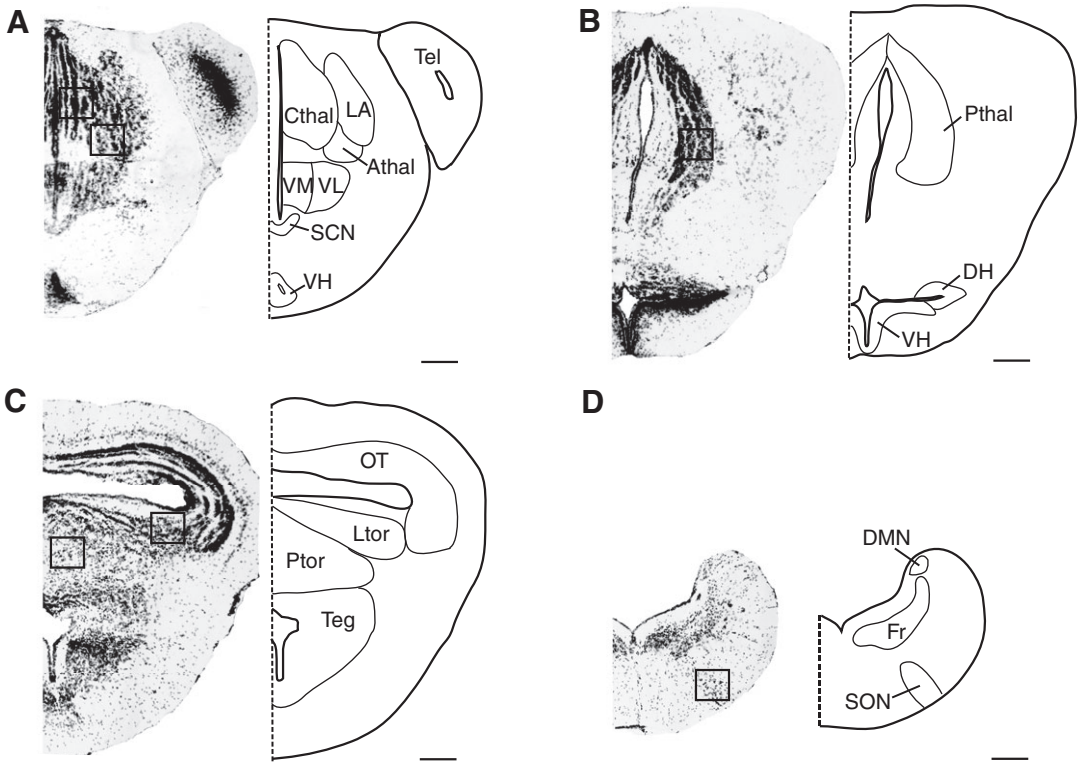

Fig. 2. Photomicrographs of Nissl-stained túngara frog brain tissue and corresponding schematic diagrams of auditory brain regions sampled (MCtor not shown). Transverse sections are arranged from rostral to caudal (A–D). Boxes indicate sampling window. See List of abbreviations for definitions. Scale bars, 200 μm.

differences among groups, only whether some difference exists. Therefore, considering each brain region separately, we next used an information-theoretic approach to identify the model that best explains variation in *egr-1* expression. This approach allows us to determine whether the data better fit one pattern of results versus another pattern. To do this, we built three models that reflected our alternative hypotheses: (1) *egr-1* expression in response to recognized stimuli was different from that in response to unrecognized stimuli, (2) *egr-1* expression in response to whine was different from that in response to tones and (3) all sounds elicited equal *egr-1* expression. For each of our three models, we used a mixed model procedure to analyze *egr-1* as a function of acoustic stimulus and used contrast coding to specify the groups that we wanted to compare. We excluded the control group (no sound) from the models because in this analysis we were interested only in the relationship of the responses to natural whines and tones stimuli. For each brain region, we compared Akaike's information criterion (AIC) values to determine which model best described the *egr-1* expression pattern we observed (i.e. has the lowest AIC value), as lower AIC values indicate a more parsimonious model that better fits the data (Burnham and Anderson, 2004). As opposed to traditional null-hypothesis testing, which uses an alpha threshold (e.g. 0.05) to test for differences (any differences) among groups, this information-theoretic approach provides more information by

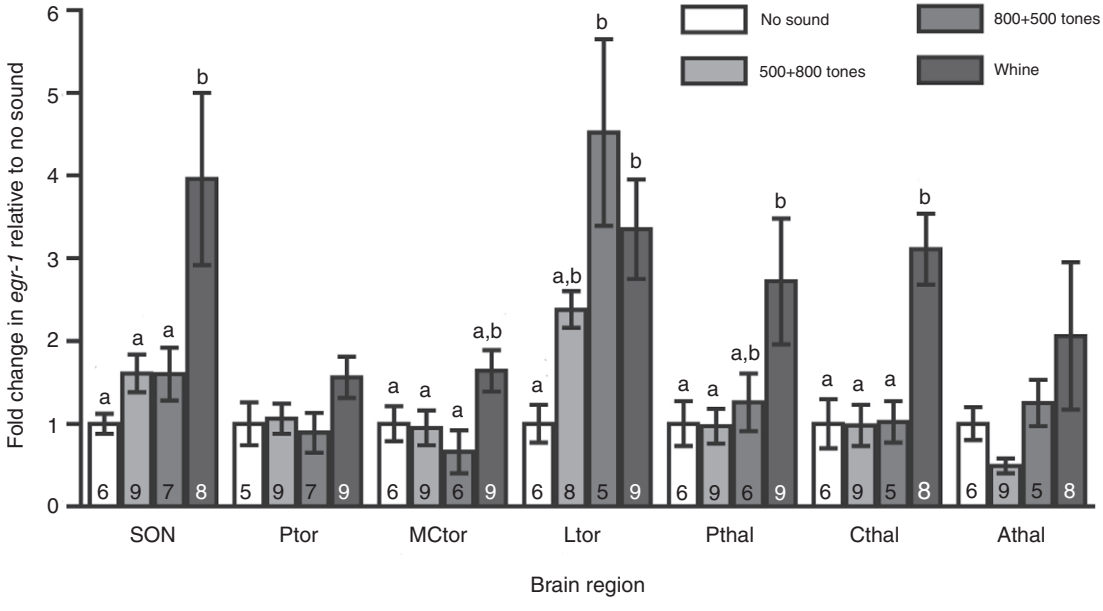

Fig. 3. Effect of whine and tone stimuli on *egr-1* expression in nuclei of the ascending auditory system. Data shown are mean (±s.e.m.) fold changes in *egr-1* expression relative to the silent (no sound) control group. Sample sizes are indicated for each group and letters above bars denote groups that are statistically different (*post hoc t*-test, $P<0.05$). See Table 1 for ANOVAs and corresponding *P*-values. See List of abbreviations for definitions of brain regions.

Table 1. Main effects of acoustic stimulus on *egr-1* expression in brain regions of the ascending auditory system of túngara frogs

Brain region	d.f.	*F*	*P*
SON	3, 26	4.89	0.008
Ptor	3, 26	1.83	0.17
MCtor	3, 26	3.18	0.04
Ltor	3, 24	5.46	0.005
Pthal	3, 26	3.04	0.046
Cthal	3, 24	10.88	0.0001
Athal	3, 24	1.78	0.18

See List of abbreviations for brain region definitions.

determining the model that best describes group differences. For example, this approach allows us to determine whether hearing a whine *versus* hearing tones explains *egr-1* expression better or worse than hearing recognized stimuli *versus* unrecognized stimuli. We used JMP version 7 (SAS, Cary, NC, USA) for ANOVAs and linear models and STATA (StataCorp, College Station, TX, USA) for AIC analyses.

RESULTS

Using null-hypothesis testing, we found that patterns of stimulus-evoked *egr-1* expression differed among brain regions (stimulus × region, $F_{11,70}$=2.16, P=0.03) and, in all brain regions except the Ptor and the Athal, we found a main effect of acoustic stimulus on *egr-1* expression (Table 1; Fig. 3). Using AIC analyses to identify the model that best explains variation in *egr-1*, we found that behavioral relevance was the best predictor of *egr-1* expression in the Ltor, but not other brain regions (Table 2). That is, in the Ltor, *egr-1* expression was elevated in females hearing acoustic stimuli that are recognized as conspecific calls compared with those hearing non-salient tones stimuli (Fig. 3; Fig. 4A–C). In addition, although not statistically distinguishable from no sound controls (P=0.10), ascending tones (500+800) elicited an intermediate level of *egr-1* expression (Fig. 3; Fig. 4C), which may reflect the fact that Ltor responds to salient frequencies in the stimulus regardless of their temporal arrangement, or that Ltor is sensitive to both ascending and descending FM signals. In contrast, in most other parts of the auditory brainstem and thalamus, *egr-1* expression patterns were best explained by the model that contrasted the whine with the tones stimuli (Table 2), indicating that the greater spectral complexity of the whine was necessary to elicit *egr-1* expression in these brain regions. One exception was the Athal, for which AIC analyses did not provide strong guidance as to which pattern of results best explains the data, as we found equal support for models contrasting recognized with unrecognized stimuli and whine with tone stimuli. In the SON, Cthal and Pthal, hearing a whine call induced an increase in *egr-1* mRNA expression compared with no sound and tones, whereas the tones failed to induce *egr-1* expression (Fig. 3), despite the fact that túngara females respond behaviorally to descending tones (800+500). Similarly, in the MCtor, Ptor and Athal, we found that hearing a whine induced an increase in *egr-1* expression compared with no sound, but the trends were not statistically significant (P=0.07, 0.12 and 0.18, respectively).

DISCUSSION

Using natural mating calls, previous studies in the túngara frog found that conspecific calls elicit *egr-1* expression throughout the auditory system and its forebrain targets but heterospecific calls do not (Chakraborty et al., 2010; Hoke et al., 2004; Hoke et al., 2008). Those results suggest that the auditory system of túngara frogs is selective for conspecific calls, but the data do not identify brain regions contributing to species recognition *per se*, because natural mating calls contain more acoustic energy than is necessary for species recognition. For example, although the whine is an FM signal with a rich harmonic structure, the energy contained in a narrow frequency channel between 900 and 500 Hz is sufficient for call recognition to occur (Wilczynski et al., 1995). In the present study, we found that a two-tone, downward frequency step that elicits recognition causes *egr-1* expression in the Ltor, but not other parts of the auditory hindbrain, midbrain or thalamus. These results suggest that the Ltor may be acting as a 'neural analyzer for call recognition' (*sensu* Wilczynski et al., 1995) because its response properties closely follow the rules that govern females' responses in behavioral tests of call recognition. In contrast, only the full spectrum whine elicited significant *egr-1* expression in other parts of the auditory brainstem and thalamus, suggesting that neural activity patterns do not correspond with females' behavioral responses in these brain regions. Thus, although a general pattern of neural responsiveness to conspecific calls is apparent throughout the auditory system and its forebrain targets, only the Ltor appears to respond specifically to stimuli that elicit species recognition behavior. Although our data cannot determine whether species recognition emerges from selectivity in the Ltor alone or whether other brain nuclei also participate, our results are consistent with the idea that Ltor activation is sufficient to explain the species recognition decisions of female túngara frogs.

Our data indicate that *egr-1* expression in the Ltor reflects species-recognition processes, but whether the Ltor's response reflects perceptual aspects of species recognition or behavioral (i.e. motor) aspects of species recognition is unclear. FM stimuli that are recognized (whine and descending tones) generated the greatest response in the Ltor, whereas FM stimuli that are not recognized (ascending tones) appeared to generate an intermediate response. As a consequence, our data are consistent with the idea that the Ltor is sensitive to the FM structure of sounds, and that the most salient FM sounds are those characterizing conspecific calls. Although some have proposed that subdivisions of the torus are dedicated to processing particular aspects of sound (i.e. temporal

Table 2. Akaike's information criterion values corresponding to three different models contrasting *egr-1* expression in female túngara frogs listening to whine (W), descending tones (800+500) or ascending tones (500+800)

	Code in statistical model			Brain region						
Model	W	800+500	500+800	SON	Ptor	MCtor	Ltor	Pthal	Cthal	Athal
Recognized *vs* not recognized	1	1	0	85.73	124.56	47.36	**107.26**	115.68	112.97	**83.82**
Whine *vs* tones	1	0	0	**79.86**	**120.69**	**41.32**	110.33	**112.57**	**100.78**	**83.70**
All sounds	1	1	1	86.55	125.26	45.07	110.64	118.64	119.10	86.10

Lower AIC values (highlighted in bold) indicate a better fit for the specified data set.
See List of abbreviations for brain region definitions.

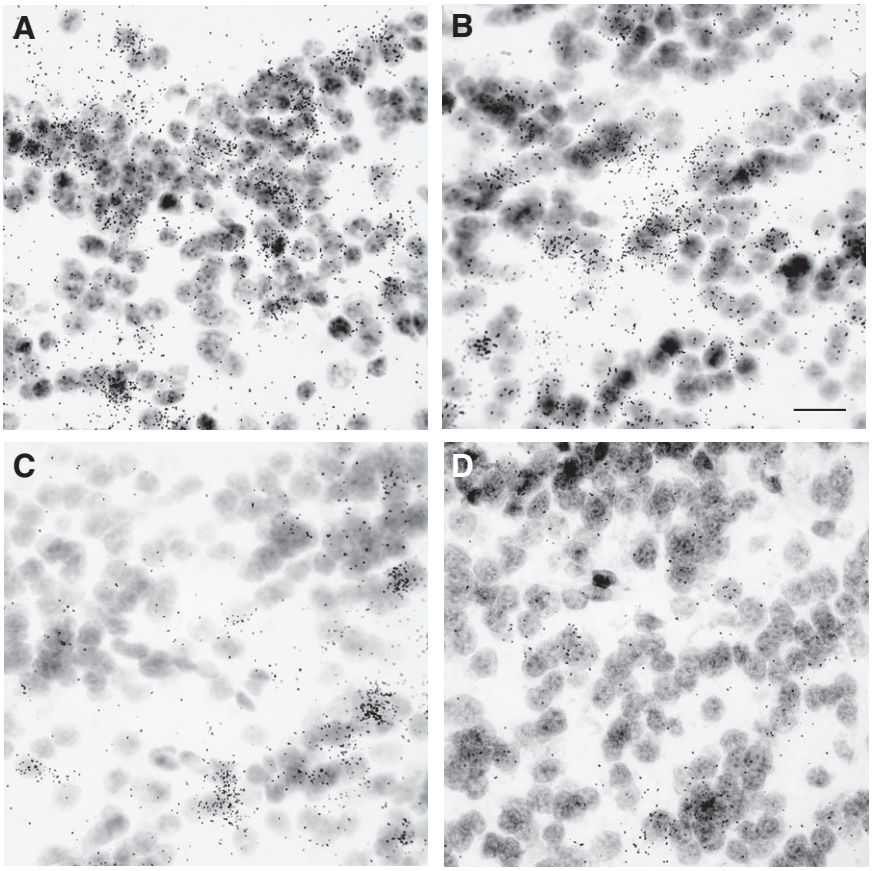

Fig. 4. Photomicrographs showing *egr-1* levels in the laminar nucleus of the torus semicircularis of female túngara frogs exposed to (A) whine, (B) 800+500 tones, (C) 500+800 tones or (D) no sound. These photomicrographs are a subset of those used for quantitative analysis of *egr-1* expression. The area covered by black spots (silver grains) represents *egr-1* expression. Cell bodies are stained with thionin. Photomicrographs were taken with a 60× objective. Scale bar, 20 μm.

and spectral information) (Feng and Lin, 1991), to our knowledge none have speculated on a specialization for FM processing. Alternatively, our data are consistent with a scenario in which *egr-1* expression in the Ltor reflects the audio-motor integration underlying the behavioral decision to approach a conspecific call. Anatomy and physiology support the idea that the Ltor is an audio-motor integrator (Endepols and Walkowiak, 1999; Endepols and Walkowiak, 2001; Neary, 1988). Furthermore, the Ltor receives widespread neuromodulatory input, including serotonin, dopamine and estradiol (Endepols et al., 2000). If the response patterns of the Ltor reflect behavioral (i.e. motor) rather than sensory aspects of species recognition, then it should also show differential *egr-1* expression in response to recognized stimuli in species whose mating calls are not frequency modulated. Additional studies in other species and complementary electrophysiology experiments would both contribute significantly to clarifying the functional role of the Ltor in species recognition.

Our results, combined with the behavioral studies of Wilczynski et al. (Wilczynski et al., 1995), indicate that the relevant acoustic feature of the conspecific call that elicited an *egr-1* response in the Ltor is likely to be the direction of frequency modulation over time. However, because the durations of our 800 and 500 Hz tones were linked with their temporal order (i.e. the tone that came first was always shorter than the tone that came second), it is possible that *egr-1* in the Ltor was influenced by the durations of the tones, not their sequence. For instance, the Ltor might be more sensitive to longer stimulation at 500 Hz, which would elicit a response to whines and 800+500 tones, but not 500+800 tones. Although this remains a possibility, the temporal arrangement of frequencies in the whine has been demonstrated to be a more relevant feature for call recognition than the duration of low-frequency stimulation. Females will still approach a whine with the final 150 ms deleted (which includes frequencies between 520 and 400 Hz), but they do not recognize full-spectrum whines that are reversed (Ryan, 1983; Wilczynski et al., 1995). Nevertheless, future studies should test whether the Ltor is sensitive to the specific durations of the spectral components in the túngara frog whine in order to better characterize the response properties of the Ltor.

Interestingly, ascending tones (500+800) elicited a small but non-significant increase in *egr-1* expression in the Ltor, indicating that some neurons in the Ltor are responding to the presence of those frequencies alone, without the species-typical temporal cues. Indeed, a small proportion of torus semicircularis neurons are so broadly tuned that they will fire in response to almost any frequency in the frog's hearing range (Fuzessery, 1988). The intermediate *egr-1* response to 500+800 Hz tones could also be a result of inhibitory interactions. For example, hearing a 500 Hz tone before an 800 Hz tone may inhibit many Ltor neurons from firing action potentials, leading to decreased levels of *egr-1* in response to ascending tones when compared with descending tones. A similar mechanism has been invoked to explain selectivity for the downward sweep of the echolocation pulse in the auditory midbrain of the pallid bat, where early low-frequency stimulation triggers inhibitory potentials, thus preventing neural responses to upward sweeps (Fuzessery et al., 2006). The same may be true for the anuran auditory system, as a majority of neurons in the torus semicircularis tuned to higher frequencies exhibit low-frequency inhibition (500 Hz or below), and *vice versa* (Fuzessery and Feng, 1982; Walkowiak, 1980). Thus, perhaps the relative timing of inhibitory and excitatory inputs allows Ltor neurons to respond selectively to FM sweep direction.

In spite of the fact that túngara females themselves respond behaviorally to descending tones, we found no significant *egr-1* induction in response to 800+500 Hz stimuli in the SON or the thalamus, suggesting that these brain regions need the whine, with its greater spectral complexity, to mount a significant genomic response. The significance of this remains unclear without complementary electrophysiological data. However, it may simply reflect the distribution of spectral energy in the stimuli. At the periphery, the full-spectrum whine will evoke responses from a greater proportion of auditory units and it may be this greater magnitude of response that is required by the SON and thalamus to generate an *egr-1* response. For example, the SON as a whole is broadly tuned to a wide range of frequencies in the frog hearing range, but a majority of cells in the SON have V-shaped tuning curves centered around a single frequency or narrow range of frequencies (reviewed in Fuzessery, 1988) and none have been found to require multiple tone stimulation in order to fire (Fuzessery and Feng, 1983a). Thus, if 800 Hz or 500 Hz tones stimulated too few neurons in the SON, we might have failed to detect significant changes in *egr-1* expression because we took an average of the number of silver grains over a group of cells. In the thalamus, we predicted that whines and whine-like tone combinations (800+500) would elicit a similar level of neural activity, primarily because of the complex physiological response properties of thalamic neurons. For example, neurons in the Cthal have broad frequency responses and are selective for the temporal features of complex signals (Hall and Feng, 1986; Hall and Feng, 1987), whereas Pthal cells have narrower frequency tuning, but respond preferentially to specific two-tone combinations that characterize conspecific mating calls in *Rana pipiens* and *Hyla cinerea* (Fuzessery and Feng, 1983b; Hall and Feng, 1987; Mudry and Capranica, 1987). At this point, it is unclear why the thalamus failed to respond to whine-like tones in túngara frogs. However, *egr-1* expression in the Cthal and the Pthal is selective for conspecific calls over heterospecific calls (Chakraborty et al., 2010; Hoke et al., 2007) and covaries with movement (Hoke et al., 2007), indicating that the thalamus may still play an as yet undefined, but important, role in call recognition. It is also possible that the thalamus contributes to other aspects of auditory processing not specifically related to species recognition.

In summary, previous studies have reported auditory responses to natural conspecific calls in the túngara frog and have described a remarkable selectivity for conspecific sounds (Chakraborty et al., 2010; Hoke et al., 2004). We, too, found that natural conspecific calls elicit robust responses from the auditory hindbrain, midbrain and thalamus. However, only the Ltor was selective for the acoustic features that elicit species recognition. The data contrasted with our expectations of a hierarchical model in which higher stations within the auditory processing stream have greater complexity and selectivity for behaviorally relevant components of acoustic signals. Instead, the picture that is emerging from *egr-1* expression patterns (this study), anatomy (Endepols et al., 2000; Feng and Lin, 1991) and physiology (Endepols and Walkowiak, 2001) is one where the Ltor is at the center of both ascending and descending streams, integrating a variety of information and directly influencing acoustically guided behavior.

LIST OF ABBREVIATIONS

AP	amphibian papilla
Athal	anterior thalamus
BP	basilar papilla
Cthal	central thalamus
DH	dorsal hypothalamus
DMN	dorsal medullary nucleus
Fr	reticular formation
LA	lateral amygdala
Ltor	laminar nucleus of torus semicircularis
MCtor	magnocellular nucleus of torus semicircularis
OT	optic tectum
Pthal	posterior thalamus
Ptor	principal nucleus of torus semicircularis
SCN	suprachiasmatic nucleus
SON	superior olivary nucleus
Teg	tegmentum
Tel	telencephalon
Tor	torus semicircularis
VH	ventral hypothalamus
VL	ventrolateral thalamus
VM	ventromedial thalamus

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