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Familiarity with a vocal category biases the compartmental expression of Arc/Arg3.1 in core auditory cortex

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Learning to recognize a stimulus category requires experience with its many natural variations. However, the mechanisms that allow a category’s sensorineural representation to be updated after experiencing new exemplars are not well understood, particularly at the molecular level. Here we investigate how a natural vocal category induces expression in the auditory system of a key synaptic plasticity effector immediate early gene, Arc/Arg3.1, which is required for memory consolidation. We use the ultrasonic communication system between mouse pups and adult females to study whether prior familiarity with pup vocalizations alters how Arc is engaged in the core auditory cortex after playback of novel exemplars from the pup vocal category. A computerized, 3D surface-assisted cellular compartmental analysis, validated against manual cell counts, demonstrates significant changes in the recruitment of neurons expressing Arc in pup-experienced animals (mothers and virgin females “cocaring” for pups) compared with pup-inexperienced animals (pup-naïve virgins), especially when listening to more familiar, natural calls compared to less familiar but similarly recognized tonal model calls. Our data support the hypothesis that the kinetics of Arc induction to refine cortical representations of sensory categories is sensitive to the familiarity of the sensory experience.

The ability to recognize novel exemplars of behaviorally relevant sensory categories is critical to navigating our environment and communicating with others. A sensory system must presumably assess whether a stimulus matches the collection of features that statistically define a recognized category. At the same time, variation present in new exemplars must themselves be incorporated into the sensory system’s representation of that category. The neural mechanisms that underlie such categorization at the electrophysiological level are being elucidated in both the visual and auditory modalities (DiCarlo et al. 2012; Bizley and Cohen 2013; Shepard et al. 2015), but the molecular mechanisms that support the learning and memory of sensory categories remain poorly understood.

One promising molecular coordinator of activity-dependent changes in synaptic efficacy, which has been suggested to underlie learning and memory, is the effector immediate early gene (IEG) Arc/Arg3.1 (Arc), a “master regulator” of synaptic plasticity during information processing (Shepherd and Bear 2011). Arc is expressed in hippocampal neurons that then undergo synaptic plasticity as a result of a behavioral experience (Guzowski et al. 1999; Vazdarjanova and Guzowski 2004; Fletcher et al. 2006). It can also be induced in sensory systems after sensory experiences (Tagawa et al. 2005; Carpenter-Hyland et al. 2010; Ivanova et al. 2011; Morin et al. 2011). Moreover, the levels of Arc expression correlate with hippocampus-dependent learning (Guzowski et al. 2001), plays essential roles in homeostatic plasticity and the long-term consolidation of memories (Gao et al. 2010). Recent studies suggest this may stem from its apparent preferential “inverse tagging” in activated neurons of the subset of inactive synapses, which then undergo long-term depression by endocytosis of AMPA receptors (Waung et al. 2008; Okuno et al. 2012). Arc’s action explains how repeated experiences with the same visual stimulus leads to a progressively smaller population of visual cortical neurons that is more robustly activated by that stimulus (Wang et al. 2006), thereby helping to refine the stimulus specificity of sensory neurons. In the real world though, the exact same experience or stimulus is not usually encountered. Instead, natural stimuli are more likely to fall into the same behaviorally relevant stimulus category, but differ in their physical detail from exemplar to exemplar. Given Arc’s role in refining synaptic connectivity for a neuron’s stimulus specificity, how might it be deployed to support plasticity for stimulus generalization to novel exemplars of a familiar stimulus category?

We address this question in the context of natural auditory categorization of behaviorally relevant communication sounds in a mouse model. Maternal females recognize the ultrasonic vocalizations (USV) of displaced mouse pups as behaviorally important, and will seek out and retrieve the lost pup to the nest. On the other hand, nonmaternal, virgin females do not show a behavioral preference for pup calls over neutral sounds, until they have had
experience “cocaring” for pups—suggesting that the behavioral relevance of pup USVs is acquired (Ehret et al. 1987; Lin et al. 2013). These whistle-like calls are naturally variable along various acoustic dimensions, such as frequency, duration, frequency modulation, and bandwidth (Liu et al. 2003; Grimsley et al. 2011), but nevertheless form a distinct acoustic category that can be categorically perceived by maternal mice (Ehret and Haack 1981; Ehret 1992). Importantly, excitatory plasticity in a subset of putative pyramidal neurons in the core auditory cortex of maternal females enhances the spiking response to favor the combination of acoustic features that are statistically predictive of the pup USV category, an effect not observed in nonmaternal virgins (Shepard et al. 2015). Hence, for maternal but not nonmaternal mice, pup USVs form a familiar, behaviorally relevant sound category that core auditory cortical activity “learns” to systematically differentiate from other sound categories.

The mounting electrophysiological evidence for plasticity in auditory cortex in this vocalization recognition paradigm provides a basis for studying the molecular mechanisms involved (Liu and Schreiner 2007; Galindo-Leon et al. 2009; Cohen et al. 2011; Lin et al. 2013; Marlin et al. 2015). In particular, it opens a unique opportunity to investigate in a natural context how Arc is expressed in auditory cortex when new sounds (models of pup USVs) either do (maternal mice) or do not (nonmaternal mice) conform to a familiar, behaviorally relevant category. We assessed this using a cell compartmental analysis of Arc gene transcription by fluorescent in situ hybridization and 3D surface reconstruction (Guzowski et al. 1999; Vazdarjanova et al. 2002; Vazdarjanova and Guzowski 2004), in which a small number of Arc mRNA strongly labeled cells can always be detected (Fig. 2A). Here and in our previous study (Ivanova et al. 2011) we used computer-generated 3D surface reconstruction of nuclei and Arc mRNA (Fig. 2B) by objectively thresholding fluorescence in the confocal z-stacks.

Results

In the present study, we used the maternal recognition paradigm (Fig. 1A,B) to investigate how familiarity with sounds (Fig. 1C) can change compartmentalization of Arc mRNA (Fig. 2) in auditory cortical neurons. The dentate gyrus was used as a positive control area for confocal microscopy (Guzowski et al. 1999; Vazdarjanova et al. 2002; Vazdarjanova and Guzowski 2004), in which a small number of Arc mRNA strongly labeled cells can always be detected (Fig. 2A). Here and in our previous study (Ivanova et al. 2011) we used computer-generated 3D surface reconstruction of nuclei and Arc mRNA (Fig. 2B) by objectively thresholding fluorescence in the confocal z-stacks.

To validate our calculations against the typical approaches taken in the literature, we compared our 3D surface counting results (Fig. 3, Ims1) to manual counting by methods previously described by Chawla et al. (2004). For such manual counting, 3D-counting methods are labor-intensive and time-consuming.

![Figure 1](image_url)

**Figure 1.** Experimental paradigm. (A) Preexperiment timeline for each of three groups of adult female mice. Dams and Cocs were cohoused for several days before pups were born, and then screened on P5 or P6 for successful retrieval of scattered, vocalizing pups in the home cage. Pups were weaned on P21, after which Dams and Cocs were separated into individual cages for 24 h. Vrgs, matched to Dams and Cocs, had no adult contact with pups, and were group housed in the colony over the same period of time before being placed into an individual cage 24 h prior to the experiment. (B) Timeline for sound exposure on experiment day. An individual mouse in its home cage was placed into a silent anechoic chamber for a 4-h habituation in silence, followed by a 5-min period of sound stimulation by Tone (65 kHz), USV in Noise, or Noise. Mice were decapitated immediately afterwards (Sac 0 min) or with a 30-min delay (Sac 30 min). (C) (Left) Waveform of the first 3 sec of the random sequence of pure tone pips at 65 kHz, a synthetic model of pup USVs. (Center) Waveform (top) and spectrograms (bottom) of the first 3 sec of the USV and the matched Noise (Right) stimulus. Gray scale indicates higher amplitude with whiter pixels.
Both foci and perinuclear cytoplasmic expression (Fig. 3B, Both, Ims2-lms1, and lms2-M), resulting in an overall significant under-count for Arc-positive neurons (Fig. 3B, Arc-positive, Ims2-lms1, and lms2-M). No effect on Perinuc-only counts was found for such a change in threshold, consistent with those neurons’ expression primarily being determined by large regions of Arc fluorescence. These results imply that there are tradeoffs in choosing thresholds for what is considered nonbackground expression. Importantly, the use of the computer-generated 3D surfaces makes these tradeoffs objective and explicit rather than subjective, and should enhance reproducibility of Arc counting. In any case, for all analyses presented here, cell count percentages based on 3D surfaces are compared across groups treated equally, minimizing the effect that any systemic bias would have in altering our conclusions.

Moving beyond methodological validation, we turned next to measuring expression differences dependent on familiarity with the acoustic features of pup USVs. A key acoustic feature characterizing the pup USV category is call frequency (Liu et al. 2003), a parameter that maternal mice can use as a basis for their categorical perception of USVs (Ehret and Haack 1982). We therefore started by asking whether playback of pure tones at 65 kHz (Fig. 1C, left), the approximate frequency most commonly found in natural CBA/CaJ mouse pup calls (Liu et al. 2003), would induce Arc expression differently in core auditory cortical neurons of Dams versus Vrgs. The stimulus consisted of randomly generated sequence of 65 kHz pure tone-pip bouts, with temporal properties (pip duration, intercall interval, interbout interval, and number of calls in a bout) spanning their ranges found in natural USV bouts (Fig. 1B, center). For both animal groups (Dam and Vrg), this particular synthetic tonal stimulus had never been encountered previously, though for the Dam, the tone frequency’s closeness to natural USVs could affect its perception as pup-like (Ehret & Haack 1982). After animals were given a 4-h period of silence in an anechoic chamber (Fig. 1B), they were either held in silence for an additional 30 min (Silence group), or were played a 5-min, 65 kHz bout of tone stimuli before being sacrificed immediately (0 min), or after a delay in silence (30 min).

Silence itself was associated with ~16% Arc-positive expression (Foci-only + Both + Perinuc-only) in auditory cortex in both Dams and Vrgs, indicating a relatively high basal level of Arc expression in cortical layers III–VI (Fig. 4A, left bars). Tonal 65 kHz stimulation led to a main effect of time in increasing the percentage of total Arc-positive neurons (time: $F_{2,113} = 69.53, P = 2.1 \times 10^{-20}$, $2 \times 3$ ANOVA). However, there was no main effect of animal group (animal: $F_{2,113} = 0.07, P = 0.80$; time $\times$ animal: $F_{2,113} = 0.31, P = 0.73, 2 \times 3$ ANOVA). By the 30-min post-stimulus time point, Arc mRNA was expressed in nearly 60% of core auditory cortical neurons irrespective of animal group (Fig. 4A, right bars), affirming sound-induced expression of Arc. Significant post hoc comparisons are shown in Figure 4A.

For cells expressing Arc only as Foci in the nucleus (Fig. 4B), 65 kHz tone stimulation led to a main effect of time but not animal group, and only a trending interaction (time: $F_{2,113} = 4.85, P = 0.0096$; animal: $F_{1,113} = 2.19, P = 0.14$; time $\times$ animal: $F_{2,113} = 2.86, P = 0.06, 2 \times 3$ ANOVA). At the 0-min time point (Fig. 4B, middle bars), the sound had elevated Foci-only expression significantly in Vrgs ($P = 0.0138$, Tukey) and was trending in Dams ($P = 0.071$, Tukey). Intriguingly, the foci-only expression in Vrgs increased by 30-min time point compared with silence level ($P = 0.0185$, Tukey), but no such increase was observed in Dams ($P = 1.0$, Tukey).

Next, the percentage of neurons with Arc expressed only in the perinuclear cytoplasm following 65 kHz stimulation showed a significant main effect of time, animal group, and interaction...
between time and animal group (Fig. 4C; time: F(3,113) = 67.64, P = 2.1 × 10^{-20}; animal: F(1,113) = 5.61, P = 0.0195; time × animal: F(3,113) = 7.69, P = 0.0007; 2×3 ANOVA). At 0 min immediately after the stimulation, Perinuc-only expression was low, and did not significantly differ between the groups (Vrg 0 min: 3.9 ± 2.2%, Dam 0 min: 3.3 ± 2.5%, P = 1.0, Tukey). By 30-min though, the percentage of Perinuc-only neurons rose in both animal groups, but Dams had a higher percentage compared with Vrgs (Dam: 30.9 ± 2.5%, Vrg: 17.6 ± 2.2%, P = 2.7 × 10^{-4}, Tukey). Hence, even though Arc-positive expression did not differ between animal groups at any time point (Fig. 4A), the cell compartmental composition of this total expression did (Fig. 4B, C). Under the assumption that 65 kHz tones sound sufficiently like pup USVs to mothers (Ebert and Haack 1982), this difference in expression is consistent with the interpretation that a larger population of neurons with Perinuc-only Arc expression provides a molecular trace of whether new exemplars sound more familiar (Ivanova et al. 2011).

However, the fact that the expression difference was observed at the 30-min but not at 0-min time point raises some questions about the above interpretation, since we previously observed an increase in Perinuc-only expression 0-min after rehearing a (slightly longer) 10-min familiar stimulus (Ivanova et al. 2011). Therefore, as an independent test, our next experiment exposed animals to 5 min of either prerecorded pup USVs (Fig. 1B, center), or to the control, microphone noise (Nse) from the recording (Fig. 1B, right), and examined Arc expression immediately afterwards. Natural pup USVs should sound more familiar to and be recognized by mothers but not by naïve virgins, even if the specific exemplars are novel.

Overall Arc-positive expression was slightly higher for the USV compared with Nse stimulation (Fig. 5A; stimulus: F(1,17) = 7.51, P = 0.0076; animal: F(1,17) = 1.18, P = 0.28; stimulus × animal: F(1,17) = 4.56, P = 0.036, 2×2 ANOVA). This result was carried mainly by a large percentage of Arc-positive neurons in Dams hearing USV (Fig. 5A), though multiple-comparison corrected post hoc testing indicated this was not significantly higher than for Vrgs hearing USV (P = 0.11, Tukey’s). At this 0-min time point after playback, there was again no significant difference in Foci-only Arc expression (Fig. 5B), either between animal groups or across stimuli (stimulus: F(1,17) = 0.67, P = 0.41, animal: F(1,17) = 0.48, P = 0.49, stimulus × animal: F(1,17) = 2.25, P = 0.14, 2×2 ANOVA).

Critically though, significant differences were found while comparing Perinuc-only expression (Fig. 5C). An interaction between stimulus and animal group (stimulus × animal: F(1,17) = 5, P = 0.028, 2×2 ANOVA) and main effects of each (stimulus: F(1,17) = 5.57, P = 0.021; animal: F(1,17) = 5.84, P = 0.018, 2×2 ANOVA) were entirely due to a much higher percentage of Perinuc-only expressing neurons in Dams hearing USV compared with all other groups (Fig. 5C; Dam-USV: 11.0 ± 1.4%; versus Vrg-Nse, 4.0 ± 1.5%, P = 0.0051; versus Vrg-USV, 4.2 ± 1.5%, P = 0.0077; versus Dam-Nse, 4.3 ± 1.5%, P = 0.0076, Tukey’s). Importantly, the fact that the background recording of Nse stimulus elicited no significant differences between Vrgs and Dams for any of our Arc measures indicates that...

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**Figure 3.** Comparative analysis of 3D-imaris and manual count of Arc expression in auditory cortex. (A) Comparison of 3D surface-assisted cell compartmental counts (Ims1, black bar) to manual counts (M, white bar). Percentage reflects the fraction of cells in a z-stack classified as having Arc mRNA in the specified compartment(s) (13 z-stacks, 777 cells). No significant differences, except marginally for Foci-only cells (no multiple comparison correction). Ims1 versus M paired t-test: Foci-only, P = 0.045; Perinuc-only, P = 0.659; Both, P = 0.54; Total, P = 0.59. (B) Paired comparisons of compartmental cell counts. Comparisons of Ims1, M, and Ims2 (10% higher fluorescence threshold for 3D imaris counts) suggest that increasing the fluorescence threshold for 3D surfaces reduces the number of Foci-only neurons closer to manual counts, but reduces the overall number of Arc-positive neurons because fewer neurons are classified as having Both Foci and Perinuc expression. Ims2 vs Ims1: Foci-only, P = 0.003; Perinuc-only, 0.76; Both, P = 0.42; Total, P = 0.023. Ims2 versus M: Foci-only, P = 0.95; Perinuc-only, 0.56; Both, P = 0.27; Total, P = 0.05. Asterisks (*) indicate significant paired t-tests (P < 0.05). Error bars represent standard error.

**Figure 4.** Arc mRNA induction in core auditory cortex 0 min or 30 min after playback of 5 min of 65 kHz pure tone sequences. Neurons in layers III-VI of core auditory cortex were classified as Arc-positive (A), Foci-only (B), and Perinuc-only (C) for Vrgs (white bar, 23 z-stack images) and Dams (black bar, 22 z-stacks) at 0-min after stimulation, and for Vrgs (18 z-stacks) and Dams (18 z-stacks) at 30-min after stimulation. First two left bars in each panel shows Arc-labeled cells after 4.5 h silence. Of note, 65 kHz pure tone induces greater Foci-only expression in Dams and Vrgs immediately after playback tone, without any differences between groups. At 30 min, the percentage of Foci neurons in Dams (10.3 ± 1.6%) becomes comparable to the silence level (9.56 ± 0.8%), but the percentage of Foci in Vrgs remains larger than in Dams (Vrg 19.2 ± 3.4%, P = 0.026). Tone models of pup USVs induce greater Perinuc-only expression in Dams versus Vrgs, but only at 30 min, without any differences in overall Arc-positive expression. Significance in the two-way ANOVA is indicated for main effects of time (t), animal group (a), or interactions between the two (t,a,i). Asterisks (*) indicate significant multiple comparison-corrected post hoc tests (P < 0.05). Error bars represent standard error.

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As with the 65 kHz tones, group, and there were no significant differences (F(2,75) = 4.48, P = 0.03, 1 × 3 ANOVA) and Perinuc-only (F(6C; F(2,75) = 5.89, P = 0.0042, 1 × 3 ANOVA) expression. The percentage of Foci-only cells in Dams (10.5 ± 1.4%) was similar with their level for silence, and was not different from that seen 30 min after 65 kHz playback (Fig. 4B, 10.3 ± 1.6%). In contrast, Vrgs (16.1 ± 1.3%) had the highest Foci-only percentage of all animal groups, which was significantly larger than for Dams (P = 0.005 Fisher’s), but not than Coc (13.8 ± 1.3%, P = 0.26, Fisher’s).

Furthermore, after a 30-min delay Vrgs (21.9 ± 1.8%) had a significantly lower percentage of Perinuc-only expressing neurons compared with Dams (30.1 ± 1.6%; P = 0.0011, Fisher’s) and Coc (26.9 ± 1.7%, P = 0.046, Fisher’s). Hence the pattern of compartmental Arc expression in Coc appeared to more closely align with that of Dams, suggesting that neither a maternal hormonal trajectory nor sustained salience of pup USVs are needed to be able to see a bias toward greater Perinuc-only Arc expression (summarized in Fig. 6, bottom). This result therefore supports the hypothesis that compartmental Arc expression in core auditory cortical neurons can provide a molecular trace of the experience-dependent familiarity of a sound category, even when the exact exemplars heard are novel.

Finally, pooling sound-stimulated data across animal groups, we found that a sound as short as 5 min induces changes in the transcription of Arc mRNA in the auditory cortex, as shown by averaging compartmental expression across all stimuli (Fig. 7A; time: F(2,131) = 114.46, P = 3.3 × 10^{-43}, 1 × 3 ANOVA). These changes are in fact observed for each of the stimuli (Fig 7B): Tone (time: F(6;131) = 84.17, P = 3.1 × 10^{-24}, 1 × 3 ANOVA), Noise (time: F(6;112) = 122.61, P = 6.2 × 10^{-29}, 1 × 3 ANOVA), and USVs (time: F(6;167) = 123.14, P = 1.4 × 10^{-34}, 1 × 3 ANOVA). These results suggest that brief sound exposures in general recruit a continuing cascade of new Arc mRNA (reflected in the combined Both + Foci-only category) in auditory cortex extending beyond the typical ~10 min window after experience to which hippocampal CA1 Arc expression is usually confined. Apparently, the time course of Arc expression in this

Figure 5.  *Arc* mRNA expression in core auditory cortex 0 min after playback of 5 min of Nse or USV. Neurons in layers III–VI of core auditory cortex were classified as Arc-positive (A), Foci-only (B), and Perinuc-only (C) for Vrgs (20 z-stack images) and Dams (20 z-stacks) exposed to Nse, and for Vrgs (19 z-stacks) and Dams (22 z-stacks) listening to USV. Natural pup USV induces greater Perinuc-only expression in Dams versus Vrgs, which cannot be explained by a response to microphone noise in the recording. Also after USV stimulation Dams had significantly higher level of Arc-positive cells versus Nse, and versus Vrgs after Nse. Significance in two-way ANOVA is indicated for main effects of stimulus (s), animal group (a), or interactions between the two (i). Asterisks (*) indicate significant multiple comparison-corrected post hoc tests (P < 0.05). Error bars represent standard error.

the animal differences observed for Perinuc-only expression elicited by the USV stimulus must be due to the presence of natural USVs in the recording. Since this was found as quickly as 5 min after the onset of sound stimulation, our data are consistent with the possibility that the time course of Arc mRNA expression and/or detectability in core auditory cortex is accelerated when acoustically realistic, familiar stimuli are heard. Alternatively, one might argue that the differences observed between Dams and Vrgs might simply be due to dissimilarities in their history of reproductive hormones, even though at the post-weaning time point of sound playback here, both Dams and Vrgs would be cycling normally. It may also be that pup USVs are far more salient to Dams than to Vrgs, irrespective to whether they are familiar or not. To address these possibilities within our natural paradigm, we next investigated a third animal group of Cocs, that is, virgin cocarers. These females gain the same duration of pup care and USV exposure as Dams, but do not experience the hormones of pregnancy, parturition, and lactation to the same degree (Fig. 1A). Furthermore, pup USVs apparently have a diminished degree of behavioral salience for Cocs compared with Dams after pups are weaned (Ehret and Kohl 1989; Lin et al. 2015). Hence, Cocs and Dams should be similar in terms of their level of acoustic familiarity with pup USVs through their common pup care experience, but differ in their hormonal trajectories and degree of pup USV salience post-weaning, which for post-weaning Cocs are more similar to Vrgs.

To determine whether Cocs are more similar to Dams or Vrgs in their pattern of Arc expression after hearing pup USVs, we played the natural calls to animals from all three groups and sacrificed them after a 30-min delay (Guzowski et al. 2001; Khodadad et al. 2015). As with the 65 kHz tones, *Arc* mRNA was expressed in nearly 60% of core auditory cortical neurons irrespective of an animal group, and there were no significant differences (Fig. 6A; F(2,75) = 0.99, P = 0.38, 1 × 3 ANOVA). However, at the compartmental level, animal group significantly modulated both the percentage of Foci-only (Fig. 6B; F(2,75) = 4.48, P = 0.01, 1 × 3 ANOVA) and Perinuc-only (Fig. 6C; F(2,75) = 5.89, P = 0.0042, 1 × 3 ANOVA) expression. The percentage of Foci-only cells in Dams (10.5 ± 1.4%) was similar with their level for silence, and was not different from that seen 30 min after 65 kHz playback (Fig. 4B, 10.3 ± 1.6%). In contrast, Vrgs (16.1 ± 1.3%) had the highest Foci-only

Figure 6. Pup care experience and not simply maternal hormonal history and call salience biases Perinuc-only expression of *Arc* mRNA in core auditory cortex 30 min after playback of 5 min of USV. Neurons in layers III–VI of core auditory cortex were classified as Arc-positive (A), Foci-only (B), and Perinuc-only (C) for Vrgs (25 z-stack images), Cocs (26 z-stacks), and Dams (28 z-stacks). Coc mice exhibit a pattern of compartmental Arc expression that is not significantly different from Dams, but is significantly different from Vrgs. (D) Recruitment of *Arc*-transcribing neurons is accelerated by hearing exemplars of a familiar stimulus category. Sacrificing Vrgs 30-min after playback of USV leads to a steady rate of recruitment of new neurons transcribing Arc, as seen in the roughly equal proportion of neurons classified as Perinuc-only (earliest response, black), Both (intermediate response, dark gray) and Foci-only (most recent response, light gray). Prior experience with vocalizing pups biases the recruitment toward earlier transcription, so that a larger proportion of neurons are classified as Perinuc-only (black) in Cocs and Dams. Asterisks (*) indicate significant multiple comparison-corrected post hoc tests (P < 0.05). Error bars represent standard error.
sensory cortical network can be different from that seen in the hippocampus.

**Discussion**

Neural plasticity to encode behaviorally relevant stimuli is a well-established mechanism for sensory learning and memory, yet studies of this have generally overlooked the question of how variable stimuli within a learned category engage plasticity mechanisms. A balance has to be found between plasticity to incorporate stimulus features of new exemplars, and stability to maintain previously learned representations. This could be achieved if plasticity mechanisms themselves were sensitive to the history of prior experience with similar stimuli, even when those stimuli are not identical to those experienced earlier. Here we have shown that the intracellular compartmentalization of a key synaptic plasticity effector immediate early gene, Arc/Arg3.1, in core auditory cortex exhibits such flexibility even if animals hear new examples of familiar natural vocal category.

An important methodological point is that our compartmental analysis utilized manually supervised, computer-generated 3D surfaces (Fogarty et al. 2013; Lee et al. 2015) to classify cells based on the location where Arc mRNA is expressed, which differs from prior approaches (Chawla et al. 2004; Ramirez-Amaya et al. 2005). Our analysis is based on z-stack confocal imaging of DAPI nuclear and Arc fluorescence staining, as with all compartmental analysis methods. However, our image analysis software then allows the generation (guided by manual verification) of artificial 3D surfaces for both, using parameters tailored to approximate the real fluorescence seen in the original z-stacks. These artificial surfaces greatly simplify a less biased definition of the nuclear edge, especially in cases where this edge is not well delineated in all layers of the original z-stack. The ability to rotate each nuclear surface to verify the relative location of its Arc fluorescence also enhances confidence in the compartmental classification. Moreover, even though we, like others (Chawla et al. 2004; Ramirez-Amaya et al. 2005), cannot assign Arc fluorescence far from the nucleus as belonging to a specific neuron without the use of a marker for cell membranes, the use of 3D surfaces at least provides a systematic way to capture perinuclear Arc expression from the overlap of Arc and nuclear surfaces. The importance of such an objective method is apparent from the fact that increasing/decreasing the quantitative fluorescence threshold for 3D surface generation can bias (~1% discrepancy) the compartmental percentages toward equivalent/higher Foci-only and simultaneously lower/equivalent Both expression relative to manual counts (Fig. 3B). While every method has its advantages and disadvantages, the key point here is that our comparisons across animal groups were analyzed by the same methods in a blind fashion.

In our study, we used the ultrasonic communication system between mouse pups and adult female mice to test whether prior experience with localizing pups alters the pattern of Arc expression elicited by both synthetic and natural exemplars of the pup USV category. In none of our experiments was the “total” percentage of Arc-positive neurons in core auditory cortex sensitive to prior experience hearing and/or interacting with mouse pups. Critically though, the compartmental expression of Arc was. When listening to novel exemplars of natural calls, mothers show a more rapid and sustained increase in the population of neurons with Perinuc-only expression compared with pup-naïve virgins (Figs 5C, 6C), which could arise from Arc mRNA being generated either earlier or with much greater magnitude. When listening to pure tone models of pup USVs, which are still salient to mothers (Ehret and Haack 1982) though acoustically less similar to the natural pup USVs they heard during pup rearing, a higher Perinuc-only percentage was again observed, albeit after a longer delay (Fig. 4C). Hence, compartmental Arc expression is sensitive to prior experience with the “category” of pup USVs, extending our previous result based on replaying "identical” familiar stimuli (Ivanova et al. 2011).

As a further test of whether this category familiarity, and not just a mother’s hormonal history and/or the immediate salience of the USVs, biases the Perinuc-only expression, we also played back natural USVs to cocaring females after pups had been weaned. Cocarers also showed a higher Perinuc-only population than naïve virgins (Fig. 5C). Altogether, our results are consistent with the interpretation that prior experience hearing exemplars of a sound category helps a larger population of core auditory cortical neurons to accumulate Arc mRNA in just the perinuclear cytoplasmic compartment once new category exemplars are heard. We speculate that the engagement of this molecular mechanism for plasticity is therefore itself plastic and sensitive to experience.

Our results fit within an emerging conceptual framework for understanding Arc's function in modifying synaptic connectivity after experience, and extend this framework by considering new sensory experiences that are congruent with but not identical to previous experiences. Completely novel stimuli can trigger a temporally extended program of Arc transcription within the nuclei of sensory cortical and hippocampal neurons, typically peaking 15–30 min after a brief experience, depending on the type of sensory experience and brain area (Guzowski et al. 2001; Kelly and Deadwyler 2002, 2003; Ramirez-Amaya et al. 2013; Khodadad et al. 2015; Nakayama et al. 2015). In core auditory cortex, this is seen within 5 min of the onset of an unfamiliar stimulus category through the ~30% of Arc-positive neurons in Vrgs listening to pure tones (Fig. 4A, middle white bar), Vrgs and Dam- listening to noise (Fig. 5A, left white and black bars, respectively), or Vrgs listening to pup USVs (Fig. 5A, right white bar). According to the current understanding of Arc kinetics (Vazdarjanova et al. 2002), initially transcribed Arc mRNA is fully transported from the nucleus into...
the perinuclear cytoplasm by ~30 min later, and is being shuttled into the dendrites or degraded (Das et al. 2003). Since cells with Arc mRNA only in the dendrites or disconnected from the nuclear membrane were not counted by our methods, an initial 27% (35%) Foci-positive (Foci-only + Both) cell population in Vrgs seen for tone (pup USV) stimulation at 0 min became a population of only 17% (22%) of Perinuc-only cells by 30 min. This ~37% reduction for both novel stimulus categories is consistent with decays that have previously been reported for this time point, suggesting that initial auditory cortical Arc expression in Vrgs for novel stimuli follows the well-characterized kinetics seen in hippocampus CA1 (Vazdarjanova et al. 2002).

Importantly though, in light of this self-consistent result for novel stimuli in Vrgs, the decays for natural pup and tonal model USVs in Dams, the experimental group with prior familiarity with pup USVs, are noticeably slower than in Vrgs. For 65-kHz tone (Fig. 4), Dams initially had 25% Foci-positive (Foci-only + Both) cells at 0 min, and a statistically indistinguishable 31% Perinuc-only cells by 30 min (P = 0.23, t-test). For pup USVs (Figs. 5C, 6C), an initial 39% Foci-positive population at 0-min decayed to 30% at 30-min, a significant 23% decrease (P < 0.005, t-test). This smaller stimulus-dependent decay in mothers relative to virgins (23% versus 37%) was a surprise—one that we did not initially hypothesize—but shows how Arc expression unfolds across the neural network based on prior experience (Abraham 2008). Hence, not only is the initial expression of Arc across the core auditory cortical population apparently faster, but it seems to decay more slowly when a sound category is somewhat familiar.

Furthermore, our data from the 30-min time point also show that sound-induced expression of Arc in naïve mice (Vrg and Coc) continues for longer than in Dams while listening to the tones (Fig. 4B, right bars) or USVs (Fig. 6B). Since silence itself produces no more than 10% Foci-only and ~3% Perinuc-only neurons in auditory cortex (Fig. 7), with no difference between animal groups, comparable to our earlier study (Ivanova et al. 2011), we suggest that these neurons with newly transcribed Arc mRNA 30 min after sound presentation are likely responding (late) to the sound, and not simply exhibiting baseline transcription.

Why there may be a temporally extended window for recruiting more neurons to transcribe Arc is not entirely clear, but it may reflect neuronal diversity in different brain areas in the time constants for the molecular cascades that lead up to Arc transcription (Saha et al. 2011; Ramirez-Amaya et al. 2013). That diversity might, for example, arise from time constants that effectively vary with the strength of initial neuronal depolarization by stimuli, which presumably depends on how strongly a neuron is connected with-in the neural network ultimately driven by the stimulus (Sheng et al. 1990). Alternatively, permissive neuromodulatory inputs that are temporally diffuse (Gu 2002) or delayed recurrent network activity (Yasuda et al. 2007) may gradually increase the population of neurons newly transcribing Arc. In this light, we recently found that muscimol injected into auditory cortex immediately after an auditory fear conditioning session impairs the consolidation of the acoustic cue, suggesting that nonstimulus locked auditory cortical neural firing after a sound experience is necessary for learning (Banerjee et al. 2017). Furthermore, the possibility for late transcription was also observed in the dentate gyrus (but not the CA1) of rats, where Arc transcription was sustained for hours following spatial exploration (Ramirez-Amaya et al. 2013). At the 30-min time point in core auditory cortex, the timeline of neuronal transcription can be inferred from the compartmental expression (Fig. 2; Zelikowsky et al. 2014). The neurons expressing just “Perinuc-only” Arc must have been recruited earliest during the stimulation, with those showing “Both” nuclear and perinuclear cytoplasmic Arc and those showing “Foci-only” Arc mRNA being recruited progressively later, respectively. For a completely novel stimulus category, such as Vrgs listening to USVs, recruitment appears to be relatively steady over time based on the approximately equal proportions of the Arc-positive neural population in these different classes (Fig. 6, bottom, Vrg). Interestingly, recruitment appears capped at ~60% of neurons in core auditory cortex, perhaps due to baseline, intrinsic connectivity, since Arc-positive expression appears generally limited to this irrespective of the stimulus (Fig. 4A, right bars; Fig. 6A).

The function of this Arc is presumed to help consolidate the auditory memory traces triggered by the sound so as to better reactivate relevant neurons when the same sound is encountered in the future. Through its role in weakening less active synapses (i.e., those that were not well-driven by the stimuli) in an activated neuron (Okuno et al. 2012), and consolidating memories associated with potentiated synapses (Guzowski et al. 2000; Messaoudi et al. 2007; Korb et al. 2013; Ramirez-Amaya et al. 2013), Arc is hypothesized to improve the signal to noise in the ability of stimulus-dependent synaptic inputs to drive a neuron (Morin et al. 2015). At the same time, it may be paring away connectivity in neurons that are much less driven by the stimulus, consistent with a role for Arc in stimulus selectivity in the visual cortex (Wang et al. 2006).

These functions of Arc would ultimately alter how the population of neurons subsequently responds electrophysiologically to the presentation of the identical stimulus. Once such a “familiar” stimulus is encountered, many of the neurons that previously expressed Arc mRNA would now be better driven by the specific sound, and we speculate that there might be some nuclear history, perhaps in the form of epigenetic marks, that facilitate Arc transcription more rapidly. This could explain the early accumulation of Perinuc-only neurons in animals that heard sounds that were more familiar from prior experience (i.e., Dams and Cos listening to USVs, Fig. 6, bottom, respectively). Alternatively or in addition, neurons from animals with prior experience might have more efficient processing or export of preexisting and/or newly induced Arc mRNA (Johnson et al. 2009). Furthermore, the degree to which new exemplars of the same familiar category of sound engage an overlapping set of synapses that were previously potentiated by that sound category would determine how much of a familiarity effect would be observed in the Perinuc-only population. This could explain the late 30 min, but not 0 min, familiarity effect seen in Dams (compared with Vrgs) listening to the tonal models of pup USVs.

Our results support the view that the continual engagement of a plasticity gene such as Arc, even when a stimulus category is familiar, helps incorporate new information about a stimulus in the behavioral contexts it is encountered (Miyashita et al. 2009; Morin et al. 2015), much like the process of memory reconsolidation (Dudai 2012; Bozon et al. 2003). This could be an essential mechanism by which the cortex builds up a more robust representation of stimulus categories from different experiences with that category, allowing hippocampal mechanisms of pattern recognition to better recall those events, even when the stimuli are incomplete or degraded (Rolls 2016). Other paradigms have also found that familiar stimuli or environments still engage Arc expression, sometimes even more so than novel contexts (Miyashita et al. 2009; Ivanova et al. 2011; Morin et al. 2011). In our studies here, we presume that the Arc-positive expression in Dams listening to USVs was as high as in Vrgs because of the novel environment in which the sounds were played back (anechoic chamber, without actual pups). An interesting question to investigate in the future would be whether some repeated number of identical trials might lead to a saturation of what new information can be gained in an experience (habituation), leading eventually to a reduced percentage of neurons expressing Arc. This might perhaps explain why baseline home cage expression is generally low, and
why a well-learned tone elicits less Arc expression (Carpenter-Hyland et al. 2010). In any case, irrespective of why the total number of neurons transcriptionally activated remains high in familiar conditions, our results provide new evidence at the population level that the expression of Arc mRNA is nevertheless altered between initial and later experiences. Therefore, this work underlines the possibility that Arc mRNA may play a role in the process of incorporating new stimulus information about already familiar sounds, and enabling the subtle refinement of existing sensorineuronal representations.

Materials and Methods
Experimental design
All procedures were approved by the Emory Institutional Animal Care and Use Committee. Experiments were performed on CBA/CaJ female mice (15–16 wk old), which were housed at least two per cage in a colony under reversed 14-h light/10-h dark cycle. Animals had access to food and water ad libitum, with experiments conducted during their dark cycle.

Figure 1A illustrates our experimental timeline for each of three groups of adult female mice (3–4 animals per group). Primiparous Mothers (Dam), which were initially paired at the age of 9 wk with a male for mating and then separated into a new cage at least 10 d before pups were born, had at least 21 d of caring for their pups experience. Cocarers (Coc), which were added to a littermate Mother’s home cage on the same day Mother was separated from the male, had the same full-term experience of caring for pups, but without physiological changes associated with pregnancy, parturition, and lactation. All Mothers and Cocarers were screened to check for successful retrieval of scattered, vocalizing pups in the home cage over a 10-min period on postnatal day P5 or P6. Pups were weaned when they reached P21, after which Mothers and Cocarers were separated into individual cages for 24 h. Naïve virgins (Vrg), which were adult female mice (age-matched to Dam and Coc) with neither mating experience nor physiological changes associated with pregnancy, parturition, and lactation, had no adult contact with pups. Virgin mice were group housed in the colony and placed into an individual home cage 24 h before experiments.

On a given experiment day, an individual mouse in its home cage was placed into a silent anechoic chamber (44 × 27 × 24 cm, W × D × H inner dimensions, Acoustic Systems) for 4 h for habituation, followed by a 5-min test period of sound stimulation (Fig. 1B). Mice were decapitated immediately or with 30-min delay after sound stimulation, and brains were processed for in situ hybridization experiments targeting the immediate early gene Arc.

Acoustic stimulation
Sounds were generated by a TDT (Tucker Davis Technologies) RX6 digital signal processor at a sampling rate of 233 kilosamples/sec- ond, attenuated by a PAS programmable attenuator (Tucker Davis Technologies), and played through an EMIT speaker (Infinity, EMIT). Acoustic stimuli included 5-min bouts of ultrasonic pure tones, prerecorded ultrasonic pup vocalizations (USV) and broadband intrinsic microphone recording noise (Nse). Pure tones (Fig. 1B, left) consisted of dynamic bouts of a random number of 65 kHz tone pips (mean of 4, range 2–6), with random durations (60 ± 24 msec, mean ± standard deviation) and inter-tone intervals (206 ± 49 msec) within a bout, and random interbout intervals (755 ± 146 msec between the end of one bout and the start of the next). This provided a tonal model of mouse pup calls with a frequency matching the most commonly found in these USVs (Liu et al. 2003). Natural pup USVs (Fig. 1B, center) were previously recorded from P6–P7 pups (Liu et al. 2003) using a 1/4” calibrated microphone (Bruel and Kjaer). The 5-min recording was selected so that the mean frequency of detected pup calls in the sound file was close to 65 kHz. The recording was high-pass filtered above 25 kHz. And third (Fig. 1B, right), 5-min of microphone thermal prerecorded noise (wide-bandwidth, arising from the intrinsic noise floor of the microphone) was used as a control sound for the USV recording. Pup calls were removed from the recording, and those segments were replaced with random quiet background noise segments from the remaining recording. The resulting sound file was high-pass filtered above 25 kHz and scaled by the same factor as the original USV to match its background noise.

Tissue processing and fluorescent in situ hybridization (FISH)
Immediately after decapitation, brains were rapidly removed, covered with OCT media (VWR International), and frozen promptly in liquid nitrogen. Frozen brains were stored at −80°C prior to cryosectioning. Serial 20 μm coronal sections cut by cryostat (Leica) were captured on Super-frost slides (Fisher Scientific), and stored at −80°C until fluorescent in situ hybridization (FISH). FISH was performed on frozen slide-mounted brain sections following protocols previously described in detail elsewhere (Guzowski et al. 1999; Muddashetty et al. 2007; Ivanova et al. 2011). Briefly, Digoxigenin-labeled Arc antisense and sense riboprobes (NCBI accession number NM_018790.2, nucleotides 273–1369) were prepared using a commercial kit (Roche Molecular Biochemicals). After riboprobe hybridization (16 h, 56°C), slides were treated with RNase A (10 mg/mL) and 3% hydrogen peroxide, and followed by incubation in block buffer containing anti-digoxigenin-POD, Fab fragments (Roche Diagnostics). Arc probes were detected with TSA-Direct Cyanine-3 fluorescence amplification kit (TSA Amp Kit, PerkinElmer). Nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI, Electron Microscopy Sciences).

Confocal microscopy and cell counting
Image acquisition was performed using a Zeiss LSM 510 (Carl Zeiss Jena GmbH) or in the Olympus FV1000 (FluoViewFV1000 Spectral Confocal Laser Scanning Microscope, Olympus Corporation of the Americas) confocal microscope. Coronal sections were matched to a standard mouse atlas ( Paxinos and Franklin 2001) to identify the anatomically labeled primary auditory cortex (Au1); sections fell between −2.30 and −2.80 mm relative to Bregma. The regions of auditory cortex and cortical layers in the coronal slices were identified based on nuclear DAPI staining with a 10× objective (Anderson et al. 2009). Then, using a 63× oil objective, confocal z-stacks composed of 0.5 μm thick optical sections were collected through the regions of interest. All images were saved in a 12 bit TIFF format at 512 × 512 or 1024 × 1024 pixels. The dentate gyrus was used as a positive control area for confocal microscopy, in which a small number of Arc mRNA strongly labeled cells can always be detected. By 30 min, Arc mRNA was located predominately within the perinuclear cytoplasm, as expected based on prior literature from the hippocampus (Guzowski et al. 1999; Vazdarjanova et al. 2002; Vazdarjanova and Guzowski 2004). The Figure 2A shows a typical Arc mRNA distribution in DG at 30 min delay after 5 min sound stimulation.

Our analysis focused on cortical layers corresponding to the thalamorecipient layers III–IV and infragranular layers V–VI in auditory cortex (Cruikshank et al. 2002; Winer et al. 2005). For each layer (layer III–IV and V–VI), usually two nonoverlapping z-stacks at a given cortical depth were imaged per slide. A confocal z-stack consisted of ~30–34 optical sections, spaced 0.5 μm apart. Typically 12–24 z-stacks were collected per experimental group. Arc mRNA fluorescence was analyzed in images of sections incubated with sense or anti-sense riboprobes. Sense-riboprobe images, which usually showed very weak intensity, were used to confirm the specificity of the probe to bind Arc mRNA. A 3D image volume was reconstructed from sequential z-sections and analyses were performed with Imaris software (Bitplane Scientific Software), which generated artificial surfaces around both DAPI-stained nuclei and fluorescently labeled Arc mRNA that allowed for a less biased estimate of the physical extent of these signals. Artificial surfaces were created by thresholding absolute intensity and smoothing, and were visually compared with the original z-stack. The software’s nucleus diameter setting was initially determined by using the line tool to measure the diameter of a random subset
Comparison of 3D surface-assisted quantification of Arc expression to manual counting

To validate our 3D surface-assisted counting methods against existing manual counting methods, we separately counted cells in a subset of images using a reconstructed sequential confocal z-stack (Fig. 1B, top; Chawla et al. 2004). The manual image analysis was performed using the Slice View application in the IMARIS software. We used this application to view individual z-sections, and navigate to any position (XY, XZ, and YZ) within each 3D stack. Z-stacks were then evaluated for Arc-positive (Foci-only, Both, or Perinuc-only) cells. The thresholds for foci size and intensity, and thresholds for perinuclear cytoplasmic distribution were set and equal to the threshold we used for 3D IMARIS. For a cell to be designated as having a “foci” of Arc expression in the nucleus, a minimum intensity threshold had to be present in consecutive planes; in most cases, this was at least three planes. For perinuclear labeling cells were considered Arc-positive when the Arc signal spread around the nucleus over at least three planes, were designated as having “perinuclear cytoplasmic” expression.

Statistics

Data were analyzed either by one-way or two-way ANOVA in MATLAB (Mathworks). In the case of two-way ANOVAs, post hoc tests were carried out by Tukey’s honestly significant difference (HSD) to control for multiple comparisons. Differences were considered significant at \( P < 0.05 \).

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