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Arc/Arg3.1 mRNA expression reveals a sub-cellular trace of prior sound exposure in adult primary auditory cortex

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Abstract

Acquiring the behavioral significance of a sound has repeatedly been shown to correlate with long term changes in response properties of neurons in the adult primary auditory cortex. However, the molecular and cellular basis for such changes is still poorly understood. To address this, we have begun examining the auditory cortical expression of an activity-dependent effector immediate early gene (IEG) with documented roles in synaptic plasticity and memory consolidation in the hippocampus: *Arc/Arg3.1*. For initial characterization, we applied a repeated 10 minute (24 hour separation) sound exposure paradigm to determine the strength and consistency of sound-evoked *Arc/Arg3.1* mRNA expression in the absence of explicit behavioral contingencies for the sound. We used 3D surface reconstruction methods in conjunction with fluorescent in-situ hybridization (FISH) to assess the layer-specific sub-cellular compartmental expression of *Arc/Arg3.1* mRNA. We unexpectedly found that both the intranuclear and cytoplasmic patterns of expression depended on the prior history of sound stimulation. Specifically, the percentage of neurons with expression *only* in the cytoplasm increased for repeated versus singular sound exposure, while intranuclear expression decreased. In contrast, the total cellular expression did not differ, consistent with prior IEG studies of primary auditory cortex. Our results were specific for cortical layers 3–6, as there was virtually no sound driven *Arc/Arg3.1* mRNA in layers 1–2 immediately after stimulation. Our results are consistent with the kinetics and/or detectability of cortical sub-cellular *Arc/Arg3.1* mRNA expression being altered by the initial exposure to the sound, suggesting exposure-induced modifications in the cytoplasmic *Arc/Arg3.1* mRNA pool.

Keywords

immediate early gene; mouse; catFISH; novelty; familiarity; synaptic plasticity

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Response properties of sensory cortical neurons change as a stimulus gains behavioral relevance (Weinberger, 2004). On short time scales, such changes may represent attentional effects (Fritz et al., 2007), but on longer time scales, they provide a basis for the distributed storage of sensory memories (Sutherland and McNaughton, 2000; Kilgard et al., 2002; Sacco and Sacchetti, 2010). The molecular mechanisms underlying such long term cortical plasticity and how each sensory experience engages these mechanisms are poorly understood. Sensory stimuli likely induce plasticity-related genomic responses (Mello et al., 1992; Velho et al., 2005; Pinaud et al., 2008; Dong et al., 2009), but these responses have not been well investigated in mammalian sensory cortex compared to electrophysiological measures of plasticity.

In considering such molecular mechanisms, *Arc/Arg3.1* (hereafter referred to as *Arc*) is of particular interest since it acts as a key regulator of translation-dependent synaptic plasticity in the hippocampus (reviewed by (Bramham et al., 2010)). *Arc* mRNA can be rapidly induced by synaptic activity (Link et al., 1995; Lyford et al., 1995) and transported into dendrites (Dynes and Steward, 2007), accumulating specifically near activated synapses (Moga et al., 2004). *Arc* protein then becomes enriched at the site of local synaptic activity (Steward et al., 1998; Yin et al., 2002; Moga et al., 2004; Rodríuez et al., 2005), where it regulates glutamate receptor trafficking, postsynaptic density remodeling and spine morphology (Chowdhury et al., 2006; Rial Verde et al., 2006; Messaoudi et al., 2007; Peebles et al., 2010). A single, brief exploratory experience can drive an initial and a temporally delayed (8–24 hour (h)) wave of hippocampal *Arc* protein expression, possibly representing a reactivation of a subnet of neurons encoding that experience (Wilson and McNaughton, 1994; Ramirez-Amaya et al., 2005).

Arc's role in hippocampal-based memory consolidation (Guzowski et al., 2000; Plath et al., 2006) has therefore made it a promising target for investigating molecular mechanisms of long term cortical plasticity (Mahlke and Wallhäusser-Franke, 2004; Sun et al., 2005; Wang et al., 2006; Tan et al., 2007; Carpenter-Hyland et al., 2010; Gao et al., 2010; Gusev and Gubin, 2010). However, basic knowledge about *Arc*'s pattern of expression across layer-dependent cortical networks is incomplete. Our objective was to characterize both the baseline and stimulus-induced layer-specific *Arc* expression in a simple stimulus *exposure* paradigm to lay the groundwork for future studies using a more explicit associative learning context. Our data revealed a significant relation between the history of prior sound exposure and the compartmentalization of the evoked *Arc* mRNA expressed in the thalamorecipient and infragranular layers, demonstrating that a single exposure to a stimulus can leave a sub-cellular molecular trace in primary sensory cortical neurons.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Emory Institutional Animal Care and Use Committee. Experiments were performed on CBA/CaJ mice (15–18 weeks old). Mice were kept under a reversed light cycle and housed individually at least one day prior to the start of experiments, performed during the dark cycle. On a given experiment day, a mouse in its home cage was placed into a silent anechoic chamber (44" × 27" × 24", W × D × H inner dimensions, Acoustic Systems, Austin, TX) for 4 h, followed by a 10 minute (min) test period of either additional silence or continuous sound stimulation. The latter consisted of a dynamic sequence of 32 kHz tones at 40 dBSPL, with random durations (60 ± 24 ms, mean ± standard deviation) and inter-tone intervals (206 ± 49 ms). Sounds were generated by an RX6 digital signal processor and attenuated by a PA5 programmable attenuator (Tucker Davis Technologies, Alachua, FL). Further details are described in the Results.

Tissue preparation

Mice were sacrificed by CO_2 inhalation. In most cases, their brains were then removed rapidly, covered with OCT media (VWR International, West Chester, PA), and frozen immediately in liquid nitrogen. Serial 20 um coronal sections cut by cryostat (Leica, Richmond, IL) were mounted onto slides and stored at −80° C. In some cases where a tangential slice through the auditory cortex was desired, mice were instead perfused with phosphate buffer saline (pH 7.4) for 2–3 min after CO_2 inhalation. The brain was removed and divided into left and right hemispheres. The left hemisphere was flattened between two glass slides, and together with a block from the right hemisphere, postfixed in 4% paraformaldehyde for 1 h followed by soaking in 30% sucrose overnight. Both hemispheres were then covered with OCT media, frozen in liquid nitrogen, and stored at −80°C. Tangential (left hemisphere) or coronal (right hemisphere) sections 40 um in thickness were cut by serial cryostat, mounted onto slides and stored at −80° C.

FISH for Arc mRNA

FISH was performed on slide-mounted brain sections following protocols previously described in detail elsewhere (Guzowski et al., 1999; Muddashetty et al., 2007). Briefly, frozen sections were fixed in 4% paraformaldehade for 5 min.; slides were rinsed in cold 2× SSC (sodium citrate chloride), and treated with 0.5% acetic anhydride in 0.1M triethanolamine-HCl buffer (pH 8.0) and dehydrated in acetone:methanol (1:1) at room temperature. After washing again in 2× SSC, slides were incubated with prehybridization buffer (2× SSC, 25% formamide, 1% Denhardt's reagent, 50% dextran sulfate, 25mg/ml yeast tRNA, and 10 mg/ml of denatured salmon sperm DNA) in wet chamber at room temperature for 1 h. Digoxigenin-labeled *Arc* antisense and sense riboprobes (NCBI accession number NM_018790.2, nt 273–1369) were prepared using a commercial kit (Roche Molecular Biochemicals, Nutley, NJ). After riboprobe hybridization (16 h, 56°C), slides were washed with 2× SSC and treated with RNaseA (10 mg/ml) at 37°C for 30 min. After a graduated series of washes in SSC, slides were incubated in 3% hydrogen peroxide for 15 min, followed by incubation in block buffer TNB (0.1% Tris HCl, pH 7.5; 0.15M NaCl; 5% Blocking-Reagent (Roche)) for 30 min, and then TNB containing anti-digoxigenin-POD, Fab fragments (Roche Diagnostics, Indianapolis, IN), for 2 h at room temperature. *Arc* probes were detected with TSA-Direct Cyanine-3 fluorescence amplification kit (TSA Amp Kit, PerkinElmer, Boston, MA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Electron Microscopy Sciences, Hatfield, PA).

Confocal microscopy and cell counting

Stained slides were analyzed using a Zeiss LSM 510 confocal microscope. For tangential sections through layer 4, auditory cortex was identified by relative alignment with the barrel cortex (Caviness and Frost, 1980). 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 mm and −2.80 mm relative to Bregma. The shape of the hippocampus was an excellent guide for localization, as validated in additional experiments in adult female mice where auditory cortex was located electrophysiologically. Details about electrophysiological methods can be found in our previous publications (Galindo-Leon et al., 2009; Lin and Liu, 2010). Positions of cortical layers in the coronal slices were determined by aligning the DAPI staining of nuclei to layers previously delineated by Nissl staining (Anderson et al., 2009). Our analysis focused only on 3 groups of cortical layers corresponding to the supragranular (layers 1–2), thalamorecipient (layers 3–4 in auditory cortex (Cruikshank et al., 2002; Winer et al., 2005)) and infragranular (layers 5–6) layers of the auditory cortex, due to the labor-intensive nature of the 3D quantification (see Results). For consistency, images were targeted to the middle of each of these 3 groups rather than across their entire widths.

In coronal sections, the threshold level for *Arc* positive fluorescence could be set by referencing to the positive-labeled cells in the hippocampus. For each experimental group and layer, typically 2 non-overlapping z-stacks at a given cortical depth were imaged per slide, for a median of 6 (range 5–9) z-stacks per group per layer. Each z-stack, imaged at 63X, consisted of single planes spaced 0.5 μm apart. The median image thickness was 16.5 um (34 planes per z-stack). Each z-stack was then viewed in the Imaris 3D software (Bitplane Scientific Software, Zurich, Switzerland), which generated artificial surfaces for DAPI-stained nuclei and fluorescently labeled *Arc* mRNA. The threshold for the DAPI surfaces was set so that the surface would just fully encompass the stained volume. The threshold for the *Arc* surfaces was determined by building the *Arc* surface in a random sample of the images to determine an average value that created a good visual match between the *Arc* surface and the raw *Arc* signal; this was then applied consistently across images.

Since glial cells are not known to express *Arc* (Cirelli and Tononi, 2000), we excluded glial-like nuclei based on the intensity, texture and homogeneity of the DAPI stain, following previously published criteria (Chawla et al., 2004); nuclei that were not spherical or oval-shaped were also suspected to be glial cells and were often removed from consideration. In quantifying images, nuclei that were substantially cut off at any of the 6 edges of the image volume were also removed. Remaining cells were classified as either being positive or negative for *Arc* mRNA. Positive neurons were sub-classified as having *Arc* mRNA expressed in “intranuclear foci *only*,” in the perinuclear “cytoplasm *only*,” or simultaneously in “both” compartments (see Results). To suppress unspecific background fluorescence in our quantification, we required *Arc* mRNA fluorescence surfaces to be within (i.e. intranuclear) or outside but in contact with (i.e. perinuclear cytoplasmic) the DAPI surface. Percentages of expressing cells were computed for each layer’s images based on these compartmental pattern-specific values. Percentages were also computed for nuclear-positive (sum of each image’s “intranuclear foci *only*” and “both” percentages), cytoplasm-positive (“cytoplasm *only*” and “both” percentages) and total cellular expression (sum of all three compartmental pattern percentages). The total cellular expression plus the negative staining percentage totaled 100%. Percentages were analyzed by ANOVA, with post-hoc multiple comparisons carried out by the Tukey-Kramer Honestly Significant Difference (HSD) test. Differences at the $p \leq 0.05$ level were considered significant.

RESULTS

We first tested whether our sound stimulus (dynamic 32kHz, 40dBSPL tone) could evoke measurable *Arc* mRNA expression in the auditory cortex. Mice (3 animals) received 10 min of sound stimulation in an anechoic chamber after a 4 h silent habituation period and were kept for an additional 30 min in silence before decapitation to ensure robust expression (Guzowski et al., 1999; Velho et al., 2005). Successful FISH for *Arc* mRNA was confirmed by observing cellular cytoplasmic expression within the granule cell layer of the dentate gyrus (Fig. 1A) (Guzowski et al., 1999). Such expression was *Arc* mRNA-specific since tissue processed with *Arc* sense riboprobes showed no staining (Fig. 1B). Extensive *Arc* mRNA expression after sound stimulation was seen across a flattened tangential section through layer 4 of primary auditory cortex (Fig. 2A, magenta arrowhead, Brodmann area 41). In contrast, spontaneous *Arc* mRNA expression (Fig. 2B) from animals kept in the anechoic chamber without sound stimulus was negligible.

Sound-evoked expression in the tangential slice was sufficiently distributed to be expected to be observed in several serial coronal slices near the center of primary auditory cortex. This center was targeted in coronal slices by comparing the shape of the hippocampus and thalamic nuclei to a standard mouse atlas (Paxinos and Franklin, 2001). Separate *in vivo*

electrophysiological experiments validated the presence of primary-like tone responses (Fig. 3A) at such a location, which was histologically marked with electrolytic lesions after recordings (Fig. 3B). Note that this anatomically-defined Au1 presumably includes both the anterior auditory field and the primary auditory field (Stiebler et al., 1997). In slices subjected to FISH, those with a nuclear DAPI stain (Fig. 3C) matching the target region (see Experimental Procedures) were then imaged at high resolution in the middle of the supragranular (1–2), thalamorecipient (3–4) and infragranular (5–6) cortical layers (Fig. 3D).

Using these coronal slices, we examined whether sound-induced auditory cortical *Arc* mRNA expression in adult mice shows changes after simply repeating sound exposure. We compared the layer-specific and compartmental pattern of *Arc* mRNA expression across four groups of animals (3–4 mice per group). All mice received two test epochs, separated by 24 h (with 4 h silent habituation periods), before being sacrificed immediately after Test 2 (Fig. 4). A familiar sound group received stimuli during both Tests 1 and 2 (S1S2) for 10 min each, which was sufficient to induce both nuclear and cytoplasmic expression. A novel sound group received stimulation with sound only during Test 2 (S2). As a control, one group received sound during Test 1 but not Test 2 (S1), to determine whether initial sound stimulation affects *Arc* mRNA expression 24 h later in the absence of further stimulation (Ramirez-Amaya et al., 2005). Finally, a no sound (SØ) control group experienced the anechoic environment without any sound stimulation, thus providing a baseline for auditory cortical expression in silence.

For each confocal z-stack, a 3D reconstruction of fluorescence surfaces was performed, and the sub-cellular compartmental location of the *Arc* mRNA expression was determined (see Experimental Procedures). Figure 5A–B illustrates examples of cells labeled as having *Arc* mRNA fluorescence in “intranuclear foci” (Fig. 5A, white arrowheads), and in the perinuclear “cytoplasm *only*” (Fig. 5B, yellow arrowheads). The top row shows the projection of the full z-stacks into 2D images, while the middle row plots the reconstructed 3D surfaces. Figure 5C shows a cell with “both” intranuclear (white arrowhead) and cytoplasmic (yellow arrowhead) expression. Figure 5D demonstrates the utility of the 3D reconstruction, which allows the rotation of an image to conclusively separate intranuclear from extranuclear (i.e. cytoplasmic or background) *Arc* fluorescence signals.

Upon quantifying the expression in this way, our experiments revealed that the sub-cellular pattern of *Arc* mRNA expression immediately after sound stimulation depends on both the specific layer within the auditory cortex and the sound history. A 3-way ANOVA on the percentage of cells with different compartmental patterns of *Arc* mRNA expression showed significant main effects of group ($F(3,204)=14.35$, $p\ll0.001$), compartment ($F(2,204)=24.29$, $p\ll0.001$) and layer ($F(2,204)=37.96$, $p\ll0.001$). Importantly, we also found interactions between group × compartment ($F(6,204)=14.39$, $p\ll0.001$), compartment × layer ($F(4,204)=5.24$, $p\leq0.0005$), and group × compartment × layer ($F(12,204)=2.65$, $p\leq0.005$), indicating that factors were not simply independent. In particular, the nature of *Arc* mRNA expression in layers 1–2 differed fundamentally from that of 3–4 and 5–6. Unlike the latter, expression in the former was never significantly different from silent controls for any group in any compartment (HSD, $p>0.05$). This was also true of the total cellular expression (2-way ANOVA for group and layer; group effect $F(3,68)=14.59$, $p\ll0.001$; layer effect $F(2,68)=38.59$, $p\ll0.001$; pairwise HSD for layers 1–2, $p>0.05$). Our sound stimulation thus induced layer-specific *Arc* mRNA expression, with groups that received sound just prior to sacrifice (S2 and S1S2) showing significantly higher expression in layers 3–4 and 5–6 versus 1–2 (Fig. 6A, HSD, $p<0.05$), in contrast to a lack of layer differences in the silent control (SØ, HSD, $p>0.05$). Intriguingly, even S1 had significantly elevated layers 5–6

expression compared to layers 1–2, the first indication that prior experience with sound in the anechoic chamber left a long-lasting molecular signature on primary auditory cortex.

To investigate this in more detail, we dropped layers 1–2 and pooled layers 3–4 and 5–6 by group for compartmental analysis. This was justified because post-hoc comparisons between them for any matching combination of group and compartment were not different (HSD, $p>0.05$). Note that the absolute level of expression was on average slightly higher for layers 5–6 compared to 3–4 (noticeable for each group in Fig. 6A, albeit not significant), although the effects of group and compartment appeared to be similar for the two (0.90 correlation coefficient, $p«0.001$). A 2-way ANOVA on the pooled layers 3–6 data remained significant for group ($F(3,147)=12.16$, $p«0.001$), compartment ($F(2,147)=22.56$, $p«0.001$) and their interaction ($F(6,147)=13.96$, $p«0.001$).

The interaction implies that the pattern of *Arc* mRNA expression across the animal groups depended on the sub-cellular compartment. The percentage of neurons with *Arc* mRNA appearing as “intranuclear foci *only*” was not significantly different between any group (HSD, $p>0.05$,data not shown) The percentage of neurons labeled as having expression in “both” the nucleus and cytoplasm was also not different between groups, except between S2 and SØ (HSD, $p<0.05$). Analyzing the overall percentage of nuclear-positive neurons (those with expression in “intranuclear foci *only*” and “both” compartments) showed a significantly higher level of expression for S2 compared to SØ (Fig. 6B, 1-way ANOVA $F(3,49)=5.53$, $p\leq0.005$; HSD, $p<0.05$). In this case, S2 was also greater than S1S2 (Fig. 7, bottom row, cyan asterisks), which was not different from SØ, demonstrating that even though initial (i.e. novel) stimulation increased total intranuclear expression, this evoked response was suppressed after a repeat stimulation 24 h later. Finally, the nuclear-positive percentage for S1 was as large as S2 (HSD, $p>0.05$), and significantly greater than S1S2 and SØ (HSD, $p<0.05$), again indicating initial sound exposure affects subsequent sub-cellular processes within primary auditory cortical neurons.

In contrast to results for the intranuclear compartment, the percentage of neurons with *Arc* mRNA expressed in the “cytoplasm *only*” was highly differentiated across groups (Fig. 6C). In this case, recent sound exposure was needed to evoke sound-induced expression, since S1 was not different from SØ (HSD, $p>0.05$), but both were significantly less than either S2 or S1S2 (HSD, $p<0.05$). More importantly, the “cytoplasm *only*” pattern of compartmental expression was observed significantly more often for S1S2 than S2 (Fig. 6C, HSD, $p<0.05$; Fig. 7, bottom row, pink asterisks), even though neither the overall cytoplasm-positive percentage (those with expression in “cytoplasm *only*” or “both” compartments) (Fig. 6D, 1-way ANOVA $F(3,49)=17.14$, $p«0.001$; HSD, $p>0.05$) nor the total cellular mRNA expression (Fig. 6A, HSD, $p>0.05$) was different between the two. Since the difference between “cytoplasm *only*” and cytoplasm-positive expression was the proportion of neurons with *Arc* mRNA expressed in both the nuclear and cytoplasmic compartments, our data suggests the earlier exposure to the sound may change the kinetics and/or detectability of cytoplasmic *Arc* mRNA expression in primary auditory cortex.

DISCUSSION

We found the history of sound stimulation affects the sub-cellular distribution of an mRNA encoding an important synaptic plasticity effector IEG, *Arc*, across the layers of the adult mammalian primary auditory cortex. This novel finding demonstrates that *compartmental* analysis of *Arc* mRNA, which has been used for neuronal activity mapping in Cellular compartmental Analysis of Temporal activity by FISH (catFISH) paradigms (Guzowski et al., 1999; Ramirez-Amaya et al., 2005; Barot et al., 2008; Marrone et al., 2008), can be a sensitive tool for differentiating the transcription-dependent response of cortical neurons

even when using just a *single recent* stimulus experience (Test 2). Our main discovery was the percentage of layers 3–6 neurons expressing *Arc* mRNA only in the cytoplasmic compartment depended significantly on whether this recent sound exposure was familiar or novel. This result was unanticipated since sound exposure alone without behavioral contingency does not induce long term receptive field plasticity within the adult primary auditory cortex (Recanzone et al., 1993; Polley et al., 2006), and thus was also not expected to modulate *Arc* mRNA expression. In fact, an earlier study in rats that assayed only total cellular expression of the transcription factor IEG, *c-Fos*, found no effect of familiarity in primary auditory cortex (Wan et al., 2001). Consistent with this, we observed that the total cellular *Arc* mRNA expression was not different between S1S2 and S2 (Fig. 3B). Hence, whereas total cellular expression is independent of the sound stimulation history, a previous sound experience increases a neuron's capability to accumulate *Arc* mRNA in the cytoplasm in response to the same sound 24 h later. As discussed below, this *Arc* mRNA sensitization may prime the molecular machinery underlying synaptic plasticity and facilitate learning if a sound is re-experienced with behavioral contingencies.

Our S1S2 vs. S2 differences might be explained by a combination of two non-exclusive hypotheses. First, prior experience with the sound may have predisposed neurons to have a sound-evoked genomic response (consisting of transcriptional and posttranscriptional events underlying gene induction) early in Test 2, so that after 10 min, much of the *Arc* mRNA transcribed in the nucleus was already targeted into the cytoplasm. In support of this, nuclear-positive *Arc* expression for S1 was indeed significantly higher than SØ (Fig. 6B), suggesting that baseline activation of primary auditory cortical neurons during silence was elevated for animals placed back in an environment in which a sound was originally experienced. This presumably occurred throughout both the silent habituation and Test 2 period. The mRNA transcribed earlier during silence may then have been targeted into the cytoplasm, giving rise to a significantly higher cytoplasm-positive (Fig. 6D) and total cellular (not shown for pooled layer 3–6 data, but can be inferred from Fig. 6A) expression for S1 than SØ, even though neither was stimulated with sound just before sacrifice. Repeat stimulation (S1S2) further enhanced the cytoplasmic expression (Figs. 6C–D), yet significantly reduced the nuclear-positive expression (Fig. 6B) compared to S1, which might be attributed to the enhanced processing and/or export of *Arc* mRNA into the cytoplasm.

However, rather than affect the kinetics of the genomic response in the nucleus, sound familiarity might have instead changed the detectability of *Arc* mRNA within the cytoplasm, possibly by affecting the distribution of mRNA in the cytoplasm. *Arc*, like other dendritically-targeted mRNAs, can have their sub-cellular distribution altered by activity (Bramham and Wells, 2007). In response to high frequency stimulation, *Arc* mRNA can be transported into distal laminae and accumulate at activated synaptic sites (Steward et al., 1998); yet in the presence of NMDA receptor antagonists, *Arc* mRNA is only diffusely distributed within dendrites and fails to accumulate at discrete laminae (Steward and Worley, 2001). The sorting of *Arc* mRNA within the somatodendritic compartment *in vivo* is influenced by numerous signaling pathways, such as Rho kinase, MAP kinase and extracellular signal-regulated kinase, ERK, as well as the actin cytoskeleton (Huang et al., 2007). The precise cellular mechanisms involved in activity-dependent regulation of *Arc* mRNA localization in the cytoplasm are unclear, but seem to involve the assembly of *Arc* mRNA molecules into transport granules which traffic from the soma into dendrites of cultured neurons (Dynes and Steward, 2007). *Arc* RNA granules are heterogeneous in size, suggesting varying amounts of *Arc* mRNA within each packet. Previous studies on other types of RNA granules in vitro have suggested that they assemble in the soma, and that their size and distribution within the somatodendritic compartment can be regulated by neuronal activity and signaling pathways (Tiruchinapalli et al., 2003; Kiebler and Bassell, 2006). Hence, one possibility is that initial exposure to the sound left low and/or diffuse levels of

Arc mRNA in the cytoplasm, rendering it undetectable by our FISH methods until the second stimulation. Existing *Arc* mRNA could then have redistributed or clustered in the cytoplasm to produce a stronger FISH signal, possibly by stimulating the recruitment of mRNAs into granules and their trafficking throughout the soma and into dendrites (Tiruchinapalli et al., 2003). The assembly and trafficking of mRNAs in granules appears, at least in vitro, to be an essential prerequisite for activity dependent local protein synthesis at synapses. It will therefore be interesting in the future to relate such studies to the present data suggesting differential levels of *Arc* mRNA redistribution in the cytoplasm in response to novel and familiar experiences.

Arc was previously shown to be necessary for several types of synaptic plasticity and learning and memory (e.g. (Plath et al., 2006; Waung et al., 2008). In particular, recent studies suggest an important role of *Arc* for synaptic homeostasis and network stability by regulating GluR1 internalization (Chowdhury et al., 2006; Shepherd et al., 2006; Gao et al., 2010; Peebles et al., 2010), for example during visual experience. Here we report that acoustic stimulation induces *Arc* mRNA expression in an experience- and cell compartment-dependent manner. In the future it will be interesting to assess the importance of *Arc* expression for auditory plasticity by testing whether auditory learning is impaired in *Arc* knockout mice (Plath et al., 2006). The necessity of auditory cortical *Arc* for forming behaviorally-relevant auditory memories could also be tested by blocking *Arc* transcription (Guzowski et al., 2000) in an operant learning paradigm (Carpenter-Hyland et al., 2010) or in the natural context of acquiring a communication sound's significance (Liu et al., 2006; Liu and Schreiner, 2007; Galindo-Leon et al., 2009). Furthermore, *Arc*'s role in network homeostasis suggests that *Arc* expression must be tightly regulated during synaptic plasticity. Apart from its induction following synaptic activity, the stability and/or translation of the mRNA might also be regulated, for example by specific mRNA-binding proteins. *Arc* mRNA was shown to associate with FMRP (Zalfa et al., 2005) and to be prone to nonsense-mediated decay (Giorgi et al., 2007). To assess their possible role for sound-induced *Arc* regulation and auditory experience, it will be interesting to analyze the sound-induced localization of FMRP and other mRNA-binding proteins known to be present in dendritic granules (e.g. Staufen) (Martin and Ephrussi, 2009).

Although finding a sub-cellular *Arc* mRNA dependence on sound exposure history in *primary* auditory cortex was unexpected, the modulation of plasticity-related IEGs by stimulus familiarity has been observed in *higher-order* auditory areas. In rats (Wan et al., 2001) and songbirds (Jarvis et al., 1995; Mello et al., 1995), repeated sound stimulation decreased overall cellular expression of transcriptional regulators *c-Fos* or *zenk* (*zif268/egr1/ngfi-a/krox-24*) in auditory association areas hypothesized to be responsible for recognition memory. In the case of the rat, this area, TE3, receives direct input from TE1 (LeDoux et al., 1991), which encompasses the primary auditory cortical area probed here. Hence, future studies should investigate whether higher order auditory areas might show even larger changes in compartmental expression of *Arc*. In the case of songbirds, debate continues as to the precise mammalian analog of the relevant area (caudomedial neopallium, NCM) (Jarvis et al., 2005), but it is clearly beyond the main target of auditory thalamic projections (Field L2a). Interestingly, Field L2a does not express *zenk* or *Arc* in response to hearing song (Mello et al., 1992; Velho et al., 2005), a striking difference from the *Arc* mRNA expression in thalamorecipient layers 3–4 of primary auditory cortex that we observed in mice (Fig. 3B). This species difference might be reconciled though if the actual thalmorecipient neurons within layers 3–4 in the mouse are not the ones expressing *Arc* mRNA.

The cortical layer dependence of *Arc* expression has previously been studied (Burke et al., 2005; Gusev and Gubin, 2010), but not at the sub-cellular compartmental level. In the auditory cortex, thalamic input from the ventral division of the medial geniculate body

mainly forms synapses in layers 3 and 4 (Cruikshank et al., 2002; Winer et al., 2005), thus motivating their combination into a thalamorecipient layer group. This was justified over grouping layer 3 with layer 2, as is often done, because evidence in mice suggests that pyramidal cells in the former are distinct from those in the latter (Oviedo et al., 2010). Instead, to keep our labor-intensive image 3D analysis manageable, we grouped layer 2 with layer 1 as a supragranular layer group, and layer 5 and 6 were combined into an infragranular layer group. The absence of *Arc* mRNA expression immediately after stimulation in our supragranular layers is consistent with a visual cortex study using *Arc*-GFP mice that showed a substantially delayed *Arc* protein response there after visual input, while the thalmorecipient and infragranular layers exhibited a protein response within 30 min, as expected (Wang et al., 2006). The similarity of the *Arc* response in these two groups despite the former's role as input and the latter's role as subcortical output of a cortical column is consistent with the strong interlaminar connection from layers 3 and 4 to layer 5 in the mouse auditory cortex (Llano and Sherman, 2009).

In summary, although analysis of compartmental and temporal *Arc* mRNA expression has been studied extensively during spatial learning paradigms, our study demonstrates for the first time that a differential compartmental analysis of activity-regulated transcripts can be useful to study the details of neural changes that may underlie auditory cortical synaptic plasticity. Our findings showing a correlation between the layer-specific sub-cellular *Arc* mRNA distribution and previous sound stimulation will motivate further experiments to analyze the molecular mechanisms underlying synaptic plasticity in the auditory cortex. Finally, we speculate that the subtle, sensory-induced sensitization of Arc mRNA may provide a substrate to bias the participation of specific neurons (Ramirez-Amaya et al., 2005) within the cortical network to store the long-term, distributed memory trace of a sensory event (Sutherland and McNaughton, 2000).

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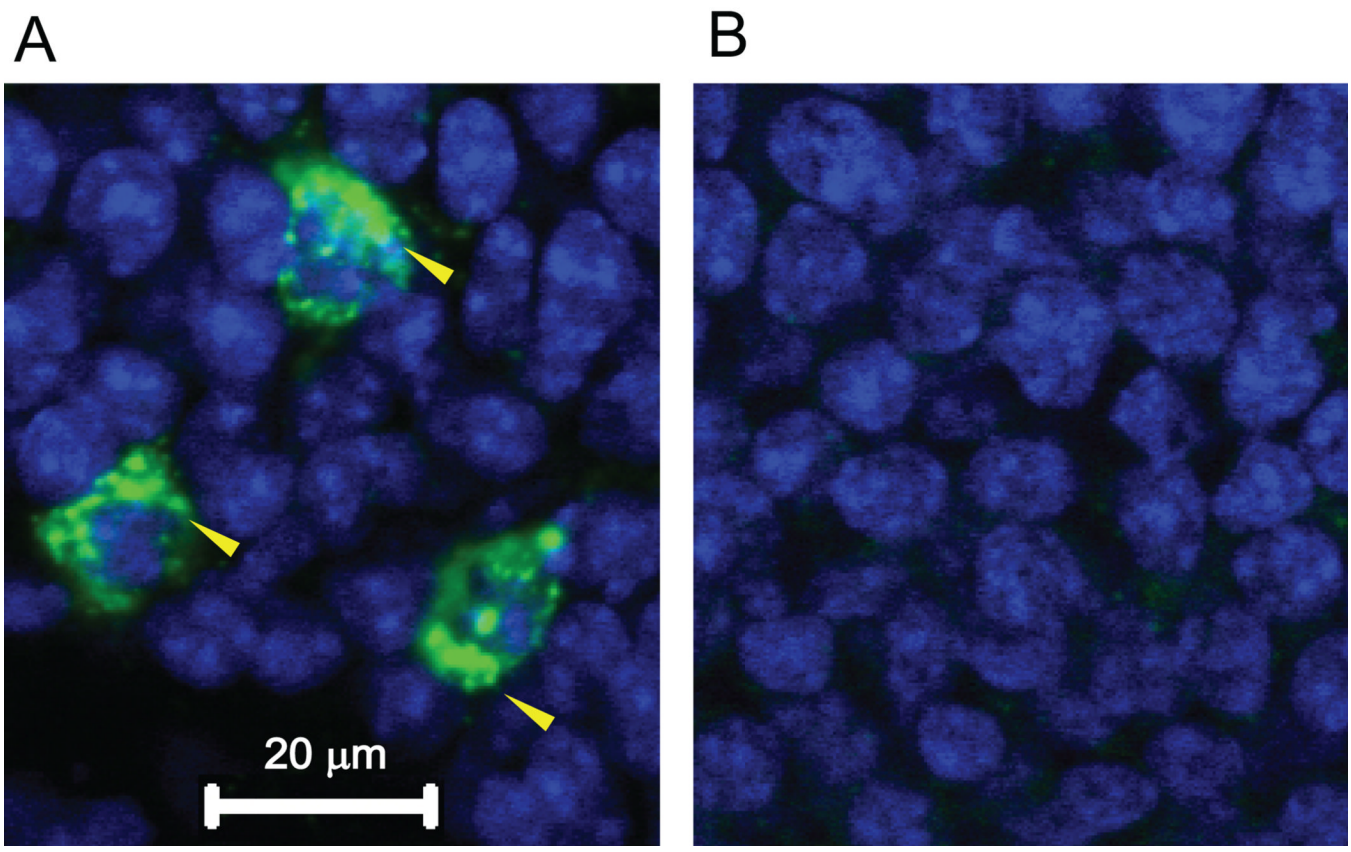

Figure 1.
Arc mRNA expression in the dentate gyrus after sound stimulation. (A) Image (63X) of a coronal section through the dentate gyrus processed for FISH using a digoxigeninlabeled riboprobe (antisense) shows *Arc* mRNA expression (yellow arrowheads) around granule cell soma. (B) Image after hybridization using the *Arc* sense probe remained unstained.

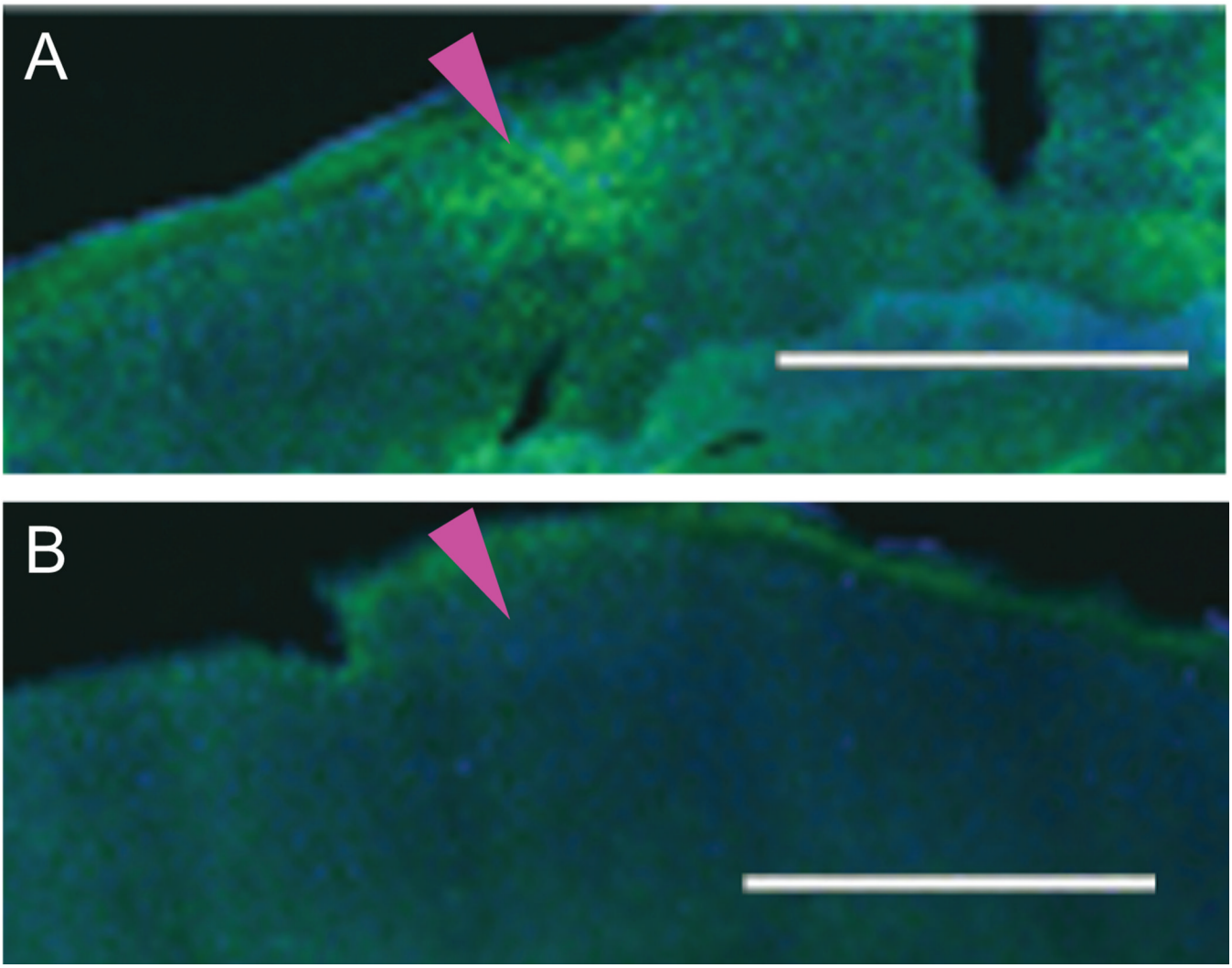

Figure 2.
Single plane images of *Arc* mRNA expression in a tangential section (40 μm) through cortical layer 4 of auditory cortex (magenta arrowhead) after (A) sound stimulation and (B) silence. Blue represents DAPI staining of nuclei. Green represents *Arc* mRNA expression. Scale bar is 1000 μm.

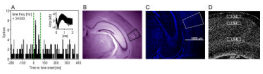

Figure 3.
Location within primary auditory cortex (Au1) of confocal image analyses. (A) Peristimulus time histogram of responses of a neuronal unit (all action potential waveforms depicted in the Inset) to a 34 kHz pure tone (duration of tone marked by dashed vertical green lines). (B) Cresyl violet stain (2X magnification) of lesioned sites (dashed circle) within areas showing primary-like auditory responses (A), presumably corresponding to primary auditory cortex (black box). This site corresponds to −2.54 mm re. Bregma according to the Paxinos and Franklin (2001) mouse brain atlas. (C) DAPI nuclear staining viewed under the confocal microscope at 10X magnification through an anatomically similar slice through primary auditory cortex (white dashed box). (D) Expanded grayscale view of the boxed area in (C) showing nuclear density variations similar to that seen in the cresyl violet stain (B). This enabled targeting of specific, identifiable regions in the middle of the supragranular (layers 1–2), thalamorecipient (layers 3–4) and infragranular (layers 5–6) layers for confocal image analysis (rectangular zones).

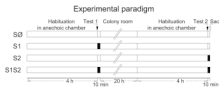

Figure 4.

Experimental paradigm. Segments indicate amount of time in each behavioral state. Filled and unfilled Test segments indicate presence or absence, respectively, of a 32 kHz dynamic tone stimulus.

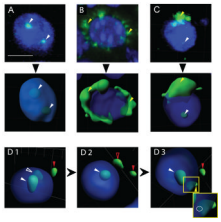

Figure 5.

Classification of *Arc* mRNA expression. Examples of *Arc* mRNA expressed in (A) intranuclear foci *only*, (B) perinuclear cytoplasm *only*, or (C) both intranuclear foci and perinuclear cytoplasm. Top panels are each confocal z-stacks (projection onto 2D) from a tissue section subjected to FISH. Middle panels represent their respective 3D surface reconstructions. These high-magnification views (63X) illustrate individual nuclear foci (white arrowheads) and diffuse perinuclear cytoplasmic signals (yellow arrowheads). Blue represents DAPI staining of nuclei. Note the detection of two *Arc* mRNA foci within the nucleus in (A), suggesting active transcription at each allele. (D1–3) The classification advantage of 3D reconstruction is evident from successively rotated views of the same nucleus. The solid red arrowhead in each panel points to a fluorescence signal labeled as background and left unclassified, since it cannot be associated with a specific cell. The solid white arrowhead in each panel indicates a fluorescence signal classified as a focal point of intranuclear *Arc* expression, since it remains wholly within the nucleus in all views. The open white arrowhead in D1 indicates a potential site of intranuclear expression based on the transparent view, but upon rotation (D2), this fluorescence appears to fall outside the nucleus, potentially representing background expression (open red arrowhead). Further rotation (D3) reveals that the fluorescence surface intersects the DAPI stained nuclear surface (highlighted by white ring around intersection in the Inset, which expands the image within the yellow box in D3), resulting in its classification as a point of perinuclear cytoplasmic expression (yellow arrowhead). This cell is therefore classified as having “both” intranuclear and cytoplasmic expression. Common scale bar shown in (A) is 10 μm.

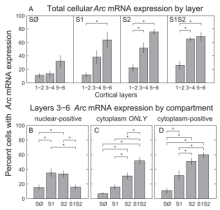

Figure 6.

Arc mRNA expression across behavioral groups, cortical layer and sub-cellular compartments. (A) Average percentage of cells positive for *Arc* mRNA (in nucleus and/or cytoplasm) in images from cortical layers 1–2, 3–4, and 5–6 for each behavioral group. The difference in layer-dependent expression between SØ and S1 mice, both of which only had silence just before sacrifice, indicates an effect of prior sound exposure. (B–D) Average percentage of *Arc* mRNA expression in each compartment, was pooled across layers 3–6, according to group. Nuclear-positive (cytoplasm-positive) cells have expression in the nucleus (cytoplasm), but may also have cytoplasmic (nuclear) expression. Cytoplasm *ONLY* cells express *Arc* mRNA only in the cytoplasm. Differences between S2 and S1S2 in panels B and C, and SØ and S1 in B and D indicate an effect of prior sound exposure. Asterisks in each graph represent significance by posthoc HSD test ($p<0.05$) for indicated pairs. Error bars represent standard error.

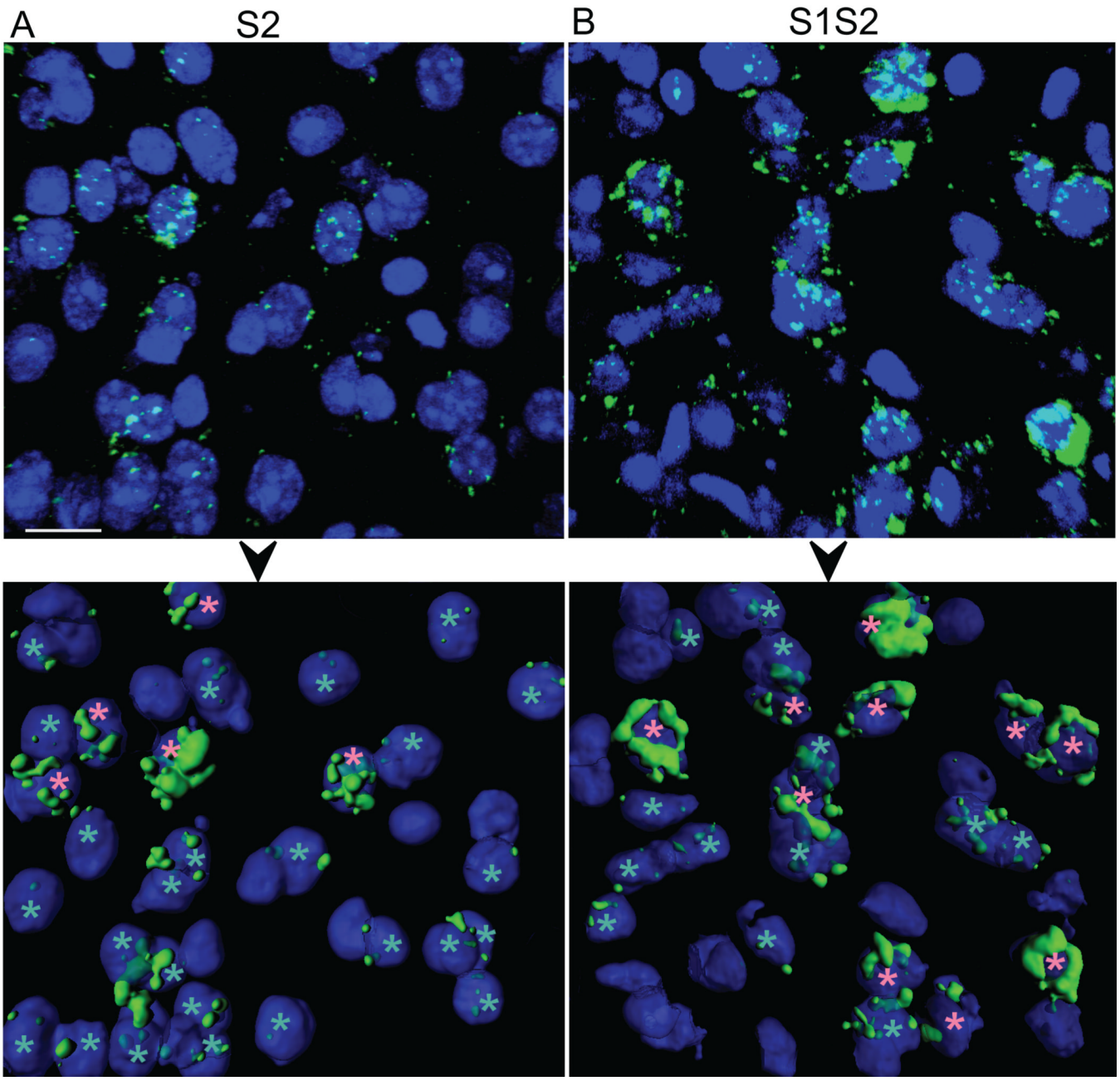

Figure 7.

Examples of sub-cellular compartmental *Arc* mRNA expression in layers 5–6 for sound-stimulated groups (A) S2 and (B) S1S2. Top panels show raw confocal z-stacks (63X) from tissue sections subjected to FISH. Bottom panels represent the respective 3D surface reconstruction, retaining only probably neuronal (and not glial) cell bodies that are predominantly within the edges of the image. Pink asterisks mark cells with *Arc* mRNA expression in the perinuclear "cytoplasm *only*"; cyan asterisks mark those with nuclear-positive *Arc* mRNA expression (either "intranuclear foci *only*," or "both"). Blue represents DAPI staining of nuclei. Scale bar is 15 μm.