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Acute high-intensity noise induces rapid Arc protein expression but fails to rapidly change GAD expression in amygdala and hippocampus of rats: Effects of treatment with D-cycloserine

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ABSTRACT

Tinnitus is a devastating auditory disorder impacting a growing number of people each year. The aims of the current experiment were to assess neuronal mechanisms involved in the initial plasticity after traumatic noise exposure that could contribute to the emergence of tinnitus and to test a potential pharmacological treatment to alter this early neural plasticity. Specifically, this study addressed rapid effects of acute noise trauma on amygdalo-hippocampal circuitry, characterizing biomarkers of both excitation and inhibition in these limbic regions, and compared them to expression of these same markers in primary auditory cortex shortly after acute noise trauma. To assess excitatory plasticity, activity-regulated cytoskeleton-associated (Arc) protein expression was evaluated in male rats 45 min after bilateral exposure to acute high-intensity noise (16 kHz, 115 dB SPL, for 1 h), sufficient to cause acute cochlear trauma, a common cause of tinnitus in humans and previously shown sufficient to induce tinnitus in rat models of this auditory neuropathology. Western blot analyses confirmed that up-regulation of amygdalo-hippocampal Arc expression occurred rapidly post-noise trauma, corroborating several lines of evidence from our own and other laboratories indicating that limbic brain structures, i.e. outside of the classical auditory pathways, exhibit plasticity early in the initiation of tinnitus. Western blot analyses revealed no noise-induced changes in amygdalo-hippocampal expression of glutamate decarboxylase (GAD), the biosynthetic enzyme required for GABAergic inhibition. No changes in either Arc or GAD protein expression were observed in primary auditory cortex in this immediate post-noise exposure period, confirming other reports that auditory cortical plasticity may not occur until later in the development of tinnitus. As a further control, our experiments compared Arc protein expression between groups exposed to the quiet background of a sound-proof chamber to those exposed not only to the traumatic noise described above, but also to an intermediate, non-traumatic noise level (70 dB SPL) for the same duration in each of these three brain regions. We found that non-traumatic noise did not up-regulate Arc protein expression in these brain regions. To see if changes in Arc expression due to acute traumatic noise exposure were stress-related, we compared circulating serum corticosterone in controls and rats exposed to traumatic noise at the time when changes in Arc were observed, and found no significant differences in this stress hormone in our experimental conditions. Finally, the ability of D-cycloserine (DCS; an NMDA-receptor NR1 partial agonist) to reduce or prevent the noise trauma-related plastic changes in the biomarker, Arc, was tested. D-cycloserine prevented traumatic noise-induced up-regulation of Arc protein expression in amygdala but not in hippocampus, suggesting that DCS alone is not fully effective in eliminating regionally-specific early plastic changes after traumatic noise exposure.

Abbreviations: ACTB, Beta actin; Arc (Arg 3.1), Activity-regulated cytoskeleton-associated protein; A1, primary auditory cortex; CA1, hippocampal Cornu Ammonis area 1; CA3, hippocampal Cornu Ammonis area 3; dB SPL, decibels Sound Pressure Level; DCS, D-cycloserine; ELISA, enzyme-linked immunosorbent assay; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; IP, intraperitoneal; NMDA, N-Methyl-D-aspartate; NR1, N-Methyl-D-aspartate receptor subunit 1

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1. Introduction

Tinnitus is the most prevalent auditory disorder in humans (Rauschecker et al., 2010). Most forms of chronic subjective tinnitus are caused by abnormally functioning neural circuits generating a phantom perception of sound in the absence of a corresponding external sound (Jastreboff, 1990; Møller, 2011a). The pathology of tinnitus has been linked to activation of harmful neural plasticity after exposure to loud noise or to input deprivation in aging, injuries, diseases or disorders affecting the conductive apparatus of the ear, cochlear receptor neurons, the VIIIth cranial nerve, and/or neurons in the central auditory system (Møller, 2011a) which can lead to a re-routing of central sensory information that may be explained by an abnormal activation of non-classical sensory pathways (Møller, 2011a, 2011b). Noise trauma is a common cause of tinnitus and is the primary focus of this experimental study, though it is important to note that people with normal hearing also can suffer from the disorder (Rauschecker et al., 2010). The current study aims to characterize potential components of pathological neuronal plasticity in an animal model system resulting from auditory overstimulation caused by 1 h exposure to high-intensity noise. Importantly, although the present research was conducted with an established paradigm used to induce tinnitus in rats, the time frame for this study was aimed at measuring early and transient changes in protein expression occurring rapidly after noise trauma. Similar to humans, not all rats exposed to noise trauma experience tinnitus; therefore, changes observed in the present study indicate very specific immediate outcomes from exposure to traumatic noise. In other words, early plasticity in non-auditory brain regions immediately post-noise trauma are not necessarily indicative of tinnitus, but could significantly contribute to its development.

Although considerable tinnitus research has focused on classical auditory structures in order to better understand the mechanisms involved in specific forms of tinnitus (Rajan and Irvine, 1998; Schwaber et al., 1993; Muhlnickel et al., 1998; Wienbruch et al., 2006; Engineer et al., 2011), multiple studies have also shown involvement of non-classical auditory structures within the limbic system in both humans (Møller et al., 1992; Lockwood et al., 1998; Mirz et al., 1999, 2000; De Ridder et al., 2006; Eichhammer et al., 2007) and animal models (Wallhauser-Franke et al., 2003; Goble et al., 2009; Kraus et al., 2010; Chen et al., 2015). Eggermont and Roberts (2004) and Møller et al. (1992) speculated that tinnitus-related plasticity occurring in the limbic system is due to an activation of the extralemniscal auditory pathway, a system typically active developmentally, i.e. in children but not in normal, healthy adults (Møller and Rollins, 2002). This non-classical pathway receives bilateral auditory input from both ears as well as from other sensory modalities (Møller, 2011b). By way of the thalamic medial geniculate nucleus, the non-classical ascending auditory pathway projects to the basolateral complex of the amygdala, triggering a chain of activation (including hippocampus and primary auditory cortex) that may contribute to the pathology of tinnitus (Singer et al., 2013), as the amygdala is known to influence plasticity in these regions (McIntyre et al., 2005; Møller, 2011a, 2011b; Farmer and Thompson, 2012).

In the context of rapid post-noise exposure plasticity in non-classical pathways, our laboratory has shown that the stable location-specific representation of spatial environments encoded by hippocampal place cells (cf. Thompson and Best, 1989, 1990; Best and Thompson, 1989; Donzis et al., 2013) in the CA1 region of hippocampus are disrupted within 15 min after high-intensity noise exposure (Goble et al., 2009), with plasticity persisting for at least 24 h afterwards. Our laboratory has also shown that auditory stimuli associated with aversive consequences can also rapidly induce plasticity in these hippocampal place cells, and that this plasticity is dependent upon functional connectivity between the CA1 region of hippocampus and the basolateral complex of the amygdala (Donzis et al., 2013). It has also been postulated that noise trauma could produce indirect effects such as emotional stress (Shulman et al., 2009), and that brain regions involved in processing emotional salience (including the amygdala and hippocampus) could be more rapidly vulnerable to stressful effects resulting from noise trauma than less emotionally-salient processing regions like primary auditory cortex (A1). Cheng et al. (2016) empirically tested the effects of moderate noise exposure (80 dB SPL; 2 h per day) on hippocampus and on A1, and saw increased oxidation and tau phosphorylation after only 1 wk of moderate noise exposure in hippocampus, an effect that required 3 wk exposure in A1. Similar to this emotionally-salient hypothesis discussed by Shulman et al. Cheng et al. speculated that their results were due to an acoustic stimulus being processed differentially in limbic regions than in A1, since the stimulus was being processed as a salient emotional stimulus (despite noise levels considered “moderate” rather than “traumatic”), i.e. not as a purely acoustic sensory stimulus.

Our current experiments aimed to extend this hypothesis of Cheng et al. (2016) to investigate if limbic regions would differentially process short term exposure (rather than exposure over the course of 1 wk or more) to traumatic noise (rather than to moderate noise) by characterizing rapid plasticity-related changes within the amygdalo-hippocampal circuitry post-acute noise trauma (our laboratory earlier reported electrophysiological evidence for hippocampal plasticity immediately after noise trauma in hippocampus: Goble et al., 2009), and testing whether or not similar changes would also occur at this same time interval in A1. To assure that changes were due to the traumatic (emotionally salient) sound, we also tested the effects of non-traumatic noise using an ambient sound intensity level similar to normal conversational levels in humans (Moore, 2013). We then further explored the magnitude of this high-intensity noise exposure as a stressful stimulus by measuring serum corticosterone levels at this same time point as we explored plasticity-related changes in protein expression in these brain regions.

We assessed expression of Arc (also known as Arg3.1) as a biomarker of plasticity. Arc (activity-regulated cytoskeleton-associated protein) is an immediate early-gene protein product whose expression is up-regulated in excitatory projection neurons within 45 min of exposure to stimuli that induce plasticity (Link et al., 1995; Guzowski et al., 2000, 2005; McIntyre et al., 2005; Bloomer et al., 2008). Arc is expressed during memory consolidation; up-regulation is often NMDA receptor-activation dependent, and is localized to active synaptic sites (Steward and Worley, 2002; Holloway and McIntyre, 2011). During active information processing, Arc transcription is rapidly induced and translation and transport occurs within the principal cells of the hippocampus (CA1 pyramidal place neurons) as well as in other brain regions (Guzowski et al., 1999; Tzingounis and Nicoll, 2006; Bramham et al., 2008, 2010; Penner et al., 2011; Donzis and Thompson, 2014). Arc was, thus, well-suited for use in the current study as a biomarker of rapid excitatory plasticity within the amygdalo-hippocampal circuitry shortly after acute high-intensity noise exposure.

Possible rapid changes in inhibition were also examined by assessing expression of the GABAergic biomarker glutamate decarboxylase (GAD) 45 min after acute noise trauma. GAD, the biosynthetic enzyme catalyzing the decarboxylation of glutamate into GABA, is expressed in 2 isoforms in inhibitory neurons: GAD 65 and GAD 67 (Erlander and Tobin, 1991; Esclapez et al., 1994; Suzuki et al., 1995; Hossein et al., 2005; Malfatti et al., 2007). Both GAD 65 and GAD 67 have been shown to decline with age in the central

auditory cortex, and this decrease has been linked to hearing loss (Burianova et al., 2009). Since no prior work has assessed GAD expression shortly after acute traumatic noise exposure, we aimed to characterize this biomarker of potential changes in inhibition within the amygdalo-hippocampal circuit and in A1 subsequent to intense noise trauma.

Finally, we examined the efficacy of D-cycloserine (DCS), a drug which readily crosses the blood-brain barrier, as a potential preventative treatment for the initiation of early noise-induced amygdalo-hippocampal plasticity which could contribute to the development of tinnitus. DCS is a partial agonist for the glycine/serine binding site on NR1 subunits of NMDA receptors (Hood et al., 1989; Donzis and Thompson, 2014). The NMDA receptor has been well characterized both in synaptic plasticity and in memory functions of the amygdalo-hippocampal circuit (Thompson et al., 1992; Link et al., 1995; Thompson and Disterhoft, 1997; Guzowski et al., 1999, 2000; Plath et al., 2006; Lonergan et al., 2010). Krings et al., 2014 reported in a pilot study (2015) that DCS was able to help patients cope better with tinnitus by improving tinnitus-related cognitive deficits when paired with a computer-based cognitive training program. The drug was well-tolerated, but DCS failed to improve patients' perception of tinnitus itself. This could be due to the fact that the drug in that study was given to patients who had already developed stable, long-term chronic tinnitus, rather than treatment given at an earlier stage of the condition. Currently, there is no cure for tinnitus. While there are many treatments in use, none have proven fully effective in all types of sufferers over the long-term. Treatment strategies that impact tinnitus during early stages of development of tinnitus may prove beneficial, since tinnitus becomes increasingly resistant to treatment as time passes after its initial onset (Møller, 2011a).

2. Materials and methods

2.1. Subjects

All animal uses were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee (IACUC), in accordance with USDA guidelines. 32 young adult (2–3 mo) male Long-Evans rats were socially housed on a 12 h light/dark cycle in a temperature-controlled vivarium, with food and water available *ad libitum*. For western blots, 6 rats were assigned to each of the 5 groups: control, non-traumatic noise exposure, traumatic noise exposure, DCS-treated with no noise exposure, and DCS-treated with traumatic noise exposure. For ELISA corticosterone assays, blood serum was collected from the same rats assigned to the control group and the traumatic noise-exposed group, plus 1 additional rat per group, totaling 7 rats for controls and 7 rats for the traumatic noise-exposed group. Behavioral procedures for all groups are defined below, and the timeline of all experimental procedures is illustrated in Fig. 1.

2.2. Handling and vehicle injections

Rats were handled and acclimated to a sound-controlled chamber (22.5″ height × 18.5″ width × 19.5″ depth; speakers 12″ above floor of holding cage (8″ height × 8.5″ width × 8.5″ depth)) and to intraperitoneal (IP) injections of normal saline (0.9%) daily for 3 wk. This lengthy handling procedure, combined with maintenance of constant environmental conditions, was used to minimize potential confounds and variability in protein expression due to environmental variability or novelty, and to assure that the observed changes were due to noise trauma and/or to DCS treatment. IP injections (0.1 ml total volume) of normal saline vehicle were given daily to each rat 15 min prior to going into the chamber.

2.3. Drug treatment and noise exposure session

On the final day, 15 min prior to going into the chamber, rats received IP injections of either the saline vehicle or DCS (6 mg/kg, pH 7.4). This dose of DCS was chosen for its nootropic effectiveness when given as a single dose, and is well-tolerated in freely-behaving animals (Thompson et al., 1992; Thompson and Disterhoft, 1997; Donzis and Thompson, 2014). For 12 freely-behaving rats (traumatic noise-exposed; half vehicle controls, half DCS-treated), acute noise trauma was induced bilaterally (16 kHz, 115 dB SPL) for 1 h in the same chamber in which they had been habituated. This frequency, intensity and duration were chosen based on methods used to inducing tinnitus in rat models reported by Turner et al. (2006) and Engineer et al. (2011). Six freely-behaving rats (non-traumatic noise-exposed) were bilaterally presented with the same pure tone for 1 h at an ambient noise level of 16 kHz, 70 dB SPL within the same chamber as habituation took place, as a control for sound intensity as a stressor. This intensity was chosen because it is only slightly elevated from the background noise which the rats were consistently exposed to in the animal facility, and it is below 85 dB SPL, a level known to have both auditory and non-auditory effects in rodents, such as eosinopenia, increased adrenal gland weights, and reduced fertility (Suckow et al., 2005; National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). Sound intensity was calibrated before each session using a sound pressure level meter (Radioshack) located in the center of the chamber 3.5" above floor level. 12 freely-behaving rats (controls) spent the same amount of time in the chamber for the duration of the experiment, but received no noise exposure; half served as vehicle controls, half were DCS-treated. Rats from each condition were individually-exposed in the sound chamber.

2.4. Western immunoblotting and analysis for Arc and GAD 65 + 67

Rats were deeply anesthetized using isoflurane and quickly decapitated for brain removal 45 min after exiting the chamber, a time point when Arc protein expression is transiently enhanced following brief experimental events (McIntyre et al., 2005; Czerniawski et al., 2011; Holloway-Erickson et al., 2012). Brains were flash frozen in 2-methylbutane submerged in a mixture of dry ice and ethanol and stored at −80 °C until processed. 500 μm sections were taken on a cryostat, and 1.22 mm diameter tissue punches collected from 3 regions (amygdala, dorsal hippocampus, and A1) verified using a rat atlas (Paxinos and Watson, 1998). Regional punches were homogenized in a 0.1 M phosphate buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). A Qubit fluorometer and protein assay kit (Life Technologies) was used to determine the concentration of protein in each sample. 15 μg samples were heated in a sample buffer with reducing agent, loaded into wells, and run on an SDS-PAGE gel for ~55 min at 165 V and 120 mA per gel using Novex Bolt Mini Gel Tanks (Life Technologies). On any specific gel, samples from the same region from different rats and from each different condition were run simultaneously. A technical replicate for each tissue sample was performed to verify reproducibility of the reported observations, but not included in the final analyses. Gels were transferred onto nitrocellulose membranes via the iBlot dry blotting system (LifeSciences) for 7 min. Membranes were washed with Tris-buffered saline, and blocked with 5% skim milk for 24 h on a rotator. Membranes were probed for 48 h with the primary antibodies of interest (Arc (1:6000, Synaptic Systems), GAD 65 + 67 (1:1000, Abcam), and β-Actin (1:1000, Proteintech)) diluted in 5% skim milk and Tris-buffered saline and Tween-20 (TBS-t) (15 mL TBS-t and 0.75 g powdered skim milk solution). Membranes were

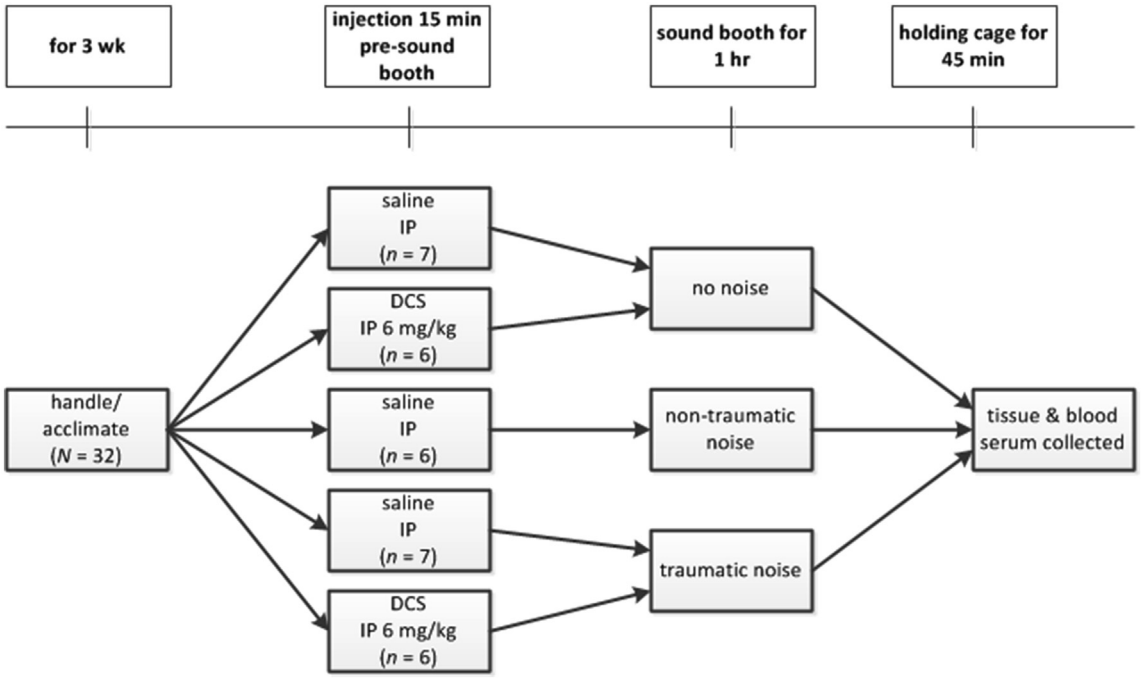

Fig. 1. Behavioral paradigm for noise exposure and treatment with D-cycloserine (DCS). Flow diagram of the experimental sequence used in this study. Well-handled rats were injected with either a nootropic dose of DCS (6 mg/kg, ip) or saline vehicle 15 min before exposure to one of three conditions: high-intensity (traumatic) noise exposure, low-intensity (non-traumatic) noise exposure or confinement in the same sound chamber without noise exposure (no noise). 45 min later, brain tissue was collected and frozen for processing to assay plasticity in protein expression, and serum samples collected, processed and frozen at the same time for later ELISA assays.

washed, then incubated in a secondary HRP-linked antibody (1:2424, Abcam) for 1 h followed by a Pierce ECL chemiluminescent substrate (Thermo Scientific) for detection of immunoreactivity. Membranes were placed on films (GE Healthcare Limited) with varying exposure times (3 min for Arc and β-Actin, 1 min for GAD 65 + 67), developed, and scanned at high resolution (400 dpi, 24-bit depth) into a computer for analysis. Western blot densitometry was analyzed using ImageJ 1.46r software. β-Actin was used as a loading control for normalizing Arc and GAD 65 + 67 expression within individual lanes. To assure that β-Actin expression was stable across all groups (i.e. to assure its reliability as a loading control in these experiments), a one-factor analysis of variance was performed for both limbic regions and found no significant main effects of condition (amygdala: $F(3,20) = 0.70$, $p = 0.56$; dorsal hippocampus: $F(3,20) = 0.56$, $p = 0.65$. In region A1, statistical comparisons between samples from controls and traumatic-noise exposed rats for β-Actin also showed no significant difference in mean optic density between control and traumatic-noise-exposed groups using an independent samples *t*-test, $t(10) = 1.27$, $p = 0.24$. Western blot densitometry was analyzed using ImageJ 1.46r software.

2.5. ELISA analysis for circulating corticosterone levels

Using the same methods reported by Underwood and Thompson (2016; per the ELISA kit's manufacturer's instructions), trunk blood was taken at the time of sacrifice, and centrifugation was performed to collect serum samples. Serum samples were aliquoted into 500 μL tubes and frozen at −20 °C until use to avoid repeated freeze/thaw cycles. Serum was thawed at room temperature for 1 h and diluted appropriately for the ELISA assay. Corticosterone was assessed with a rat corticosterone (CSCI) ELISA kit (Abcam) using an ELx800 plate reader (BioTek) with Gen5 software.

2.6. Statistical analyses

All statistical comparisons were made using R v3.1.2 software. One-factor analysis of variance (ANOVA) tests were run to test for overall group differences in protein expression of Arc and GAD 65 + 67, with the exception of GAD 65 + 67 expression in A1 (which only compared 2 groups). An independent samples *t*-test was used to compare controls with traumatic-noise exposed rats to check for significant differences in A1 GAD 65 + 67 expression. To analyze *a priori* expected changes in Arc and GAD 65 + 67 protein expression within these brain regions, a series of planned comparisons (controls versus traumatic noise-exposed; controls versus non-traumatic noise-exposed; controls versus DCS; controls versus DCS plus traumatic noise-exposed) were made using Dunnett's tests to assess any variation from noise exposure and drug treatment compared to controls. Western blot densitometry results are displayed as a normalized ratio of Arc or GAD 65 + 67 over β-Actin (ACTB). Corticosterone levels were compared between controls and traumatic-noise exposed rats using an independent samples *t*-test. A probability level of $p < 0.05$ was considered significant for all statistical testing.

3. Results

This study investigated the immediate effects of acute noise trauma and of non-traumatic noise on protein expression within the amygdaloid complex and in dorsal hippocampus (both non-classic auditory structures involved in emotional learning and memory) and in A1 (a prominent auditory structure) that could contribute to long-term maladaptive plasticity resulting in tinnitus. Arc protein expression was used as a biomarker of excitatory plasticity, while GAD 65 + 67 protein expression was used as a biomarker of GABAergic inhibition. Rats were exposed bilaterally either to acute low-intensity noise, to acute high-intensity noise

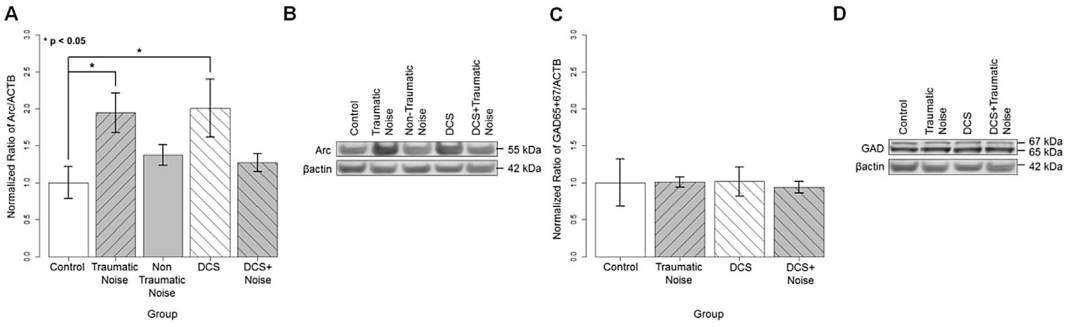

Fig. 2. Comparisons of changes in expression of Arc and of GAD 65 + 67 after noise exposure and treatment with DCS in the amygdaloid complex. (A) Significantly greater Arc expression was observed in western blots from rats exposed to traumatic noise compared to controls, $^{*}p = 0.04$, but not in rats exposed to non-traumatic noise. Significantly greater Arc expression was observed in rats treated with 6 m/kg DCS compared to controls, $^{*}p = 0.03$. Treatment with 6 mg/kg DCS reversed the effects of noise trauma, with Arc expression similar to that seen in controls, $p = 0.85$. **(B)** One representative blot each for Arc and β-actin (3 min exposure) expression are displayed. **(C)** Neither noise trauma nor DCS treatment effected GAD 65 + 67 protein expression, with no significant changes across any groups. **(D)** One representative western blot for GAD 65 + 67 (1 min exposure) and β-actin (3 min exposure) expression are displayed.

trauma, or served as controls. Rats either received equivolume injections of vehicle or of 6 mg/kg of DCS to investigate the drug's potential to block any plasticity resulting from noise trauma. Finally, circulating corticosterone was assessed to further characterize the involvement of stress in this early plasticity after noise trauma. Data are graphically presented as means ±*SEM*.

3.1. Arc and GAD 65 + 67 protein expression within amygdala

A one-factor ANOVA performed to test for an overall effect of noise exposure and DCS treatment conditions on Arc protein expression within amygdala revealed a significant main effect, $F(4,25) = 3.17$, $p = 0.03$, $MSe = 0.36$. To examine differences in Arc protein expression within the amygdala between experimental conditions compared to controls, a series of planned comparisons were made using Dunnett's tests (see Fig. 2A, representative blots are shown in Fig. 2B).

Rats exposed to acute high-intensity noise showed significantly increased expression of Arc protein compared to basal expression in controls ($p = 0.04$), while rats exposed to non-traumatic noise showed no significant increase ($p = 0.66$). Thus, increased Arc expression was induced only by traumatic noise, not by the novelty of a particular sound. Rats given DCS alone showed significantly increased expression of Arc protein compared to controls ($p = 0.03$). Rats given DCS treatment before acute high-intensity noise exposure, however, showed no significant increase in Arc protein expression compared to controls ($p = 0.85$). While treatment with DCS or traumatic noise exposure each up-regulated Arc protein expression within amygdala, the combination of DCS treatment with noise trauma prevented this up-regulation.

A one-factor ANOVA performed to test for an overall effect of noise exposure and DCS treatment conditions on GAD 65 + 67 protein expression within amygdala revealed no significant main effects, $F(3,20) = 0.03$, $p = 0.99$, $MSe = 0.23$ (see Fig. 2C, representative blots are shown in Fig. 2D). GAD 65 + 67 protein was not changed by traumatic noise exposure or DCS treatment.

3.2. Arc and GAD 65 + 67 protein expression within dorsal hippocampus

A one-factor ANOVA performed to test for an overall effect of noise exposure and DCS treatment conditions on relative Arc protein expression within dorsal hippocampus revealed a significant main effect, $F(4,25) = 3.46$, $p = 0.02$, $MSe = 0.20$. To examine differences in Arc protein expression within the dorsal hippocampus between experimental conditions, a series of planned comparisons were made using Dunnett's tests (see Fig. 3A, representative blots are shown in Fig. 3B).

Rats exposed to acute high-intensity noise showed significantly increased expression of Arc protein compared to basal expression in controls ($p = 0.03$), while rats exposed to non-traumatic noise showed no significant increase ($p = 0.93$). As in the amygdala, increased Arc expression in dorsal hippocampus was induced only by traumatic noise, not by the novelty of a particular sound. Rats

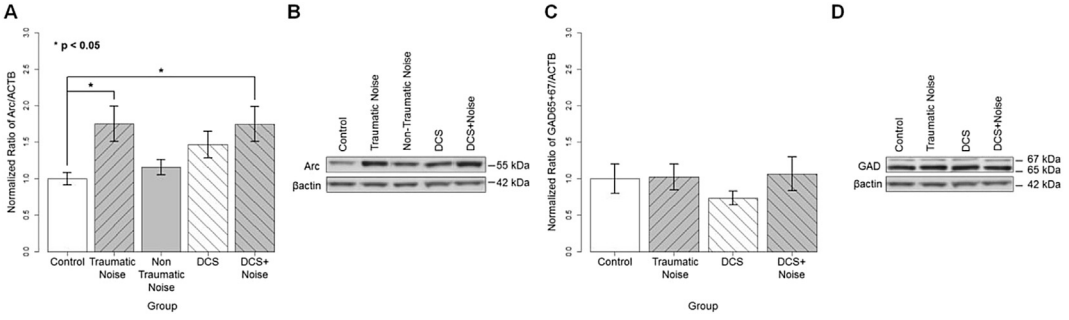

Fig. 3. Comparisons of changes in expression of Arc and of GAD 65 + 67 after noise exposure and treatment with DCS in the dorsal hippocampus. (A) Significantly greater Arc expression was observed in western blots from rats exposed to noise trauma compared to controls, $^{*}p = 0.03$, but not in rats exposed to non-traumatic noise. DCS alone did not significantly up-regulated Arc expression in dorsal hippocampus. There was no statistically significant difference observed in rats treated with DCS compared to controls, $p = 0.24$. Treatment with 6 mg/kg DCS did not block the effects of noise trauma, with Arc expression significantly elevated compared to controls, $p = 0.03$. **(B)** One representative blot for Arc and β-actin (3 min exposure) expression are displayed. **(C)** Neither noise trauma nor DCS treatment effected GAD 65 + 67 protein expression, with no significant changes across any groups. **(D)** One representative blot for GAD 65 + 67 (1 min exposure) and β-actin (3 min exposure) expression are displayed.

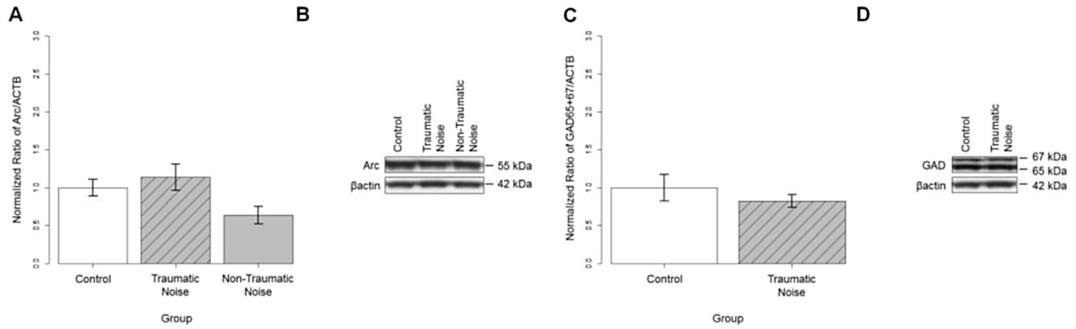

Fig. 4. Lack of changes in expression of Arc and of GAD 65 + 67 after noise exposure in the primary auditory cortex (A1). (A) Unlike in the amygdala and dorsal hippocampus, there were no observed changes in Arc protein expression in western blots from A1 after noise exposure compared to controls. **(B)** One representative blot for Arc and β-actin (3 min exposure) expression are displayed. **(C)** Similar to the amygdala and dorsal hippocampus, there were no observed changes in GAD 65 + 67 protein expression in A1 after noise exposure compared to controls. **(D)** One representative blot for GAD 65 + 67 (1 min exposure) and β-actin (3 min exposure) expression are displayed.

given DCS alone showed no significant changes in Arc protein expression compared to controls ($p = 0.24$). Rats given DCS treatment paired with acute high-intensity noise exposure expressed a significant up-regulation in Arc protein expression compared to controls ($p = 0.03$), so unlike in the amygdala, DCS treatment did not block noise-induced Arc up-regulation.

A one-factor ANOVA performed to test for an overall effect of noise exposure and DCS treatment conditions on relative GAD 65 + 67 protein expression within dorsal hippocampus revealed no significant main effects, $F(3,20) = 0.68$, $p = 0.57$, $MSe = 0.20$ (see Fig. 3C, representative blots are shown in Fig. 3D). GAD 65 + 67 protein was not changed by traumatic noise exposure or DCS treatment.

3.3. Arc and GAD 65 + 67 protein expression within primary auditory cortex

A one-factor ANOVA performed to test for an overall effect of noise exposure conditions on Arc protein expression within A1 revealed a significant main effect (prior to rounding), $F(2,15) = 3.75$, $p = 0.05$, $MSe = 0.11$. However, when differences in Arc protein expression within A1 between experimental groups were examined using a series of planned comparisons with Dunnett's tests (see Fig. 4A, representative blots are shown in Fig. 4B) no significant results for pairwise comparisons were found. Arc protein expression was not changed by traumatic noise exposure compared to controls ($p = 0.70$), nor by non-traumatic noise exposure ($p = 0.13$). Since Arc expression was not altered by noise exposure, effects of DCS treatment were not assayed (i.e. no *a priori* hypotheses were made for this case).

To assess differences in GAD 65 + 67 protein expression within A1 between traumatic noise-exposed rats and controls, an independent samples *t*-test was performed and showed no significant changes, Rats given acute high-intensity noise exposure for 1 h showed no significant changes, $t(10) = 0.91$, $p = 0.40$ (see Fig. 4C, representative blots are shown in Fig. 4D). Since GAD 65 + 67 was not changed by traumatic noise, effects of non-traumatic noise or DCS treatment were not tested.

3.4. Serum corticosterone analysis

To assess effects of acute traumatic noise exposure on circulating serum corticosterone (a possible stress response) at the same time point as Arc protein expression was up-regulated in the amygdala and dorsal hippocampus, an independent samples *t*-test was performed and found no significant difference between controls and traumatic-noise exposed rats, $t(12) = -0.98$, $p = 0.35$ (see Fig. 5). Thus, traumatic noise did not significantly alter the output of the hypothalamic-pituitary-adrenal axis in this paradigm.

4. Discussion

The results of the present study extend those of earlier experiments from our laboratory (Goble et al., 2009; Donzis et al., 2013; Donzis and Thompson, 2014) and those of others (Mahlke and Wallhäusser-Franke, 2004; De Ridder et al., 2006; Landgrebe et al., 2009; Shulman et al., 2009; Crippa et al., 2010; De Ridder et al., 2011; Gunby et al., 2015) linking amygdala and hippocampal excitability and showing evidence of early plasticity in these regions shortly after acute intense noise exposure. Within both amygdala and dorsal hippocampal regions, Arc protein expression was up-regulated 45 min after rats were exposed to acute high-intensity sound exposure. The heightened expression of this immediate-early gene (IEG) product is indicative of early excitatory plastic changes occurring within these limbic regions as a result of noise trauma. The hippocampus and basolateral amygdala are reciprocally connected (Richter-Levin and Akirav, 2001; McIntyre et al., 2003; Pitkänen et al., 2000). This connection is necessary

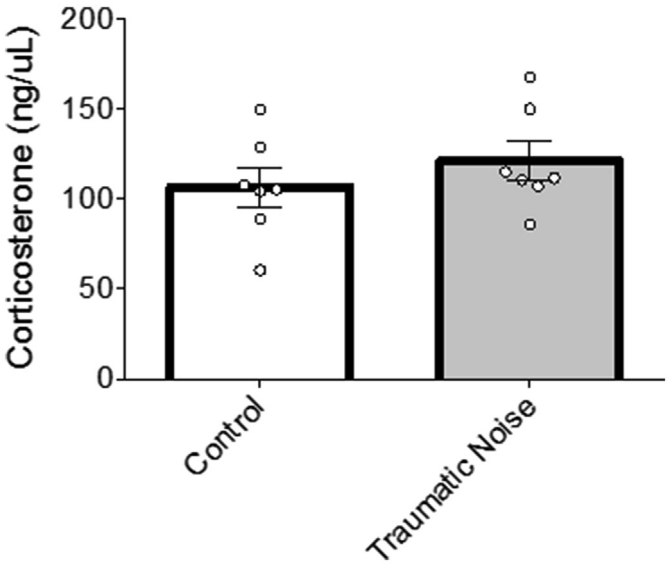

Fig. 5. Acute high-intensity noise trauma failed to alter circulating corticosterone. There was no observed change in corticosterone in serum from rats exposed to traumatic noise ($n = 7$) compared to controls ($n = 7$), $t(12) = -0.98$, $p = 0.35$. Both means ±*SEM* and actual data points are shown.

for enhancing long-term memory with emotional salience (LeDoux, 2007; Farmer and Thompson, 2012; Donzis et al., 2013; Lovitz and Thompson, 2015). An up-regulation of Arc expression in both regions after noise trauma is, thus, consistent with other experimental findings of early non-classical pathway plasticity in the context of tinnitus. This activation shows early plasticity in circuits well described for memory function that could play a developmental role (i.e. over a time course of days, weeks, and months) contributing to the initiation and maintenance of the chronic disorder, tinnitus.

The present research also found that Arc protein expression remained unchanged in A1 at this early time point post-noise trauma. This denotes that A1 was initially less susceptible to plastic changes after noise trauma. Wallhauser-Franke et al. (2003), however, observed an up-regulation of c-fos protein expression (another immediate-early gene biomarker for plasticity) in A1 at two early time points (1 h and 7 h) after exposure to low and high doses of salicylate or to impulse noise trauma. The observed differences could be due to differences in regulation of expression for Arc as compared to c-fos. For example, Arc plays a significant role in glutamate AMPA receptor trafficking in neural plasticity (Chowdhury et al., 2006), while specific roles for c-fos are not well-defined. Mahlke and Wallhäusser-Franke (2004) reported an increase in both c-fos and Arc immunoreactivity in both A1 and amygdala simultaneously 3 h after impulse noise exposure and 5 h after salicylate injections. Another investigation by Chen et al. (2015) used salicylate to induce tinnitus and assessed electrophysiological, behavioral and fMRI measures 2 h post-treatment to show that A1 is a major hub in the tinnitus network. This same study also reported increased connectivity between A1, hippocampus and amygdala 2 h after salicylate injections. These reports suggest that the mechanism for induction of tinnitus is more diverse and complex than simply activating classic auditory structures.

Expression of GAD 65 + 67, the enzyme responsible for converting glutamate into the inhibitory neurotransmitter GABA, remained stable in the amygdala, hippocampus and A1 at the same time point (45 min after acute noise exposure) that Arc was up-regulated in these limbic forebrain regions. This was a somewhat surprising finding, as it was hypothesized that GAD expression would be altered adaptively to maintain homeostatic inhibitory regulation (Stolzberg et al., 2012) in compensation for the changes in Arc expression, which can be directly linked to increases in excitability in hippocampus (Guzowski et al., 1999, 2000; Vazdarjanova et al., 2002; Donzis and Thompson, 2014). Zheng et al. (2015) found that, several months after tinnitus-inducing noise trauma, there were still no changes in GAD expression within brainstem dorsal and ventral cochlear nuclei. Whereas the timeline of the current study was based around previous work on the time course of changes in Arc/Arg 3.1 protein expression, Zheng et al. waited until after behavioral evidence of tinnitus was exhibited (22 wk post-noise exposure) to probe for plasticity in GAD expression, and they assayed different regions. Nonetheless, these two studies (ours immediately after noise trauma and Zheng et al.'s after behavioral signs of tinnitus were well-established) indicate that GAD expression may be less susceptible to change due to tinnitus-related plasticity. Other findings, however, suggest that down-regulation of inhibitory neurotransmission could be responsible for observed neuronal hyperactivity during the development of tinnitus (Milbrandt et al., 2000; Middleton et al., 2011; Wang et al., 2011; Richardson et al., 2012). With the exception of Richardson et al. (2012), these latter studies assayed GAD expression at intermediate time points between the present study and Zheng et al. While it is unclear precisely the time point assayed by Richardson et al. (it is only stated that experiments were conducted after behavioral evidence of tinnitus was observed), more experimental work on the contribution of GABAergic signaling to the development and maintenance of tinnitus is required.

As noted, our experimental observations for Arc and GAD 65 + 67 showed no noise-induced changes in A1 early after noise trauma. The results of Zheng et al. (2015) were based on studies of the brainstem dorsal and ventral cochlear nuclei, which, like A1, are parts of the classic (lemniscal) auditory pathway. The present study examined A1 in the classic pathway, along with key limbic regions that have been assessed for plasticity in studies of non-classical auditory pathways (Møller et al., 1992; De Ridder et al., 2006; Escartí et al., 2010; Chen et al., 2014, 2015). Our data support the hypothesis that initial excitatory or inhibitory plasticity resulting from acute traumatic noise exposure does not occur in A1, while early excitatory plasticity does occur in hippocampus and amygdala.

Many authors, including Møller (2011a) hypothesize that tinnitus results from abnormal neural plasticity high-jacking many of the mechanisms used routinely for formation of memories. In tinnitus, consolidation of such memories can occur over a long time course, as rats exposed to acute high-intensity noise exposure can often take several weeks to develop behavioral evidence of tinnitus (Engineer et al., 2011; Mohankumar et al., 2015; Zheng et al., 2015). Given the necessary time constraints to capture changes in Arc protein expression (rapid transcription, transport, translation, and subsequent degradation; Guzowski et al., 1999), the presence of tinnitus in experimental animals used in this study could not be tested; however, the consistent early up-regulation of Arc expression is indicative of early neuronal activity within these limbic regions resulting from exposure to acute traumatic noise. ABR thresholds have been shown to change throughout the development of tinnitus in a rat model, and such changes remain dynamic for at least a year post-noise trauma (Mohankumar et al., 2015). Unpublished data from our own lab, and data from other labs using the same noise trauma protocol (Engineer et al., 2011) indicate that the noise exposure experienced here does evoke behavioral signs of tinnitus, typically at a much later (weeks later) time interval, although less than half of all noise-exposed rats develop tinnitus even after long intervals. While several studies show that traumatic noise exposure associated with significantly delayed and/or gradual development of tinnitus (Coles et al., 2000; Gates et al., 2000), other studies report signs of either a rapid, transient onset of tinnitus after traumatic noise induction or a rapid onset of chronic tinnitus. Again, divergent findings are most readily interpreted to indicate that the overall time course for tinnitus is complex and can vary greatly between models and condition (e.g. Degeest et al., 2014; Turner and Larsen, 2016). Nonetheless, the early plasticity in Arc observed here, consistent with our earlier report (Goble et al., 2009) of disrupted place-field activity in dorsal hippocampus immediately after acute noise trauma, suggest that interventions targeting periods early in the development of tinnitus have particular clinical utility.

Although much is known about potential causes of tinnitus induction, less is known about the complete time course of the pathophysiology of the disorder. As a result, there is currently no effective treatment or cure for tinnitus. Guitton and Dudai (2007) investigated how certain neural components of tinnitus may be linked to memory formation. Their study showed that treatment with an NMDA antagonist (ifenprodil, which blocks the NR2B subunit), delivered into the cochlea for 4 d immediately following noise trauma, prevented development of behavioral signs of tinnitus, while later identical treatment was ineffective, providing evidence for their hypothesis that tinnitus is the end-product of abnormal neural plasticity, sharing some underlying mechanisms with memory formation, and producing some similar neural

correlates. In the present study, it was hypothesized that targeting the NR1 glycine/serine subunit of NMDA receptors with a partial agonist, D-cycloserine (DCS), 15 min prior to onset of the 1 h exposure to high-intensity sound could decrease NMDA receptor activation. NMDA receptor activation is a mechanism commonly hypothesized to play a necessary role in memory consolidation (Thompson et al., 1992; Moyer et al., 1996; Riedel et al., 2003; Morris, 2013), and hypothetically could also be involved in the aberrant plasticity of tinnitus. DCS treatment has already been approved by the FDA for human use (Davis et al., 2006). Several studies demonstrate that DCS is an effective cognitive enhancer, reversing age-dependent learning deficits (Thompson and Disterhoft, 1997) and enhancing extinction of fear memories (Ledgerwood et al., 2003; Davis et al., 2006). The present study hypothesized that DCS could modulate homeostatic plasticity, potentially preventing the heightened expression of Arc shortly after acute noise trauma exposure. Rats were treated with a single acute does of DCS, as evidence suggests that the drug's effects are decreased over repeated treatment sessions (Parnas et al., 2005; Davis et al., 2006; Norberg et al., 2008; Grillon, 2009).

The results from the present study show that acute DCS, given shortly before high-intensity noise exposure, succeeded in preventing up-regulation of Arc protein expression resulting from traumatic noise in the amygdala. In this condition, DCS treatment maintained Arc expression at basal levels in noise-exposed rats, rather than decreasing expression to lower, potentially detrimental, levels. Preventing up-regulation of this activity-dependent plasticity-related protein within the amygdaloid complex may be clinically relevant because the amygdala, which lies outside the classic auditory pathway, responds robustly to emotionally salient sounds (Romanski and LeDoux, 1993; Stutzmann et al., 1998; Chen et al., 2014), and increased functional connectivity occurs between auditory cortex and amygdala in tinnitus patients (Kim et al., 2012; for a review, see Kraus and Canlon, 2012). Since no changes in Arc expression were observed in auditory cortex at this early interval after traumatic noise exposure, DCS could potentially prevent this increased coupling between auditory cortex and amygdala, effectively shunting later manifestation of tinnitus after traumatic noise exposure. Directly testing this possibility was beyond the scope of the current experiments, but should be validated using multiple experimental approaches in the future. To start, future experiments should test how DCS paired with noise trauma could influence Arc expression in A1 and in hippocampus, amygdala, and perhaps other regions at multiple intervals over a longer time scale, allowing assessment if later changes in A1 reported in other studies could be prevented by early treatment with DCS. Additionally, other pharmacological manipulations can be assessed, since our data from the hippocampus suggests that DCS alone may not be the optimal early intervention to slow or reverse initiation of tinnitus.

As aforementioned, prior studies have shown that DCS can enhance fear extinction and improve age-related cognitive deficits (Thompson and Disterhoft, 1997; Ledgerwood et al., 2003; Davis et al., 2006; Kuriyama et al., 2011). Donzis and Thompson (2014) further showed that the same dose of DCS used here both increases intrinsic excitability and Arc protein expression in dorsal hippocampus. The effects of DCS also appear to depend on the context of treatment, consistent with reports of the drug's ability to homeostatically regulate memory-related mechanisms in various contexts (Gabriele and Packard, 2007; Vervliet, 2008; Kalisch et al., 2009; Baker et al., 2012). As reported here, DCS treatment in the absence of traumatic noise exposure produced effects similar to those of acute high-intensity noise exposure only in hippocampus (replicating earlier findings of Donzis and Thompson, 2014), but reversed noise-induced Arc expression in amygdala. Future investigation can compare the current results of DCS treatment with manipulations of serine (the endogenous ligand for NR1 subunits of NMDA receptors) or other compounds, to further address whether NMDA receptor-mediated plasticity is required at this stage. At later time points (days or weeks post-noise trauma), once behavioral evidence of tinnitus is present, acute treatments could also be tested by pairing them with tones surrounding the frequency of which the tinnitus is manifested, in a paradigm similar to fear extinction treatment with DCS (Walker et al., 2002).

Future studies could also investigate a longer time course to assess plasticity in GAD expression within relevant brain regions in order to address whether changes would occur at a later time point. Also, despite no observed changes in GAD expression in the current study, potential changes in inhibitory neurotransmission should still be further considered; despite no observed changes in GAD expression, there may still be changes in GABAergic inhibition. Additional pharmacological studies using other NR1 ligands are also needed, since DCS treatment is known to affect GABA release (Lehner et al., 2010; Wisłowska-Stanek et al., 2011).

Another important point to note is that the neural mechanisms of established chronic tinnitus (i.e. with behavioral signs) are very likely to differ significantly from those in the immediate post-exposure period tested here. Since many individuals report transient tinnitus-like symptoms immediately after high-intensity noise exposure, but not all develop chronic tinnitus, future studies also should aim to further characterize potential similarities and differences in neural mechanisms between chronic and acute tinnitus (Gilles et al., 2012; Degeest et al., 2014; Chen et al., 2015, 2016).

After establishing that the observed changes in Arc resulted from high-intensity sound exposure rather than from the novelty of a new sound (as shown in the present study with intermediate low-intensity sound exposure) we expected to see a strong relation between stress and Arc expression. Auditory information arrives in the basolateral amygdala, then to hippocampus, and to A1; therefore, it can directly activate the hypothalamic-pituitary-adrenal axis if it is perceived as stressful (and would be detected via corticosterone serum levels) (Chavez et al., 2009). Contrary to expectation, we did not see such an effect at this early time frame (45 min post-noise trauma). Singer et al. (2013) also reported changes in Arc expression in amygdala, hippocampus as well as in A1 in a model of noise-induced tinnitus in rats, although these changes were observed at a much later time point (6–30 d post noise-exposure) than the current study observed. They found that changes in Arc were also moderated by stress that occurred for the duration of the 2 d leading up to noise exposure, induced by changing rats' littermates (with more stress causing less Arc expression and moderate stress causing more Arc expression). While it has been hypothesized that noise-trauma, itself, would be a stressor (especially in our rats which were not anesthetized), our data does not bear this out. It may be that the time frame for changes in corticosterone levels would become apparent at a later time point. It may also be possible that the noise trauma paradigm used in this study was enough to induce changes in Arc but not in corticosterone.

5. Conclusions

The aims of this study were fourfold. First, investigate the immediate early effects of acute noise trauma on a well-established excitatory biomarker of neural plasticity (Arc) and on an inhibitory biomarker (GAD 65 + 67) in prominent limbic regions (the amygdala and hippocampus) critically involved in many forms of neural plasticity, which have also exhibited tinnitus-related plasticity in prior studies, and to compare these to the same biomarkers in primary auditory cortex (A1). Second, demonstrate whether the observed significant changes in Arc protein expression were due

specifically to high-intensity noise exposure rather than simply a response to novel ambient noise. Third, assess whether D-cycloserine, an NDMA receptor partial agonist, could prevent any observed changes in protein expression resultant from the high-intensity noise exposure. Finally, determine if stress was a potential contributing factor to changes resulting from acute exposure to traumatic noise. These findings aim to contribute to a better understanding of underlying changes (or lack thereof) involved shortly after acute traumatic noise exposure.

Acute noise trauma has been previously shown to cause tinnitus in rat models (Zheng et al., 2014, 2015; Brozoski et al., 2013; Bauer et al., 2013; Engineer et al., 2011) and is strongly suspected as a cause in humans (Mrena et al., 2002; Nottet et al., 2006; Møller, 2011b). In the present study, acoustic noise trauma produced rapid increases in Arc protein expression within the amygdaloid complex. This enhancement in Arc is indicative of immediate early plasticity occurring within this limbic region. Acute systemic administration of D-cycloserine, an NMDA receptor partial agonist, effectively prevented this plasticity-related up-regulation of Arc protein expression resulting from high-intensity noise exposure. DCS alone (without noise exposure) appears to have functioned as a partial agonist within this region, since DCS alone significantly up-regulated Arc. These mixed agonist/antagonist effects corroborate those of other studies demonstrating DCS's nootropic effects (Donzis and Thompson, 2014; Kaplan and Moore, 2011; Shaw et al., 2009; Thompson and Disterhoft, 1997; Thompson et al., 1992). There were no observed changes in inhibition within the amygdaloid complex 45 min post-noise exposure, as characterized by GAD 65 + 67 protein expression.

Acute noise trauma also significantly increased Arc protein expression within dorsal hippocampus at this immediate early time point. Unlike our results obtained in the amygdala, the effects of noise trauma were not blocked by DCS treatment. When noise trauma was paired with DCS treatment, there was still an up-regulation of Arc expression. These regional differences require further study of the role of glial release of the endogenous ligand, serine, in the initial stages of tinnitus induction and of noise trauma-evoked plasticity. As in the amygdala, there were no observed changes in the inhibitory biomarker, GAD 65 + 67, in hippocampus at this early time point after acute noise trauma.

Contrary to our results from these limbic regions, we found no significant changes in Arc protein expression 45 min post-acute high intensity noise exposure in A1. There was also no rapid alteration in GAD 65 + 67 protein expression in A1.

An additional set of sound-exposure controls were used to strengthen our findings that observed changes in Arc protein expression were due specifically to acute high-intensity sound exposure rather than to exposure of novel sounds in general. Acute low-intensity sound did not produce significant changes in Arc protein expression in amygdala, dorsal hippocampus nor in A1 when compared to controls.

A final aim of this study was to assess the potential of acute noise trauma on a direct measure of stress in rats at the same time point as plasticity in immediate-early gene protein expression was assayed. Elevated corticosterone levels could potentially influence/activate maladaptive pathways. Specifically, it has been shown that maladaptive changes can occur after audiogenic stress (Campeau and Watson, 1997). Given that we found up-regulation of Arc expression resulting from traumatic noise exposure, we also hypothesized that these changes could be mediated by stress; however, we failed to find any relation between changes in Arc expression and changes in circulating corticosterone levels 45 min post-traumatic noise exposure.

Our results substantiate findings of several other studies, supporting the hypothesis that limbic regions, i.e. non-classical auditory brain regions, are involved in immediate early neural plasticity resulting from noise trauma which may play a developmental role in later manifestation of tinnitus (De Ridder et al., 2006; Goble et al., 2009; Vanneste et al., 2010; Chen et al., 2012, 2014), whereas A1, a prominent classical auditory structure, does not appear to utilize these same early mechanisms. Other studies have also reported changes in limbic regions occurring earlier than changes in A1, e.g., Cheng et al. (2016) compared levels of oxidative stress and tau phosphorylation resulting from exposure to moderate environmental noise and found changes occurred much earlier in hippocampus than in A1. Plasticity fostered by amygdalo-hippocampal activation may contribute to the long-term, maladaptive memory formation/stabilization characteristic of chronic tinnitus (De Ridder et al., 2006; Ulanovsky and Moss, 2008; Zhang et al., 2016). Treatments targeting limbic brain regions involved in processing emotional salience and other more subtle aspects of noise-induced plasticity as well as in initial stages of memory formation should continue to be investigated in models of tinnitus.

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