

Acoustically Evoked Immediate Early Gene Expression in the Pallium of Female Túngara Frogs

Lisa A. Mangiamele Sabrina S. Burmeister

Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, N.C., USA

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Key Words

Auditory processing · Telencephalon · Pallium · Túngara frog · *egr-1* · Immediate early gene

Abstract

In anurans, much is known about the role of the auditory midbrain in processing conspecific calls, but comparatively little is known about the role of the pallium. To address this deficiency, we investigated the induction of the immediate early gene *egr-1* by natural mate chorus in the medial, dorsal, lateral, and ventral pallium of female túngara frogs. We found strong acoustically evoked *egr-1* expression in the dorsal medial pallium ($p < 0.01$) and ventral pallium ($p = 0.02$), with a weaker effect in the lateral pallium ($p = 0.05$). In the ventral pallium, acoustically induced *egr-1* expression was stronger in the anterior portion. Measures of movement and olfactory activity could not explain a significant portion of acoustically evoked pallial *egr-1* expression. In contrast, *egr-1* expression in the auditory midbrain covaried with *egr-1* expression in the dorsal medial pallium and ventral pallium, suggesting that their activity was coupled with auditory activity. Taken together, these results suggest that the acoustically evoked *egr-1* expression in the dorsal medial pallium and ventral pallium were a direct result of auditory stimulation. Furthermore, although both anatomical and electrophysiological evidence demonstrate that multiple

modalities overlap in the frog pallium, our results show that a multimodal stimulus is not required to activate pallial neurons. Although the functional role of the frog pallium is not known, our results demonstrate that species-specific sounds activate spatially segregated and anatomically distinct areas of the frog pallium, inviting further investigation into the role of the frog pallium in acoustic communication.

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Introduction

Acoustic communication plays an essential role in mediating reproductive behavior in anuran amphibians (frogs and toads). Males produce advertisement calls during the breeding season primarily to attract females to mate [Gerhardt and Huber, 2002]. For females, who must distinguish among males of different species and conspecifics of varying quality, the perception and discrimination of male calls is vital to ensuring reproductive success [Andersson, 1994]. Understanding how females' brains process sexual signals is therefore important in elucidating the proximate mechanisms of mate choice behavior and, ultimately, sexual selection.

Anurans are good models for studying the neural mechanisms of acoustic communication because much is known about the neural substrate underlying call pro-

cessing and discrimination, particularly in the midbrain and thalamus. Single-unit physiological recordings reveal that auditory neurons in the midbrain and thalamus are tuned (i.e. respond best) to the calls of conspecifics [Fuzessery and Feng, 1983] and can perform temporal and frequency discrimination [Fuzessery and Feng, 1982; Hall and Feng, 1986; Gooler and Feng, 1992; Edwards et al., 2002]. Some researchers have proposed that the midbrain torus semicircularis also plays a direct role in acoustically guided behavior, such as female phonotaxis [Endepols and Walkowiak, 2001; Endepols et al., 2003]. For example, in female gray treefrogs (*Hyla versicolor*), lesions of the torus semicircularis abolish phonotaxis, whereas deep lesions of the thalamus leave this behavior largely intact and do not alter female call preferences [Endepols et al., 2003]. Only superficial lesions of the thalamus affect female call preference in some two-choice tests [Endepols et al., 2003], suggesting that dorsal thalamic nuclei are not critical for phonotaxis. These results imply that forebrain targets of the auditory system are not necessary for the regulation of sexual behavior in female anurans, and that recognition and complex feature analysis of acoustic signals are performed at the level of the midbrain. This view has influenced much of the current research on the neural bases of mating call recognition and discrimination in frogs [Endepols and Walkowiak, 2001; Hoke et al., 2004].

Unlike the pallium of amniotes, the anuran pallium appears to lack differentiated fields that specialize in processing sensory information [Butler and Hodos, 1996]. Instead, the dorsal and medial pallia are thought to be multimodal sensory processing and integration centers [Neary, 1984; Northcutt and Ronan, 1992; Laberge and Roth, 2007], whereas the lateral pallium is thought to primarily process olfactory input [Scalia et al., 1968; Northcutt and Royce, 1975]. Although the medial pallium is generally accepted as the homologue of mammalian hippocampus, some controversy exists over the organization and homology of the other pallial regions. Recently, developmental gene expression studies have revealed a fourth pallial region in frogs, the ventral pallium [Bachy et al., 2002; Brox et al., 2003, 2004]. The ventral pallium incorporates areas formerly known as the ventral lateral pallium and striatopallial transition area (SPTA) [Roth et al., 2004; also named anterior amygdala by Marin et al., 1998], and it might be homologous to parts of the anterior dorsal ventricular ridge in birds and part of the claustramygdalar complex in mammals [Brox et al., 2002; Molnar and Butler, 2002; Moreno and Gonzalez, 2004].

Whether the frog pallium plays a role in conspecific call recognition or in regulating the behavioral responses to calls remains to be determined. Auditory information probably reaches all pallial regions via afferents from the anterior nucleus of the dorsal thalamus, which terminate predominantly in the rostral half of the pallium [Kicliter and Northcutt, 1975; Kicliter, 1979; Neary, 1984, 1990; Northcutt and Ronan, 1992; Roth et al., 2003; Laberge et al., 2008]. These anterior thalamic projections might carry auditory, visual, and/or somatosensory information [Neary, 1990; Roth et al., 2003; Laberge and Roth, 2007]. In addition, auditory information reaches the medial pallium and ventral pallium (previously called the ventral part of the lateral pallium) from the ventromedial and central thalamic nuclei, respectively [Neary, 1990; but see Laberge et al., 2008]. Strong connections among pallial divisions [Northcutt and Ronan, 1992; Roth et al., 2007] also raise the possibility that auditory information from the thalamus is redistributed after it has arrived in the pallium. However, despite ample opportunity for auditory influences in the pallium, there is little physiological evidence for it. This gap is probably due, in part, to the fact that so few studies have addressed the question. To date, the medial pallium is the only pallial subdivision known to respond to acoustic stimuli [Mudry and Capranica, 1980]. In general, the pallium responds equally to electrical stimulation of auditory, somatosensory, and visual nerves [Laberge and Roth, 2007]. Yet it remains unclear whether these electrically evoked responses reflect the role of the pallium in processing natural sensory stimuli. Thus, many questions remain regarding how the anuran brain represents conspecific vocalizations.

Using immediate early gene (IEG) expression to create neural activity maps is an effective way to discover the function of brain regions. Immediate early genes can be induced in response to neural activity, and their expression is linked to synaptic stimulation [Worley et al., 1991; Stripling et al., 1997; Clayton, 2000; Jarvis, 2004]. Yet neurophysiological activity in the brain is not always accompanied by gene expression [Stripling et al., 1997; Clayton, 2000], presumably because expression of IEGs is restricted to those cells that possess the appropriate signal transduction mechanisms to facilitate IEG transcription. One of the most highly conserved and well-characterized immediate early genes is *egr-1* (also known as *zif268*, *NGFI-A*, *krox-24*, *ZENK*). In birds and mammals, *egr-1* can be induced in specific brain nuclei by a variety of natural stimuli [e.g., hearing birdsong, Mello et al., 1992; Jarvis et al., 1998; exposure to regular light

cycles, Kaczmarek and Chaudhuri, 1997] and by the production of behaviors [e.g., singing birdsong, Jarvis and Nottebohm, 1997]. In male túngara frogs, mating calls induce widespread *egr-1* expression in the torus semicircularis [Hoke et al., 2004, 2007] and diencephalon, but not in the telencephalon [Hoke et al., 2007]. However, in the medial pallium acoustically evoked locomotion correlates with *egr-1* expression [Hoke et al., 2007]. Whether *egr-1* expression in the dorsal, lateral, and ventral pallia responds to acoustic stimuli or evoked locomotion is unknown.

To map acoustic responses in the frog pallium, we measured *egr-1* expression in the pallium of female túngara frogs after the presentation of either conspecific mating calls or no sound. Based on a previous electrophysiological study [Mudry and Capranica, 1980], we expected to find acoustic responsiveness in the medial pallium, but previous studies do not lead to clear predictions regarding acoustically evoked IEG induction in the dorsal, lateral, or ventral pallia. Based on recent neurophysiological evidence [Laberge and Roth, 2007], we also anticipated that we would find greater auditory responses in the anterior pallium when compared with the posterior pallium. To determine how activity in the pallium relates to the activation of pallial sensory inputs, we correlated *egr-1* expression in the pallium with *egr-1* expression in the auditory midbrain to test whether the observed *egr-1* expression was consistent with a direct effect of auditory system activity. Because of strong olfactory inputs to the pallium, we also examined the correlation of *egr-1* expression in the pallium with *egr-1* expression in the olfactory system. Finally, in order to account for behavioral responses to the acoustic stimulus, which itself involves activation of sensory and motor systems, we examined the correlation between locomotion and pallial *egr-1* expression.

Materials and Methods

The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved the research presented here and The Republic of Panama's National Authority for the Environment (Autoridad Nacional del Ambiente) permitted the collection and export of the subjects in our study. We have previously reported data collected from the tissue in this experiment [Burmeister et al., 2008].

Animals

Female túngara frogs (*Physalaemus (Engystomops) pustulosus*) were captured in amplexus near Gamboa, Panama in June 2005 between 19:00 and 22:00 h. We transported females to the labora-

tory of the Smithsonian Tropical Research Institute and isolated them in dark acoustic chambers for 6 or 24 h. The floors of the chambers were lined with wet paper towels to prevent dehydration and the animals were enclosed within perforated plastic circular arenas. After the acclimation period, we exposed half of the females ($n = 10$) to a playback of natural mate chorus for 30 min followed by sacrifice. The other females ($n = 10$) received no acoustic stimulation and were sacrificed immediately following the acclimation period. The mate chorus stimulus was recorded near Gamboa, Panama, and consisted of a 15-min recording looped once. Peak amplitude of the playback was set at 82 dB (re 20 μ Pa) at ~ 0.5 meter from the speaker.

Tissue Preparation and in situ Hybridization

Following decapitation, heads were embedded in OCT, rapidly frozen in liquid nitrogen, and stored at -80°C until sectioning at our UNC-CH laboratory. We sectioned brains on a cryostat at 16 μm thickness in 3 series and mounted them onto slides (Superfrost Plus, Fisher Scientific, Pittsburgh, Pa., USA). Radioactively labeled (S-35, GE Healthcare, Piscataway, N.J., USA) *egr-1* mRNA probes were reverse transcribed from plasmids containing *P. pustulosus egr-1* cDNA (GenBank Accession No. AY562993). All slides were processed simultaneously to eliminate variation between procedures. We performed in situ hybridization of the radioactive probe according to the protocol described in Burmeister et al. [2008]. To visualize the bound riboprobe, slides were dipped in Kodak NTB emulsion, allowed to dry, and stored in lightproof boxes at 4°C for 14 days. We developed slides with Kodak D-19 developer and Kodak fixer and then counterstained the tissue with thionin.

Quantification of *egr-1* Expression

We identified the medial pallium (MP), dorsal pallium (DP), lateral pallium (LP), and ventral pallium (VP) in transverse sections based on clearly identifiable cytoarchitectural boundaries and cell morphology (fig. 1). Where possible, we followed the nomenclature of Roth et al. [2007]. Within the MP, we separately sampled the dorsal (dMP) and ventral parts (vMP) because of clear differences in cell size, morphology, and connectivity between the two subdivisions [Westhoff and Roth, 2002; Roth et al., 2007]. Although Roth et al. [2007] identify medial and lateral subdivisions of the DP in fire-bellied toads, these were not reliably apparent in our tissue and thus we did not attempt to sample them separately. Within the VP, our sampling window captured part of both dorsal and ventral portions, which have similar connectivity and morphology to one another [Roth et al., 2007].

For all brain regions, *egr-1* mRNA expression was quantified using a 100 \times objective on one hemisphere of the brain chosen at random. We began sampling from rostral sections at a level posterior to the accessory olfactory bulb and continued sampling caudally from every other section in each brain region, for a total of 6 sections each spaced 96 μm apart on average. The caudal limit of our sampling was approximately at the level of the beginning of the preoptic area and was rostral to the anterior commissure. To compare activity-dependent expression in the pallium to auditory and olfactory activity, we also sampled *egr-1* expression from the torus semicircularis and the dorsal half of the granular cell layer of the main olfactory bulb. For each of three subdivisions of the torus semicircularis (laminar, principal, and magnocellular nuclei), we calculated mean *egr-1* expression from 4 consecu-

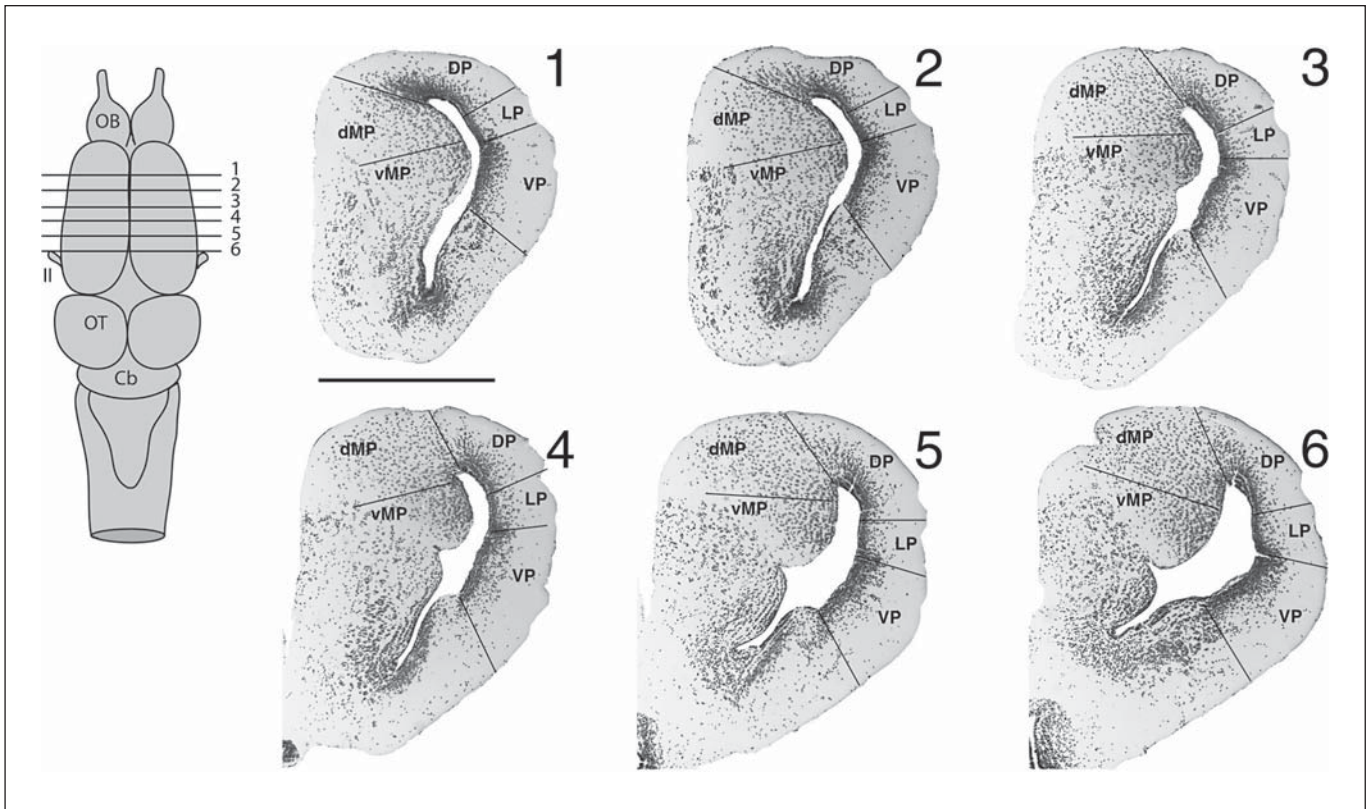


Fig. 1. Representative photomicrographs of transverse sections 1–6 (rostral to caudal) showing cytoarchitecture of the túngara frog pallium in the areas sampled. See inset for approximate level of sections through the telencephalon. Abbreviations: II = optic nerve; dMP = dorsal part of medial pallium; Cb = cerebellum; DP = dorsal pallium; LP = lateral pallium; OB = olfactory bulb; OT = optic tectum; vMP = ventral part of medial pallium; VP = ventral pallium. Scale bar = 400 μm .

tive sections spaced 48 μm apart [torus semicircularis data have been reported previously in Burmeister et al., 2008]. For the main olfactory bulb, we calculated mean *egr-1* expression from 3 consecutive sections spaced 48 μm apart.

We quantified *egr-1* expression from digital photomicrographs taken with a Leica DFC480 camera attached to a Leica DM 4000B 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'). In the blue-filtered images, exposure, brightness and contrast settings were the same for each picture of a given section. We used Image J (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 used the point selection

tool in Image J to mark and count all visible cells in the color image of the region of interest. Our final measure of *egr-1* mRNA expression for each section was the number of silver grains above background per cell. Because we counted all cells in the field of view, including those that had no silver grains, the number of silver grains per cell represents an average over the region of interest and is generally low (see fig. 2).

Quantification of Behavior

We videotaped females' behavior for 30 min prior to their sacrifice using an infrared camera (Infrared Microvideo Camera, Super Circuits, Austin, Tex., USA) and quantified their rate of movement (hops per minute) by counting the number of times the animal hopped to a new position over the observed time period. Distance traveled with each movement was limited by the small size of the enclosure (24 cm diameter) and females were prevented from physically contacting the speaker broadcasting the mate chorus. We also used a stopwatch to quantify the total amount of time (in seconds) the animal spent in motion. Behavioral data from these animals has been reported previously in Burmeister et al. [2008].

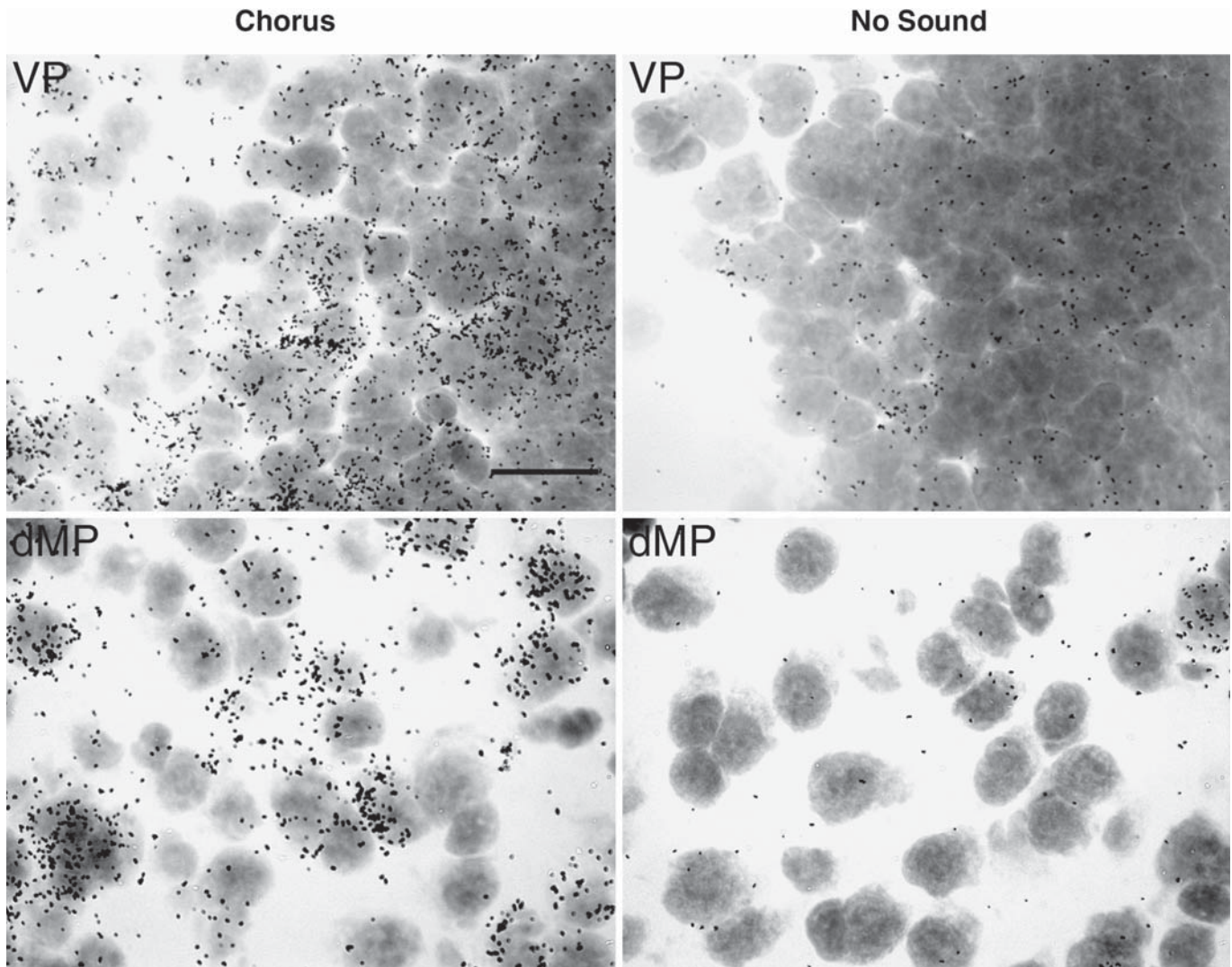


Fig. 2. Photomicrographs showing *egr-1* mRNA levels in response to a mating chorus and no sound in the ventral pallium (VP) and dorsal part of the medial pallium (dMP). Scale bar = 20 μ m.

Statistical Analyses

We used SAS V8 (SAS, Cary, N.C., USA) and SPSS 15.0 (SPSS, Chicago, Ill., USA) for statistical analyses. A Shapiro-Wilk test revealed that the data were normally distributed ($W > 0.90$ for all variables). To test whether exposure to an acoustic stimulus influenced *egr-1* expression in the pallium overall and whether this effect varied among brain regions, we first conducted an analysis of variance (ANOVA) with acoustic treatment (chorus or no sound) as a between-subjects factor and brain region (dMP, vMP, DP, LP, VP) and section number (1–6) as within-subjects factors. Individual was modeled as a random factor. We nested brain region and section within individual because measurements of *egr-1* levels from different pallial regions and different brain sections of the same animal are not statistically independent. We modeled our within-subjects data using an auto-regressive covariance structure, which assumes that the variance in two adjacent

measures will be more correlated than between measures that are farther apart in space. This avoids the problem of assumed sphericity in repeated measures ANOVAs. Some animals in each acoustic treatment group were acclimated for 6 h and some for 24 h; therefore, we also included acclimation period as a between-subjects factor in this model. We initially included all three- and four-way interactions but we excluded them from the model presented here because they were non-significant and they greatly increase the complexity of the model.

Because our results suggest that the effect of the acoustic stimulus varied among brain regions, we next conducted five separate ANOVAs (dMP, vMP, DP, LP, VP) in order to determine the magnitude of the acoustic effect in each pallial region. For each of these tests, acoustic treatment was a between-subjects factor and section was nested within brain region of interest. We tested for violations of the assumption of sphericity using Mauchly's test,

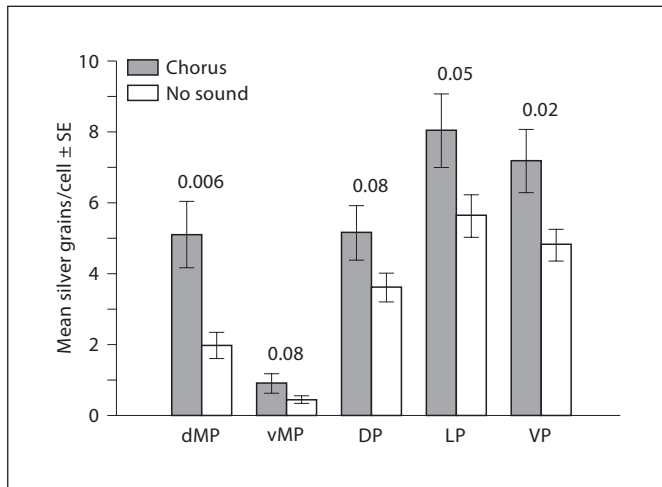


Fig. 3. Effect of mate chorus on *egr-1* expression in five regions of the túngara frog pallium. Data are shown as mean (\pm SE) silver grains/cell. Numbers above bars refer to p values of independent ANOVAs for each brain region. Abbreviations: dMP = dorsal part of medial pallium; DP = dorsal pallium; LP = lateral pallium; vMP = ventral part of medial pallium; VP = ventral pallium.

Table 1. Four-factor analysis of variance showing the effects of acoustic treatment, acclimation period, brain region, section number, and their two-way interactions on *egr-1* expression in the pallium

Source	F-statistic (d.f.)	p
Treatment	$F_{(1, 514)} = 8.62$	0.004
Acclimation	$F_{(1, 514)} = 2.02$	0.16
Treatment \times Acclimation	$F_{(1, 514)} = 0.27$	0.61
Region	$F_{(4, 514)} = 77.90$	<0.0001
Region \times Treatment	$F_{(4, 514)} = 4.66$	0.001
Region \times Acclimation	$F_{(4, 514)} = 1.56$	0.18
Section	$F_{(5, 514)} = 12.90$	<0.0001
Section \times Treatment	$F_{(5, 514)} = 3.12$	0.009
Section \times Acclimation	$F_{(5, 514)} = 0.29$	0.92
Region \times Section	$F_{(19, 514)} = 0.91$	0.57

and in cases where violations were detected we report the Huynh-Feldt corrected degrees of freedom and corresponding p values for within-subjects effects (vMP and LP only). In both cases, the corrected p values were similar to uncorrected values and therefore did not change our interpretation of the results. We excluded acclimation period in order to simplify the models and because the initial ANOVA that included all brain regions suggested that acclimation period did not affect acoustically evoked *egr-1* expression in the pallium. When a section \times treatment interaction was found, we conducted post-hoc t tests between group means (no sound vs. chorus) at each section level to determine which

sections expressed significantly different *egr-1* levels in response to chorus.

Because an animal's behavior might also influence *egr-1* expression, we used Pearson's correlations to test for a relationship between *egr-1* expression in each pallial subdivision and rate of movement or total time the animal spent in motion. Behavioral responses to the mate chorus will change aspects of the sensory environment that might result in *egr-1* expression in the pallium that is not a direct consequence of activation of the auditory system. Although locomotion presumably causes changes in vestibular, proprioceptive, and somatosensory systems, we examined those effects only indirectly by correlating the magnitude of the behavioral response itself. Although we took care to ensure that the sensory environment was as uniform as possible across treatment groups, it is conceivable that females hearing the chorus released an odorant or became more sensitive to ambient odorant molecules in the chambers, which could induce *egr-1* expression in pallial regions known to receive strong inputs from the olfactory system. Therefore, we used Pearson's correlation to examine whether the patterns of *egr-1* expression that we observed in the pallium could be better explained by activity in olfactory or auditory processing regions of the brain. However, because the animals were housed in the dark (except for infrared emitted by video cameras), and *egr-1* expression in the optic tectum was undetectable in 19 of 20 animals (data not shown), we did not examine correlations between *egr-1* expression in the visual system and the pallium. For the correlations, we used means of *egr-1* expression of multiple sections (6 sections for each pallial subdivision; 4 sections for each subdivision of the torus semicircularis; 3 sections for the olfactory bulb). Sample sizes were 20 in all correlation analyses except in the correlations including behavior ($n = 17$) and the olfactory bulb ($n = 19$), due to missing data.

Results

Acoustic Exposure Increases *egr-1* Expression

To determine whether acoustic stimulation had an effect on activity-dependent *egr-1* expression in the túngara frog pallium, we compared animals that heard a mating chorus to animals exposed to silence. Overall ANOVA results are shown in table 1. We found that *egr-1* expression increased in the pallium in response to the acoustic stimulus (treatment, $p = 0.004$; fig. 3) and that this effect varied among pallial divisions (treatment \times region, $p = 0.001$; fig. 3). In addition, we found that, independent of treatment, the magnitude of *egr-1* expression varied among brain regions (region, $p < 0.0001$) and along the rostral-caudal axis (section, $p < 0.0001$). In general, *egr-1* expression was highest in the anterior pallium and decreased as we sampled posteriorly (fig. 4). In contrast, we found no evidence that the effect of acoustic treatment on *egr-1* expression was modulated by acclimation period (treatment \times acclimation, $p = 0.61$). Taken

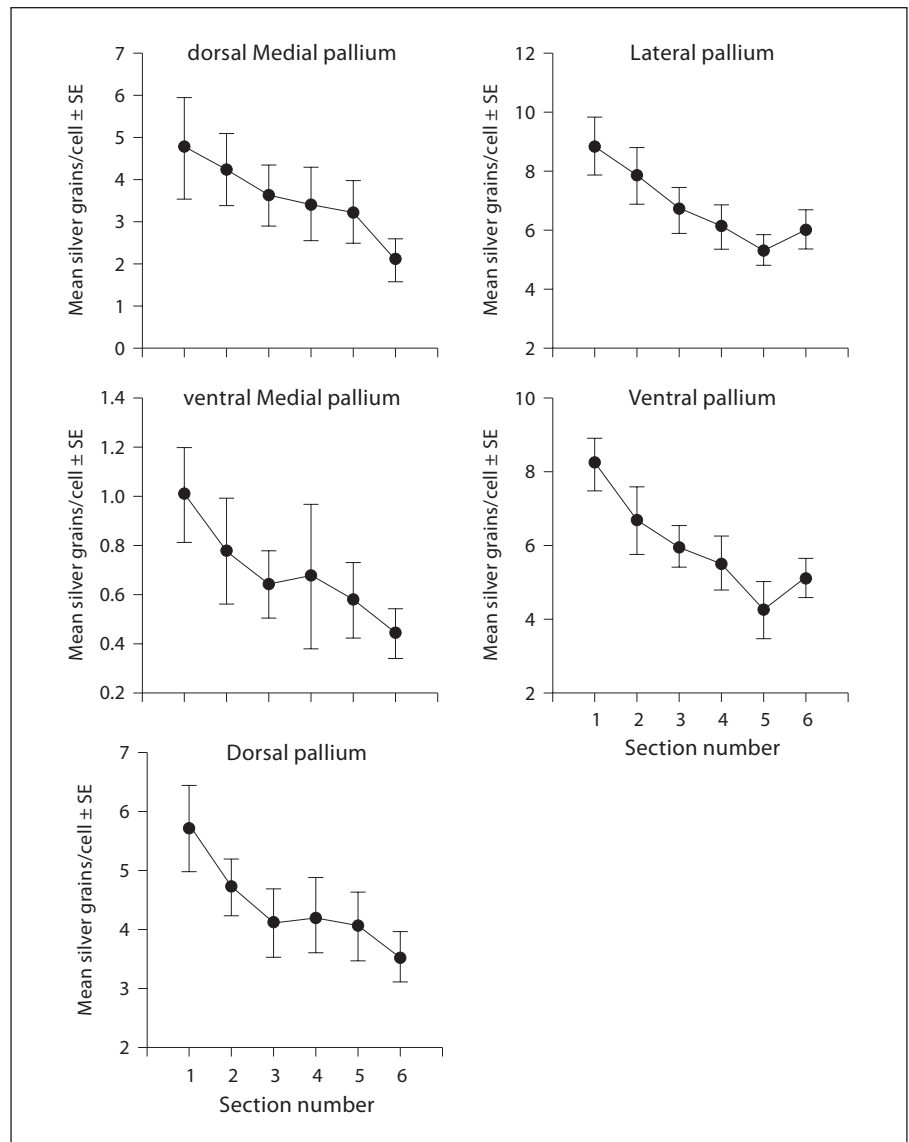


Fig. 4. Spatial variation in *egr-1* expression in the pallium. Data are shown as mean (\pm SE) silver grains/cell by section number (1–6, anterior to posterior). Note that Y-axes vary among panels.

together, these results demonstrate that the response of the pallium to a relevant acoustic stimulus, although widespread, had spatial specificity.

Because we found that the effect of chorus on *egr-1* expression in the pallium varied among brain regions, we next conducted separate ANOVAs for each of the five pallial regions (ANOVA results are shown in table 2). In the MP, we found that hearing mate chorus induced a 2.5-fold increase in *egr-1* mRNA expression in the dorsal part (treatment, $p = 0.006$), but not in the ventral part (treatment, $p = 0.08$; fig. 3). The effect of the chorus on *egr-1* expression in the MP did not vary across the rostral-caudal axis (section \times treatment, $p = 0.32$ and 0.80). In the

DP, mating chorus failed to evoke a strong change in *egr-1* expression (treatment, $p = 0.08$; treatment \times section, $p = 0.73$), although *egr-1* expression levels did vary along the rostral-caudal axis (section, $p = 0.008$; fig. 4). In the LP, the mating chorus induced higher *egr-1* expression (treatment, $p = 0.05$) and overall *egr-1* expression levels varied across the rostral-caudal axis (section, $p < 0.0001$) where it was higher in the anterior sections compared to the posterior sections (fig. 4). Finally, we found a strong effect of the chorus on *egr-1* expression in the VP (treatment, $p = 0.02$; fig. 3) that varied along the rostral-caudal axis (section \times treatment, $p = 0.04$; fig. 5). Post-hoc analysis for each section showed that the effect of

Table 2. Effects of acoustic treatment, section number, and their interaction on *egr-1* expression in pallial brain regions

	d Medial pallium	v Medial pallium	Dorsal pallium	Lateral pallium	Ventral pallium
Treatment	$F_{(1, 18)} = 9.60 (0.006)$	$F_{(1, 18)} = 3.37 (0.08)$	$F_{(1, 18)} = 3.39 (0.08)$	$F_{(1, 18)} = 4.38 (0.05)$	$F_{(1, 16)} = 6.10 (0.02)$
Section	$F_{(5, 90)} = 2.05 (0.08)$	$F_{(3, 53)} = 1.72 (0.17)$	$F_{(5, 90)} = 3.37 (0.008)$	$F_{(4, 73)} = 6.01 (<0.0001)$	$F_{(5, 80)} = 7.38 (<0.0001)$
Section \times Treatment	$F_{(5, 90)} = 1.19 (0.32)$	$F_{(3, 53)} = 0.32 (0.80)$	$F_{(5, 90)} = 0.55 (0.73)$	$F_{(4, 73)} = 1.12 (0.35)$	$F_{(5, 80)} = 2.46 (0.04)$

p values are given in parentheses after each F-statistic.

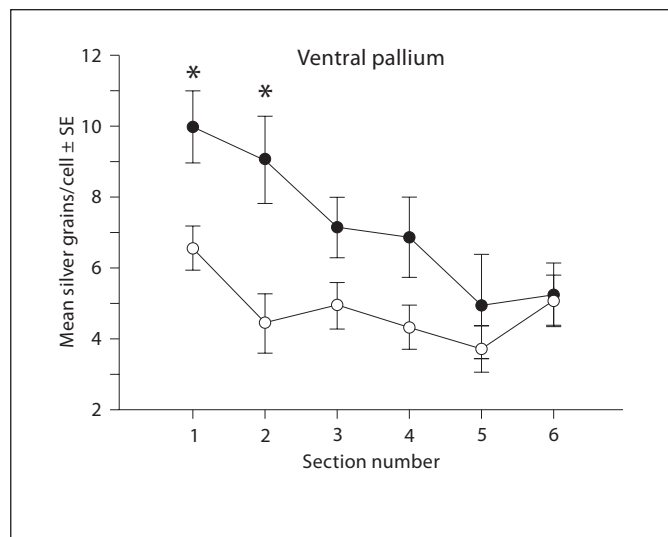


Fig. 5. The effect of chorus on *egr-1* expression in the ventral pallium varied spatially. Data are shown as mean (\pm SE) silver grains/cell by section number (1–6, anterior to posterior) in females exposed to chorus (filled circles) or no sound (open circles). Asterisks denote differences between treatment groups where $p < 0.05$ in the mean value of a particular section level.

chorus was stronger in the two rostral-most sections of the VP (fig. 5).

Because variation in motor behavior might also influence *egr-1* expression in the brain, we correlated movement with mean *egr-1* level in each pallial region (table 3). We found no strong evidence that rate of movement varied with *egr-1* expression in any pallial region. We also found no evidence for a relationship between *egr-1* expression and total time spent in motion.

Correlations with Auditory Midbrain and Olfactory Bulb

In addition, we wanted to know whether the *egr-1* expression we saw in the pallium is specifically related to sensory inputs that are activated by acoustic stimulus ex-

posure, or whether it can be explained by other sensory input to the pallium. Because some pallial regions (e.g., LP and VP) are known to receive strong inputs from the olfactory system, we asked whether variation in *egr-1* expression in the pallium could be better explained by activity in the olfactory or auditory systems. We found that mean *egr-1* expression in the MP, DP, and VP correlated significantly with mean *egr-1* expression in at least one of the nuclei of the torus semicircularis (table 4). In contrast, mean *egr-1* expression in the olfactory bulb did not correlate with mean *egr-1* expression in any pallial area (table 4), suggesting that olfactory stimulation probably does not explain the chorus-induced patterns of *egr-1* induction that we observed in the pallium.

Interestingly, the relationship between *egr-1* expression in the torus and pallium varied among divisions. For example, the dMP had a strong relationship with each nucleus of the torus. In contrast, *egr-1* expression in the VP only had a strong relationship with the principal nucleus. Furthermore, even though our acoustic stimulus failed to induce a strong response from the vMP and DP, *egr-1* levels in the laminar nucleus were good predictors of *egr-1* levels in these pallial divisions. Finally, although the mating chorus induced higher levels of *egr-1* expression in the LP, we did not find strong evidence for a relationship between activity in the torus and activity in the LP. These results are consistent with the hypothesis that acoustically evoked *egr-1* expression in the dMP and VP was a direct effect of auditory activity.

Discussion

Our results demonstrate that a natural, conspecific acoustic stimulus can induce *egr-1* mRNA expression in the pallium of a female anuran. Despite the lack of anatomical evidence for dedicated auditory projections to the pallium, we found acoustically evoked responses in the pallium that varied spatially. Acoustically induced

Table 3. *egr-1* expression in the pallium did not correlate with rate or duration of movement

	d Medial pallium		v Medial pallium		Dorsal pallium		Lateral pallium		Ventral pallium	
	r ²	p	r ²	p	r ²	p	r ²	p	r ²	p
Rate of movement	0.11	0.17	<0.01	0.82	0.01	0.63	<0.01	0.96	<0.01	0.84
Duration of movement	0.05	0.40	<0.01	0.75	<0.01	0.95	<0.01	0.93	<0.01	0.85

The Pearson correlation coefficient (r^2) and corresponding p value were determined between mean *egr-1* values for each individual in five subdivisions of the pallium and each individual's rate of movement (hops per minute) and duration of movement (time in seconds).

Table 4. *egr-1* expression in the pallium correlated with *egr-1* expression in the auditory midbrain, but did not correlate with *egr-1* expression in the olfactory bulb

	d Medial pallium		v Medial pallium		Dorsal pallium		Lateral pallium		Ventral pallium	
	r ²	p	r ²	p	r ²	p	r ²	p	r ²	p
<i>Ptor</i>	0.21	0.04	0.07	0.26	0.13	0.10	0.13	0.11	0.37	0.004
<i>Ltor</i>	0.37	0.004	0.28	0.02	0.31	0.01	0.09	0.21	0.14	0.10
<i>Mctor</i>	0.22	0.04	0.003	0.81	0.11	0.15	0.02	0.53	<0.001	0.96
MOB	0.02	0.50	0.03	0.41	<0.001	0.68	<0.001	0.90	<0.001	0.98

The Pearson correlation coefficient (r^2) and corresponding p value were determined between mean *egr-1* values for each individual in five subdivisions of the pallium, three nuclei of the auditory midbrain, and the olfactory bulb. *Ltor* = Laminar nucleus of torus semicircularis; *Mctor* = magnocellular nucleus of torus semicircularis; MOB = main olfactory bulb; *Ptor* = principal nucleus of torus semicircularis.

egr-1 expression was strongest in the dMP and the VP. In the VP, acoustically induced *egr-1* expression was more pronounced in the anterior portion compared to the posterior portion, which is consistent with recent evidence that electrically evoked sensory potentials are greater in the anterior pallium of the fire-bellied toad [Laberge and Roth, 2007]. However, our results are not consistent with a previous study of male túngara frogs which found that *egr-1* levels in the MP were better explained by motor behavior than acoustic treatment [Hoke et al., 2007]. The reasons for this difference are not clear, but they could reflect a sex difference in the function of the MP, or a difference in sampling procedures. For example, in the earlier study [Hoke et al., 2007], *egr-1* expression in the dorsal and ventral parts of the MP were not analyzed separately, which could have obscured an effect of acoustic treatment.

Our results suggest that the *egr-1* expression we observed in the dMP and VP was caused by the acoustic stimulus and not by other sensory stimuli the frogs might

have experienced during the test period. Measures of movement and olfactory activity could not explain a significant portion of acoustically evoked *egr-1* expression in any region of the pallium. Moreover, the spatial distribution of *egr-1* expression we observed is not consistent with observed patterns of electrically evoked olfactory activity. The largest olfactory responses have been recorded from regions in the caudal pallium [Laberge and Roth, 2007], whereas we observed the lowest level of *egr-1* expression in the caudal pallium. In contrast, *egr-1* expression in the auditory midbrain covaried with *egr-1* expression in the dMP and VP, suggesting that their activity was coupled with auditory activity and indicating that these brain regions are part of a functional network that plays a role in processing mating call stimuli. However, complex acoustic stimuli have the potential to activate a variety of brain regions; in primates, for example, sounds cause widespread cortical activation that includes regions of primary visual cortex [Poremba et al., 2003]. Thus, we cannot conclude that the acoustically

responsive regions of the frog pallium are strictly auditory brain regions. Acoustically evoked pallial *egr-1* responses could reflect other processes related to conspecific call presentation, such as increased arousal or motivation. This might explain, for example, why chorus evoked higher *egr-1* expression in the LP, but *egr-1* expression there did not correlate with auditory *egr-1* expression. In addition, our results leave open the possibility that other sensory experiences could evoke similar patterns of *egr-1* expression in the pallium. Future studies are necessary to understand the specificity of acoustically evoked *egr-1* expression in the frog pallium and its implications for neural processing of communication signals.

Anatomical and electrophysiological evidence both demonstrate that, within the pallium, ascending sensory input from multiple modalities overlap. However, given the paucity of electrophysiological recordings from pallial neurons, the function of these multimodal inputs is unclear. For example, a recent study in mammalian auditory cortex demonstrated that convergence from multiple modalities does not necessarily produce multisensory integration as traditionally defined. Although neurons exhibited clear spiking responses to both auditory and visual stimuli, they did not always transmit more information in their firing pattern when combined visual-auditory stimulation was used [Bizley et al., 2007]. Our results show that in the frog a multimodal stimulus is not required to activate pallial neurons. This raises the possibility that, as in the mammalian neocortex [Bizley et al., 2007], pallial auditory neurons receive input from more than one modality, but nonetheless are preferentially dedicated to processing a single modality. Alternatively, it is possible that most neurons in the frog pallium contribute to processing multiple sensory modalities using a complex ensemble-type code. Of course, our data cannot distinguish among these and other possibilities. Nonetheless, the finding that an acoustic stimulus can evoke neural responses in the frog pallium suggests that those neurons are contributing to the processing of auditory signals.

To date, the role of the pallium in sensory processing is not well understood and virtually nothing is known about its function in modulating behavior in frogs. Our results indicate that neurons in the pallium respond to species-specific vocalizations and therefore could play a role in auditory processing. However, an alternative hypothesis is that the pallium is involved in cognitive and emotional functions, as it is in other vertebrates. Known anatomical connections of the frog MP and VP suggest

that this is possible, and that the pallium could act as a 'selection system' that participates in information processing and influences behavioral and physiological responses to sensory stimuli [Veenman et al., 1989]. In anurans, all pallial regions receive multimodal sensory input from the thalamus [Neary, 1990; Northcutt and Ronan, 1992; Moreno and Gonzalez, 2004; Laberge et al., 2008], but only the MP and VP have extra-pallial descending connections [Roth et al., 2007], making them the sole output zones of the pallium. For instance, both the MP and part of the VP (formerly the ventral part of the lateral pallium) project heavily to the hypothalamus and preoptic area [Neary, 1995; Roth et al., 2007], brain areas well-known to regulate sexual behavior in vertebrates. In particular, the MP of anurans is thought to be part of an 'audiolimbic interface' [Wilczynski and Endepols, 2007] that may influence motivational state and reward-seeking behavior in response to sensory stimuli via projections to the medial amygdala and nucleus accumbens [Northcutt and Ronan, 1992; Westhoff and Roth, 2002]. The MP also has direct projections to the striatum [Neary, 1990], a brain area known to be involved in motor control and in modulating female phonotaxis behavior in anurans [Walkowiak et al., 1999]. Given their increased neural response to mating chorus in túngara frogs [this study], the anuran dMP and possibly VP might be involved in interpreting the biological significance of signals and modulating brain areas that generate behavioral and physiological responses to those signals. In light of known connections, we hypothesize that neural activity in the pallium during mate call reception could participate in neural circuitry involved in regulating acoustically mediated sexual behavior.

The anuran MP is considered homologous to the mammalian hippocampus, a brain area known to be involved in memory formation [Neary, 1990; Northcutt and Ronan, 1992; Westhoff and Roth, 2002], and some behavioral evidence suggests that the MP is involved in conditioned visual learning tasks in toads [Finkenstadt and Ewert, 1988; Ewert et al., 1994]. Such studies raise the hypothesis that the MP of anurans might also play a role in auditory memory. Although we can only speculate at this point, the formation of auditory memories could play a role in mate choice behavior in anurans whose females sequentially assess multiple calling males, a behavior that has been observed in several species including the túngara frog [Ryan, 1985; Robertson, 1986; Arak, 1988]. In conclusion, we do not yet know the relationship between acoustically evoked *egr-1* expression in the frog pallium and complex neural processes. Yet, because acoustic com-

munication plays an essential role in the reproductive behavior of anurans, future experiments are likely to provide important new insights into the relationship of the pallium to auditory discrimination and mate choice behavior.

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