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Tinnitus-inducing noise trauma and D-cycloserine alter amygdalo-hippocampal excitatory biomarkers

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Introduction

Tinnitus, a phantom perception of sound afflicting 1 in 5 people, is a pathology most commonly caused by noise trauma. Patients with chronic tinnitus are difficult to treat, with poor characterization of the mechanisms initiating and maintaining the condition. Our lab demonstrated dorsal hippocampal plasticity in the early stages of tinnitus (Goble et al., 2009), and showed a strong causal linkage between the amygdala and hippocampus in auditory-linked plasticity (Farmer & Thompson, 2012; Donzis & Thompson, 2013). Our current aims were to further characterize the initial plasticity mechanisms leading to this disorder and to determine if D-cycloserine (DCS), a partial NMDAR-agonist, can reduce or prevent this plasticity. Specifically, our study addressed amygdalo-hippocampal involvement in the early stages of tinnitus and looked at DCS as a potential pharmacological intervention for this disorder in an experimental rat model. To assess excitatory and inhibitory signaling in these limbic regions, Arc and GAD 65+67 protein expression were evaluated in male rats (n = 24) after bilateral exposure to acute high-intensity noise (16 kHz, 115 dB, for 1 hr). Western blot analyses and immunohistochemistry confirmed Arc expression, an activity-related IEG product, was significantly up-regulated in both the amygdaloid complex and dorsal hippocampus post-noise trauma. No regionally-specific changes in expression of either form of GAD, the biosynthetic enzyme required for GABAergic inhibition, occurred post-noise trauma. DCS (6 mg/kg, ip, 15 min pre-noise trauma) prevented up-regulation of Arc expression in the amygdala due to noise trauma, thereby functionally acting as a partial antagonist to compete off endogenous serine in this region. However, DCS alone up-regulated Arc expression within the dorsal hippocampus (see also Donzis & Thompson, 2014), and did not reduce Arc expression from noise trauma, suggesting that endogenous hippocampal serine was not at saturating concentrations post-noise. These regional differences require further study of the role glial release of the endogenous ligand, serine, plays in tinnitus-inducing plasticity. Our results corroborate other findings that indicate both hippocampus and amygdala, non-classical auditory nuclei, are involved in early neural plasticity underlying tinnitus.

Amygdalo-Hippocampal Circuitry Involved in Auditory Stimuli

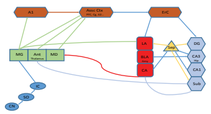


Figure 1. (adapted from Kandratavicius *et al.*, 2012) The medial geniculate nucleus of the thalamus (MG) can follow the classical pathway to primary auditory cortex, or it can branch off and activate limbic pathways more directly, rather than via the entorhinal cortex. These pathways may be stimulated simultaneously with parallel processing occuring between the amygdalo-hippocampal circuits.

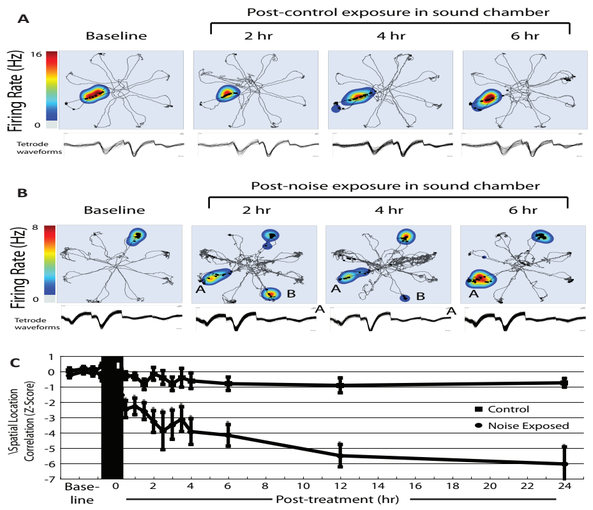


Figure 2. Examples of place-field stability (control) or plasticity (after induction of tinnitus). An overlay map of the rat's path (dark lines) on the maze is displayed along with the single-unit firing positions (black squares) and place fields (colored contours). The firing rate scale of each unit is given to the left, and each unit's tetrode waveform is displayed below each map. **A.** Example from a control rat (no noise exposure) shows a stable place field across several hours. **B.** Example from a rat before (baseline) and after noise exposure. Note the novel hippocampal place field plasticity that occurred as an immediate result of the acute noise trauma. **C.** Tinnitus altered place-field location-specific stability as a function of time after tone exposure (filled circles) and control (filled squares). The normalized spatial location correlation (expressed in Z-scores) compared to baseline is given for units from both control and sound-exposed rats (black bar represents a 30 min treatment condition). Means ± SEM are shown.

Hypotheses

1. After acute noise trauma, excitatory neurons and inhibitory interneurons within the amygdalo-hippocampal circuitry will exhibit rapid plasticity.
2. D-cycloserine (DCS), an NR partial agonist, will reduce tinnitus-related plasticity within the amygdalo-hippocampal circuitry.

Methods

Subjects: All animal uses were approved by the UTD IACUC. Male Long-Evans rats were housed on a 12 hr light/dark cycle in a temperature-controlled room, with food and water available ad libitum.

Behavior and Injections: Rats were handled and acclimated to IP injections daily for 3 wk while maintaining a constant environment to minimize confounds and to assure that observed changes in protein expression were due to noise trauma and DCS effects. IP injections (0.1 ml total volume) of either vehicle or 6 mg/kg DCS were given to the rat 15 min prior to going into a sound-controlled chamber where the rat was either exposed to noise trauma or no noise trauma.

Noise Exposure: Acute noise trauma was induced bilaterally (16 kHz, 115 dB) for 1 hr.

Western Immunoblotting and Analysis for Arc and GAD: Rats were sacrificed 1 hr after time in the sound booth ended, a time point when Arc protein expression is transiently enhanced following brief experimental events (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 and 1.22 mm diameter tissue punches collected from 9 regions identified using a rat atlas (Paxinos & Watson, 1998). Punches were homogenized in a 0.1 M phosphate buffer containing a protease inhibitor cocktail. A Qubit fluorometer and protein assay kit was used to determine the concentration of protein in each sample. 15 µg tissue samples were heated in a sample buffer with reducing agent and loaded into wells and run on an SDS-PAGE gel for ~55 min at 200 V and 200 mA per gel. Gels were transferred onto a nitrocellulose membrane via the iBlot dry blotting system (LifeSciences) for 7 min. The membrane was washed with Tris-buffered saline, and blocked with 5% skim milk for 1-24 hr on a rotator. The membrane was probed overnight with the primary antibodies of interest (Arc, GAD 65+67 and Actin) diluted in 5% skim milk. The membrane was incubated in a secondary HRP-linked antibody for 1 hr followed by an ECL chemiluminescent detection of immunoreactivity. Membranes were placed on films with varying exposure times (3 min for Arc and Actin, 1 min for GAD 65+67), developed, and scanned into a computer for analysis. Actin was used as a loading control for normalizing Arc and GAD 65+67 expression. Western blot densitometry was analyzed using ImageJ 1.46r software, and statistical comparisons were made using Statview 5.0.1 software. A series of planned comparisons were made using a between groups, two-tailed Student's *t*-test to assess how Arc and GAD varied between each of the 4 groups. A probability level of $p < 0.05$ was considered significant.

Immunohistochemistry: Brains were fixed in 4% paraformaldehyde for 10 hr and cryoprotected in 30% sucrose for 72 hr at 4° C. 40 µM tissue sections were blocked in 0.3% Triton X and 3.0% normal donkey serum (Sigma-Aldrich) in 0.1M PBS for 30 min, then incubated overnight with Arg 3.1 primary antibody (1:500, Abcam). Tissue was incubated with the secondary antibody (Donkey anti-Sheep, (DyLight 488) 1:50, Abcam) for 2 hr, then mounted with Fluoroshield Mounting Medium with DAPI, (Abcam), and imaged using an Olympus Fluoview FU1000 confocal microscope and processed in ImageJ 1.46r.

Behavioral Paradigm

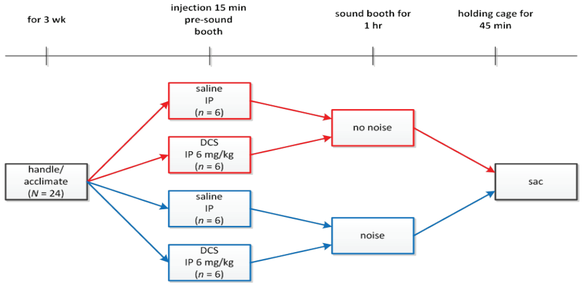
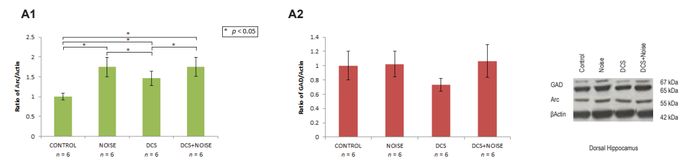


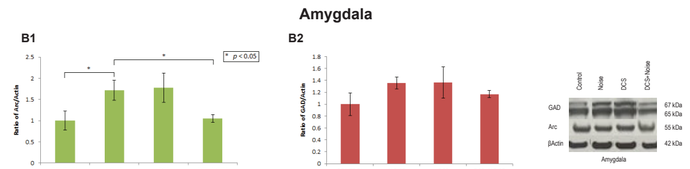
Figure 3. Regions of Interest: Dorsal Hippocampus, Amygdala and Primary Auditory Cortex

Western Immunoblotting Results

Dorsal Hippocampus



Amygdala



Primary Auditory Cortex

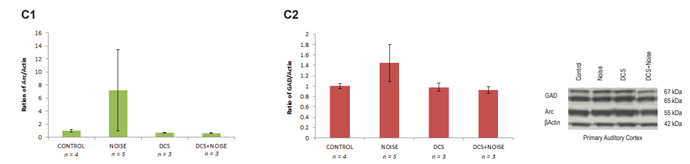


Figure 4 (A1-C2). Evidence of rapid plasticity in Arc and GAD protein expression within the amygdalo-hippocampal circuity. There was considerable regional variability, indicating that a simplistic interpretation (e.g. noise trauma induces rapid plasticity throughout the circuit) is not well supported. DCS altered Arc protein expression within these limbic regions. DCS also altered tinnitus-related plasticity within the amygdaloid complex, a region of the non-classical auditory pathway, indicating it may have value as a potential treatment for tinnitus. Normalized data are presented as means ±SEM.

Immunohistochemistry Results

Dorsal Hippocampus

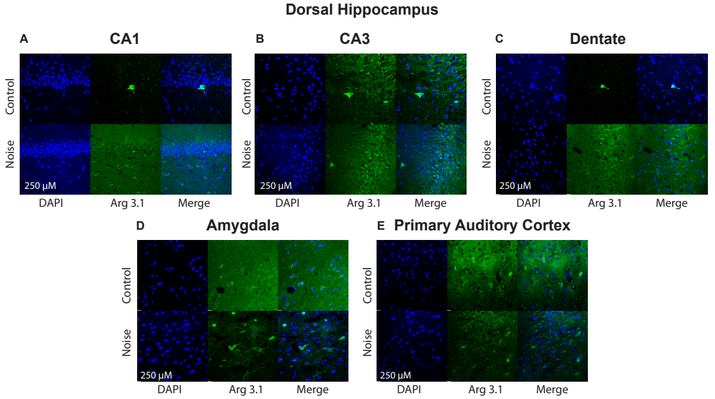


Figure 5 (A-E). Immunohistochemistry analysis of the effect of acute noise trauma on Arg 3.1 protein expression within the dorsal hippocampus, amygdala and primary auditory cortex. Acute noise trauma appears to increase Arg 3.1 immunoreactivity within the CA1 and dentate regions of dorsal hippocampus as well as within the amygdaloid complex. **A**. Control and noise-exposed sections of dorsal CA1 imaged at 60x magnification. **B**. Control and noise-exposed sections of dorsal CA3 imaged at 60x magnification. **C**. Control and noise-exposed sections of dentate imaged at 60x magnification. **D**. Control and noise-exposed sections of the amygdaloid complex imaged at 60x magnification. **E**. Control and noise-exposed sections of primary auditory cortex imaged at 60x magnification.

Conclusions

Effects of Noise Trauma Alone

Dorsal Hippocampus: Acute noise trauma significantly up-regulated Arc protein expression compared to controls. Effects were observed locally within the CA1 and dentate.

Amygdala: Acute noise trauma significantly up-regulated Arc protein expression compared to controls. Effects were observed throughout the amygdaloid complex.

Primary Auditory Cortex: Arc and GAD expression in Primary Auditory Cortex were unaffected.

Effects of Noise Trauma + DCS

Dorsal Hippocampus: DCS significantly down-regulated noise-induced Arc protein expression.

Amygdala: DCS significantly down-regulated noise-induced Arc protein expression.

Primary Auditory Cortex: Arc and GAD expression in Primary Auditory Cortex were unaffected.

Effects of DCS Alone

Dorsal Hippocampus: DCS alone significantly up-regulated Arc protein expression compared to controls.

Amygdala: DCS alone significantly up-regulated Arc protein expression compared to controls.

Future Directions

- Follow plasticity time course throughout the development of tinnitus
- Follow up with immunohistochemistry staining for location-specific expression of Arc immunoreactivity to determine if changes are generalized throughout the amygdaloid complex or specific to a subset of nuclei within this limbic region
- Assess effects of other treatment options

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Acknowledgements

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