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Noise-induced hearing loss: neuropathic pain via Ntrk1 signaling

Senthilvelan Manohar*, Kimberly Dahar, Henry J. Adler, Ding Dalian, and Richard Salvi

Center for Hearing & Deafness, State University of New York at Buffalo, Buffalo, NY 14214, United States of America

Abstract

Severe noise-induced damage to the inner ear leads to auditory nerve fiber degeneration thereby reducing the neural input to the cochlear nucleus (CN). Paradoxically, this leads to a significant increase in spontaneous activity in the CN which has been linked to tinnitus, hyperacusis and ear pain. The biological mechanisms that lead to an increased spontaneous activity are largely unknown, but could arise from changes in glutamatergic or GABAergic neurotransmission or neuroinflammation. To test this hypothesis, we unilaterally exposed rats for 2 h to a 126 dB SPL narrow band noise centered at 12 kHz. Hearing loss measured by auditory brainstem responses exceeded 55 dB from 6 to 32 kHz. The mRNA from the exposed CN was harvested at 14 or 28 days post-exposure and qRT-PCR analysis was performed on 168 genes involved in neural inflammation, neuropathic pain and glutamatergic or GABAergic neurotransmission. Expression levels of mRNA of *Slc17a6* and *Gabrg3*, involved in excitation and inhibition respectively, were significantly increased at 28 days post-exposure, suggesting a possible role in the CN spontaneous hyperactivity associated with tinnitus and hyperacusis. In the pain and inflammatory array, noise exposure up-regulated mRNA expression levels of four pain/inflammatory genes, *Tlr2*, *Oprd1*, *Kcnq3* and *Ntrk1* and decreased mRNA expression levels of two more genes, *Ccl12* and *Il1β*. Pain/inflammatory gene expression changes via Ntrk1 signaling may induce sterile inflammation, neuropathic pain, microglial activation and migration of nerve fibers from the trigeminal nerve and cuneate and vestibular nuclei into the CN. These changes could contribute to somatic tinnitus, hyperacusis and otalgia.

Keywords

Cochlear Nucleus; GABA; Glutamate; Neuropathic Pain; Sterile inflammation; Gene array; Noise exposure

*Send correspondence to: Senthilvelan Manohar Tel: +1-716-829-5310; Fax: +1-716-829-2980; smanohar@buffalo.edu.

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Introduction

Noise-induced damage to inner hair cells leads to auditory nerve fiber degeneration (Kraus et al., 2011; Salvi et al., 1978; Salvi et al., 1979; Salvi et al., 2000), thereby reducing the neural activity flowing from the auditory nerve into CN. Paradoxically, the reduced neuronal input causes a significant increase in spontaneous neuronal activity in the CN beginning 4-7 days after cochlear damage. This increase continues for 3-4 weeks (Coomber et al., 2014; Imig and Durham, 2005; Kaltenbach and McCaslin, 1996; Kaltenbach et al., 2000). Spontaneous hyperactivity in the CN has been considered as a putative mechanism for tinnitus (Dong et al., 2009; Kaltenbach and McCaslin, 1996; Kaltenbach et al., 2000) as well as a physiological manifestation of enhanced central gain.

The biological mechanisms that result in spontaneous hyperactivity and sound-evoked hyperactivity are poorly understood, but could arise from increased glutamatergic or decreased γ -aminobutyric acid (GABA) neurotransmission (Milbrandt et al., 2000; Wang and Manis, 2005). The release and uptake of glutamatergic and GABA neurotransmitters are altered in the CN following noise exposure (Muly et al., 2004; Suneja et al., 1998). Noise exposure also modulates expression of GABA receptors in the CN (Kou et al., 2013) as well as expression of genes involved in glutamatergic or GABAergic neurotransmission (e.g., NMDA receptor subunit 2A, GABA_A receptor alpha 1 subunit and GAD67) in the auditory brainstem (Browne et al., 2012; Dong et al., 2009). Previous studies show significant up-regulation of the glutamate transporter *Slc17a6* in the CN as a result of cochlear and auditory nerve degeneration. Since *Slc17a6* mRNA is expressed in trigeminal axons, the increased labeling in the CN is suggestive of axonal sprouting from the trigeminal nerve (Brozoski et al., 2002; Dehmel et al., 2012; Kaltenbach and McCaslin, 1996; Zeng et al., 2009; Zeng et al., 2012; Zhang and Kaltenbach, 1998; Zhou et al., 2007).

Tinnitus (Levine, 1999; Lockwood et al., 1998; Pinchoff et al., 1998; Simmons et al., 2008), like phantom limb pain, is thought to arise from aberrant connections among neurons in the CN and the trigeminal, cervical and/or facial nerves (Bartels et al., 2007; Dehmel et al., 2012; Stolzberg et al., 2012; Thill et al., 2012; Vass et al., 1997; Zeng et al., 2012). Intense sounds are not only perceived as painfully loud (Ades et al., 1959), but can also induce pain sensations in around the external ear (otalgia; Dominguez et al., 2006; Hebert et al., 2013; Henry et al., 2014; Kaltenbach et al., 2000; Knipper et al., 2013; McFerran and Baguley, 2007; Norena, 2011; Pienkowski et al., 2014; Tyler et al., 2014a; Van Campen et al., 1999; Westcott et al., 2013). Pain transmitting neuropeptides and receptors are present in the CN and auditory nerve (Aguilar et al., 2004; Bauer et al., 2007b; Jongkamonwiwat et al., 2003; Nguyen et al., 2014; Phansuwan-Pujito et al., 2003; Tongjaroenbuangam et al., 2006), raising the possibility that loud sounds may trigger the perception of pain through these signaling pathways.

Acoustic overstimulation and ototoxic drugs lead to the degeneration of auditory nerve fibers and synaptic losses in the CN (Kim et al., 2004; Kraus et al., 2013; Morest and Bohne, 1983). Auditory nerve fiber degeneration is strongly correlated with chronic tinnitus (Bauer et al., 2007a) and hyperacusis (Hickox and Liberman, 2014), suggesting that auditory nerve fiber degeneration may trigger spontaneous hyperactivity in the CN. Nerve fiber

degeneration elicits a chronic neuroimmune response characterized by activation of microglia, resident macrophages in the central nervous system (Amor et al., 2010; Baizer et al., 2015). Auditory nerve fiber degeneration leads to neuroinflammation in the CN as reflected by microglial activation (Campos Torres et al., 1999; Janz and Illing, 2014) and the up-regulation of proinflammatory cytokines such as *IL1 β* (Fuentes-Santamaria et al., 2013). Chronic treatment with high-dose sodium salicylate, a drug that reliably induces tinnitus, increases the expression of TNF- α in the CN (Hu et al., 2014b), a potent cytokine that elicits mechanical hyperalgesia (Sorkin and Doom, 2000). Similarly, vestibular deafferentation upregulates TNF- α in brainstem vestibular nuclei (Liberge et al., 2010).

Collectively, these studies suggest that noise-induced tinnitus and spontaneous hyperactivity in the CN may involve dysregulated expression of genes associated with pain and inflammation. To test this hypothesis, we unilaterally exposed rats to a high intensity noise that induced significant hearing loss, hair cell damage and auditory nerve fiber degeneration in the exposed ear (Baizer et al., 2015). Gene expression changes were then evaluated at both 14 and 28 days post-exposure in a large panel of glutamate and GABAergic and genes associated with inflammation and pain.

Experimental Procedures

Animals

Adult SASCO Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) with a mean body weight of 325 ± 50 gm were used for this study. Nine rats were used for RT² Profiler™ PCR Arrays, and 18 were used for immunohistochemistry and Western blots to verify the presence of proteins encoded by genes whose expression changed significantly. All experimental protocols used in this study were reviewed and approved by The University at Buffalo Institutional Animal Care and Use Committee.

Noise exposure

Acoustic trauma conditions used for this study cause extensive loss of outer hair cells (OHC), inner hair cells (IHC) and auditory nerve fibers, as described elsewhere (Baizer et al., 2015; Kraus et al., 2011; Kraus et al., 2010). Rats were anesthetized with isoflurane during noise exposure (4% induction, 1.5% maintenance). The left ear was positioned 10 mm from the mouth of a super compression driver (D-59; GMI Sound Corp., NY, USA) and exposed to a narrow band (100 Hz bandwidth) noise centered at 12 kHz for 2 h at 126 decibels sound pressure level (dB SPL). The right ear was plugged to prevent noise damage and to maintain normal hearing (Kraus et al., 2011; Kraus et al., 2010). The noise was generated by Tucker-Davis Technology (TDT) hardware and delivered to a power amplifier followed by a loudspeaker. The SPL was calibrated with a sound level meter coupled to a half-inch condenser microphone (Model 824, Larson Davis). Rats were allowed to survive for 2, 14 or 28 days following the noise exposure. Unexposed sham control rats underwent the identical procedures without noise exposure.

Hearing Assessment

Hearing loss was assessed using auditory brainstem response (ABR) testing. Rats were anesthetized intraperitoneally with a combination of ketamine (100 mg/kg) and xylazine (20 mg/kg). Body temperature was maintained at 37 °C with a heating pad during ABR recordings. ABR measurements were made with subdermal needle electrodes; the non-inverting electrode was inserted at the vertex, the inverting electrode was inserted beneath the ipsilateral pinna and the ground electrode inserted in the dorsal part of the hindlimb. ABR recordings were conducted in a sound attenuating booth. To ensure that the noise exposure caused significant hearing loss in the left (exposed) ear, but not the right (plugged) ear, the speaker was positioned 20 cm directly in front of the left ear, and the opposite ear was occluded with an ear plug; hearing sensitivity in the right ear was estimated by reversing the left-right conditions (Note: the maximal interaural sound attenuation obtained with ear plugging is approximately 40 dB). Stimuli were synthesized and generated by Tucker-Davis Technologies (TDT) hardware and software (BioSigRP) and delivered to a loudspeaker (FT28D, Fostex) as previously described, (Chen et al., 2014). Biological signals from the electrodes were routed to a TDT Headstage-4 amplifier (5020X) and filtered from 100-3000 Hz. ABRs were elicited with tone pips (0.5 ms rise/fall; 1 ms duration; 6, 12, 20, and 32 kHz, without plateau) presented 400 times at 11.1/s. ABRs were obtained at each frequency by reducing the SPL in 10 dB steps from a maximum of 90 dB to 10 dB below the intensity at which the ABR response disappeared. Threshold was defined as the lowest level at which an ABR could be recognized.

Total RNA isolation and cDNA construction

Total RNA was isolated from the ipsilateral CN (Friedland et al., 2006) in an RNase free environment using the RNeasy lipid tissue extraction kit (QIAGEN, Valencia, CA) as previously described (Manohar et al., 2014). CN tissue samples used for RT² gene profiling were obtained from the right CN of exposed rats and right CN of sham control rats. The concentration and purity of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was constructed from 500 ng of RNA using a RT² first strand cDNA kit (SABiosciences Corporation, Valencia, CA).

RT² Profiler PCR Arrays

Nine rats were used for mRNA expression analysis. Animals were divided into three groups (n=3/group), a sham control group, a group sacrificed at 14 days post-exposure and another group sacrificed at 28 days post-exposure. The mRNA harvested from these groups was evaluated with two different RT² Profiler gene expression arrays, a GABA and Glutamate RT² Profiler array containing 84 genes involved in glutamatergic and GABAergic synaptic transmission (GABA & Glutamate RT² Profiler PARN-152Z PCR Array; QIAGEN, Valencia, CA) and an RT² Profiler array with 84 genes involved in neuropathic pain and inflammation (RT² Profiler PARN-162Z; QIAGEN). Both arrays had primers for five housekeeping genes (*Actb*, *B2m*, *Hprt1*, *Ldha* and *Rplp1*) to facilitate normalization, a genomic DNA primer to detect genomic DNA contamination, three reverse transcription controls, and three positive PCR controls.

Using the RT qPCR Master Mix (SA Biosciences), real-time PCR was performed with a two-step cycling program with a hot start to activate the DNA polymerase at 95 °C for 10 minutes, followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). PCR was carried out using a Bio-Rad CFX Connect Real Time PCR System which measured the cycle threshold (CT) values. Noise-induced changes in mRNA expression levels in the CN were calculated using the QIAGEN online data analysis resource by uploading raw Ct values (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Fold changes with a p-value less than 0.05 were considered as statistically significant (Hu et al., 2009; Manohar et al., 2014).

The p values were calculated based on a Student's t-test of the replicate $2^{(-\Delta \text{Ct})}$ values for each gene in the sham group versus the same gene in the 14 or 28 days noise-exposed groups. Distributions showing the differential expression of genes from the focused gene array between the groups with replicated data points [Log2 (Fold-Change) on the X-axis were plotted against Log10 (p-Value) on the Y-axis]. Eighty percent of the genes that showed significant changes ($p < 0.05$) in mRNA expression were further validated using immunofluorescence and immunoblotting.

Immunolabeling

Since noise exposure greatly increased *Gabrg3* mRNA expression levels in the CN in the present gene array study, we performed immunolabeling studies with antibodies that recognized the proteins coded by these genes in order to determine the location of Gabrg3 protein as well as to co-localize Gabrg3 with Slc17a8 proteins in CN. Three sham control rats and three noise-exposed rats were used for immunolabeling; the rats were transcardially perfused 28 days post-exposure with saline followed by 10% formalin as described previously (Manohar et al., 2012). Brains were removed and post-fixed in 10% formalin for 48 h, cryoprotected with 30% sucrose in Tris-buffered saline (TBS) for 24 h and sectioned every 40 μm on a cryostat as described previously (Manohar et al., 2012). Sections were incubated in a blocking solution [TBS, 1% normal horse serum, 1% bovine serum albumin (BSA) and 0.1% Triton-X100 (T-X)] for 30 min.

The sections were split into two groups, one of which was incubated in 1:100 rabbit polyclonal anti-Gabrg3 (cat no. AP4800a; Abgent, Inc. USA). The other group was subjected to labeling in the same rabbit anti-Gabrg3 antibody and 1:200 guinea pig polyclonal anti-Vglut3 antibodies (cat no. AB5421, EMD Millipore, USA). Primary antibodies were incubated overnight at 4 °C followed by three rinses in 0.1% T-X TBS. The sections were further incubated with biotinylated anti-rabbit secondary antibody at room temperature for 2 h (Vector laboratories, USA), followed by avidin-biotin-peroxidase complex for the first group or a combination of donkey anti-rabbit Alexa Fluor 594 (1:1000) and donkey anti-guinea pig Alexa Fluor 488 (1:1000) to visualize for the second group (Alexa Fluor antibodies purchased from Life Technologies, USA). Secondary antibody combinations were diluted in TBS with 1% normal horse serum, 1% BSA and 0.1% T-X. For the first group, immunoreactivity was visualized using the glucose oxidase-diaminobenzidine (Sigma-Aldrich) method. Then sections were rinsed and mounted on Fisher "Superfrost" polarized slides (Fisher Scientific), allowed to dry and coverslipped with

DePex mounting medium (Manohar et al., 2012) and photographed using a SPOT digital camera (SPOT Insight; Diagnostic Instruments, Inc). For the second group of double staining, sections were mounted with Prolong Antifade mounting medium (Invitrogen, Carlsbad, CA) and examined with a Carl Zeiss Laser Scanning Systems LSM 510/Zeiss Axio confocal Microscope.

Immunoblot analysis

Noise-exposed rats or sham controls were allowed to survive for 2, 14, and 28 days to determine if the changes in mRNA levels observed in the gene arrays were correlated with a similar change in levels of protein encoded by its respective gene. A 2 day survival time was added to test for earlier changes in Ntrk1, Slc17a6 and Kcnq3 protein expression in addition to the 14 and 28 day survival times used for gene profiling. Cochlear nuclei were dissected and homogenized in RIPA buffer (Thermo Scientific). The homogenates were centrifuged at 12,000 rpm for 20 min and total protein concentration of the supernatant was measured using the Bradford method. The extracted protein samples (10 µg) were separated on a 4–12% gradient NuPage gel (Life Technologies, USA), transferred to a polyvinylidene difluoride membrane (Life Technologies, USA), and blocked with 5% dry non-fat milk at room temperature for 1 hr. The membrane was incubated overnight with 1:200 rabbit anti-Ntrk1 (Alomone Labs, Jerusalem, Israel), 1:5000 mouse anti-Slc17a6 (cat no. MAB5504; EMD Millipore, USA), 1:1000 rabbit polyclonal anti-Kcnq3 (cat no. NBP1-46666; Novus Biologicals, USA) or 1:5000 rabbit anti-actin (cat no; MAB1501; EMD Millipore, USA.) at 4 °C, followed by a 2-hr incubation with appropriate hydrogen peroxidase conjugated secondary antibody (Pierce Chemical Co., Rockford, IL). Protein bands were visualized using the chemiluminescence detection method (Pierce Chemical Co.) and samples were evaluated using a Fuji model LAS 1000 imaging system (Fujifilm Medical Systems USA, Stamford, CT). The membrane was then stripped and re-probed with an antibody against actin to facilitate normalization. ImageJ software (NIH, Bethesda, MD) was used for densitometric analysis (Manohar et al., 2014) and one-way analysis of variance was used to determine the significance of differences in protein expression between the groups. Tukey's multiple comparison post-test was carried to determine the significance of difference between the recovery periods.

Results

Hearing Loss

ABR thresholds were measured in both ears of the noise-exposed rats and the sham control rats at 6, 12, 20 and 32 kHz. In the rats exposed to narrow band noise ABR thresholds were ranged from 90-100 dB SPL in the exposed ear across all frequencies tested at 14 and 28 days post-exposure whereas thresholds in the plugged ear ranged from 38-42 dB SPL (Fig. 1). Mean thresholds in the left and right ears of the unexposed, sham control ears were approximately 40 dB SPL. While these results indicate that the thresholds in the exposed ear were 50-55 dB higher than in the unexposed ear, the actual hearing loss is likely substantially higher because of bone-conducted cross-talk to the intact ear. This interpretation is consistent with our previous studies in which we observed massive hair cell

loss throughout most of the cochlea after the 126 dB exposure (Baizer et al., 2015; Kraus et al., 2011).

Gene Expression Profiles

For the purpose of documentation and to aid future investigations, Tables 1 and 2 show the relative abundance of the mRNAs associated with the 84 genes in the GABA/Glutamatergic array and the 84 genes (3 not detected) in the Neuropathic Pain/Inflammatory array respectively (gene abundance normalized to the highly abundant actin housekeeping gene). The five most abundant genes (bold text) in the GABAergic and glutamatergic gene array were *Glul*, *App*, *Slc1a2*, *Gabbr1* and *Itp1*. In the neuropathic pain and inflammatory gene array the five most abundant genes (bold text) were *Ptges3*, *Mapk1*, *Mapk3*, *Dbh* and *Adora1*.

Noise-induced changes in GABAergic and Glutamatergic Genes

Table 3 and Figure 2A-B showed the mean (n=4 replicates) GABAergic and glycinergic gene expression changes in the CN measured at 14 and 28 days post-exposure. The volcano plots in Figure 2A presented the GABAergic/glutamatergic gene expression changes at 14 days post-exposure. Two genes in the noise-exposed rats increased expression by more than >50% (> 0.5 fold up-regulation, red dots) and 18 other genes decreased expression by > 50% (> -0.5 fold down-regulation, blue dots) relative to those genes in the CN of sham controls (Fig. 2A). However, none of these noise-induced expression changes were significantly different from those in the controls at 14 days post-exposure (Fig. 2A). At 28 days post-exposure, the expression of eight genes increased by > 50% (i.e., > 0.5 fold) whereas expression of 18 genes decreased by >50% (> -0.5 fold) (Fig. 2B). Of the changes, only two genes [*Slc17a6* (also known as Vglut2, ~60%, ~0.6 fold) and *Gabrg3* (~150% increase, 1.5 fold)] demonstrated a statistically significant increase relative to controls (p < 0.05, Fig. 2B).

Noise-induced changes in Neuropathic Pain and Inflammatory Genes

Table 4 and Figure 2C-D showed the mean (n=4 replicates) neuropathic pain/ and inflammatory gene expression changes in the CN measured at 14 and 28 days post-exposure. Noise exposure caused statistically significant changes in mRNA expression levels of neuropathic pain and inflammatory genes in the exposed CN. Three significant changes occurred at 14 days post-exposure (Figure 2D) while four occurred at 28 post-exposure (Figure 2D). mRNA expression levels for the G protein-coupled δ -opioid receptor (*Oprd1*) and toll-like receptor 2 (*Tlr2*) exhibited statistically significant upregulation of >50% (>1.5 fold) at 14 days post-exposure (Fig. 2C). Genes associated with neuropathic pain [e.g., neurotrophic tyrosine kinase, receptor type 1 (*Ntrk1*); potassium voltage-gated channel, KQT-like subfamily, member 3 (*Kcnq3*)] presented a statistically significant increase of >50% (>1.5 fold) at 28 days post-exposure (Fig. 2D). Down-regulation of mRNA expression levels occurred in several inflammatory genes at 14 and 28 days post-exposure. Chemokine (C-C) ligand 12 (*Ccl12*) exhibited a statistically significant decrease of ~60% (-0.6 fold) at 14 days post-exposure and roughly a 100% decrease (-1.0 fold) at 28 days post-exposure (Fig. 2C, D). mRNA expression levels of interleukin 1 β (*Il1 β*) showed a statistically significant decrease of 130% (-1.3 fold) at 28 days post-exposure (Fig. 2D).

Immunoblot analysis

We targeted genes that exhibited upregulation at a longer recovery period (i.e., 28 days post exposure) in order to further determine permanent changes in their protein expression. Protein expression levels of Ntrk1/TrkA, Slc17a6 (also known as vesicular transporter protein 2, Vglut2), Gabrg3 and Kcnq3 were analyzed by Western blotting. Tissue samples were harvested at 2, 14 and 28 days post-exposure. Ntrk1 (Fig. 3A), Kcnq3 (Fig. 3C), and Gabrg3 (Fig. 3D) were significantly down-regulated in the noise-exposed CN at all recovery durations compared to control CN tissues ($p < 0.001$). Protein expression levels of Ntrk1 and Kcnq3 at 28 days post-exposure was significantly recovered from 2 days post-exposure ($p < 0.05$; Fig. 3A, C). Slc17a6 (Vglut2) protein expression levels demonstrated an increase in the CN at all three post-exposure time points compared to sham controls (Fig. 3B). The largest increase in protein occurred at 14 days post-exposure ($p < 0.05$); this was followed by a slight reduction at 28 days post-exposure compared to that at 2 and 14 days post-exposure (Fig. 3B). Protein expression levels of Slc17a6 at 2, 14 and 28 days post-exposure were not significantly different from one another.

Immunolabeling

A recent population study on DNA polymorphisms has identified variation in GABRG3 DNA sequence as a quantitative auditory trait in normal hearing subjects (Giroto et al., 2011). A subsequent study reported strong Gabrg3 immunoreactive expression in inner hair cells in the normal mouse organ of Corti (Giroto et al., 2014). In support of this previous study (Giroto et al., 2014), noise exposure greatly increased *Gabrg3* mRNA expression levels in the CN. Here, we performed immunohistochemistry to localize and identify the cells that expressed Gabrg3 protein in the cochlear nuclei.

Figure 4 displayed the immunolabeling of Gabrg3 (gamma-aminobutyric acid (GABA) A receptor, gamma 3) positive cells in ventral cochlear nucleus (VCN) at 28 days post-exposure. In sham controls, Gabrg3 labeling was present at the periphery of or in the soma of VCN neurons (Figure 4A, arrows). At a higher magnification, Gabrg3 appeared as puncta surrounding the soma of numerous neurons (Figure 4C). At 28 days post-exposure, Gabrg3 labeling in the noise-exposed VCN was slightly intense at the periphery of soma (Figure 4B) and was present as dark puncta in the soma and to a lesser extent the surrounding neuropils (Figure 4D) consistent with previous findings (Fyk-Kolodziej et al., 2011).

Figure 5 demonstrated fluorescent co-labeling of Gabrg3 (red) with Slc17a8 (vesicular glutamate transporter 3, Vglut3, green) in the VCN of sham controls (Figure 5A-C) and in the VCN at 28 days post-exposure (Figure 5D-F). Even though three isoforms of glutamate transporters exist, Vglut3 was targeted because of its role in hearing and pain (Akil et al., 2012; Flores et al., 2015; Ruel et al., 2008; Seal et al., 2009). Here, immunofluorescence was performed to determine whether or not Vglut3 positive neurons would also be Gabrg3 positive. In controls, Slc17a8 (Fig. 5A) and Gabrg3 (Fig. 5B) immunolabels were prevalent throughout the VCN. Slc17a8 labeling was heavily expressed in the soma and lightly expressed in the surrounding neuropils whereas Gabrg3 labeling yielded granular rings or puncta surrounding the soma consistent with previous findings (Fyk-Kolodziej et al., 2011; Schafer et al., 2002). The merged image exhibited extensive Gabrg3 labeling surrounding

the soma of Slc17a8 positive cells (Fig. 5C, insert) as well as Gabrg3 puncta in the surrounding neuropil (Fig. 5C). In the 28 day post-exposure VCN, the patterns of both Slc17a8 and Gabrg3 immunolabeling were similar to those in controls (Slc17a8 – Fig. 5A vs. 5D; Gabrg3 – Fig. 5B vs. 5E). In Fig. 5C (control) and 5F (28 days post-exposure), Gabrg3 labeling (red) was evident at the periphery of Slc17a8 (green) positive neurons. Surrounding neuropils showed an overlap of Slc17a8 and Gabrg3 (Fig. 5F, insert) in VCN. These observations suggest two things. First, Gabrg3 protein was expressed not only in sensory hair cells of the undamaged organ of Corti (Giroto et al., 2014) but also in VCN. Second, noise exposure appeared to alter Gabrg3 protein expression in the whole cochlear nucleus (Fig. 3D) as well as Gabrg3 translocalization in VCN (compare Fig. 5F with 5C).

Discussion

High intensity noise causes degeneration of auditory nerve fibers and extensive synaptic modifications in the CN (Baizer et al., 2015; Kim et al., 2004; Kraus et al., 2013; Lin et al., 2011; Morest and Bohne, 1983) including modification of synaptic inputs from non-auditory areas associated with multisensory integration (Dehmel et al., 2012; Levine, 1999; Zhou et al., 2007). Despite the loss of auditory input, noise-induced hearing loss leads to a significant increase in spontaneous activity in the CN. These electrophysiological and neuroanatomical changes have been linked to tinnitus as well as hyperacusis and ear pain (Bauer et al., 2007a; Dong et al., 2009; Flores et al., 2015; Hickox and Liberman, 2014; Kaltenbach et al., 2000). The biological mechanisms that give rise to spontaneous hyperactivity and these perceptual disturbances remain poorly understood. To elucidate the biological mechanisms underlying these functional changes, we exposed rats to an intense noise that caused significant hearing loss and extensive auditory nerve fiber degeneration (Baizer et al., 2015) and then observed changes in gene expression in the CN within the GABAergic/glutamatergic signaling pathway that might contribute to noise-induced spontaneous hyperactivity as well as changes in gene expression in neuropathic pain and inflammation pathways that could contribute to hyperacusis and/or otalgia.

The 126 dB SPL noise produced significant hearing loss (~50-60 dB) over a broad range of frequencies (Fig. 1; see also (Kraus et al., 2011; Kraus et al., 2010)), consistent with our earlier studies, showing massive hair cell loss throughout the cochlea, extensive loss of auditory nerve fibers and increased expression of microglia in the CN (Baizer et al., 2015). Despite these profound electrophysiological and neuroanatomical changes, the gene expression changes in GABAergic/glutamatergic and neuropathic pain/inflammatory signaling pathway were restricted to eight of 168 genes examined. Only six genes showed significantly enhanced mRNA expression and the other two yielded lower expression levels at 14 or 28 day post-exposure. Among the 84 genes in the GABAergic/glutamatergic array, only *Gabrg3* and *Slc17a6* genes were significantly up-regulated at 28 days post-exposure; none of the other genes showed any significant change in expression at 14 or 28 days post-exposure.

Among the 84 genes in the neuropathic pain/inflammatory array, two genes, *Tlr2* and *Oprd1*, were significantly upregulated at 14 days post-exposure while two additional genes, *Kcnq3* and *Ntrk1*, significantly increased their expression at 28 days post-exposure. Gene

expression of *Ccl12* was significantly reduced at both 14 and 28 days post-exposure whereas only *Il1 β* gene expression was significantly depressed at 28 days post-exposure. *Ntrk1*, *Kcnq3* and *Oprd1* play important roles in neuropathic pain (Benbouzid et al., 2008; Dondio et al., 2001; Dost et al., 2004; Fritch et al., 2010; Ma et al., 2010; Shinoda et al., 2007; Ugolini et al., 2007) while *Ccl12*, *Tlr2*, and *Il1 β* are involved in inflammation (Gundra et al., 2011; Mojsilovic-Petrovic et al., 2007; Niven et al., 2015; Oh et al., 2012; Yang et al., 2009). *Ntrk1* also may be a part of a negative feedback loop linking neuropathic pain and inflammation.

Slc17a6

Slc17a6 codes for a membrane transport protein, VGLUT2 that carries glutamate into synaptic vesicles. VGLUT2 is expressed on glutamatergic somatosensory neurons (Zeng et al., 2012; Zhou et al., 2007) and auditory neurons in the superior olivary complex, inferior colliculus and cochlear nucleus (Altschuler et al., 2008; Blaesse et al., 2005; Toyoshima et al., 2009). We found that *Slc17a6* mRNA was significantly up-regulated in the CN 28 days post-exposure (Fig. 2-3) and Slc17a6 protein expression was significantly upregulated at 2, 14 and 28 days post-exposure (Figure 3B), suggesting a compensatory role of Slc17a6 following noise-induced deafferentation. Our results are consistent with prior studies showing that cochlear damage (Barker et al., 2012; Zeng et al., 2009; Zeng et al., 2012) increases the expression of VGLUT2 in the CN; this increase is associated with putative new inputs from the lateral vestibular nuclei, spinal trigeminal nucleus and cuneate nucleus. The putative increase in glutamatergic input from non-auditory regions could be a major factor leading to spontaneous hyperactivity in the CN. Spontaneous hyperactivity has been proposed as a neural correlate of tinnitus and the modulation of spontaneous activity in the CN by trigeminal nerve stimulation provides a mechanism through which oral-facial movements can modulate the pitch and loudness of tinnitus (Dehmel et al., 2012; Levine, 1999; Shore et al., 2000; Simmons et al., 2008; Zeng et al., 2009).

Gabrg3 and Slc17a8 (VGLUT3)

Gabrg3 encodes a subunit of the GABA_A receptor for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). When GABA binds to its receptor, it increases the influx of chloride ions resulting in neuronal hyperpolarization and inhibition of neural activity (Watanabe et al., 2002). *Gabrg3* mRNA expression levels increased significantly in the CN at 28 days post-exposure. This increase in mRNA was associated with more widespread immunolabeling of Gabrg3 in VCN (Fig. 2, 5). However, Gabrg3 protein expression via immunoblotting in the whole cochlear nucleus decreased at 2, 14, and 28 days post-exposure (Fig. 3D). This decrease in protein expression could induce a rapid recruitment of intercellular components to the plasma membrane of the noise-affected neurons via translocation, and this in turn could trigger *Gabrg3* gene transcription (Luscher et al., 2011; Wan et al., 1997).

Gabrg3 was highly expressed at the periphery of putative glutamatergic neurons (Fig. 5F, insert) whose soma were intensely immunolabeled with Slc17a8. Qualitatively there was little change in the pattern and intensity of Slc17a8 immunolabeling at 28 days post-exposure consistent with earlier findings (Fyk-Kolodziej et al., 2011). On the other hand,

Gabrg3 immunoreactivity has been recently reported in the mouse inner ear. Inner hair cells were strongly Gabrg3 immunoreactive compared to outer hair cells (Giroto et al., 2014). Here, we showed Gabrg3 expression in Vglut3 positive neurons in VCN. This suggests that the function of Vglut3 positive neurons in VCN might be strongly modulated by Gabrg3 or vice versa. However, the increase in Gabrg3 inhibitory receptor immunolabeling on putative glutamatergic-excitatory neurons expressing Slc17a6/ Slc17a8 could be interpreted as an effort to compensate for the loss of inhibitory glycinergic activity following deafening (Asako et al., 2005) and/or an attempt to suppress spontaneous or sound-evoked hyperactivity in the CN following acoustic trauma (Kaltenbach and McCaslin, 1996; Kaltenbach et al., 2000; Zhang and Kaltenbach, 1998).

Ntrk1

The high-affinity nerve growth factor (NGF) binding receptor *Ntrk1* (also known as TrkA) is a member of a Trk sub-family of protein kinases which includes TrkB and TrkC. *Ntrk1* gene expression was significantly upregulated at 28 days post-exposure, but the functional significance of this is unclear. *Ntrk1* autophosphorylates after binding NGF; this activates the MAPK pathway, which promotes differentiation in developing and regenerating neurons (Sen et al., 2013). Since the binding of NGF to *Ntrk1* promotes neurite and axonal outgrowth (Arevalo and Wu, 2006; Reichardt, 2006), increased expression of *Ntrk1* may help to promote neuroplasticity and re-myelination in the CN (Richner et al., 2014). NGF levels are elevated in inflamed tissues and this is accompanied by an increase in spontaneous activity in nociceptive neurons (Koltzenburg et al., 1999). This raises the possibility that NGF and/or *Ntrk1* promote spontaneous hyperactivity in the CN. NGF is expressed at high levels in damaged and inflamed tissue, but after it binds to TrkA, it reduces inflammation and neuropathic pain by interfering with toll-like receptor activity (Prencipe et al., 2014; Ugolini et al., 2007). Interestingly, *Tlr2* mRNA expression was significantly upregulated at 14 days post-exposure, but not at 28 day post-exposure when *Ntrk1* mRNA expression was upregulated. These results suggest that the failure to upregulate *Ntrk1* following noise-induced damage and/or the loss of regulation on toll-like receptor could conceivably cause moderately intense sounds to be perceived as painful loud (Ades et al., 1959; Tyler et al., 2014b).

Unexpectedly, *Ntrk1* mRNA and *Ntrk1* protein showed opposite patterns of expression. *Ntrk1* mRNA expression levels were significantly upregulated in the CN 28 days post-exposure (Fig. 2), yet immunoblot analysis revealed lower protein expression levels of *Ntrk1* between 2 and 28 days post-exposure (Fig. 3). One potential explanation for this discrepancy is that the *Ntrk1* protein is rapidly degraded due to the degeneration of auditory nerve fibers innervating the CN (Baizer et al., 2015; Kraus et al., 2011). The recovery of *Ntrk1* protein levels at 28 days post-exposure (Fig. 3A) is consistent with the upsurge of *Ntrk1* mRNA at this time suggesting that *Ntrk1* and/or NGF protein expression could increase further with an extended recovery period. Interestingly, increased NGF expression is associated with increased numbers of trigeminal sensory neurons raising the possibility that NGF/*Ntrk1* signaling contributes to the invasion of trigeminal neurons into the CN (Krol et al., 2001; Shore et al., 2000; Zhou et al., 2007).

Kcnq3

At 28 days post-exposure, *Kcnq3* mRNA expression was up-regulated in the CN (Fig. 2D, 3C). The *Kcnq3* gene encodes Kv7.3, a channel protein that in association with other Kcnq proteins forms part of the M channel (Kv7.2/3; (Cooper, 2011; Kalappa et al., 2015; Miceli et al., 2008), a slow voltage-gated channel that modulates potassium ionic currents (Adams and Brown, 1980; Brown and Adams, 1980; Browne et al., 2012). Mutations in Kcnq3 contribute to neuronal hyperexcitability (Singh et al., 2003; Uehara et al., 2008) and epilepsy (Soldovieri et al., 2014; Zara et al., 2013). Interestingly, Ntrk1 modulates potassium currents in injured neurons (Gamper et al., 2003; Jia et al., 2008), increasing their sensitivity to pain (Mousa et al., 2007). Kcnq3 and Ntrk1 proteins were depressed after the noise exposure, but recovered in parallel (Figure 3A, C) raising the prospect that these proteins may collaborate to modulate spontaneous activity in the CN following noise exposure.

Oprd1

Oprd1 codes for delta-type, G-protein coupled opioid receptors (Pinello et al., 2010; Sen et al., 2013). Delta opioid receptors have a high affinity for endogenous enkephalins. When the ligand binds to the receptor, it reduces the conductance of calcium ions and enhances potassium currents, functional changes that could influence pain perception and opiate analgesia (Sen et al., 2013). Opioid receptors, which are expressed in the auditory brainstem and olivocochlear efferent neurons, have been implicated in tinnitus and hyperacusis (Sahley et al., 2013; Sahley et al., 1999). *Oprd1* mRNA expression was significantly up-regulated at 14 days post-exposure (Fig. 2C). The noise-induced increase in delta opioid receptor gene expression might serve to modulate pain sensations associated with degenerating nerve fibers in the cochlea nucleus (Baizer et al., 2015) or the invasion of trigeminal nerve fibers that express delta opioid receptors (Hwang et al., 1986; Mansour et al., 1994). Given the increase in *Ntrk1* gene expression it is interesting to note that NGF-induced pain is suppressed by non-selective opiate antagonists (Apfel et al., 1995; Shen and Crain, 1994).

Tlr2 and Ccl12

Chemokine (C-C motif) ligand 12 is a small cytokine that attracts several types of immune cells such as eosinophils, monocytes, and lymphocytes (Jia et al., 1996) while toll-like receptor 2 (Tlr2) is a protein that recognizes foreign pathogens and activates the immune system (Vollmar et al., 2010). Microglia, which express both Tlr2 (Liu et al., 2009; Shah et al., 2009) and Ccl12 (Goldmann and Prinz, 2013; Selenica et al., 2013; Steel et al., 2014), act as neuroimmune cells in the central nervous system (Baizer et al., 2015; Coull et al., 2005; Janz and Illing, 2014; Moss et al., 2007). We recently reported that the traumatizing noise used in this study strongly activated microglia in the CN; the location and time course of microglia activation was closely correlated with the pattern of auditory nerve fiber degeneration in the CN (Baizer et al., 2015). We found that *Tlr2* mRNA expression was significantly up-regulated in the CN 14 days post-exposure (Fig. 2C), whereas *Ccl12* mRNA was significantly down-regulated in the CN at 14 and 28 days post-exposure (Fig. 2C, D). These changes in mRNA expression suggest that our traumatizing exposure triggers a central immune response due to the release of inflammatory molecules from degenerating auditory nerve fibers and synapses similar to that observed in other ototraumatic events (Hu

et al., 2014a). The disruption of cells and their matrices in the absence of microorganisms is known as sterile inflammation (Fleshner, 2013; Moller et al., 2014; Rubartelli, 2014). Thus, the strong upregulation of *Tlr2* mRNA along with *Ntrk1* suggests that noise-induced cochlear damage may induce a sterile-inflammatory response in the CN. This interpretation is consistent with previous results showing that *Ntrk1* alters TLR expression levels in human monocytes (Prencipe et al., 2014). These observations suggest that *Ccl2*, *Tlr2*, and *Ntrk1* are part of a negative feedback loop that may protect the CN against sterile inflammation following noise trauma.

Interleukin 1 β

We observed a significant decrease in *Il1 β* at 28 days post-exposure (Fig. 2D). *Il1 β* is a cytokine and an important mediator of inflammatory responses that affect apoptosis (Kaur et al., 2014) and cell proliferation (Morioka et al., 2000). *Il1 β* modulates pain caused by perineural inflammation (Eliav et al., 2009), and regulates the synthesis of nerve growth factor (NGF; (Clary et al., 1994; Holtzman et al., 1995; Lindholm et al., 1987; Strijbos and Rothwell, 1995). In human monocytes, interactions between NGF and *Ntrk1* lower the production of inflammatory cytokines (including *Il1 β*) and enhance anti-inflammatory mediators (Prencipe et al., 2014). It is conceivable that such an interaction could occur in the CN since *Il1 β* mRNA expression decreased at the same time when *Ntrk1* expression increased at 28 days post-exposure, as compared to 2 & 14 days (Fig. 2D). The NGF-*Ntrk1* complex sensitizes primary afferent nociceptors during tissue inflammation (Koltzenburg et al., 1999). Hyperalgesia, which is often induced by chemokines and cytokines including *Il1 β* , has been observed in mice following sciatic nerve transection (Ramer et al., 1997) and neuropathic pain can be enhanced by inflammatory molecules such as *Il1 β* (Scholz and Woolf, 2007; Woolf and Ma, 2007).

In summary, the 126 dB noise exposure used in this study caused significant hearing loss (Figure 1), extensive hair cell loss and massive auditory nerve fiber degeneration in the CN (Baizer et al., 2015; Kraus et al., 2011). Despite these large structural and functional changes, only 8 of 168 (4.8%) genes in the GABAergic, glutamatergic, neuropathic pain and inflammatory arrays changed significantly. *Gabrg3* and *Slc17a6* genes were upregulated at 28 days post-exposure suggesting that the proteins encoded by these inhibitory and excitatory genes may alter spontaneous and driven activity in the CN. Within the neuropathic pain/inflammatory array *Tlr2* and *Oprd1* were upregulated at 14 days post-exposure and *Kcnq3* and *Ntrk1* were increased at 28 days post-exposure. *Ccl12* was reduced at 14 and 28 days post-exposure and *Il1 β* was reduced at 28 days post-exposure. The proteins encoded by these genes could conceivably contribute to hyperacusis and/or painful sensations evoked by loud sound, neuroinflammation and microglia activation and migration of nerve fibers into the CN from the trigeminal, cuneate and vestibular nuclei. However, changes in chemokines (*Ccl12*) and cytokines (*Il1 β*) might sensitize afferent nociceptors (*Oprd1*) and increase the migration of trigeminal nerve fibers (*Vglut2*) into the CN via *Ntrk1* signaling. Also, interactions between *Ntrk1* and toll-like receptor (*Tlr2*) might induce neuropathic pain in noise induced hearing loss.

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Abbreviations

CN	Cochlear Nucleus
Ntrk1	Neurotrophic Tyrosine Kinase, Receptor, Type 1
GABA	γ -aminobutyric acid
FC	Fold changes
ABR	Auditory Brainstem Response
Vglut	Vesicular glutamate transporter
NGF	Nerve growth factor

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HIGHLIGHTS

- Severe to profound noise-induced hearing loss altered mRNA expression of eight of 168 genes involved in GABAergic, glutamatergic, neuropathic pain and inflammation in the rat cochlear nucleus.
- mRNA expression of *Slc17a6* and *Gabrg3*, involved in excitation and inhibition respectively, increased significantly. This suggested a possible role in spontaneous hyperactivity linked to tinnitus.
- Noise exposure increased mRNA expression of *Tlr2*, *Oprd1*, *Kcnq3* and *Ntrk1*, but decreased mRNA expression of *Ccl12* and *Il1 β* gene involved in neuropathic pain/inflammation. These changes suggested a role for the signaling molecules in neuroinflammation, microglia activation, hyperacusis and/or otalgia.
- Time-specific changes were observed in Ntrk1, Kcnq3 and Slc17a6 protein expression in the cochlear nucleus following noise induced hearing loss.
- Ntrk1 signaling could play a vital role in both noise-induced sterile inflammation and neuropathic pain in the cochlear nucleus.

Figure 1.

Mean ($\pm$ SEM) ABR thresholds in the right-exposed ear ranged from 90-100 decibels sound pressure level (dB SPL) between 6 and 32 kHz at 14 and 28 days post-exposure (open symbols; $n=6$). ABR thresholds in the left-plugged sham control ear were approximately 40 dB SPL (green, filled symbol, $n=6$). ABR thresholds measured in the anesthetized, but unexposed control animals (filled symbols) were approximately 40 dB at 14 days and 28 days post-anesthesia ($n=6$).

Figure 2.

Gene array volcano plots for GABA and glutamate (upper half) and neuropathic pain and inflammatory (lower half) gene arrays. Each volcano plot shows the Log₂ (Fold-Change) in gene expression on the X-axis versus Log₁₀ (p-Value) of statistical significance on the Y-axis (mean of 4 replicates). Red dots indicate fold increases above 0.5 (increases >50%) while the blue dots indicate fold decreases below -0.5 (decreases >50%). Data points above the dotted horizontal line indicate statistically significant gene expression changes (p < 0.05). Fig. 2 A and B show GABA and glutamate gene expression changes obtained at 14 days (A) and 28 days post-exposure (B). Fig. 2 C and D demonstrate neuropathic pain and inflammatory gene expression changes obtained at 14 days (C) and 28 days (D) post-exposure.

Figure 3.

Western blots and histograms depict noise-induced changes in (A) Ntrk1, (B) Slc17a6 (C) Kcnq3 and (D) Gabrg3 protein expression at 2, 14 and 28 days post-exposure. The protein bands for Ntrk1, Slc17a6, Kcnq3 and Gabrg3 protein were normalized with the housekeeping protein actin. Ntrk1, Kcnq3 and Gabrg3 protein expression levels were significantly down-regulated relative to control ($p < 0.001$) at 2, 14 and 28 days post-exposure while Slc17a6 protein expression was significantly upregulated relative to control ($p < 0.05$) at 14 days post-exposure. Asterisks (*) denote significant differences between sham and noise exposure groups, while the pound symbol (#) indicates significant differences between recovery durations. ns = non-significant.

Figure 4.

Immunolabeling of Gabrg3 in the ventral cochlear nucleus (VCN). Representative photomicrographs from an unexposed sham control rat at low (A) and high (C) magnifications and from a rat that recovered 28 days after noise at low (B) and high (D) magnifications. Low magnification images marked with a grey dotted border line in Fig. 4 A and B inserts show the overview of VCN (D-dorsal, L-lateral, GCL-Granule cell lamina, DCN Dorsal cochlear Nucelus) with a black star symbol inside the border. This star indicates the location of each high magnification image. These high magnification images show dark Gabrg3 puncta in and around the periphery of the soma and the surrounding neuropils.

Figure 5.

Representative fluorescent photomicrographs exhibit Slc17a8 (A, D), Gabrg3 (B, E) immunolabeling and Slc17a8/Gabrg3 merged images (C, F) taken from the ventral cochlear nuclei (VCN) of an unexposed control rat (A-C) and of a 28 day post-exposure rat (D-F). In Fig. 5A & D, Slc17a8 (green) is heavily expressed in the soma (arrows) of neurons; while scattered puncta and patches are present in the surrounding neuropils. The overview of VCN anatomy is shown in Fig. 5 B & E inserts marked with a white dotted border line (D-dorsal, M-Medial). A star symbol in the insert localizes where the high magnification image is obtained. Slc17a8 labeling in 28 day post-exposure rat (D) is similar to that in control rat (A). In Fig. 5B & E, Gabrg3 (red) immunolabels surround numerous soma of neurons (arrows) and also are present in patches and puncta in the surrounding neuropils. In Fig. 5C & F, a merge of Slc17a8 and Gabrg3 labels demonstrates that Gabrg3 co-localizes the perimeter of Slc17a8-positive neurons in the cochlear nucleus of both control and noise-exposed rats at 28 days post-exposure (as indicated by red-filled arrows). Inserts in Fig. 5C and F depict higher magnification images of Gabrg3 engulfing most, if not all, of the Slc17a8-positive neurons in both sham (C) and 28 days recovered rat VCN. Scale bar = 50 μm.

Table 1

Relative abundance of mRNA (Ct) for glutamatergic and GABAergic genes relative to actin in CN. Five most abundant genes are in bold.

Gene	C _t	Gene	C _t	Gene	C _t
		-			
Abat	-4.3	Gabrr1	13.2	Homer1	-5.6
	-		-		
Adcy7	-10.2	Gabrr2	-12.2	Homer2	-9.5
Adora1	-5.3	Gad1	-4.1	Il1b	-11.2
Adora2a	-7.6	Gls	-4.7	Itpr1	-2.8
Aldh5a1	-5.7	Glu1	-1.0	Mapk1	-2.9
App	-1.1	Gnai1	-3.0	Nsf	-3.3
	-				
Avp	15.3	Gnaq	-4.4	P2rx7	-8.4
	-				
Bdnf	10.4	Gphn	-5.6	Phgdh	-5.0
Cacna1a	-7.8	Gria1	-4.9	Pla2g6	-7.7
Cacna1b	-7.6	Gria2	-4.0	Plcb1	-5.6
Cdk5r1	-5.8	Gria3	-5.5	Prodh	-6.0
Cln3	-8.7	Gria4	-4.4	Shank2	-7.1
Dlg4	-6.2	Grik1	-4.3	Slc17a6	-4.4
		-			
Gabbr1	-2.4	Grik2	10.2	Slc17a7	-3.4
Gabbr2	-5.3	Grik4	-5.3	Slc17a8	-12.1
Gabra1	-4.0	Grik5	-8.1	Slc1a1	-5.1
Gabra2	-7.0	Grin1	-6.6	Slc1a2	-1.4
Gabra4	-8.5	Grin2a	-6.9	Slc1a3	-3.5
Gabra5	-7.1	Grin2b	-7.2	Slc1a6	-11.3
		-			
Gabra6	-4.1	Grin2c	10.0	Slc1a7	-14.3
Gabrb1	-6.9	Grm1	-5.8	Slc32a1	-6.9
Gabrb3	-5.2	Grm2	-9.4	Slc38a1	-3.8
Gabrd	-6.2	Grm3	-4.8	Slc6a1	-4.9
	-				
Gabre	14.5	Grm4	-6.9	Slc6a11	-3.9
Gabrg1	-4.3	Grm5	-9.5	Slc6a12	-10.8
		-			
Gabrg2	-3.8	Grm6	15.5	Slc6a13	-6.9
Gabrg3	-8.6	Grm7	-8.3	Sncg	-5.0
	-				
Gabrq	12.1	Grm8	-7.1	Srr	-5.2

Table 2

Relative abundance of mRNA (C_t) for neuropathic pain & inflammation genes relative to actin in CN. Five most abundant genes are in bold.

Gene	C_t	Gene	C_t	Gene	C_t
Ace	-6.2	Grm1	-6.7	P2rx7	-8.8
		-	-		
Adora1	-6.0	Grm5	10.0	P2ry1	-7.5
		-	-		
Adrb2	-9.3	Htr1a	10.1	Pdyn	-10.6
	-	-	-		
Alox5	11.9	Htr2a	11.2	Penk	-10.5
		-	-		
Bdkrb1	ND	Il10	16.7	Pla2g1b	-13.4
	-				
Bdnf	11.6	Il18	-8.7	Pnoc	-8.9
	-	-	-		
Cacna1b	11.2	Il1a	-11.2	Prok2	-13.3
		-	-		
Calca	-9.8	Il1b	11.8	Ptger1	-10.0
Cck	-8.7	Il2	ND	Ptger3	-10.7
	-	-	-		
Cckbr	11.4	Il6	14.1	Ptger4	-10.6
	-	-	-		
Ccl12	13.4	Itgam	10.1	Ptges	-10.6
	-				
Ccr2	13.9	Itgb2	-8.6	Ptges2	-6.1
Cd200	-9.5	Kenip3	-7.1	Ptges3	-3.6
Cd4	-8.7	Kcnj6	-6.8	Ptgs1	-9.9
Chrna4	-8.1	Kcnq2	-6.5	Ptgs2	-10.2
Cnr1	-6.3	Kcnq3	-8.9	Scn10a	-9.6
	-				
Cnr2	13.4	Maob	-6.0	Scn11a	ND
Comt	-6.0	Mapk1	-3.8	Scn3a	-8.9
Csf1	-7.9	Mapk14	-7.0	Scn9a	-10.2
Cx3cr1	-7.5	Mapk3	-4.8	Slc6a2	-12.4
Dbh	-5.1	Mapk8	-6.2	Tac1	-7.3
	-	-	-		
Edn1	10.3	Ngf	12.0	Tacr1	-11.5
	-	-	-		
Ednra	10.4	Ntrk1	14.1	Tlr2	-9.7
Faah	-9.3	Oprd1	-13.1	Tlr4	-8.7
	-				

Gene	C _t	Gene	C _t	Gene	C _t
Gch1	-10.2	Oprk1	-10.5	Tnf	-13.4
	-				
Gdnf	13.0	Oprm1	-8.7	Trpa1	-12.4
		-			
Grin1	-6.7	P2rx3	11.3	Trpv1	-11.3
Grin2b	-7.6	P2rx4	-7.3	Trpv3	-9.4

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Table 3

Glutamatergic and GABAergic gene expression changes in CN at 14 and 28 days post-exposure relative to control. Mean (n=4 replicates) log2 fold gene expression changes shown for 14 and 28 days post-exposure. Statistically significant changes are indicated in bold

Gene	14 d	28 d	Gene	14 d	28 d	Gene	14 d	28 d
-	-	-	-	-	-	-	-	-
Abat	0.06	-0.13	Gabrr1	-0.13	1.04	Homer1	0.14	-0.04
-	-	-	-	-	-	-	-	-
Adcy7	0.63	0.42	Gabrr2	0.07	0.97	Homer2	-0.36	-0.29
-	-	-	-	-	-	-	-	-
Adora1	0.38	-0.41	Gad1	-0.87	0.39	Il1b	-0.16	-1.16
-	-	-	-	-	-	-	-	-
Adora2a	0.60	0.68	Gls	0.18	0.21	Itpr1	-1.20	-2.01
Aldh5a1	0.00	0.24	Glul	0.33	0.56	Mapk1	0.15	0.21
-	-	-	-	-	-	-	-	-
App	0.09	-0.03	Gnai1	-0.01	0.22	Nsf	-0.16	0.09
-	-	-	-	-	-	-	-	-
Avp	0.03	0.04	Gnaq	-0.14	0.35	P2rx7	0.16	0.26
-	-	-	-	-	-	-	-	-
Bdnf	0.85	-0.30	Gphn	0.07	0.22	Phgdh	-0.13	-0.06
-	-	-	-	-	-	-	-	-
Cacna1a	0.80	-1.11	Gria1	-0.57	1.01	Pla2g6	-0.35	-0.14
-	-	-	-	-	-	-	-	-
Cacna1b	1.40	-0.09	Gria2	0.02	0.07	Plcb1	0.35	0.26
-	-	-	-	-	-	-	-	-
Cdk5r1	-0.37	-0.15	Gria3	0.37	0.52	Prodh	-0.38	0.02
-	-	-	-	-	-	-	-	-
Cln3	0.41	-0.13	Gria4	0.09	0.35	Shank2	-0.18	-0.26
-	-	-	-	-	-	-	-	-
Dlg4	0.08	-0.27	Grik1	0.05	0.35	Slc17a6	0.20	0.69
-	-	-	-	-	-	-	-	-
Gabbr1	0.40	0.28	Grik2	-0.56	0.94	Slc17a7	-0.28	-0.23
-	-	-	-	-	-	-	-	-
Gabbr2	0.02	0.11	Grik4	-0.06	0.42	Slc17a8	-0.10	0.74
-	-	-	-	-	-	-	-	-
Gabra1	0.46	-0.20	Grik5	-0.29	0.62	Slc1a1	0.23	0.47
Gabra2	0.45	0.50	Grin1	-0.12	0.24	Slc1a2	0.17	0.48
-	-	-	-	-	-	-	-	-
Gabra4	0.61	-0.95	Grin2a	-0.41	0.40	Slc1a3	-0.31	-0.77
-	-	-	-	-	-	-	-	-
Gabra5	0.02	0.79	Grin2b	0.14	0.42	Slc1a6	-1.59	-2.28
-	-	-	-	-	-	-	-	-

Gene	14 d	28 d	Gene	14 d	28 d	Gene	14 d	28 d
Gabra6	0.53	-0.65	Grin2c	-0.96	1.23	Slc1a7	-0.64	-0.58
					-			
Gabrb1	0.02	0.14	Grm1	-0.33	0.33	Slc32a1	-0.41	-0.32
					-			
Gabrb3	0.11	0.25	Grm2	-0.42	0.32	Slc38a1	0.09	0.15
					-			
Gabrd	1.42	-1.69	Grm3	0.16	0.18	Slc6a1	-0.39	-0.42
					-			
Gabre	0.50	1.71	Grm4	-0.85	1.45	Slc6a11	-0.01	0.72
					-			
Gabrg1	0.16	0.13	Grm5	-0.22	0.19	Slc6a12	-0.95	-2.32
					-			
Gabrg2	0.75	0.01	Grm6	-0.41	0.64	Slc6a13	-0.85	-2.84
Gabrg3	1.14	1.48	Grm7	0.13	0.51	Snca	-0.18	-0.03
Gabrq	0.12	1.50	Grm8	0.13	0.47	Srr	0.02	0.002

Table 4

Ne 28 days pos expression c changes ind uropathic t-exposu hanges icated in pain a re relati shown f bold nd inflamma ve to contro or 14 and 28 tion gene ls. Mean days po express (n=4 rep st-expos ion change licates) log ure. Statis s in CN at 2 fold gene tically signi 14 and ficant

Gene	14 d	28 d	Gene	14 d	28 d	Gene	14 d	28 d
Ace	1.16	0.62	Grm1	0.34	0.64	P2rx7	0.85	0.57
Adora1	0.31	0.67	Grm5	0.47	0.56	P2ry1	-0.03	0.06
					-			
Adrb2	0.00	-0.15	Htr1a	-0.21	0.17	Pdyn	-1.59	-1.66
					-			
Alox5	0.26	0.09	Htr2a	-0.07	0.25	Penk	0.18	0.31
					-			
Bdkrb1	0.19	-0.02	Il10	-0.19	0.02	Pla2g1b	-0.10	0.01
					-			
Bdnf	0.76	0.23	Il18	0.08	0.00	Pnoc	-0.49	-0.27
Cacna1b	0.08	0.65	Il1a	-0.20	0.18	Prok2	-0.82	-0.11
					-			
Calca	0.31	-0.62	Il1b	-0.12	1.26	Ptger1	0.82	0.39
					-			
Cck	0.10	-0.28	Il2	-0.19	0.02	Ptger3	0.25	-0.29
Cckbr	0.78	0.72	Il6	0.30	0.74	Ptger4	-0.13	0.30
					-			
Ccl12	0.59	-0.95	Itgam	0.65	0.59	Ptges	-0.19	-0.66
Ccr2	0.30	-0.54	Itgb2	0.77	0.36	Ptges2	0.19	0.48
Cd200	0.33	0.55	Kcnip3	0.21	0.48	Ptges3	0.10	0.14
Cd4	0.41	-0.21	Kcnj6	0.10	0.42	Ptgs1	0.57	0.17
Chrna4	0.69	0.92	Kcnq2	0.71	0.89	Ptgs2	-0.15	0.12
					-			
Cnr1	0.04	0.70	Kcnq3	0.55	0.71	Scn10a	0.66	0.77
Cnr2	0.71	-0.28	Maob	0.41	0.07	Scn11a	-0.19	-0.02
Comt	0.15	0.25	Mapk1	0.12	0.25	Scn3a	0.22	0.40
Csf1	0.56	0.34	Mapk14	0.08	0.33	Scn9a	0.10	-0.09
Cx3cr1	0.37	0.35	Mapk3	0.42	0.38	Slc6a2	-1.23	0.40
					-			
Dbh	0.02	0.23	Mapk8	0.18	0.21	Tac1	-1.67	-2.92
					-			
Edn1	0.33	-0.23	Ngf	0.31	0.27	Tacr1	-0.92	-0.33
					-			
Ednra	0.14	-0.50	Ntrk1	1.33	1.30	Tlr2	0.57	0.31
Faah	0.75	0.19	Oprd1	1.48	1.51	Tlr4	0.27	-0.01
					-			
Gch1	0.13	0.22	Oprk1	0.33	0.45	Tnf	0.29	0.78

Gene	14 d	28 d	Gene	14 d	28 d	Gene	14 d	28 d
Gdnf	0.05	1.06	Oprm1	-0.20	0.15	Trpa1	0.21	-0.06
	-				-			
Grin1	-0.26	0.55	P2rx3	0.31	0.01	Trpv1	0.60	0.59
Grin2b	0.27	0.28	P2rx4	0.45	0.68	Trpv3	0.25	-0.12