Characterization of the Plasticity-Related Gene, *Arc*, in the Frog Brain

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ABSTRACT: **In mammals, expression of the immediate early gene *Arc/Arg3.1* in the brain is induced by exposure to novel environments, reception of sensory stimuli, and production of learned behaviors, suggesting a potentially important role in neural and behavioral plasticity. To date, *Arc* has only been characterized in a few species of mammals and birds, which limits our ability to understand its role in modifying behavior. To begin to address this gap, we identified *Arc* in two frog species, *Xenopus tropicalis* and *Physalaemus pustulosus*, and characterized its expression in the brain of *P. pustulosus*. We found that the predicted protein for frog Arc shared 60% sequence similarity with Arc in other vertebrates, and we observed high *Arc* expression in the forebrain, but not the midbrain or hindbrain, of female túngara frogs sacrificed at breeding ponds. We also examined the time-course of *Arc* induction in the medial pallium, the homologue of the mammalian hippocampus, in response to a recording of a *P. pustulosus* mating chorus and found that accumulation of *Arc* mRNA peaked 0.75 h following stimulus onset. We found that the mating chorus also induced *Arc* expression in the lateral and ventral pallia and the medial septum, but not in the striatum, hypothalamus, or auditory midbrain. Finally, we examined acoustically induced *Arc* expression in response to different types of mating calls and found that *Arc* expression levels in the pallium and septum did not vary with the biological relevance or acoustic complexity of the signal.** © 2010 Wiley Periodicals, Inc. Develop Neurobiol 70: 813–825, 2010

Keywords:* *Arc*; *Arg3.1*; neural plasticity; *Xenopus tropicalis*; *Physalaemus pustulosus

INTRODUCTION

Activity-dependent genes link neural activity to the long-term cellular changes that underlie synaptic plasticity, learning, and memory. In particular, the activity-regulated cytoskeleton-associated (*Arc*) gene (also known as *Arg3.1*) has been implicated in directly coupling stimulus-evoked neural activity to the physical modification of synapses (Bramham et al., 2008). Unlike other activity-dependent genes, *Arc* mRNA localizes to dendrites, accumulating specifically at recently activated synapses (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). The accumulation of Arc protein in dendritic spines strongly suggests that it is translated locally (Moga et al., 2004; Rodriguez et al., 2005), and Arc has been found to interact with synaptic proteins, such as dynamin and endophilin (Chowdhury et al., 2006). Local translation of Arc protein is required for some types of neural plasticity (Park et al., 2008; Waung et al.,

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Table 1 Primers (5′ to 3′) and PCR Conditions Used to Generate cDNA Sequences

	Forward	Reverse	Annealing Temperature (°C)	Species
Pair 1	AGC GTT CCA TAA AGG CTT GTT	TTT GAT GGC CTC TCT AAC C	52.5	*X. tropicalis*
Pair 2	GAA TTT AGA AAG GTG GGT CAA	CTC CCA CCA CTT CTT AGC TG	52.6	*P. pustulosus*
Pair 3	AGC GTT CCA TAA AGG CTT GTT	AGG GCT CCC AGC GTC T	54.2	*X. tropicalis, P. pustulosus*

2008), and blocking Arc protein synthesis inhibits long-term memory consolidation (Guzowski et al., 2000; Plath et al., 2006; Messaoudi et al., 2007). These characteristics suggest that *Arc* plays an especially important role in coordinating information storage.

It has been hypothesized that the expression of activity-dependent genes in behaviorally relevant situations ensures that information associated with biologically significant events, such as recognizing an appropriate mate or learning a new task, is selectively processed and stored (Clayton, 2000). *Arc* may play an important role in this process because the expression of *Arc* mRNA in the brain is highly specific. For example, listening to conspecific song selectively increases *Arc* in the auditory forebrain of adult songbirds compared to birds listening to tones or white noise (Velho et al., 2005). In rats, levels of *Arc* mRNA are elevated in the hippocampus of individuals learning a new spatial task relative to those performing a familiar task (Guzowski et al., 2001). Furthermore, *Arc* is induced in distinct neuronal ensembles when rats are exposed to different environments, suggesting that *Arc* gene expression can encode hippocampal place fields (Guzowski et al., 1999).

To date, most of what is known about *Arc*'s function is based on studies of mammalian hippocampus, and no studies have examined the expression of *Arc* in vertebrates other than mammals and birds, which limits our ability to understand the functional role of *Arc*-mediated neuroplasticity in modifying behavior. Has *Arc* expression evolved as a mechanism of synaptic plasticity only in vertebrates that exhibit complex, learned behaviors, or is it expressed in all vertebrates in a variety of biologically important contexts? To begin to address these questions, we identified frog *Arc* and characterized its expression in the brains of female túngara frogs (*Physalaemus pustulosus*). A classic model for understanding the evolution of communication behavior (Ryan, 2005), the túngara frog has recently emerged as an important model system for studying activity-dependent gene expression within a neuroethological context (Hoke et al., 2004, 2005, 2007; Burmeister et al., 2008; Mangiamele and Burmeister, 2008). During the breeding season, female túngara frogs visit ponds where males are calling in order to select a mate. Neural and behavioral plasticity might have adaptive value in this species because choosing a mate involves simultaneous evaluation of multiple males' calls and it could require females to engage spatial memory (Akre and Ryan, 2010), as females have been observed sequentially assessing several potential mates before choosing one (Ryan, 1985).

We first cloned frog *Arc* from *Xenopus tropicalis* because the genome sequence is publicly available, and then used the same primers to clone *Arc* in *P. pustulosus*. We conducted Northern blot analysis to determine the size of the full-length *P. pustulosus* transcript and to partially characterize its tissue-specificity. We next examined spatial variation in *Arc* mRNA expression in the brains of wild-caught female *P. pustulosus* to identify brain regions that are capable of expressing *Arc* and to determine if there were any broad neuroanatomical patterns of *Arc* expression in frogs in their natural environment. Next, we examined the time-course and spatial distribution of *Arc* expression in response to a mating chorus to test whether *Arc* was induced in specific brain nuclei where synapses are likely to be modified by species-typical signals. Finally, we tested the stimulus specificity of *Arc* mRNA induction by exposing laboratory-reared túngara frogs to mating calls that vary in their acoustic complexity and their attractiveness to females in order to ask whether calls with different biological meanings could induce differential *Arc* expression.

METHODS

Identification of Frog *Arc*

First, to identify frog *Arc*, we queried the translated *X. tropicalis* genome (Joint Genome Institute *X. tropicalis* Genome Assembly, version 4.1; available at: http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) with rat Arc protein. Our search yielded a single genomic sequence that we used to design primers (Table 1) to amplify *Arc* cDNA from *X. tropicalis* and *P. pustulosus*. We extracted mRNA from brain using TRIzol® (Invitrogen, Carlsbad, CA), syn-

Table 2 Percent Sequence Similarity (and Identity) in Predicted Arc Protein Sequence among Vertebrates

	Xenopus tropicalis[a]	*Physalaemus pustulosus*[b]
Chicken[c]	60 (40)	60 (41)
Zebra finch[d]	61 (41)	62 (41)
Rat[e]	59 (41)	59 (42)
Human[f]	57 (41)	57 (42)

[a] Genbank No. FJ577656.
[b] Genbank No. EU437548.
[c] Genbank No. AJ272062.
[d] Genbank No. EF076776.1.
[e] Genbank No. AAA68695.1.
[f] Genbank No. AF193421.1.

thesized cDNA by reverse transcription using a poly-dT primer, and amplified two fragments of *Arc* cDNA using the following thermocycling protocol: 2 min at 94°C followed by 35 cycles of 20 sec at 94°C, 10 sec at the annealing temperature (Table 1), 30 sec at 65°C, and concluding with a final elongation of 65°C for 1 min. We transformed the fragments into bacterial cells (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) for sequencing. To determine sequence similarity across species, we compared the hypothetical Arc protein sequences among the two frogs, rat, human, chicken, and zebra finch (Table 2).

To estimate the size of the *Arc* transcript in *P. pustulosus*, we conducted Northern blot analysis on total RNA from túngara frog brain and liver. We extracted RNA using TRIzol® and ran 10 μg each of brain and liver RNA on a 1.5% MOPS/formaldehyde agarose gel before transferring the RNA to a nylon membrane (NorthernMax, Ambion, Austin TX). We synthesized a ^{32}P-labeled *Arc* antisense probe by *in vitro* transcription of plasmids containing the 593 bp fragment of *P. pustulosus Arc* cDNA. We hybridized the blot overnight at 65°C in 1 mL hybridization solution (Ultrahyb, Ambion) containing 1×10^6 cpm of ^{32}P-labeled *Arc* antisense probe. We removed the unbound probe by washing twice in low stringency wash solution (2× SSC, 0.1% SDS) at room temperature, followed by two high stringency washes (0.1× SSC, 0.1% SDS) at 68°C. Finally, we exposed the blot to film using an intensifying screen for 7 days at −80°C.

Neuroanatomical Distribution of *Arc* Expression in the Frog Brain

Next, to identify brain regions capable of expressing *Arc*, we qualitatively assessed *Arc* mRNA expression levels in wild-caught female *P. pustulosus*. We caught females (n = 6) at mating ponds where males were calling near Gamboa, Panama between 19:50 and 21:00 h, and while they were in a mating clasp with a male. We removed the male and immediately sacrificed the females, embedded females' heads in TissueTek OCT (Sakura Finetek, Torrance, CA), and rapidly froze them in liquid nitrogen.

We sectioned brains on a cryostat at 12-μm thickness in 4 series. We prepared ^{35}S-labeled *Arc* mRNA sense and antisense probes by *in vitro* transcription, performed *in situ* hybridization, and visualized the bound riboprobes according to the procedures described in Burmeister et al. (2008), except that we exposed slides to emulsion for 24 days before development and counterstaining with thionin. To assess relative levels of *Arc* expression, we examined the tissue under darkfield illumination and categorized expression as low, moderate, high, or very high. We also noted the absence of binding in control slides hybridized with sense strand riboprobe under identical hybridization conditions [Fig. 1(A)].

Temporal Profile of *Arc* Induction by Sound

To determine the temporal profile of *Arc* induction in response to sound, we captured female túngara frogs at breeding ponds in Gamboa, Panama, isolated them in dark acoustic chambers for 6 h, and either sacrificed females immediately (0 h), or exposed them to 30 min of mating chorus and sacrificed them at the following time points relative to stimulus onset: 0.25, 0.5, 0.75, 1, 2 h (n = 4), and 4 h (n = 5 each, except where noted). The mating chorus consisted of a 15-min recording looped once and played back at 82 dB (re 20 μPa) peak amplitude. We videotaped animals with infrared cameras for the last 30 min before sacrifice and quantified their movement by counting the number of hops. We processed the brain tissue as described above, except that we sectioned brains at 16-μm thickness in three series. We quantified *Arc* expression in the medial pallium, the frog homolog of the hippocampus, focusing on the

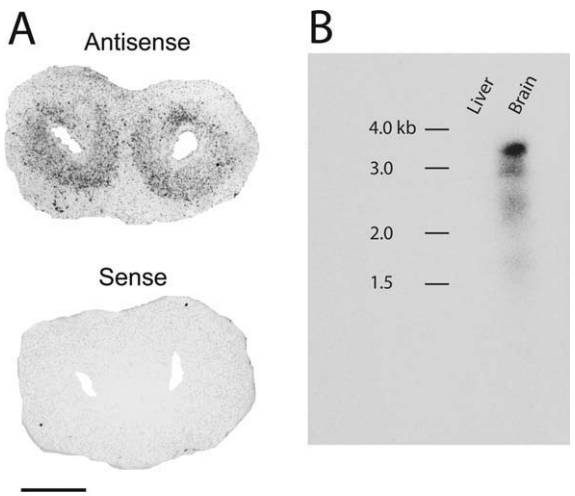

Figure 1 Specificity of our *Arc* riboprobe. (A) Inverted darkfield images of transverse sections of the olfactory bulb in chorus stimulated animals hybridized with antisense or sense riboprobes (scale bar represents 500 μm). Excess tissue surrounding brain section was removed for clarity. (B) Northern blot of total liver and brain RNA hybridized with an *Arc* antisense riboprobe. Approximate positions of molecular size markers indicated (left). Images were adjusted for contrast.

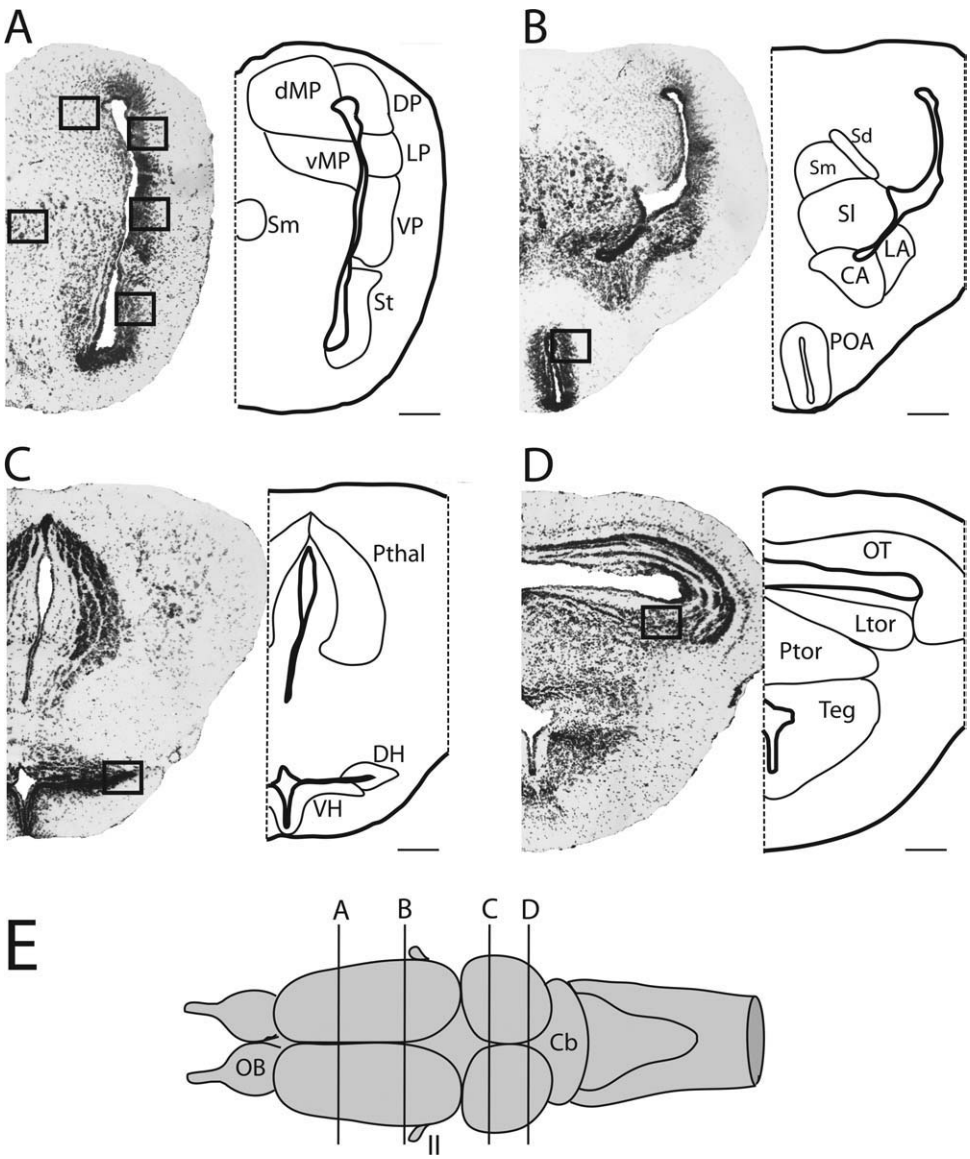

Figure 2 Photomicrographs of Nissl-stained túngara frog brain tissue and corresponding schematic diagrams showing cytoarchitecture of areas in which *Arc* was sampled in the telencephalon (A), preoptic area (B), hypothalamus (C), and auditory midbrain (D). Boxes indicate sampling window. Bottom panel (E) shows the approximate level of transverse sections shown in A–D. Photomicrographs taken with a 5× objective. Scale bar represents 200 μm.

dorsal portion of the medial pallium (dMP). We collected data from an average of seven alternating sections typically spaced 96 μm apart and quantified *Arc* expression (mean number of silver grains per cell above background) following procedures described in Mangiamele and Burmeister (2008).

Spatial Variation in *Arc* Induction by Sound

To further characterize acoustically induced *Arc* expression, we asked whether *Arc* is induced in brain regions that play a role in sexual communication and/or that showed clear *Arc* expression in túngara frog females caught at mating ponds (Fig. 2). In addition to the dorsal portion of the medial pallium, we sampled from the lateral pallium and ventral pallium of the telencephalon. In the basal forebrain, we sampled *Arc* expression in the striatum (incorporating both dorsal and ventral parts), medial septum, preoptic area, and dorsal hypothalamus. Finally, we sampled *Arc* expression in the laminar nucleus of the torus semicircularis (homolog of the inferior colliculus). We measured *Arc* expression in animals that heard no sound and those that were sacrificed 0.75 h following the onset of chorus, which corresponded to the peak level of *Arc* mRNA accumulation

in the dorsal medial pallium. We followed the same procedure for quantifying grains per cell above background as described for the medial pallium, except we sampled from the following numbers of sections: lateral pallium, seven alternating sections; ventral pallium and medial septum, five alternating sections; preoptic area, six consecutive sections; striatum, five consecutive sections; dorsal hypothalamus, four consecutive sections; laminar nucleus of the torus semicircularis, three consecutive sections.

Stimulus Specificity of the *Arc* Response

To characterize the stimulus specificity of acoustically induced *Arc*, we measured *Arc* expression in response to heterospecific calls or conspecific calls that vary in their attractiveness, using laboratory bred túngara frogs. In order to synchronize the reproductive status of the subjects, we injected 24 adult females and males with 500 IU of human chorionic gonadotropin (Sigma, St. Louis, MO) and allowed them to make nests. Ten days after the females had oviposited, a time when endogenous sex steroids are low, we injected them with 0.07 μg of estradiol per g body weight (Sigma, St. Louis, MO), a dose that is sufficient to induce normal sexual behavior in female túngara frogs (Chakraborty and Burmeister, 2009). We then placed females in acoustic isolation chambers for a 24-h acclimation period. After acclimation, we exposed females to a *P. pustulosus* whine advertisement call, *P. pustulosus* whine-chuck advertisement call (whine +3 chucks), or the advertisement call of a closely related species, *Physalaemus enesefae*, for 30 min followed by 15 min of silence before sacrifice ($n = 8$ for all groups). In behavioral choice tests, female túngara frogs prefer *P. pustulosus* calls over *P. enesefae* calls (Ryan et al., 2003; Chakraborty and Burmeister, 2009), and they prefer whines with chucks to whines without chucks (Ryan, 1980, 1985). We presented each mating call at a rate of one call every 2 sec at 82 dB (re 20 μPa). We quantified relative *Arc* expression levels as described above only in the areas that showed significant induction by mating chorus (medial, lateral, and ventral pallia and medial septum).

Statistical Analysis

To test whether survival time influenced the level of *Arc* mRNA expression in the medial pallium, we performed a one-way analysis of variance (ANOVA) with survival time (0, 0.25, 0.5, 0.75, 1, 2, 4 h) as a between-subjects factor. We used Fisher's least significant difference *post hoc* analyses to compare the level of *Arc* expression at each time point to the 0 h group. To test whether other brain regions show acoustically induced *Arc* expression 0.75 h after stimulus onset, we used a two-way ANOVA with survival time (0, 0.75 h) as a between-subjects factor and brain region (medial pallium, lateral pallium, ventral pallium, medial septum, striatum, preoptic area, dorsal hypothalamus, laminar nucleus of the torus) as a within-subjects factor. Because we were interested in whether, within each brain region, *Arc* was induced by sound, we followed up the two-way ANOVA with *post hoc t*-tests for each brain region. In addition, because variation in motor behavior might also affect *Arc* expression, we tested for a difference in the rate of movement (hops per min) between the 0 h and 0.75 h group using a *t*-test. We also used Pearson's correlations to test for a relationship between *Arc* expression in each brain region and the rate of movement in the 0.75 h group only. Finally, to test whether acoustically responsive brain regions showed stimulus-specific *Arc* induction, we conducted one-way ANOVAs for each brain region (medial pallium, ventral pallium, lateral pallium, and medial septum) with acoustic treatment (conspecific whine, conspecific whine-chuck, or heterospecific call) as the between-subjects factor.

RESULTS

Identification of Frog *Arc*

We identified 595- and 593-bp fragments of *X. tropicalis* and *P. pustulosus Arc* mRNA, respectively. According to our Northern blot, the full length *P. pustulosus Arc* transcript was about 3.5 kb, and was expressed in brain but not liver [Fig. 1(B)]. Both our *X. tropicalis* and *P. pustulosus* sequences code for a predicted protein of 197 amino acids. Arc protein sequences for *X. tropicalis* and *P. pustulosus* were highly similar to one another and shared over 40% identity and 60% similarity with other tetrapods (Table 2; Fig. 3). For comparison, a similarly sized fragment of zebra finch Arc has 72% identity and 82% positive similarity to rat Arc. Arc appears to be highly conserved in some regions, but is also characterized by regions of low conservation (Fig. 3). Two highly conserved regions are protein domains that may play a role in mediating interactions between Arc and other molecules. They include a known endophilin 3 binding domain (Bramham et al., 2010) and a region with sequence homology to α-spectrin where protein-protein interactions are likely to occur (Lyford et al., 1995) (Fig. 3). Our *P. pustulosus* Arc predicted protein is 75% identical to the endophilin 3 binding site on rat Arc and 50% identical with its spectrin-like region, indicating that Arc's function is likely conserved at these sites. Frog Arc shares only 24% identity with an identified dynamin 2 binding site on rat Arc (Bramham et al., 2010) (Fig. 3); however, because Arc protein sequences are more divergent in that region, it is less clear whether the dynamin 2 binding site of rat Arc is likely to be shared by Arc of other species.

Neuroanatomical Distribution of *Arc* Expression in the Frog Brain

In wild-caught female túngara frogs, we observed higher levels of *Arc* expression in the olfactory bulb,

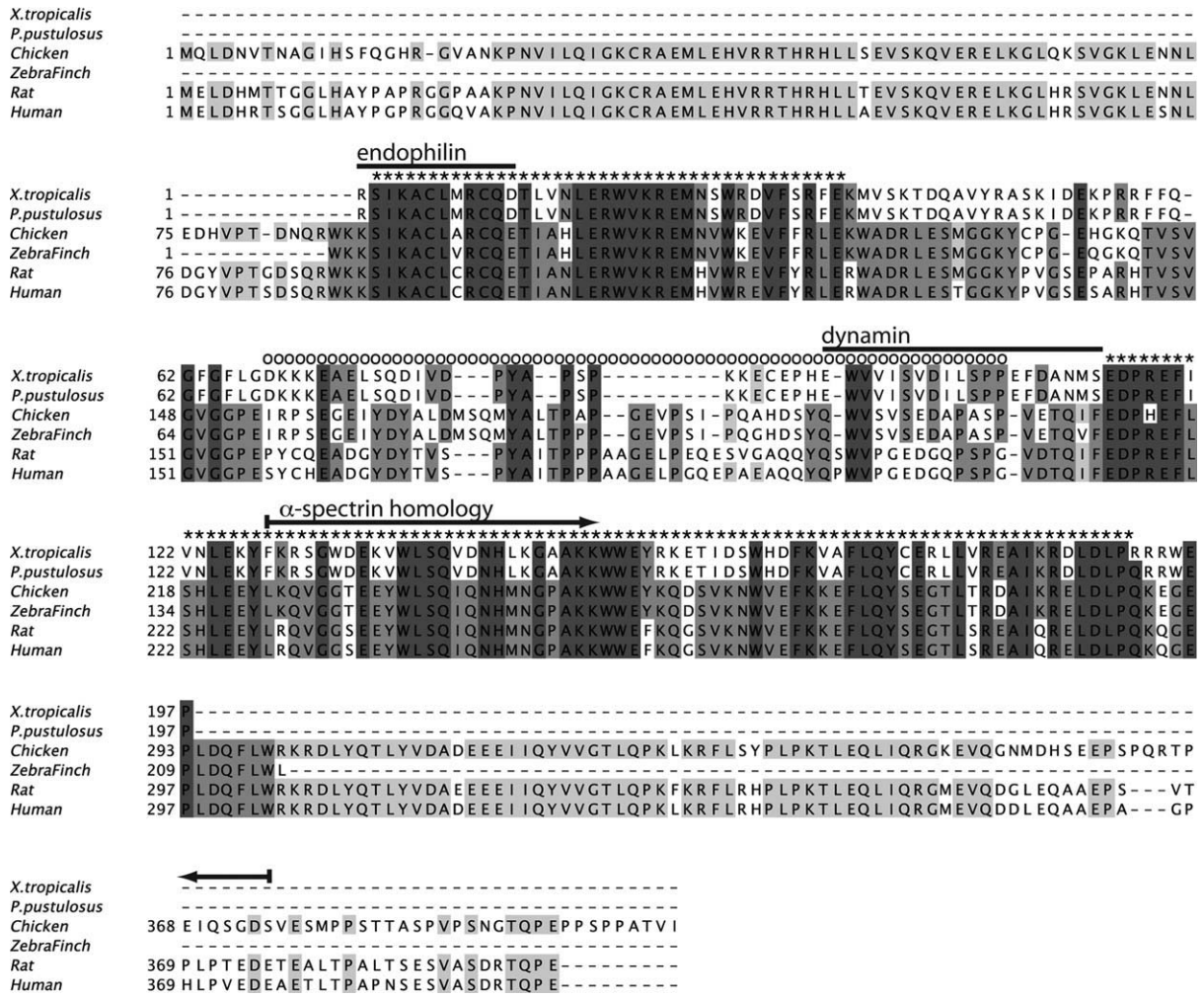

Figure 3 Comparison of *Xenopus tropicalis* and *Physalaemus pustulosus* Arc predicted protein sequence to other known vertebrate sequences. Conserved residues are shaded by their percent identity to the consensus sequence (not shown), where the darkest shade represents that >80% of residues in a column agree with the consensus sequence, medium shade represents >60% agreement, lightest shade represents >40% agreement, and no shading represents <40% agreement. Asterisks indicate regions that are conserved among all vertebrates. Open circles indicate regions that are conserved among members of the same class. See Table 2 for GenBank Accession Numbers.

pallium, septum, amygdala, and preoptic area with lower levels of *Arc* expression in the striatum, hypothalamus, and torus semicircularis (Table 3; Fig. 4). There was some variation in the level of *Arc* expression among the five subdivisions of the pallium (Table 3; Fig. 4). We also saw higher expression in the lateral amygdala than the medial amygdala (Table 3). However, we could not distinguish variation in *Arc* expression levels among the subdivisions of the septum, striatum, or hypothalamus. We saw no *Arc* expression in the thalamus, optic tectum, tegmentum, or hindbrain (Table 3). Overall, *Arc* expression was restricted to the forebrain with little to no expression in the midbrain or hindbrain. This pattern is consistent with the neuroanatomical distribution of *Arc* described in mammals (Ons et al., 2004).

Temporal Profile of *Arc* Induction by Sound

To determine the temporal profile of acoustically induced *Arc* expression, we compared *Arc* mRNA levels in unstimulated females (0 h) to those that were exposed to 30 min of mating chorus and sacrificed at various time points after stimulus onset. We found that the mating chorus induced a rapid and robust increase in *Arc* mRNA expression in the dorsal

Table 3 Relative Abundance of *Arc* mRNA Expression in Wild-Caught Female Túngara Frogs

Area	*Arc* Expression
Olfactory bulb	++++
Dorsal medial pallium	++++
Ventral medial pallium	++
Ventral pallium	++++
Lateral pallium	++++
Dorsal pallium	+++
Striatum	+
Septum (dorsal, ventral, medial, lateral)	++++
Lateral amygdala	+++
Medial amygdala	+/++
Preoptic area	+++
Hypothalamus	+
Thalamus	–
Torus semicircularis (laminar and principal nucleus)	+
Optic tectum	–
Tegmentum	–
Hindbrain	–

Undetectable, –; low, +; moderate, ++; high, +++; very high, ++++.

medial pallium (dMP) (ANOVA: $F_{(7,38)} = 6.14$, $p < 0.001$) that peaked 0.75 h after stimulus onset and that declined with further survival [Fig. 5(A)]. *Arc* expression changed dramatically over the course of the experiment. After only 30 min of exposure to the mating chorus, *Arc* expression had already doubled. At peak, the level of *Arc* expression in stimulated animals was nearly fivefold higher than in unstimulated animals. Following peak levels at 0.75 h, *Arc* expression plummeted by more than half in only 15 min. Thus, as in other vertebrates, frog *Arc* is regulated by sensory stimuli in a dynamic fashion.

Spatial Variation in *Arc* Induction by Sound

In order to determine if *Arc* can be induced by mating chorus in other regions of the brain, we quantified *Arc* expression in the pallium (medial, lateral, ventral), basal forebrain (medial septum, preoptic area, dorsal hypothalamus), striatum, and auditory midbrain. When all brain regions were considered together, we found that the mating chorus induced *Arc* expression compared with no sound controls (treatment $F_{(1,8)} = 19.90$, $p = 0.002$), but that this effect varied among brain regions (treatment × region $F_{(7,55)} = 4.71$, $p < 0.001$). When we examined each brain region separately, we found that the mating chorus induced *Arc* expression in all regions of the pallium [medial, $t(8) = -3.52$, $p = 0.007$; lateral, $t(8) = -2.25$, $p = 0.05$; ventral, $t(8) = -2.57$, $p = 0.03$; Fig. 5(B)] but in only some nuclei of the basal forebrain. The medial septum showed *Arc* expression in response to chorus ($t(8) = -2.48$, $p = 0.04$), but the dorsal hypothalamus ($t(8) = -1.14$, $p = 0.29$) and preoptic area ($t(7) = -1.98$, $p = 0.09$) did not [Fig. 5(B)]. In addition, *Arc* expression was not induced in the striatum ($t(8) = -0.02$, $p = 0.98$) or the laminar nucleus of the torus ($t(8) = -0.87$, $p = 0.41$) by hearing mating chorus [Fig. 5(B)]. Among acoustically stimulated females, we saw the highest expression in the pallium [Fig. 6(A,C,E), medial septum Fig. 6(I), and preoptic area Fig. 6(K)], and lower levels in the striatum [Fig. 6(G)]. We observed very low levels of *Arc* in the dorsal hypothalamus and laminar nucleus that appeared to be associated with only a few cells [Fig. 6(M,O)]. This distribution of stimulus induced *Arc* suggests that it may have a region-specific function in frogs.

Animals in the 0 h group remained essentially stationary (mean ± SD = 0.76 ± 1.11 hops/min), while animals in the 0.75 h group moved periodically during the test period (mean ± SD = 2.51 ± 2.63 hops/min), although not significantly more than the 0 h group ($t(7) = 1.34$, $p = 0.23$). Because locomotor activity can induce immediate early gene expression, we tested for a relationship between movement and *Arc* expression in each brain region in the 0.75 h group. We found no covariation (all $p > 0.14$; data not shown), suggesting that the *Arc* expression we observed is not related to motor output.

Specificity of Acoustically Induced *Arc mRNA*

We exposed laboratory-reared túngara frogs to different types of mating calls in order to ask whether calls with different biological meanings could induce differential *Arc* expression. We exposed females to conspecific calls (whine or whine-chuck) or heterospecific calls and measured *Arc* mRNA expression in brain regions that showed significant *Arc* induction in response to chorus playback. We found that *Arc* mRNA expression was similar among the three groups in all brain regions examined (medial pallium, $F_{(2,18)} = 0.65$, $p = 0.53$; lateral pallium, $F_{(2,18)} = 0.57$, $p = 0.58$; ventral pallium, $F_{(2,19)} = 0.37$, $p = 0.69$; medial septum $F_{(2,18)} = 0.57$, $p = 0.57$; Fig. 7).

DISCUSSION

We identified *Arc* in two frog species, *X. tropicalis* and *P. pustulosus*, and characterized its expression in

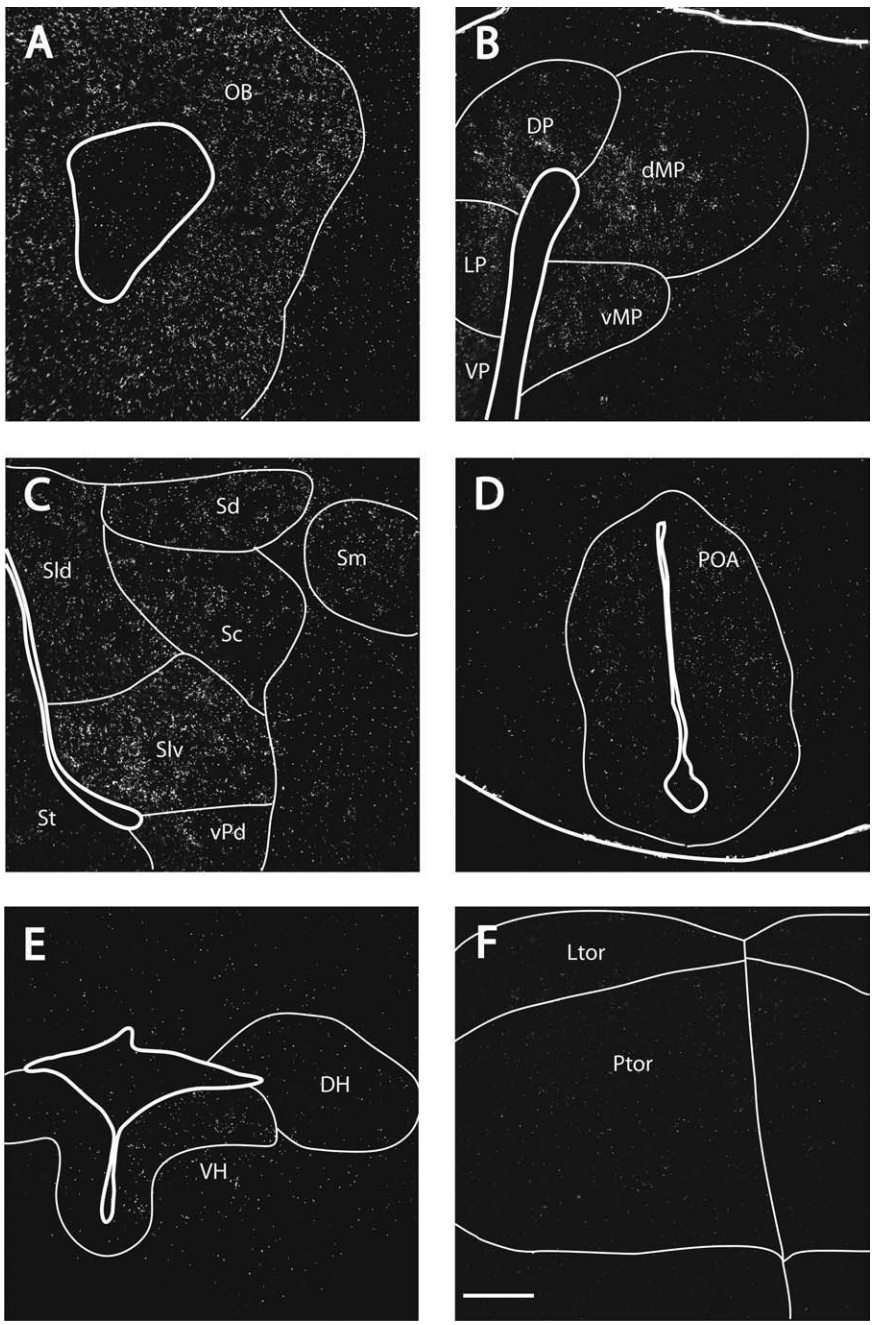

Figure 4 Darkfield images showing *Arc* expression in the olfactory bulb (A), pallium (B), septum (C), preoptic area (D), hypothalamus (E), and torus semicircularis (F) of wild-caught females. Scale bar represents 100 μm.

the brain of *P. pustulosus*. We found that the predicted protein for frog Arc shared 60% sequence similarity with Arc in other vertebrates, and *Arc* was expressed at high levels in the forebrain, but not the midbrain or hindbrain, of females sacrificed at breeding ponds. In controlled experiments, accumulation of *Arc* mRNA peaked 0.75 h following onset of a mating chorus, and the mating chorus induced *Arc* expression in the pallium and septum, but not in the striatum, hypothalamus, or auditory midbrain. Finally, *Arc* expression in the pallium and septum of female túngara frogs did not vary with the attractiveness or complexity of the acoustic mating calls that we presented. This study is the first to characterize the neuroplasticity-related gene, *Arc,* in a frog species. Our results demonstrate that expression of *Arc*

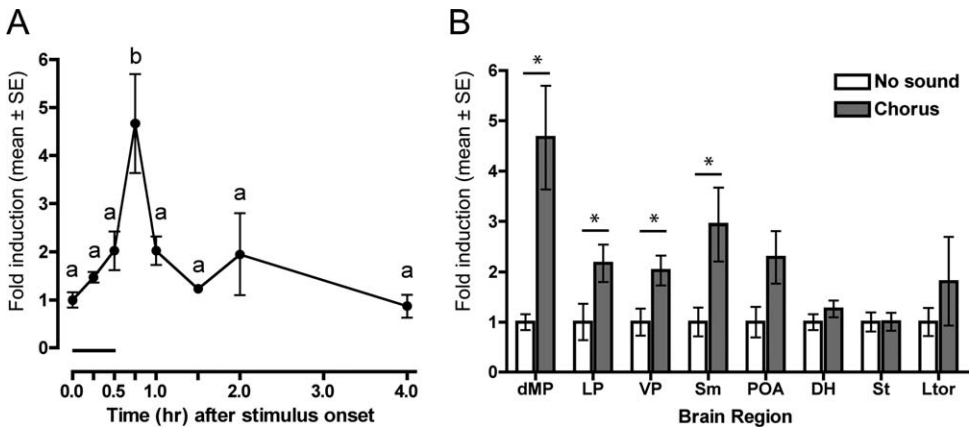

Figure 5 Temporal and spatial distribution of acoustically-induced *Arc* expression. (A) Time-course of *Arc* induction in the dorsal medial pallium in response to 30 min of mating chorus (black bar). Filled circles represent mean fold-induction (±SE) of *Arc* mRNA expression relative to 0 h. Letters above data points denote significant differences between groups (Fisher's least significant difference *post hoc* test, $p < 0.05$). (B) *Arc* mRNA induction in response to mating chorus (gray bars) relative to no sound (white bars) in select nuclei of the frog brain. Data are shown as mean fold-induction (±SE) relative to the no sound group. Asterisks above bars denote significant differences between groups ($p < 0.05$). DH, dorsal hypothalamus; dMP, dorsal medial pallium; LP, lateral pallium; Ltor, laminar nucleus of torus semicircularis; POA, preoptic area; Sm, medial septum; St, striatum; VP, ventral pallium.

in biologically significant contexts is not limited to mammals and birds, but may be a feature of the immediate early gene response in all vertebrates.

We cloned fragments of *Arc* cDNA in two frogs, and used Northern blot to determine that the full-length *P. pustulosus Arc* transcript was about 3.5 kb, which is similar in size to *Arc* in mammals (rodents, 3.2 kb; humans, 3.4 kb), but substantially smaller than zebra finch *Arc* (5.1 kb). The larger size of the bird transcript likely reflects sequence divergence in the 5′ and 3′ UTR regions (Velho et al., 2005). We also found that *Arc* was expressed in túngara frog brain but not liver, which is consistent with findings in rats (Lyford et al., 1995). Furthermore, we found that the predicted protein sequence of frog Arc shares over 40% identity with chicken and rat Arc. Because >30% identity at the amino acid level generally suggests that two proteins are structurally similar and evolutionarily related (Rost, 1999; Yang and Honig, 2000), we conclude that frog Arc is likely to share many of the functions described for other vertebrates. In particular, Arc protein appears to be highly conserved at two functional domains that are likely to be important for mediating intermolecular interactions at the synapse. For example, túngara frog predicted Arc protein shares 75% sequence identity with the endophilin 3 binding domain on rat Arc. Endophilin 3 plays an important role in receptor-mediated endocytosis in olfactory nerve terminals (Sugiura et al., 2004), and it is localized in dendritic spines (Chowdhury et al., 2006), where Arc can also be found (Moga et al., 2004). The high degree of similarity between rat and túngara frog Arc predicted protein at this binding domain suggests that Arc probably plays a similar role in mediating synaptic plasticity in all vertebrates studied to date.

We found that *Arc* expression peaked 0.75 h after onset of an acoustic stimulus in the medial pallium of túngara frogs compared to 0.5 h after stimulus onset in zebra finch mesopallium (Velho et al., 2005) and rat hippocampus (Guzowski et al., 2001). Because previous studies did not measure *Arc* expression at 0.75 h, we cannot know whether relative mRNA levels could have increased further with time. At peak, we observed chorus-induced *Arc* levels in the túngara frog medial pallium that were more than 4.5 times that of unstimulated controls. In contrast, Velho et al. (2005) observed an approximately 2.5-fold peak induction of *Arc* in zebra finches exposed to conspecific song, while Guzowski et al. (2001) found a 1.5-fold induction of *Arc* in rats after spatial water-task training. The differences among species in the magnitude of peak *Arc* mRNA induction could be due to differences in experimental design or the characteristics of the stimuli used. Alternatively, our protocol might simply provide greater resolution of a time-course of stimulus-induced *Arc* that is common to all vertebrate species and that peaks at 0.75 h. For instance, we found twofold induction of *Arc* 0.5 h post-stimulus onset, compared with 2.5-fold induc-

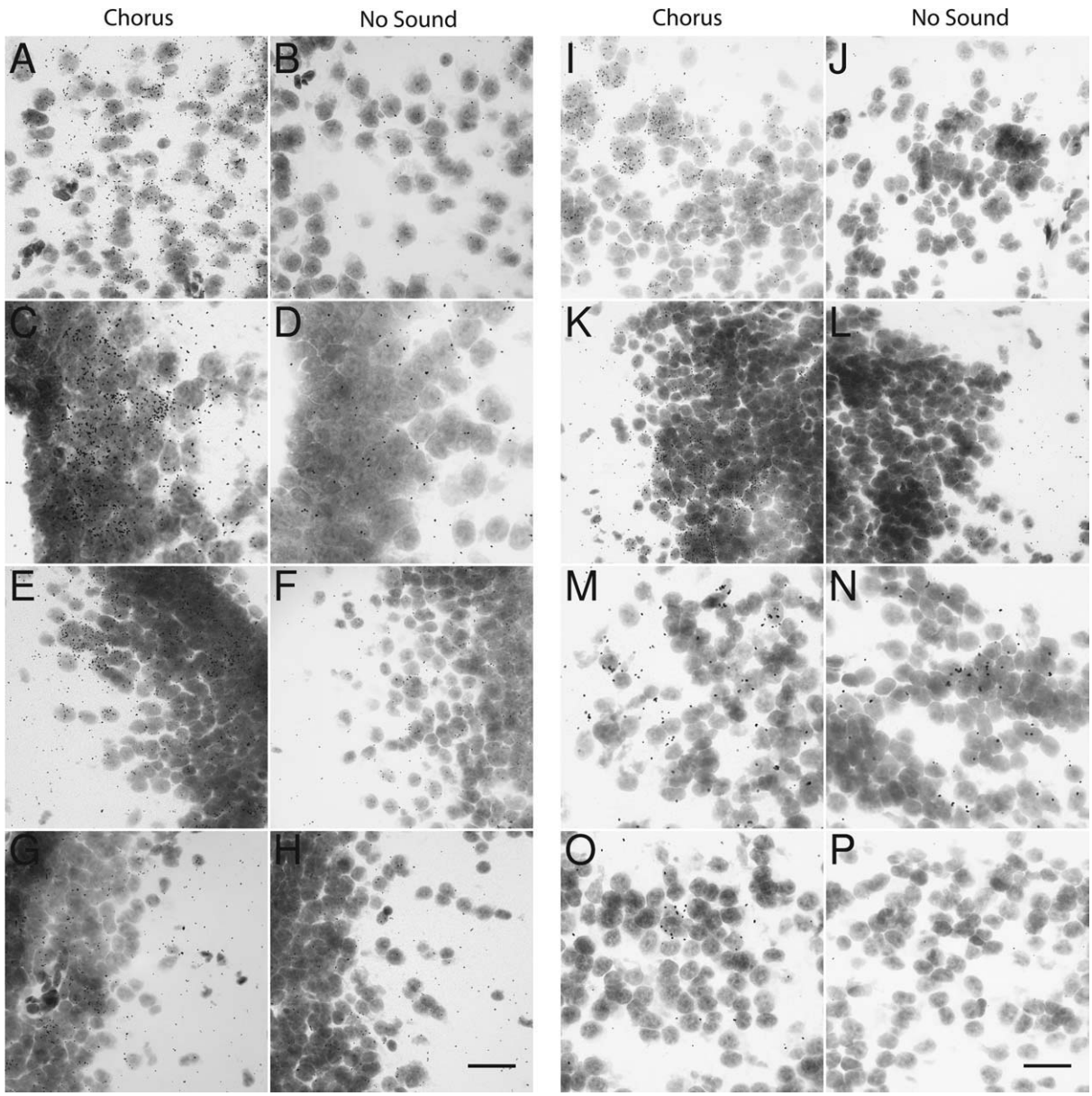

Figure 6 Photomicrographs of *Arc* mRNA expression in females exposed to a mating chorus (left column) compared to females not exposed to sound (right column) in the dorsal medial pallium (A and B), lateral pallium (C and D), ventral pallium (E and F), striatum (G and H), medial septum (I and J), preoptic area (K and L), dorsal hypothalamus (M and N), and laminar nucleus of the torus semicircularis (O and P). Images were adjusted for contrast. Scale bar represents 20 μm.

tion of *Arc* in zebra finches at the same time point (Velho et al., 2005). Our results highlight the need for a finer temporal scale when studying the time-course of an immediate early gene in a new species, particularly when species comparisons are important.

We found that *Arc* is expressed at high levels in the forebrain, but not midbrain or hindbrain, of wild-caught female túngara frogs. In addition, exposure to a mating chorus induced *Arc* expression in the pallium (medial, lateral, and ventral) and septum, but not in the striatum or auditory midbrain of females tested in the laboratory. In rat and chicken, *Arc* is expressed in the hippocampus (Guzowski et al., 1999; Lyford et al., 1995; Kelly and Deadwyler, 2003; Vazdarjanova et al., 2006), other limbic regions (e.g. nucleus accumbens and amygdala; Kelly and Deadwyler, 2003; Ons et al., 2004), and primary sensory cortices (Kelly and Deadwyler, 2003; Ons et al., 2004; Bock et al., 2005; Vazdarjanova et al., 2006). Of particular relevance to our study, in zebra

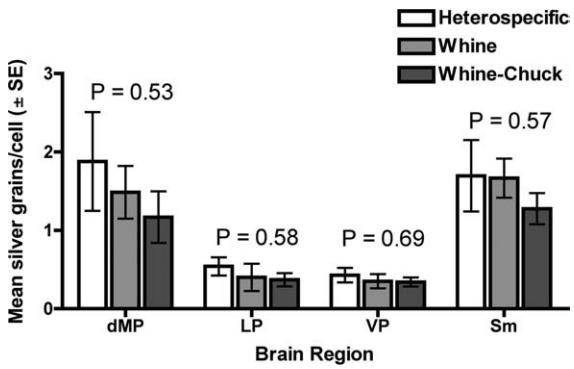

Figure 7 Lack of selectivity of acoustically induced *Arc* mRNA expression. Mean (±SE) *Arc* expression levels, shown as mean silver grains per cell, in the dorsal medial pallium (dMP), lateral pallium (LP), ventral pallium (VP), and medial septum (Sm) of female túngara frogs exposed to 30 min of heterospecific calls, conspecific whine calls, or conspecific whine-chuck calls. *p* values refer to main effect of stimulus type (one-way ANOVA).

finches, song-induced *Arc* is found predominantly in pallial regions involved in auditory learning and song discrimination (nidopallium and mesopallium), but it is not induced in the thalamus (nucleus ovoidalis) (Velho et al., 2005). In contrast to our study, several authors have reported *Arc* induction in the striatum of chickens (Bock et al., 2005) and rats (Kelly and Deadwyler, 2003; Ons et al., 2004) in response to environmental stimuli. In all of these studies, it appears that stimulus-induced *Arc* expression is restricted to the telencephalon, with no induction reported in diencephalic or mesencephalic regions. However, because few studies measure *Arc* induction in extra-telencephalic brain regions (but see Haugan et al., 2008), it is not clear whether this expression pattern is characteristic of the *Arc* response in vertebrates or whether it simply reflects the aims and sampling strategies of the experiments in which *Arc* is utilized as a marker for neural activity.

In túngara frogs, *Arc* is expressed in fewer brain regions in response to acoustic stimulation than is *egr-1*. *Egr-1* is induced by conspecific calls in the auditory midbrain, thalamus, hypothalamus, pallium, and subpallium, including the striatum (Hoke et al., 2004, 2005, 2007; Burmeister et al., 2008; Mangiamele and Burmeister, 2008), whereas *Arc* was induced only in the pallium and septum. Similarly, in mammals, *Arc* is expressed in a more restricted set of brain regions compared to other immediate early genes, such as *c-fos* (Ons et al., 2004). *Arc*'s more restricted expression pattern compared to *egr-1* and *c-fos* is probably a consequence of the fact that *Arc*, as an effector immediate early gene, has a highly specific function whereas genes like *egr-1* and *c-fos* are transcription factors with many target genes and, therefore, many different functions. For example, in rats, exploration of a novel environment elicits *Arc* induction in a subpopulation of α-CAMKII positive neurons because *Arc* is probably important only in the subset of cells that are actively maintaining or forming synaptic connections (Vazdarjanova et al., 2006). In contrast, although *egr*-1 is also important for synaptic plasticity (e.g., Bozon et al., 2003), it probably also contributes to regulating other cellular processes. Thus, *Arc* is expressed in fewer brain areas and under fewer scenarios.

In our study, acoustically induced *Arc* lacked some of the stimulus specificity that has been observed in songbirds. In zebra finches, females stimulated with zebra finch song had a greater induction of *Arc* in the caudomedial nidopallium than females stimulated with canary song (Velho et al., 2005). In contrast, in túngara frogs, heterospecific and conspecific mating calls induced similar levels of *Arc* expression in all areas of the telencephalon included in this study, even though conspecific mating calls elicit greater *egr-1* expression than heterospecific mating calls in the medial and lateral pallia (Chakraborty, Mangiamele, and Burmeister, unpublished). We also failed to find increased *Arc* expression in response to the female-preferred whine-chuck mating call compared to the less preferred whine; however, this result is similar to the *Arc* response in songbirds. In canaries, *Arc* mRNA expression in the auditory forebrain does not vary between females presented with a more attractive "sexy" song compared with a less attractive song (Leitner et al., 2005). Leitner et al. (2005) speculate that a lack of elevated *Arc* expression in female canaries in response to "sexy" song may be due to the fact that preferences for certain acoustic elements of the songs are innate, thus experience-dependent long-term synaptic memory may not be necessary for the maintenance of song preferences. Likewise, in female túngara frogs, the preference for the whine-chuck call does not require acoustic experience (Dawson, 2007) and is probably innate. Although *Arc* expression did not vary in response to mating calls that differ in their behavioral relevance, *Arc* induction may still be an important way in which the brain responds to relevant stimuli in the animal's natural environment. For example, *Arc* may be involved in mediating dynamic changes in neural connections when a female is actively making a choice between two different male signals. Alternatively, it is possible that different mating calls elicit *Arc* expression within distinct networks of cells, but that the overall level of *Arc* expression remains unchanged.

In summary, we identified frog *Arc,* showed that it is expressed in the brain, and that it behaves as an immediate early gene in that it can be rapidly induced by acoustic stimulation. We also found that the *Arc* response in túngara frogs is not selective for different categories of acoustic stimuli, as it is in some songbirds. Further investigation is needed to elucidate the significance of acoustically induced *Arc* expression in frogs and how it relates to the patterns of *Arc* expression in other vertebrate species.

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