Influence of the paraflocculus on normal and abnormal spontaneous firing rates in the inferior colliculus

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ABSTRACT

Spontaneous firing rates of neurons in the central auditory pathway, such as in the inferior colliculus, are known to be increased after cochlear trauma. This so-called hyperactivity is thought to be involved in the generation of tinnitus, a phantom auditory perception. Recent research in an animal model suggests behavioural signs of tinnitus can be significantly reduced by silencing or removal of the paraflocculus (PF) of the cerebellum. The current study investigated the effects of acute PF removal on spontaneous firing rates recorded from single neurons in the right inferior colliculus of guinea pigs with normal hearing (which did not receive acoustic trauma) or with hearing loss caused by acoustic trauma. Spontaneous firing rates were obtained at either 2 or 13 weeks after initial surgery on the left side. In half of the animals in each group the left PF was removed immediately prior to the spontaneous firing rates recordings. In the acoustic trauma groups, spontaneous firing rates in the inferior colliculus were higher when the PF was removed compared to animals with an intact PF. This effect of PF removal was not observed in animals that did not receive acoustic trauma. These results suggest that the PF has a tonic inhibitory effect on hyperactivity in the inferior colliculus in animals with hearing loss, but not on normal spontaneous firing rates in normal hearing animals.

Keywords: tinnitus, electrophysiology, hyperactivity, acoustic trauma, paraflocculus

Abbreviations:

AC	Auditory Cortex
CAP	Compound Action Potential
CF	Characteristic Frequency
CN	Cochlear Nucleus
CNIC	Central Nucleus of the Inferior Colliculus
dB SPL	decibel Sound Pressure Level (re 20µPa)
DCN	Dorsal Cochlear Nucleus
IC	Inferior Colliculus
i.m.	intramuscular
i.p.	intraperitoneal
PF	Paraflocculus
s.c.	subcutaneous
SFR	Spontaneous Firing Rate

1. INTRODUCTION

Tinnitus, a common and potentially debilitating symptom, is frequently associated with hearing loss (Axelsson et al., 1989). To date, the neural substrate for tinnitus is unconfirmed however one proposed mechanism is hyperactivity, i.e. increased spontaneous firing rates (SFRs) of neurons in the auditory pathway (Eggermont et al., 2004). Hyperactivity is observed in multiple auditory brain regions following hearing loss as demonstrated in different animal models (Finlayson et al., 2009; Kalappa et al., 2014; Mulders et al., 2009; Noreña et al., 2010; Vogler et al., 2011). Animals that show hearing loss and subsequent hyperactivity also demonstrate behavioural evidence of tinnitus (Brozoski et al., 2002; Kalappa et al., 2014; Mulders et al., 2014b). The co-occurrence of hyperactivity and tinnitus suggests hyperactivity may be involved in the generation of tinnitus.

Changes in central activity after cochlear trauma are not restricted to the auditory pathway. One of the non-auditory structures that shows hyperactivity after acoustic trauma and after development of tinnitus is the paraflocculus (PF) of the cerebellum (Brozoski et al., 2007). In addition, Mulders et al (2014a) showed altered mRNA levels of inhibitory genes in PF after cochlear trauma. Finally, other studies revealed that PF removal or silencing by glutamatergic antagonists or lidocaine resulted in reduced behaviours associated with tinnitus (Bauer et al., 2013a; Bauer et al., 2013b; Brozoski et al., 2013), whereas application of glutamatergic agonists induced tinnitus-like behaviour, which suggests a role for the PF as a modulator of tinnitus (Bauer et al., 2013b).

Traditionally, the PF has been associated with vision and movement (Azizi et al., 1990; Burne et al., 1978; Lisberger et al., 1994; Rambold et al., 2002; Stone et al., 1990). However, the cerebellum has been shown to be activated when auditory input is processed (Petacchi et al., 2005) and the PF receives and integrates multi-modal sensory inputs (Azizi et al., 1990).

Anatomical studies have suggested inputs to PF from the cochlea, cochlear nucleus and indirectly from the auditory cortex (Azizi et al., 1985; Huang et al., 1982; Morest et al., 1997). In line with this connectivity, PF neurons have been shown to be responsive to auditory stimulation (Azizi et al., 1990; Azizi et al., 1985; Sun et al., 1990) as well as electrical stimulation of the auditory cortex (Azizi et al., 1985; Sun et al., 1990).

This study investigated the effect of acute PF removal on SFRs in the IC after acoustic trauma, because 1) removal of the PF can reduce behaviours associated with the presence of tinnitus (Bauer et al., 2013a; Brozoski et al., 2013), 2) acoustic trauma induced hyperactivity may be involved in the generation of tinnitus (Eggermont, 2005; Eggermont et al., 2004; Kaltenbach et al., 2000) and 3) treatments that modulate hyperactivity also modulate tinnitus (Mulders et al., 2014b). In view of the above mentioned studies, it was hypothesized that acute PF removal would decrease the acoustic trauma induced hyperactivity in inferior colliculus. Single neuron recordings were made from guinea pig inferior colliculus (IC) either 2 or 13 weeks after acoustic trauma with or without acute unilateral removal of PF at the final time-point. In addition, the effects of PF removal were also investigated in separate group of animals that did not receive acoustic trauma.

2. METHODS

2.1 Animals

Forty-one adult pigmented guinea pigs of either sex were used. At the time of initial surgery animals weighed between 265-532g. All experimental protocols conformed to the Code of Practice of the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee of The University of Western Australia. A flow diagram showing the experimental design is shown in figure 1.

2.2 Recovery surgery

Surgery was similar to that which has been described previously (Mulders et al., 2009; Robertson et al., 2013; Vogler et al., 2014). Prior to anaesthesia, animals were injected with 0.1 ml of atropine sulphate (0.6 mg/ml, subcutaneously (s.c.)). Anaesthesia consisted of an intraperitoneal (i.p.) injection of diazepam (5 mg/kg) followed 20 minutes later by an intramuscular (i.m.) injection of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; 1 ml/kg). Deep anaesthesia was indicated by the absence of the foot withdrawal reflex. Following this, animals were placed on a heating blanket in a soundproof room and mounted in hollow ear bars. A hole was made in the tympanic bulla and an insulated silver wire was placed on the round window of the left cochlea. Compound action potential (CAP) thresholds were recorded for frequencies between 2-24 kHz and were used to assess the animals' cochlear sensitivity as previously described (Johnstone et al., 1979). All sound stimuli were presented in a closed sound system using a 0.5 inch condenser microphone driven in reverse as a speaker (Bruel and Kjaer, type 4134). A computer, equipped with a DIGI96 soundcard, synthesized pure tone stimuli. This signal was then passed to an analog/ digital interface (ADI-9 DS, RME Intelligent Audio Solution). Sample rate was 96 kHz. A custom-designed computer program (Neurosound, MI Lloyd) was used to regulate frequency

and intensity and collect single neuron data. A different data acquisition system (Powerlab 4SP, AD Instruments) was used to amplify, filter (100 Hz–3 kHz bandpass) and record CAP signals. When initial cochlear sensitivity was within the normal range (Johnstone et al., 1979), animals were randomly assigned to one of two treatment groups, i.e. no acoustic trauma (*n=21*) or acoustic trauma (*n=20*). The acoustic trauma animals were exposed to a 2 hour continuous 10 kHz pure tone at 124 dB SPL, while the contralateral ear was blocked using a plasticine plug. Post-acoustic trauma, CAP audiograms were recorded to assess the immediate loss in peripheral sensitivity. All wounds were sutured and animals were recovered for 2 or 13 weeks before the final single neuron recordings were made. All aspects of experimental design (e.g. anaesthesia, exposure surgery etc.) were identical between the no acoustic trauma animals and acoustic trauma animals, except that the no acoustic trauma group did not receive any acoustic trauma.

2.3 Non-recovery surgery for electrophysiological recordings

During the final non-recovery surgery at 2 or 13 weeks after the initial recovery surgery, half of the animals in each group (see flow diagram in figure 1) underwent an acute PF aspiration before single neuron recordings from the IC were obtained (PF- groups). In the other half of the animals in each group PF was kept intact before single neuron recordings from the IC were obtained (PF+ groups). PF aspiration was performed ipsilateral whereas IC recordings were done contralateral to the cochlea that underwent acoustic trauma.

Animals were administered 0.1ml of atropine (s.c.) immediately followed by an injection of Nembutal (pentobarbital sodium, 30 mg/kg, i.p.) and 10 minutes thereafter a 0.15 ml i.m. injection of Hypnorm. Maintenance anaesthesia consisted of administering full Hypnorm doses every hour and half doses of Nembutal every 2 hours. When deep anaesthesia was achieved (absence of the foot withdrawal) animals were placed on a heating blanket in a

sound proof room and artificially ventilated on carbogen (95% O_2 and 5% CO_2). They were mounted in hollow ear bars and the right and left cochleae were exposed. CAP thresholds were measured on both sides, using methods identical to the recovery experiment. Prior to electrophysiological recordings in the central nucleus of the IC (CNIC), animals were paralysed with a 0.1 ml i.m. injection of pancuronium bromide (2 mg/ml i.m.). Anaesthesia level was monitored thereafter using a continuous electrocardiogram and heart rate never increased above pre-paralysis levels at any stage of the experiments.

To enable recordings to be made in IC, the cortex overlying the CNIC was exposed by performing a craniotomy contralateral to the initially operated cochlea. A glass-insulated tungsten microelectrode (Merrill et al., 1972) was advanced along the dorso-ventral axis through the cerebral cortex (2-3mm) with a hydraulic microdrive. Initial electrode placement was 2.2 mm laterally from the midline and 0.6 mm rostral from the inter-aural line as described previously (Vogler et al., 2014). Sound-evoked cluster and single neuron responses were used to optimize the electrode position for individual animals. Pure tones of varying frequency and intensity were used to monitor these responses (duration 50ms, repetition rate 2/s, rise/fall time 1 ms). The transition to short latency, secure cluster responses of low characteristic frequency (CF) and a systematic progression to higher CFs with increasing depth were used as indicators of electrode entry into the CNIC. The craniotomy was covered with 4% agar in saline to aid mechanical stability.

To remove the PF ipsilateral to the initially operated cochlea, a posterior craniotomy was performed and the overlying cerebellum and all of the paraflocculus located in the lateral sub-arcuate fossa was aspirated under visual control. IC recordings commenced immediately after the PF removal. In the animals without the PF removal craniotomies were made of a similar size but no aspiration was performed.

2.4 Single neuron recordings

Recordings from IC were made as described previously in other work from our laboratory (Dong et al., 2009; Mulders et al., 2009; Mulders et al., 2011a; Robertson et al., 2013; Vogler et al., 2014). Swept pure tone bursts were used as a search stimulus along the length of each electrode track in the IC. When spikes from an individual neuron were reliably distinguishable for background noise, the neuron's CF and threshold at CF were determined audio-visually. SFRs were then measured over a 10s time window. Speaker input was turned off during this measurement to prevent low threshold neurons responding to any background "hiss" that may be emanating from the sound system. The mean number of neurons collected per animal was 110 ± 26, with numbers varying between 37 and 182 neurons per animal (n > 100 in 32 of 41 animals). Data were recorded from multiple tracks through the IC in each animal (up to 6 tracks per animal). Single neuron recordings lasted approximately 4-6 hours.

2.5 Post-experiment confirmation of PF removal

Following completion of the electrophysiological recordings animals were overdosed with 0.3ml Lethabarb (325mg/ml Pentobarbitone sodium). Some animals were then transcardially perfused with saline followed by paraformaldehyde (4% in 0.1 M phosphate buffer) to enable demonstration of the extent of the cerebellar lesion. Then the brain was removed and photographs were taken of the cerebellum showing the removal of the PF on one side only (Fig. 2).

2.6 Data analysis and Statistics

Neurons in the trauma-affected regions lose sensitivity in the region of their tuning curve tips and as a result can show apparent changes in CF. Consequently, inaccuracies may result from

audio-visually determined CFs. To avoid this, single neuron recordings of CF were converted and grouped according to nominal CF (a depth-based estimate of the original CF) using methods previously described (Mulders et al., 2011b).

Where data were distributed normally (e.g. CAP thresholds), statistical analysis was performed using *t* tests, 1-way ANOVAs and 2-way ANOVAs. For all non-Gaussian distributions and data that failed normality tests (e.g. SFRs), non-parametric tests were used, i.e. Kruskal-Wallis one-way ANOVAs (multiple groups) and Mann-Whitney tests (two group comparisons). For statistical analysis in these non-parametric tests, n referred to the number of neurons in each group. Dunn's multiple comparison tests were used for post-hoc analysis of Kruskal-Wallis 1-way ANOVAs and Bonferroni multiple comparisons test for post-hoc analysis of 2-way ANOVAs. Significance level was set at 0.05 for all statistical tests.

3. RESULTS

3.1 Peripheral thresholds: CAP

Acoustic trauma resulted in a small permanent CAP threshold loss in all animals at 2 or 13 weeks of recovery, in contrast to animals that did not receive acoustic trauma. Figure 3 demonstrates the changes to CAP thresholds with respect to initial thresholds in all groups after 2 or 13 week recovery periods. No acoustic trauma did not affect thresholds as can be seen in figure 3A, C, E and G. Figures 3A and E show the results of no acoustic trauma for the individual animals after 2 weeks and 13 weeks, respectively. Mean CAP threshold losses are shown in figures 3C and G, divided into the animals with and without acute PF removal after 2 weeks and 13 weeks, respectively. These figures also illustrate there was no difference in threshold loss, measured at the start of the non-recovery experiments, between the animals that received acute PF removal and those that did not.

Figures 3B and F show the individual CAP threshold losses for the trauma animals at 2 and 13 week recovery, respectively and figures 3D and H show the mean CAP threshold losses, divided into the animals with and without acute PF removal after 2 weeks and 13 weeks of recovery, respectively. Hearing loss showed considerable variability between animals, although all trauma animals showed some degree of hearing loss (Fig. 3B, F). These results are consistent with previous published data (Mulders et al., 2009; Vogler et al., 2011; Vogler et al., 2014).

A 2-way ANOVA revealed significant differences in threshold loss between trauma animals and the animals that received no acoustic trauma [$F(1, 216) = 152.1$, $p<0.001$]. Post-hoc tests revealed significant hearing losses for all frequencies 10 kHz and above in 2 week animals ($p<0.01$) and at all frequencies except for 18 kHz in 13 week animals ($p<0.002$). However, no significant differences in CAP threshold loss were found between the 2 and 13 week trauma

animals irrespective of PF removal. In addition no significant differences were found between the PF removal and PF intact groups at either time-point.

3.2 Spontaneous activity

Spontaneous firing rates were increased in animals that received acoustic trauma as compared to the animals that did not as we have demonstrated previously (Mulders et al., 2009; Mulders et al., 2011a; Robertson et al., 2013; Vogler et al., 2014). SFRs were measured from a total of 4458 neurons in the CNIC (2123 from no acoustic trauma and 2335 from trauma animals). Mean SFRs for the different groups are shown in Figure 4. In the animals that did not receive acoustic trauma (Fig. 4A) SFRs were not significantly different between animals with the PF intact (2 weeks recovery: 1.57 ± 0.19 spikes/s based on 519 neurons from 5 animals and 13 weeks recovery: 1.08 ± 0.11 spikes/s based on 529 neurons from 6 animals) and those with PF removed (2 weeks recovery: 1.90 ± 0.21 spike/s based on 544 neurons from 5 animals and 13 weeks recovery: 1.97 ± 0.22 spikes/s based on 531 neurons from 5 animals) . Post-hoc analysis revealed no significant differences between mean SFRs at the different time-points, either in animals with the PF intact or removed.

A Kruskal-Wallis 1-way ANOVA revealed that SFRs were elevated in trauma groups (Fig. 4B) compared to no acoustic trauma [$H(3) = -58.69$, $p < 0.001$]. Post-hoc analysis showed that, compared to no acoustic trauma, all trauma groups showed significantly elevated SFRs ($p < 0.001$), a result consistent with previously reported data (Mulders et al., 2009; Vogler et al., 2014). However, in contrast to the no acoustic trauma animals, the trauma groups showed significant increases in SFRs following PF removal (Fig. 4B) [$H(3) = 42.47$, $p < 0.001$]. Post-hoc tests showed that with the PF removed, mean SFRs in the 2 week trauma group significantly increased from 5.31 ± 0.46 (591 neurons from 5 animals) to 6.91 ± 0.59 (2 week; 559 neurons from 5 animals) spikes/s ($p < 0.05$) and in the 13 week group from 5.48 ±

0.50 spikes/s (584 neurons from 5 animals) to 7.08 ± 0.49 spikes/s (601 neurons from 5 animals) ($p<0.001$) (Fig. 4B). As with no acoustic trauma animals, post-hoc testing showed no differences in mean SFRs were reported between the 2 and 13 week trauma groups either with or without PF present

3.3 Spontaneous activity and the region of peripheral hearing loss

Hyperactivity has previously been observed to mainly occur in the CF regions in the IC tonotopic map that correspond to the frequency regions of peripheral hearing loss (Robertson et al., 2013). Therefore, neurons were grouped based on their location in the region of peripheral hearing loss (>10 kHz) or well outside the region of peripheral hearing loss (<8 kHz; see figure 3D and H) as determined from the neurons' nominal CF (Fig. 5) and SFRs between these groups were compared. Neurons with CFs between 8 and 10 kHz were excluded.

Post-hoc analysis of Kruskal-Wallis 1-way ANOVAs of no acoustic trauma groups revealed no effect of PF removal on SFRs in 2 week [$H(3) = 17.94$, $p <0.001$] or 13 weeks [$H(3) = 36.25$, $p <0.001$] of either the neurons with CF <8 kHz or CF >10 kHz (Fig. 5A and B), except for a small statistically significant difference at 2 weeks recovery between <8 kHz PF intact group compared to the <8 kHz PF removed ($p<0.02$).

Post-hoc analysis of a Kruskal-Wallis 1-way ANOVA [$H(3) = 11.24$, $p <0.02$] of 2 week trauma groups revealed PF removal did not affect the SFRs of neurons with a CF<8 kHz but did increase the SFRs of neurons with a CF >10 kHz (7.53 ± 0.79 to 10.28 ± 1.10 spikes/s), although this comparison just failed to reach statistical significance ($p<0.059$) (Fig. 5C). Post-hoc tests of in the 13 week trauma groups [$H(3) = 64.69$, $p <0.001$] revealed SFRs were significantly higher in the neurons with CF <8 kHz ($p<0.001$) and with CF >10 kHz ($p<0.001$) when the PF was removed.

Post-hoc tests revealed that all trauma groups showed higher mean SFRs in the >10 kHz group compared to the <8 kHz group (Fig. 5C-D). When PF was intact, mean SFRs for 2 week animals were 3.37 ± 0.53 and 7.53 ± 0.79 spikes/s ($p<0.05$) and for 13 weeks animals were 2.32 ± 0.48 and 6.71 ± 0.67 spikes/s ($p<0.001$) for CF <8 kHz and CF >10 kHz, respectively. In animals with PF removed, mean SFRs were also increased in the neurons with CF >10 kHz compared to neurons with CF <8 kHz of 2 week data (10.28 ± 1.10 vs 3.82 ± 0.55 spikes/s, respectively) ($p<0.002$) and 13 week data (8.71 ± 0.76 vs 4.80 ± 0.59 spikes/s, respectively) ($p < 0.002$).

4. DISCUSSION

Peripheral hearing loss in animals from the present study was consistent with previous reports (Mulders et al., 2009; Mulders et al., 2011a; Vogler et al., 2014). Animals that did not receive acoustic trauma showed no change in peripheral thresholds whereas all trauma animals did, especially at frequencies >10 kHz. Additionally, overall SFRs in CNIC were increased in trauma animals compared to no acoustic trauma animals and the increase in SFR was greatest in the frequency regions showing peripheral hearing loss (>10 kHz), in line with previous observations using the same animal model (Mulders et al., 2009; Mulders et al., 2011a; Robertson et al., 2013; Vogler et al., 2014).

Interestingly, trauma animals (at both the 2 and 13 week time-points) with the PF acutely removed showed higher SFRs than those with PF intact. However, this effect of PF removal was not observed in the animals that did not receive acoustic trauma. Higher SFRs in the trauma group with PF removed, could be the result of a difference in hearing loss between the two trauma groups, as we have shown previously (Mulders et al., 2011b) but this possibility can be excluded as no differences were found in hearing loss (measured before the PF ablations or sham surgery were performed) between the with and without PF groups et either time-point. We therefore suggest that this increased hyperactivity observed in CNIC implies a tonic inhibitory effect of the PF on the neuronal activity in CNIC after acoustic trauma.

The anatomical connectivity between PF and CNIC and the exact nature of the projection as to which neurotransmitters or receptors are involved, remains to be elucidated. Anatomical data on the projection from PF to IC are lacking from the literature. A predominantly contralateral projection from the fastigial nucleus, one of the deep cerebellar nuclei, to the external nucleus of the IC has been shown previously (Zimny et al., 1981). However, the PF does not show a direct projection to the fastigial nucleus but rather to other deep cerebellar

nuclei such as the interpositus and dentate nuclei (Gayer et al., 1988; Nagao et al., 1997). Interestingly, there is a direct projection from the interpositus nucleus to the auditory thalamus (Halverson et al., 2010), which may provide a possible indirect pathway by which PF can influence activity in CNIC. The main output neurons of the PF, the Purkinje cells, are inhibitory (Mapelli et al., 2015) but in view of the fact that the connection to the IC is likely to be poly-synaptic, the final effect of activation of the PF on IC cannot be predicted.

One possible explanation for the fact that the increased SFRs with PF removal only occur after acoustic trauma, may be that acoustic trauma results in an alteration of spontaneous activity in PF. Increased activity in PF after acoustic trauma would be in line with an earlier observation that acoustic trauma increased activity in the ipsilateral PF in rats with tinnitus, shown by manganese-enhanced magnetic resonance imaging (Brozoski et al., 2007), though this latter technique does not distinguish between an increase in excitatory or inhibitory neurons and downstream effects of this hyperactivity would therefore be impossible to predict *a priori*. Our data would suggest that the hyperactivity in PF would have an inhibitory effect on the hyperactivity observed in IC.

The mechanism for the postulated increased activity in PF remains to be elucidated, but it has been shown that neurons in PF respond to auditory stimulation (Azizi et al., 1990; Azizi et al., 1985; Sun et al., 1990), which could be the result of small direct projections from cochlea or cochlear nucleus (Huang et al., 1982; Morest et al., 1997) or of an indirect projection from auditory cortex (Azizi et al., 1985). This latter projection has been shown to be functional, as electrical stimulation of the auditory cortex resulted in altered firing patterns of PF neurons (Azizi et al., 1985). Cochlear trauma may lead therefore directly to increased activity in PF or increased activity in PF could be driven by the hyperactivity in CN or auditory cortex (Finlayson et al., 2009; Noreña et al., 2010; Vogler et al., 2011).

Although our results provide support for the notion of a modulatory influence of the PF on hyperactivity in the auditory pathway after hearing loss, the relationship to PF involvement in tinnitus is not clear. If indeed the hyperactivity in central auditory structures that is known to be present after cochlear trauma, contributes to the development of tinnitus (Eggermont et al., 2004) then our results would suggest an increase in tinnitus after PF removal. However, earlier behavioural studies showed that removal or inactivation of the PF can significantly reduce signs of tinnitus in rats exposed to acoustic trauma (Bauer et al., 2013a; Brozoski et al., 2013), but multiple factors need to be considered before directly comparing the present results and these former studies. First, our data were collected acutely under general anaesthesia (within approximately 4 hours) after PF removal, whereas the other studies investigated the presence of tinnitus (in awake behaving animals) at least 48 hours after PF removal or silencing (Bauer et al., 2013a; Bauer et al., 2013b; Brozoski et al., 2013). Plasticity can occur rapidly in the auditory system, as has been shown previously (Mulders et al., 2013; Seki et al., 2003) and in the cerebellum as well (Cesa et al., 2005; Gramsbergen, 2007; Mapelli et al., 2015) and the effect of PF removal may change over time. In addition, the relationship between hyperactivity and tinnitus may not be straightforward. Kalappa et al. (2014) showed that hyperactivity in the auditory thalamus strongly correlated with the presence of tinnitus in rats exposed to an acoustic trauma. However, when Brozoski and colleagues (2013) measured activity levels in auditory thalamus using manganese enhanced magnetic resonance imaging, 8 weeks after pharmacological silencing of the PF which eliminated established tinnitus in rats exposed to acoustic trauma, no differences were observed between exposed animals without drug treatment and exposed animals that received the treatment. These studies could perhaps be reconciled by proposing that hyperactivity is necessary for tinnitus development but not for tinnitus maintenance. Finally, we did not measure tinnitus development in our animals and therefore we do not know the effect of PF

removal on the presence of tinnitus in our animals. In our animal model tinnitus develops between 4-6 weeks after acoustic trauma in about half of the animals (Mulders et al., 2014b; Robertson et al., 2013), so it would be unlikely that the animals at 2 weeks post-trauma have tinnitus and not all animals would have tinnitus at the 13 week time-point. Further studies are required to investigate the mechanism by which the PF affects the activity in the auditory system after acoustic trauma and how this affects the development and maintenance of tinnitus.

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6. REFERENCES

Axelsson, A., Ringdahl, A. 1989. Tinnitus - a study of its prevalence and characteristics. British Journal of Audiology 23, 53-62.

Azizi, S.A., Woodward, D.J. 1990. Interactions of visual and auditory mossy fiber inputs in the paraflocculus of the rat: a gating action of multimodal inputs. Brain Research 533, 255-262.

Azizi, S.A., Burne, R.A., Woodward, D.J. 1985. The auditory corticopontocerebellar projection in the rat: inputs to the paraflocculus and midvermis. An anatomical and physiological study. Experimental Brain Research 59, 36-49.

Bagnall, M.W., Zingg, B., Sakatos, A., Moghadam, S.H., Zeilhofer, H.U., du Lac, S. 2009. Glycinergic projection neurons of the cerebellum. The Journal of Neuroscience 29, 10104-10110.

Bauer, C.A., Kurt, W., Sybert, L.T., Brozoski, T.J. 2013a. The cerebellum as a novel tinnitus generator. Hearing Research 295, 130-139.

Bauer, C.A., Wisner, K.W., Baizer, J.S., Brozoski, T.J. 2013b. Tinnitus, unipolar brush cells, and cerebellar glutamatergic function in an animal model. PLoS ONE 8, e64726.

Brozoski, T.J., Bauer, C.A., Caspary, D.M. 2002. Elevated Fusiform Cell Activity in the Dorsal Cochlear Nucleus of Chinchillas with Psychophysical Evidence of Tinnitus. The Journal of Neuroscience 22, 2383-2390.

Brozoski, T.J., Ciobanu, L., Bauer, C.A. 2007. Central neural activity in rats with tinnitus evaluated with manganese-enhanced magnetic resonance imaging (MEMRI). Hearing Research 228, 168-179.

Brozoski, T.J., Wisner, K.W., Odintsov, B., Bauer, C.A. 2013. Local NMDA Receptor Blockade Attenuates Chronic Tinnitus and Associated Brain Activity in an Animal Model. PLoS ONE 8, e77674.

Burne, R., Mihailoff, G., Woodward, D. 1978. Visual corticopontine input to the paraflocculus: a combined autoradiographic and horseradish peroxidase study. Brain Research 143, 139-146.

Cesa, R., Morando, L., Strata, P. 2005. Purkinje cell spinogenesis during architectural rewiring in the mature cerebellum. European Journal of Neuroscience 22, 579-586.

Dong, S., Mulders, W.H.A.M., Rodger, J., Robertson, D. 2009. Changes in neuronal activity and gene expression in guinea-pig auditory brainstem after unilateral partial hearing loss. Neuroscience 159, 1164-1174.

Eggermont, J.J. 2005. Tinnitus: neurobiological substrates. Drug Discovery Today 10, 1283-1290.

Eggermont, J.J., Roberts, L.E. 2004. The neuroscience of tinnitus. Trends in Neurosciences 27, 676-682.

Finlayson, P., Kaltenbach, J.A. 2009. Alterations in the spontaneous discharge patterns of single units in the dorsal cochlear nucleus following intense sound exposure. Hearing Research 256, 104-117.

Gayer, N.S., Faull, R.L.M. 1988. Connections of the paraflocculus of the cerebellum with the superior colliculus in the rat brain. Brain Research 449, 253-270.

Gramsbergen, A. 2007. Neural compensation after early lesions: a clinical view of animal experiments. Neuroscience & Biobehavioral Reviews 31, 1088-1094.

Halverson, H.E., Lee, I., Freeman, J.H. 2010. Associative plasticity in the medial auditory thalamus and cerebellar interpositus nucleus during eyeblink conditioning. The Journal of Neuroscience 30, 8787-8796.

Huang, C.-M., Liu, G., Huang, R. 1982. Projections from the cochlear nucleus to the cerebellum. Brain Research 244, 1-8.
Johnstone, J.R., Alder, V.A., Johnstone, B.M., Robertson, D., Yates, G.K. 1979. Cochlear action potential threshold and single unit thresholds. The Journal of the Acoustical Society of America 65, 254-7.
Kalappa, B.I., Brozoski, T.J., Turner, J.G., Caspary, D.M. 2014. Single unit hyperactivity and bursting in the auditory thalamus of awake rats directly correlates with behavioural evidence of tinnitus. The Journal of Physiology 592, 5065-5078.
Kaltenbach, J.A., Afman, C.E. 2000. Hyperactivity in the dorsal cochlear nucleus after intense sound exposure and its resemblance to tone-evoked activity: a physiological model for tinnitus. Hearing Research 140, 165-172.
Lisberger, S., Pavelko, T., Broussard, D. 1994. Neural Basis for Motor Learning in the Vestibuloocular Reflex of Primates I. Changes in the Responses of Brain Stem Neurons. Journal of Neurophysiology 72, 928-953.
Mapelli, L., Pagani, M., Garrido, J.A., D'Angelo, E. 2015. Integrated plasticity at inhibitory and excitatory synapses in the cerebellar circuit. Frontiers in Cellular Neuroscience 9.
Merrill, E.G., Ainsworth, A. 1972. Glass-coated platinum-plated tungsten microelectrodes. Med. & biol. Engng. 10, 662-672.
Miller, D.M., Cotter, L.A., Gandhi, N.J., Schor, R.H., Huff, N.O., Raj, S.G., Shulman, J.A., Yates, B.J. 2008. Responses of rostral fastigial nucleus neurons of conscious cats to rotations in vertical planes. Neuroscience 155, 317-325.
Morest, D.K., Kim, J., Bohne, B.A. 1997. Neuronal and transneuronal degeneration of auditory axons in the brainstem after cochlear lesions in the chinchilla: cochleotopic and non-cochleotopic patterns. Hearing Research 103, 151-168.
Mulders, W., Robertson, D. 2013. Development of hyperactivity after acoustic trauma in the guinea pig inferior colliculus. Hearing Research 298, 104-108.
Mulders, W.H., Rodger, J., Yates, C.G., Robertson, D. 2014a. Modulation of gene expression in guinea pig paraflocculus after induction of hearing loss. F1000Research 3.
Mulders, W.H.A.M., Robertson, D. 2009. Hyperactivity in the auditory midbrain after acoustic trauma: dependence on cochlear activity. Neuroscience 164, 733-746.
Mulders, W.H.A.M., Robertson, D. 2011a. Progressive centralization of midbrain hyperactivity after acoustic trauma. Neuroscience 192, 753-760.
Mulders, W.H.A.M., Barry, K.M., Robertson, D. 2014b. Effects of Furosemide on Cochlear Neural Activity, Central Hyperactivity and Behavioural Tinnitus after Cochlear Trauma in Guinea Pig. PLoS ONE 9, e97948.
Mulders, W.H.A.M., Ding, D., Salvi, R., Robertson, D. 2011b. Relationship between auditory thresholds, central spontaneous activity, and hair cell loss after acoustic trauma. The Journal of Comparative Neurology 519, 2637-2647.
Nagao, S., Kitamura, T., Nakamura, N., Hiramatsu, T., Yamada, J. 1997. Location of efferent terminals of the primate flocculus and ventral paraflocculus revealed by anterograde axonal transport methods. Neuroscience research 27, 257-269.
Noreña, A.J., Moffat, G., Blanc, J.L., Pezard, L., Cazals, Y. 2010. Neural changes in the auditory cortex of awake guinea pigs after two tinnitus inducers: salicylate and acoustic trauma. Neuroscience 166, 1194-1209.
Petacchi, A., Laird, A.R., Fox, P.T., Bower, J.M. 2005. Cerebellum and auditory function: An ALE meta-analysis of functional neuroimaging studies. Human Brain Mapping 25, 118-128.
Rambold, H., Churchland, A., Selig, Y., Jasmin, L., Lisberger, S. 2002. Partial ablations of the flocculus and ventral paraflocculus in monkeys cause linked deficits in smooth

pursuit eye movements and adaptive modification of the VOR. Journal of Neurophysiology 87, 912-924.
Robertson, D., Bester, C., Vogler, D., Mulders, W.H.A.M. 2013. Spontaneous hyperactivity in the auditory midbrain: Relationship to afferent input. Hearing Research 295, 124-129.
Seki, S., Eggermont, J.J. 2003. Changes in spontaneous firing rate and neural synchrony in cat primary auditory cortex after localized tone-induced hearing loss. Hearing Research 180, 28-38.
Stone, L., Lisberger, S. 1990. Visual responses of Purkinje cells in the cerebellar flocculus during smooth-pursuit eye movements in monkeys. I. Simple spikes. Journal of Neurophysiology 63, 1241-1261.
Sun, D., Sun, X., Jen, P.H.-S. 1990. The influence of the auditory cortex on acoustically evoked cerebellar responses in the CF-FM bat, Rhinolophus pearsonic chinesis. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 166, 477-487.
Vogler, D.P., Robertson, D., Mulders, W.H.A.M. 2011. Hyperactivity in the Ventral Cochlear Nucleus after Cochlear Trauma. The Journal of Neuroscience 31, 6639-6645.
Vogler, D.P., Robertson, D., Mulders, W.H.A.M. 2014. Hyperactivity following unilateral hearing loss in characterized cells in the inferior colliculus. Neuroscience 265, 28-36.
Zimny, R., Sobusiak, T., Deja, A., Kotecki, A. 1981. Cerebellar afferent projection to the inferior colliculus of the mesencephalic tectum. An anterograde degeneration study in the rabbit. Zeitschrift fur mikroskopisch-anatomische Forschung 95, 197-209.

FIGURE LEGENDS

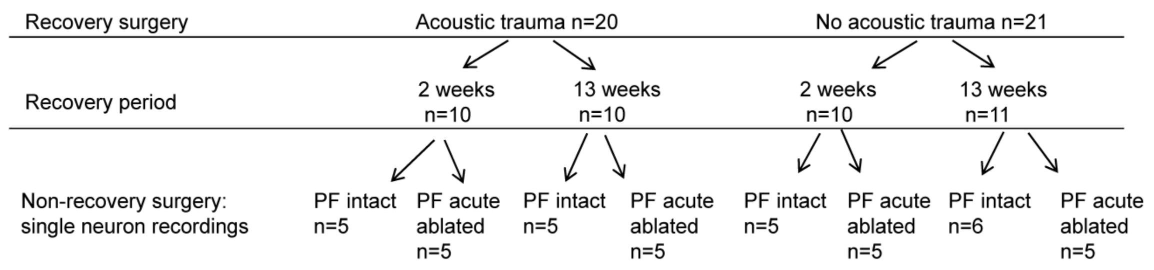
Figure 1: Flow diagram showing the experimental design of this study. Animals were first exposed to surgery to measure CAP thresholds and half of the animals then received acoustic trauma and the other ones did not (no acoustic trauma). Animals were then allowed to recover for either 2 or 13 weeks at which time-point a final electrophysiological experiment was performed during which the SFRs of neurons in the IC were recorded. In each group PF was ablated in half of the animals (PF-) and not ablated (left intact) in the other half (PF+). This resulted in a total of 8 groups to be analysed.

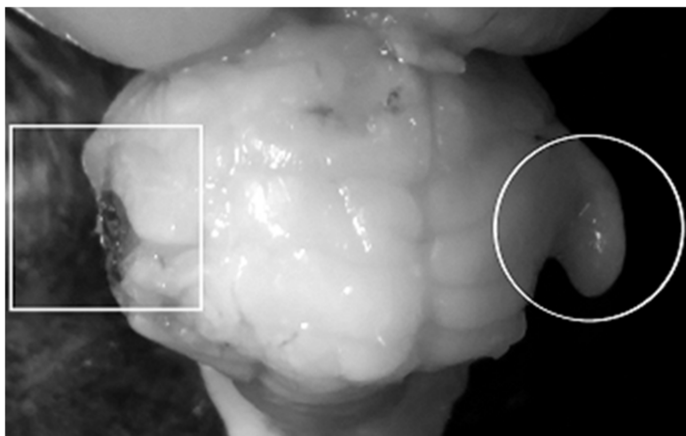
Figure 2: Photograph of guinea pig cerebellum showing the left paraflocculus removed (white square). The white circle shows the intact contralateral paraflocculus.

Figure 3: Changes in cochlear sensitivity measured as CAP thresholds in no acoustic trauma (left column) and acoustic trauma animals (right column) after 2 weeks (A-D) and 13 weeks recovery times (E-H). A, B, E and F show data from individual animals and, C, D, G and H show the average CAP threshold loss of the different groups with and without paraflocculus removal (mean ± SEM). The dotted line marks no threshold loss. Statistical significance indicated in Results.

Figure 4: Mean spontaneous firing rates (SFR) ± SEM of (A) no acoustic trauma and (B) trauma animals after 2 and 13 weeks recovery times showing the comparison between paraflocculus left intact (+PF; white bars) or paraflocculus removed (-PF; black bars). Numbers of neurons in each group are shown in the histogram. * $p < 0.05$, *** $p < 0.001$.

Figure 5: Mean spontaneous firing rates (SFR) ± SEM for neurons with nominal CFs <8 kHz and >10 kHz when PF was intact (+PF; white bars) or removed (-PF; black bars) in no acoustic trauma animals after (A) 2 weeks and (B) 13 weeks recovery periods and in trauma animals after (C) 2 weeks and (D) 13 weeks recovery periods. Numbers of neurons in each group are shown in the histogram. *** $p < 0.001$.

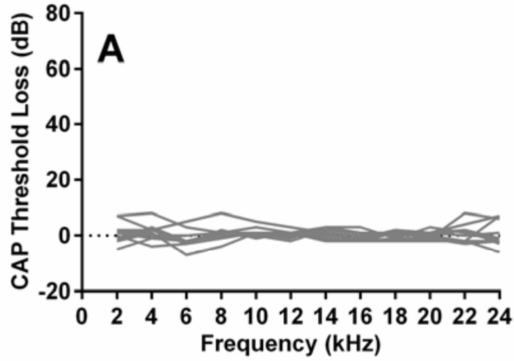

Recovery surgery
Acoustic trauma n=20
No acoustic trauma n=21
Recovery period
2 weeks n=10
13 weeks n=10
2 weeks n=10
13 weeks n=11
Non-recovery surgery: single neuron recordings
PF intact n=5
PF acute ablated n=5
PF intact n=5
PF acute ablated n=5
PF intact n=5
PF acute ablated n=5
PF intact n=6
PF acute ablated n=5

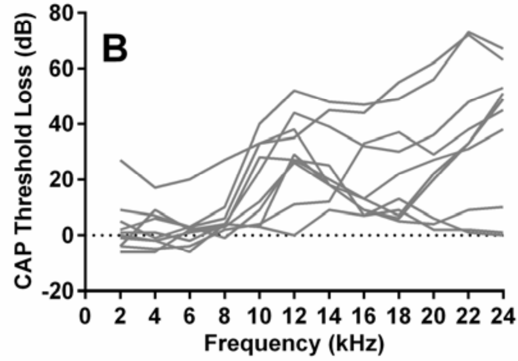


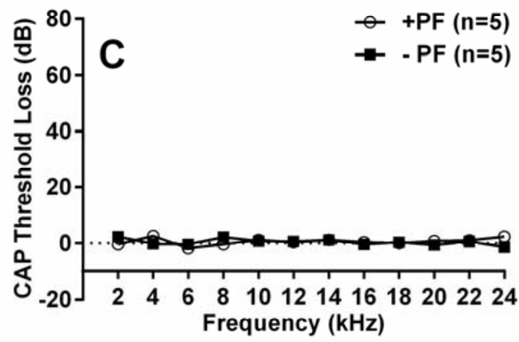
No Trauma

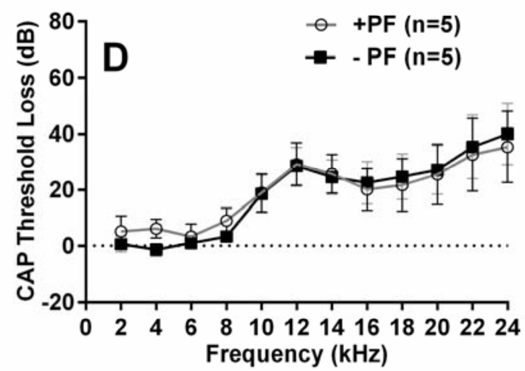
Trauma

2 weeks

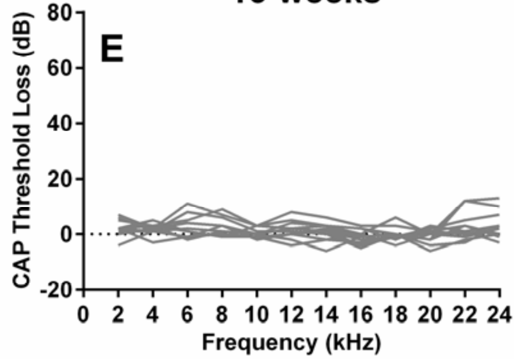


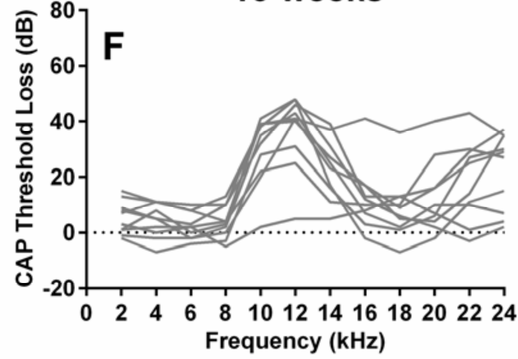


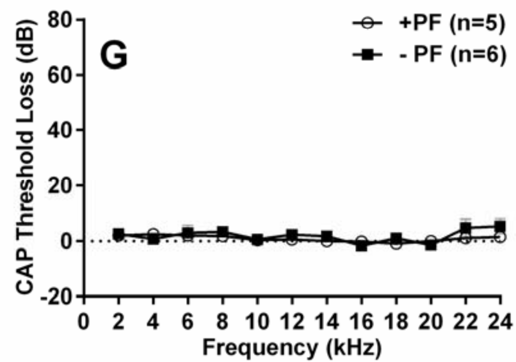


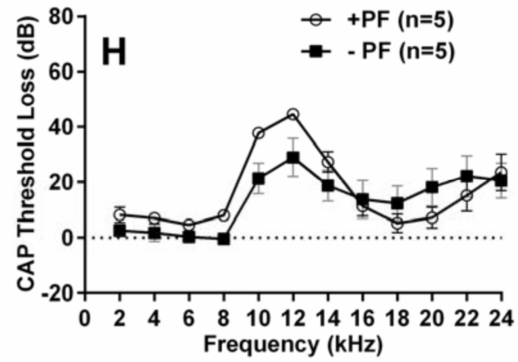


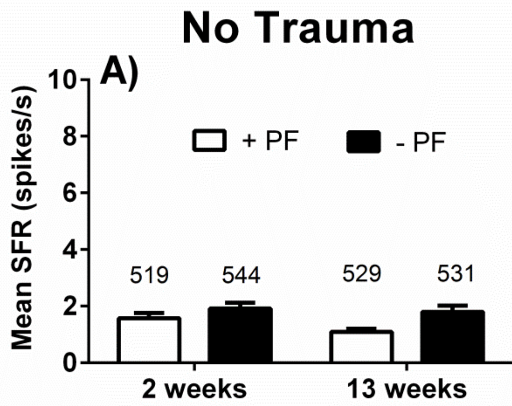
13 weeks

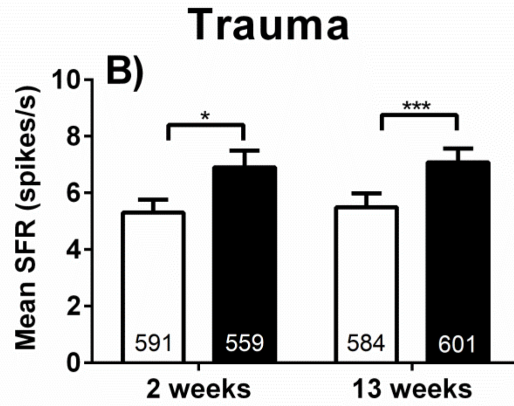


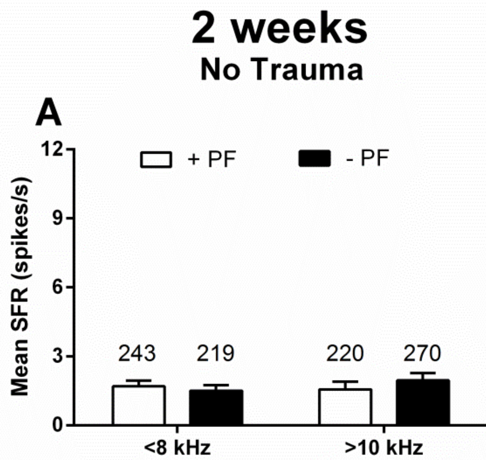


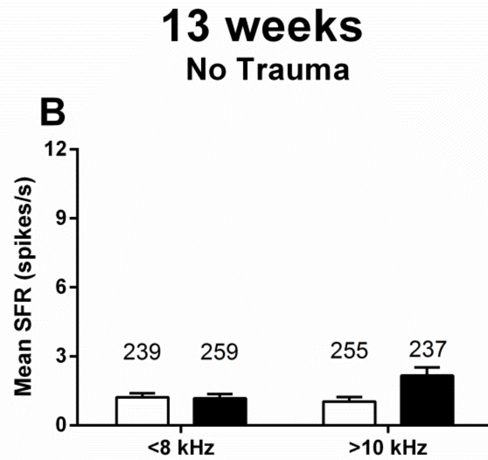


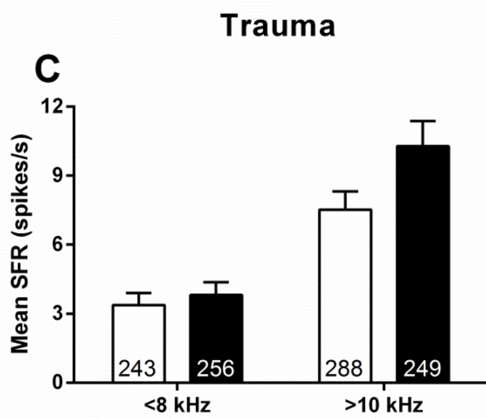


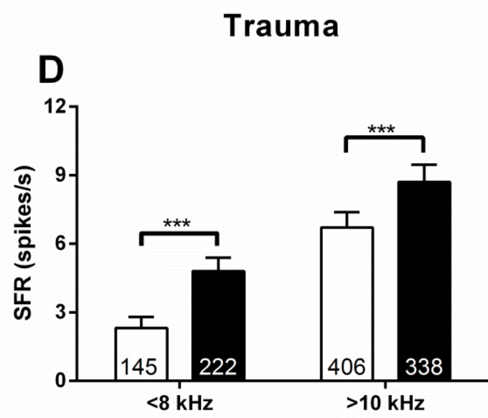

No Trauma
A)
Mean SFR (spikes/s)
+ PF
- PF
519
544
529
531
2 weeks
13 weeks


Trauma
B)
Mean SFR (spikes/s)
*
***
591
559
584
601
2 weeks
13 weeks


2 weeks
No Trauma
A
Mean SFR (spikes/s)
+ PF
- PF
243
219
220
270
<8 kHz
>10 kHz


13 weeks
No Trauma
B
Mean SFR (spikes/s)
239
259
255
237
<8 kHz
>10 kHz


Trauma
C
Mean SFR (spikes/s)
243
256
288
249
<8 kHz
>10 kHz


Trauma
D
SFR (spikes/s)
***
***
145
222
406
338
<8 kHz
>10 kHz