Contrasting Changes in Nitric Oxide Synthase Expression in the Auditory Cortex and Inferior Colliculus of Rats in a Tinnitus Model

H.V. Maxwell[1], J. Schofield[2], M. Hamann[2], B.M.J. Olthof[1], N. Pilati[3], C.H. Large[3], M.O. Cunningham[1], S.E. Gartside[1] and A. Rees[1]

[1]Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK; [2]Department of Neuroscience, Psychology and Behaviour, University of Leicester, Leicester, LE1 7RH, UK; [3]Autifony Therapeutics Ltd, Stevenage Bioscience Catalyst, Stevenage, SG1 2FX, UK

Introduction

- Nitric oxide is a signalling molecule that acts within neurons as part of an NMDA-sGC pathway, as well as presynaptically by volume transmission.
- Nitric oxide functions include modulating cell activity, synaptic plasticity and controlling excitotoxicity[1].
- The enzyme neuronal nitric oxide synthase (nNOS) catalyses the production of nitric oxide in the brain.
- In the ventral cochlear nucleus, the number of neurons expressing nNOS increases in animals with tinnitus: this upregulation occurs ipsilateral to the ear subjected to unilateral acoustic over-exposure [2,3].
- We hypothesised that changes in nNOS expression may occur in other auditory centres.
- Here we examined expression of nNOS in the inferior colliculus (IC) and auditory cortex comparing rats with noise induced tinnitus and controls.

Methods

Animals

- Male Long Evans rats were anaesthetized and either acoustically over-exposed ('tinnitus', 16kHz, 115 dB SPL for 2 hours with left ear plugged, n=6), or were not exposed to noise ('controls', n=6).
- Rats were tested for tinnitus with gap pre-pulse inhibition (PPI) of the acoustic startle reflex[4]. Tone PPI was used to exclude animals with hearing loss.

Immunohistochemistry

- Rats were deeply anaesthetized and perfused with PBS and 4% paraformaldehyde.
- Coronal sections (30 µm) were cut through the inferior colliculus and auditory cortex[5] (Fig 1 and 2).
- Sections were labelled for nNOS using a rabbit primary antibody and fluorophore labelled goat anti-rabbit secondary (shown in red). Nuclei were stained with DAPI (blue).
- Labelling was visualized using confocal microscopy.
- Image J, Imaris and MatLab scripts were used to count cells and measure fluorescence intensity in regions of interest.
- Repeated measures, 3 way ANOVA, and post hoc t-tests were used for statistical analyses within (right vs left) and between groups (control vs tinnitus).

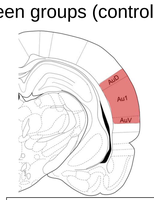


Fig 1: Auditory cortex[5]

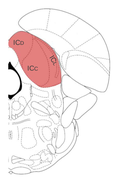


Fig 2: Inferior Colliculus[5]

nNOS in the Auditory Cortex

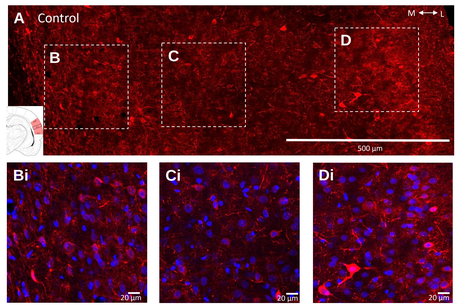


Fig 3: Control - nNOS labelling in auditory cortex (A). nNOS labelling in deep (layers V, VI) (B, Bi), mid (layers III and IV) (C,Ci) and superficial (layer II) (D,Di) regions.

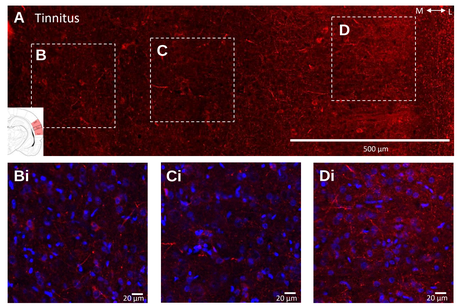
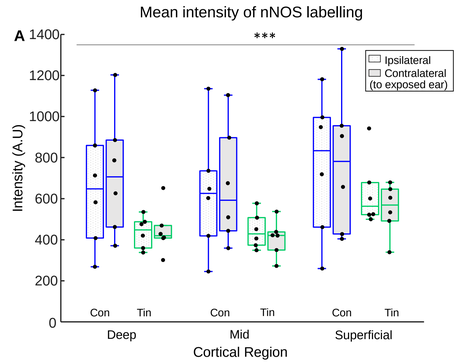


Fig 4: Tinnitus - nNOS labelling in auditory cortex (A). nNOS cells in deep (layers V, VI) (B, Bi), mid (layers III and IV) (C,Ci) and superficial (layer II) (D,Di) cortical regions.

Cortical distribution of nNOS

- nNOS is expressed across the layers of the auditory cortex (Fig. 3A). nNOS positive cells are most densely distributed in superficial (layer II) and deep (layers V, VI) regions (Fig. 3Bi, Di).
- In the mid cortex (layers III, IV), nNOS is mainly expressed in neuropil, with comparatively few nNOS labelled somata (Fig. 3Ci).



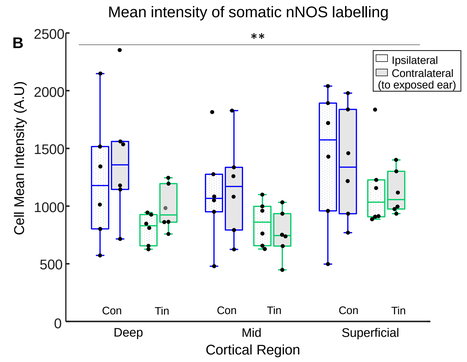


Figure 5: Intensity of nNOS labelling across the auditory cortex (A) Intensity of nNOS in somata (B).

Reduced nNOS expression in tinnitus rats

- The intensity of nNOS labelling was lower in the auditory cortex in tinnitus rats compared to controls (Fig 4A).
- Downregulation of nNOS occurred across superficial (Fig. 4B & Bi), mid (Fig. 4C & Ci) and deep (Fig. 4D & Di) regions.
- Statistical analysis revealed a significant main effect of tinnitus ($F_{(1,60)}$=14.92, p≤.001) (Fig. 4A, 5A).
- nNOS expression in tinnitus rats was significantly lower in both somata ($F_{(1,60)}$=7.04, p≤.01) (Fig. 5B), and neuropil ($F_{(1,60)}$=14.70, p≤.001) (data not shown).
- Although tinnitus rats were unilaterally noise exposed, there was no significant difference in nNOS expression between the cortices ipsilateral and contralateral to the exposed ear (Fig. 5A).

nNOS in the Inferior Colliculus

Distribution of nNOS in the inferior colliculus

- nNOS is densely expressed in the dorsal (IC_D) and lateral (IC_L) cortices of the IC (Fig. 6A). Many cells express nNOS throughout their somata and dendrites (Fig. 6B).
- In the central nucleus (IC_C), few cells express nNOS throughout their cytoplasm, but nNOS occurs in many cells as small puncta in the soma[6] (Fig. 6C) .

Increased nNOS expression in the dorsal cortex in tinnitus rats

- The number of nNOS positive cells in IC_D was significantly higher in tinnitus rats than in controls ($F_{(1,10)}$=12.14, p≤.01), as shown in thresholded images (Fig. 6D,E, 7A). However, there was no significant difference in the intensity of nNOS labelling (Fig. 7B).
- In IC_L, there was no difference in the number of nNOS positive cells between control and tinnitus animals (Fig. 7A).
- There was also no difference in the intensity of nNOS labelling in IC_L, or in IC_C (Fig. 7B).
- Across the IC, there was no difference in nNOS expression ipsilateral and contralateral to the exposed ear.

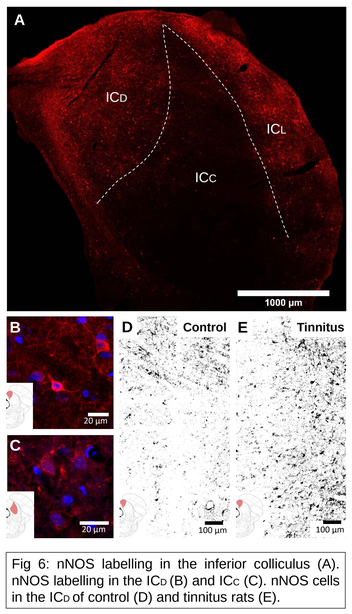


Fig 6: nNOS labelling in the inferior colliculus (A). nNOS labelling in the IC_D (B) and IC_C (C). nNOS cells in the IC_D of control (D) and tinnitus rats (E).

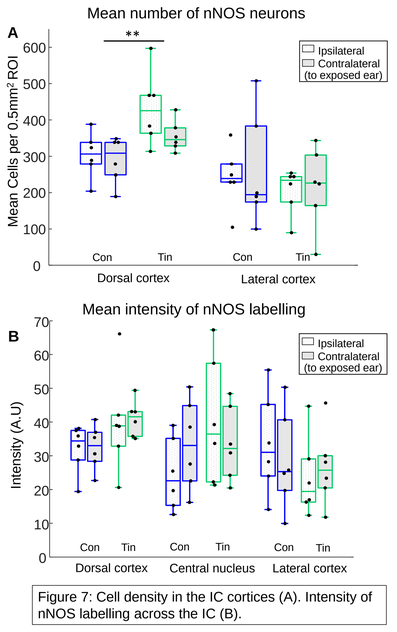


Figure 7: Cell density in the IC cortices (A). Intensity of nNOS labelling across the IC (B).

Conclusion

- We show tinnitus is associated with increased expression of nNOS in the IC and decreased expression in the auditory cortex.
- In contrast to the unilateral nNOS upregulation previously reported in the ventral cochlear nucleus[3], changes in nNOS expression in IC and auditory cortex occurred both ipsi- and contralaterally to the noise-exposed ear.
- These results suggest that nitric oxide plays a role in the pathophysiology of tinnitus. The differential direction of change in nNOS expression between brainstem and cortex suggests nNOS is involved in different mechanisms in these two divisions of the auditory pathway.

References

1. Hardingham, N. et al (2013) *Front Cell Neurosci* **7** 190
2. Zheng, Y. et al. (2006) *Brain Research* **1123** 201-206
3. Coomber, B. et al. (2015) *Front Neurol* **6** 53
4. Turner, J. et al. (2006) *Behav. Neurosci* **120** 188-195
5. Paxinos, G.,Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates (4th Edition)*. Academic Press.
6. Olthof, B. et al. (2019) *J Neurosci* **39** 876-887

Acknowledgments

Supported by a TRIH grant from Action on Hearing Loss. HM is supported by an MRC CASE studentship with Autifony Therapeutics.