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RESEARCH ARTICLE

Low-intensity repetitive transcranial magnetic stimulation over prefrontal cortex in an animal model alters activity in the auditory thalamus but does not affect behavioural measures of tinnitus

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Abstract

Tinnitus, a phantom auditory percept, is strongly associated with cochlear trauma. The latter leads to central changes in auditory pathways such as increased spontaneous activity and this may be involved in tinnitus generation. As not all people with cochlear trauma develop tinnitus, recent studies argue that non-auditory structures, such as prefrontal cortex (PFC), play an important role in tinnitus development. As part of sensory gating circuitry, PFC may modify activity in auditory thalamus and consequently in auditory cortex. Human studies suggest that repetitive transcranial magnetic stimulation (rTMS), a non-invasive tool for neurostimulation, can alter tinnitus perception. This study used a guinea pig model of hearing loss and tinnitus to investigate effects of low-intensity rTMS (LI-rTMS) over PFC on tinnitus and spontaneous activity in auditory thalamus. In addition, immunohistochemistry for calbindin and parvalbumin in PFC was used to investigate the possible mechanism of action of LI-rTMS. Three treatment groups were compared: sham treatment, LI, low frequency (1 Hz) or LI, high frequency (10 Hz) rTMS (10 min/day, 2 weeks, weekdays only). None of the treatments affected the behavioural measures of tinnitus but spontaneous activity was significantly increased in auditory thalamus after 1 Hz and 10 Hz treatment. Immunostaining showed significant effects of rTMS on the density of calcium-binding protein expressing neurons in the dorsal regions of the PFC suggesting that rTMS treatment evoked plasticity in cortex. In addition, calbindin-positive neuron density in the superficial region of PFC was negatively correlated with spontaneous activity in auditory thalamus suggesting a possible mechanism for change in activity observed.

Keywords Tinnitus · Compound action potential · Gap prepulse inhibition · Guinea pig · Audiogram

Abbreviations

CAP	Compound action potential
ECG	Electrocardiogram
GPIAS	Gap prepulse inhibition of acoustic startle
i.p.	Intraperitoneal
i.m.	Intramuscular
LI	Low intensity
MGN	Medial geniculate nucleus
PB	Phosphate buffer
PFC	Prefrontal cortex
PPI	Prepulse inhibition
rTMS	Repetitive transcranial magnetic stimulation
s.c.	Subcutaneous
SPL	Sound pressure level

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Introduction

Tinnitus is phantom auditory percept, described as a ringing of the ears, a buzzing, roaring, hissing or uncomfortable tone in the absence of physical stimulus (Axelsson and Ringdahl 1989). Many people will experience brief periods of tinnitus

at some point in their life but for approximately 10–15% of the population, it is a chronic sensation (Hoffman and Reed 2004; Bhatt et al. 2016). For 1–3% of these individuals with chronic tinnitus, the sensation is so severely uncomfortable that it causes sleep disturbance, stress, anxiety, depression, and suicidal ideation (Bhatt et al. 2017; Aazh and Moore 2018). There is currently no cure for tinnitus, however there are many treatment options, with the most successful being behavioural therapies aimed at alleviating the distress associated with tinnitus rather than the perception itself (Jastreboff 2007; Fioretti et al. 2011; Baguley et al. 2013).

The neural mechanisms underlying tinnitus are not yet completely understood, which is likely to contribute to the lack of successful treatments. Nonetheless, it is generally agreed that in response to cochlear trauma, plasticity-driven changes occur in the central auditory pathway, which lead to the perception of tinnitus (Eggermont and Roberts 2004, 2015). This is supported by human imaging and EEG studies showing abnormal neural activity in the auditory pathway in tinnitus patients (Melcher et al. 2000, 2009; Gu et al. 2010; Moazami-Goudarzi et al. 2010). Electrophysiological studies in animal models support the notion of plasticity as they show an increase in spontaneous firing rates (hyperactivity) in auditory brainstem after acoustic trauma (Kaltenbach and Afman 2000; Mulders and Robertson 2009; Vogler et al. 2011). In addition, animals with tinnitus after an acoustic trauma show hyperactivity at the level of the thalamus and auditory cortex compared to animals without tinnitus (Kalappa et al. 2014; Basura et al. 2015).

However, though cochlear damage reliably leads to changes in central auditory neuronal activity (Kaltenbach and Afman 2000; Mulders and Robertson 2009; Vogler et al. 2011), only 20–40% of individuals with hearing damage develop tinnitus (Axelsson and Ringdahl 1989; Hoffman and Reed 2004). In addition, non-auditory factors have been linked to tinnitus generation, such as pre-existing stress and anxiety (Folmer et al. 2001; Hinton et al. 2006; Gordon et al. 2016). This association between emotional state and tinnitus has led to the suggestion that dysfunctional sensory gating at the level of the auditory thalamus, the medial geniculate nucleus (MGN), is an important element of tinnitus development (Rauschecker et al. 2010, 2015; Zhang 2013; De Ridder et al. 2014, 2015).

Sensory gating involves the filtering of unnecessary sensory information, preventing it from reaching cortex and perception (Adler et al. 1998; Rauschecker et al. 2015). In the MGN this is thought to occur through inhibitory inputs from the thalamic reticular nucleus (TRN) (Pinault 2004), which, in turn, is modulated by inputs from the limbic system and prefrontal cortex (PFC) (Zikopoulos and Barbas 2006, 2007; Barbas et al. 2011; Cai and Caspary 2015; John et al. 2016). Indeed, the PFC has been proposed to control sensory information en route to cortex regulating attention and suppressing irrelevant signals (Barbas and Zikopoulos 2007; Zikopoulos and Barbas 2012). In agreement, human studies in tinnitus patients have shown that modulating the activity of the PFC using non-invasive methods such as direct current stimulation or repetitive transcranial magnetic stimulation (rTMS), can alleviate tinnitus loudness and distress (Vanneste and De Ridder 2011; De Ridder et al. 2013). However, these human studies cannot elucidate whether any beneficial effects on auditory cortex are due to direct effects from PFC, or indirect via the MGN.

Animal models provide an excellent opportunity to investigate the underlying neural substrate of tinnitus as it is possible to record neuronal activity at the single neuron level from deep structures such as MGN and measure markers of plasticity post-mortem. We therefore studied the effect of low-intensity rTMS (LI-rTMS) at low and high frequency applied over the PFC in guinea pigs with tinnitus using an animal specific coil, custom-built in our laboratory (Tang et al. 2015a, 2016b; Mulders et al. 2016). We have previously shown that LI-rTMS applied over auditory cortex was successful in attenuating tinnitus (Mulders et al. 2016), demonstrating the efficacy of LI stimulation in altering cortical excitability. We hypothesised that LI-rTMS would reduce behavioural signs of tinnitus and change the spontaneous neural activity in MGN. In addition, in order to shed light on the possible mechanism by which rTMS alters neural activity, we used immunohistochemical analysis of the calcium-binding proteins (CBPs), parvalbumin and calbindin, as previous studies have shown changes in neuronal densities of these proteins following rTMS (Benali et al. 2011; Mix et al. 2014).

General methods

Animals

These experiments and procedures conformed to the National Health and Medical Research Council's Australian code for the care and use of animals for scientific purposes and were approved by the Animal Ethics Committee of the University of Western Australia (UWA) (RA/3/100/1458). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Twenty-four tri-colour guinea pigs (Cavia porcellus) of both sexes (15 males and 9 females) were used. They were sourced from UWA bred colonies and had all passed baseline prepulse inhibition (PPI) and gap prepulse inhibition of acoustic startle (GPIAS) testing before acoustic trauma (see "Gap prepulse inhibition of acoustic startle" below for details). Animals weighed between 300 and 715 g (Mean ± SEM: 448 ± 23 g) at the time of acoustic trauma.

The experimental design involved first baseline behavioural assessment of PPI and GPIAS. This was followed by recovery surgery during which a unilateral acoustic trauma was performed. Following recovery, behavioural tests (PPI and GPIAS) were performed weekly to assess the development of tinnitus as described previously by us and others (Turner et al. 2006; Dehmel et al. 2012; Mulders et al. 2016; Leggett et al. 2018). Once guinea pigs presented with behavioural signs of tinnitus they were then treated with either low frequency (1 Hz) LI-rTMS, high frequency (10 Hz) LI-rTMS, or sham rTMS over prefrontal cortex contralateral to the acoustic trauma ear. Following 2 weeks of treatment, the behavioural tests were repeated to assess possible changes in the behavioural signs of tinnitus. This was followed by a final electrophysiological experiment during which recordings of the spontaneous firing rate of single neurons were made in the MGN of the thalamus. Animals that did not develop tinnitus were not included in further data analysis. Following the electrophysiological recordings, all animals were processed for immunohistochemical analysis of parvalbumin and calbindin in prefrontal cortex.

Gap prepulse inhibition of acoustic startle

Behavioural testing took place in a dark soundproof room. Guinea pigs were tested in batches of 4 or less, and were never mixed with guinea pigs outside their initial group. Guinea pigs were mildly restrained in polycarbon animal holders and placed on force transducing platforms. Animals were allowed to acclimatise for 5 min before each test. In order to minimise possible habituation, guinea pigs were never tested on consecutive days.

Prepulse inhibition

The PPI test consisted of 50 trials each lasting 20–30 s. Each trial contained a startle stimulus presentation (1 kHz, 0.5 kHz bandwidth; 106 dB SPL; 50 ms duration) embedded in silence. Twenty-five trials contained a prepulse (50 ms duration; starting 100 ms before startle stimulus presentation). Each test presented the prepulses as a narrowband noise at either 8 kHz (10 dB bandwidth 2.2 kHz) or 14 kHz (10 dB bandwidth 1.6 kHz). The remaining 25 trials were "non-prepulse" trials; trials presented with only the startle stimulus. The order of prepulse and non-prepulse trials was randomised. A single prepulse intensity was used for each test (66, 70 or 73 dB SPL). A startle speaker generated the startle stimulus (Radio Shack 401278B) and prepulses were generated as a narrowband noise by a separate speaker (Beyer DT 48).

Gap prepulse inhibition of acoustic startle reflex

The GPIAS consisted of 50 trials (length 20–30 s). Each trial contained a startle stimulus presentation that was the same as in the PPI test, embedded in a constant wide band background noise. Background noise was produced with the same characteristics as the prepulses in the PPI test. Twenty-five trials contained a small section of silence, a "gap", in the background sound starting 100 ms before startle stimulus presentation (50 ms duration). The other 25 trials presented no gap in the background sound, "no gap" trials. The order of gap and no gap trials within the GPIAS was randomised. The background noise was generated by the same speaker used to generate prepulses in the PPI test.

Analysis of behavioural data

Baseline movement was measured from 0.35 to 0.1 s before startle stimulus. Startle response was measured from time 0 to 0.25 s after startle stimulus. Custom software (Nathaneal Yates) produced the root mean square (RMS) of force produced during the baseline response and startle response. The force of the startle response was calculated by dividing the startle RMS by baseline RMS for each trial, producing a startle response ratio. The mean startle response ratio of prepulse trials was compared with non-prepulse (for PPI), or gap trials with non-gap trials (for GPIAS). Data were not normally distributed and therefore required non-parametric statistical analysis (Mann–Whitney *U* test). An animal was considered to "pass" if there was a significant difference ($p < 0.05$) between prepulse/no prepulse or gap/no gap trials, and "fail" is this condition was not met. The first four trials were excluded to avoid habituation bias. Before acoustic trauma, guinea pigs were required to pass the PPI test once and the GPIAS paradigm twice to indicate stable and normal baseline measures.

One week following the acoustic trauma guinea pigs were again assessed using the PPI test. A pass of the PPI test indicated that any existing hearing loss did not affect detection of the prepulse and hence background noise for GPIAS tests. Following a pass of a PPI test, guinea pigs underwent weekly GPIAS testing. If an animal failed GPIAS, the test was repeated 2 days later. If the animal passed, it would return to weekly testing, if it failed GPIAS again they were randomly allocated to one of the rTMS treatment groups. Treatment ("LI-rTMS treatment") took place over 2 weeks, Monday to Friday. The morning after the last treatment (Day 15 since beginning of treatment) animals underwent a GPIAS test once more before being anaesthetised for the final electrophysiological experiment ("Electrophysiological recordings in medial geniculate nucleus").

Acoustic trauma surgery

For anaesthesia 0.1 ml atropine (subcutaneous; s.c.) and diazepam (5 mg/kg, 25 ml; intraperitoneal; i.p.) were administered, which was followed 20 min later by an intramuscular (i.m.) injection with 1 ml/kg Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone). Lignocaine was injected s.c. at incision site (0.1 ml). When foot withdrawal reflex returned during acoustic trauma a one-third of the original Hypnorm dose was administered.

Once surgical anaesthesia was obtained, incision site was shaved and animals were placed on a heated platform with a blanket in a soundproof room and mounted between hollow ear bars. An incision was made to expose the left bulla and a small hole was made to expose the cochlea. An insulated silver recording wire was place on the round window to measure the compound action potential (CAP) thresholds. Pure tone stimuli (10 ms duration, 4/s, frequency range 4–24 kHz) were created in a closed sound system using a ½ in. condenser microphone driven in reverse (Bruel and Kjaer, type 4134). CAP signals were recorded using a custom-made computer program (sample rate 96 kHz, Neurosound; MI Lloyd). CAP signals were amplified (1000×), filtered (100 Hz–3 kHz bandpass) and recorded (Powerlab 4SP, AD instruments). Then a unilateral acoustic trauma (left ear; 10 kHz, 120 dB SPL, 2 h) was performed, with the contralateral ear blocked with plasticine. After acoustic trauma, another CAP audiogram was measured, the incision was sutured and animals were allowed to recover.

LI-rTMS treatment

LI-rTMS was delivered as described in our previous study (Mulders et al. 2016). A custom-made circular rTMS coil (Tang et al. 2015c, 2016b) was used in this study with a 8-mm outer diameter, 780 turns of insulated copper wire (0.125 mm diameter) around an air core, generating a peak magnetic field strength of 90 mT. rTMS pulses were generated by an Agilent 33500B series waveform generator, connected to a TEKNET bipolar operational power supply and amplifier.

Guinea pigs that developed behavioural signs of tinnitus ("Analysis of behavioural data") were treated with the rTMS coil placed over the right prefrontal cortex [coordinates 14 mm rostral to inter-aural line and 1 mm off the midline, based on the guinea pig atlas (Rapisarda and Bacchelli 1977)]. Positioning of the coil and the extent of the magnetic field is illustrated in Fig. 1. Guinea pigs were treated with 1 Hz biphasic, LI-rTMS (90 mT), 10 Hz biphasic LI-rTMS or sham rTMS for 10 min per day, for 2 weeks on weekdays only (Monday to Friday). The 1 Hz and 10 Hz stimulation paradigms were selected based on previous data showing excitatory effects of high frequency (> 5 Hz) and inhibitory

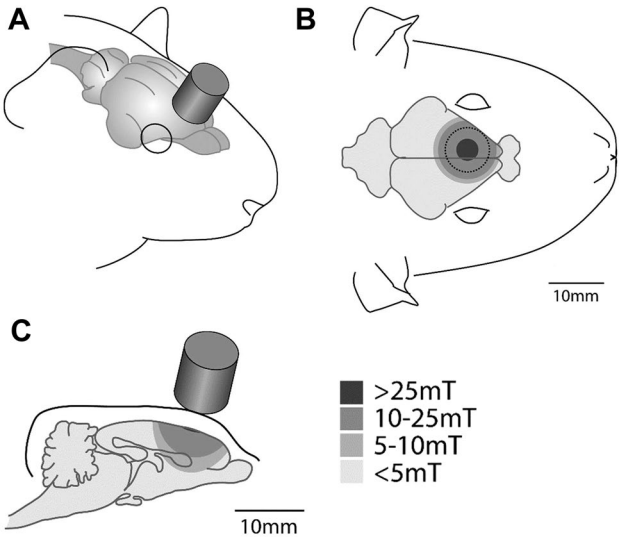

Fig. 1 Schematic drawings showing coil position and magnetic field in **a** 3D view; **b** bird's eye view and **c** sagittal view. Legend shows different magnetic field strengths at different distances from the coil based on previous published data on these coils (Tang et al. 2015a, 2016b)

effects of low frequency (1 Hz) (Wassermann and Zimmermann 2012; Tang et al. 2015b; Heath et al. 2018). The high frequency (10 Hz) rTMS resulted in a heating up of the coil and therefore sessions were separated in smaller periods (3 × 200 s with 100 s intervals) to prevent overheating.

High temperatures generated in the coil by high-frequency rTMS treatment is due to increased thermal stress in the small coil while preserving a maximum intensity of stimulation (Tang et al. 2015c, 2016b). Sham treatment used the same coil but with the coil disconnected from the power supply. In our previous publication on the use of this coil in the tinnitus model, we have calibrated the sound intensity produced by the coil and have found this to be very low (20–30 dB SPL) and hence unlikely to affect outcomes (Mulders et al. 2016).

Electrophysiological recordings in medial geniculate nucleus

For non-recovery anaesthesia, injections of i.p. pentobarbitone sodium (30 mg/kg) and sc. atropine (0.1 ml) were administered, followed 10 min later by an i.m. injection with Hypnorm (0.2 ml). Lignocaine (s.c.) was used as local anaesthetic in all incision sites. Depth of anaesthesia was assessed using the foot withdrawal reflex. Anaesthesia was maintained with 0.15 ml Hypnorm every hour and a half dose of pentobarbitone every two hours. After full anaesthesia was confirmed, a tracheotomy was performed and the animal was artificially ventilated on carbogen (95% O_2, 5% CO_2). The guinea pig was then placed on a heated

platform in a soundproof room and mounted in hollow ear bars. Bilateral CAP audiograms were measured using the method described above ("Acoustic trauma surgery").

Then a small craniotomy was performed over the cortex overlying the right medial geniculate nucleus (MGN), contralateral to the acoustic trauma ear (Rapisarda and Bacchelli 1977). The right ear was blocked with plasticine. A glass insulated tungsten microelectrode was advanced along the dorso-ventral axis through the cortex to the MGN. Exposed cortex was covered with 5% agar in saline to improve stability of electrode and prevent dehydration of neural tissue. Entry into MGN was indicated by presence of a sound-evoked cluster, approximately 6–7 mm from cortical surface. Single neuron characteristic frequency (CF) and threshold were determined audio-visually using the Neurosound software and the spontaneous firing rate of the neurons was measured during a 10 s period. Single neuron recordings continued until at least 40 neurons were recorded or until the interval between QRS complexes of the ECG (ECG interval) extended > 300 ms. We have shown previously (Cook et al. manuscript submitted) that spontaneous firing rates in MGN significantly decrease with ECG intervals > 300 ms.

Immunocytochemical analysis

At the end of the experiment animals were euthanised with Lethabarb (0.4 ml i.p.) followed by an intracardiac perfusion with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, post-fixed overnight in the same fixative and then immersed for at least 24 h in 30% sucrose in (PB), followed by sectioning (serial sections, 60 µm, 7 series) on a freezing microtome (Thermo Fisher Scientific microtome (Microm HM 420) with a temperature regulated platform (Physitemp, BFC -40MPA Controller). One series of each brain was mounted immediately following sectioning on gelatine-coated slides, dried overnight and stained with toluidine blue for reference purposes.

Two series were used for immunohistochemistry, one for parvalbumin and one for calbindin, which meant that one in every seven sections was stained for each protein. Sections were first incubated for 10 min in 3% H_2O_2 in methanol to eliminate endogenous peroxidase. In between each step of the protocol, sections were rinsed 3 × 15 min in PB. Sections were preincubated for 30 min in PB containing 0.1% BSA and 0.3% Triton X-100 and 5% donkey serum (blocking solution), followed by incubation overnight at 4 °C in either mouse-anti-calbindin (1:500 Sigma-Aldrich) or mouse-anti-parvalbumin (1:500 Sigma-Aldrich) in the blocking solution. The next day sections were incubated for 90 min in the blocking solution containing donkey anti-mouse (1:500 Chemicon), followed by a 2 h incubation in ABC (Vector ABC kit 1:800 in 0.1 M PB). Finally, sections were developed with 0.02% DAB in 0.05 M Tris-buffer (pH 7.6), mounted on gelatine-coated slides and dried overnight. The next day slides were dehydrated, cleared and coverslipped using Entellan.

Immuno-reactive neurons were counted in every cortical section (7–9 sections in total) of the PFC in the stained series using imaging software (ImageJ). In each cortical section (both left and right) 4 regions of interest (ROIs) were utilised for neuron quantification, including two dorsal ROIs (ROI 1–2) and two ventral ROIs (ROI 3–4) (Fig. 2). ROI 1 counting frame area was 1.15 mm^2 (10× objective), and ROIs 2–4 counting frame area was 0.29 mm^2 (20× objective). Frames were chosen in order to sample from superficial and deep layers of cortex, without frame overlap and to ensure equivalent placement of counting frames in each animal.

Images were captured on the NIS-elements software and immuno-reactive neurons were counted within a counting frame using the ImageJ software. Immuno-reactive neurons were characterised by a clearly stained soma and the presence of at least 2 visible dendrites. Cell counting was conducted blind to treatment. The mean density per mm^2 was calculated for each treatment group and used for statistical analysis.

Statistical analysis

GPIAS data were analysed using a two-way ANOVA with repeated measures followed by Sidak's multiple comparison tests. One-way ANOVA was used for CAP threshold loss data. Spontaneous firing rate data was non-parametric (D'Agostino–Pearson omnibus normality test) and therefore

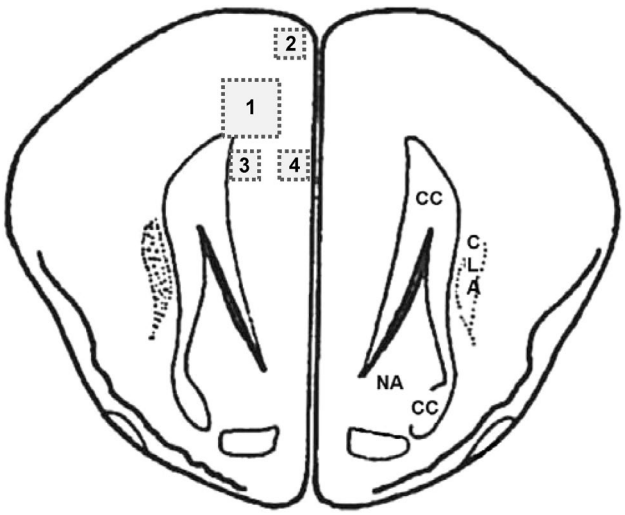

Fig. 2 Representative drawing of a transverse guinea pig section containing the PFC based on the atlas of Rapisarda and Bacchelli (1977), showing the regions of interest (1–4, light grey shaded) for counting of parvalbumin and calbindin-stained neurons. *CC* corpus callosum, *CLA* claustrum, *NA* nucleus accumbens

analysed using a Kruskal–Wallis test with post hoc Dunn's multiple comparisons. Neuronal density was analysed using one-way ANOVA and Sidak's multiple comparison tests.

Results

Behavioural analysis of tinnitus

All guinea pigs showed good PPI following acoustic trauma confirming that any hearing loss they had did not significantly affect the detection of the 8 kHz and 14 kHz centred bandpass noise used as prepulse (and as background noise in GPIAS test). However, 18 guinea pigs (of the 24 exposed to an acoustic trauma, 13 male and 5 female) showed a failure to pass the GPIAS test in two sessions within 1 week at either 8 kHz ($n=10$), 14 kHz ($n=7$) or at both frequencies ($n=1$). This GPIAS deficit occurred at different time-points varying between 3 and 10 weeks following acoustic trauma (mean 5 ± 0.5 weeks), and was interpreted as a behavioural sign of tinnitus. No difference was seen in time-point of development between males and females (Student's t test $p=0.2$).

The remaining 6 guinea pigs that had undergone acoustic trauma showed consistent good GPIAS (indicated as a pass) over the maximum time-frame in this study of 12 weeks following acoustic trauma and were therefore not deemed to have developed tinnitus. These animals were allocated to other experiments and are not discussed further.

The 18 animals with tinnitus were then randomly allocated to sham, 1 Hz or 10 Hz rTMS treatment, followed by electrophysiological recording. In some animals, electrophysiological recordings in MGN were not obtained as they were part of an earlier pilot study where recordings were made in the inferior colliculus. Seven animals (4 male, 3 female) underwent rTMS at 1 Hz (and recordings in MGN were collected in 6 of these), 6 at 10 Hz (5 male, 1 female) (and recordings in MGN were collected in 4 of these), and 5 (4 male, 1 female) received sham treatment (all 5 used for recordings in MGN). These data have been illustrated in a flow diagram (Fig. 3).

None of the treatments had an effect on GPIAS suppression, which indicates treatments did not affect tinnitus perception. Data are shown in Fig. 4, in which GPIAS suppression is shown at the background frequency at which the animals developed signs of tinnitus. The figure shows the GPIAS suppression in each group before trauma (BT), at the time of tinnitus development when GPIAS suppression was significantly reduced and at the end of experiment (EOE), which was day 15 after treatment commenced and the day of electrophysiological recordings. A two-way ANOVA with repeated measures revealed a significant effect of time ($p<0.0001$) but no effect of treatment. Sidak's multiple comparison tests showed significant differences in each group between before trauma and tinnitus as well as between before trauma and end of experiment, but no difference between tinnitus time-point and end of experiment.

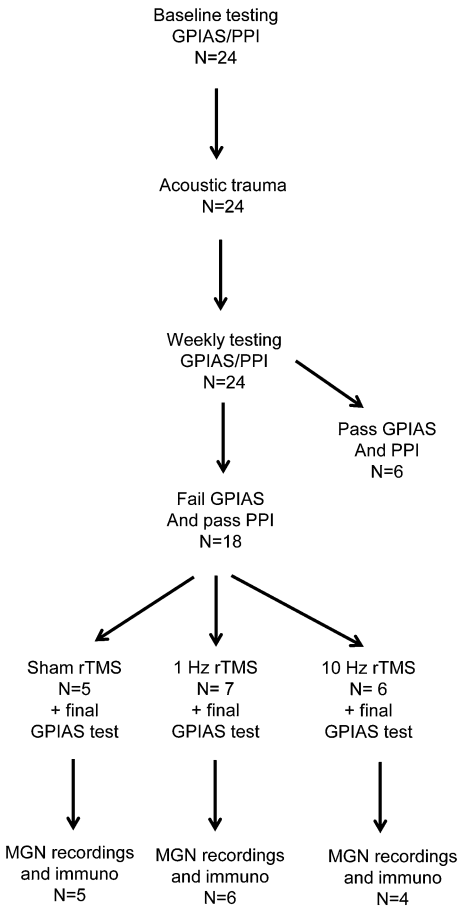

Fig. 3 Flow diagram of experimental design. Animals were tested for GPIAS and PPI to obtain baseline measures. They then underwent an acoustic trauma. After acoustic trauma weekly testing of GPIAS and PPI commenced. When animals passed weekly testing continued, when animals failed GPIAS twice but passed PPI they were deemed to show behavioural evidence of tinnitus and the first Monday after this occurred, rTMS treatment commenced (2 weeks Monday to Friday). On the Monday after these 2 weeks animals were put through a final GPIAS test and underwent a final electrophysiological experiment during which the spontaneous firing rates in MGN were recorded. At the end of this experiment animals were euthanised and tissue for immunostaining of PFC was collected

CAP audiograms

Compound action potential (CAP) audiograms were measured from the round window of the cochlea. Hearing loss patterns both immediately after acoustic trauma and after recovery were similar as described extensively in our previous publications using this animal model (Mulders and Robertson 2009, 2011; Mulders et al. 2011, 2016). Immediately

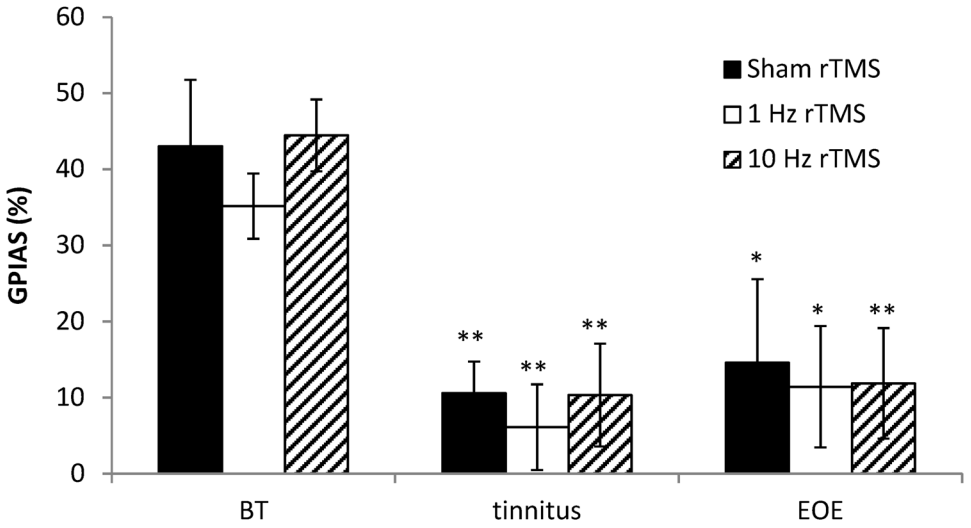

Fig. 4 Percentage GPIAS (mean ± SEM) at the tinnitus frequency (for the animal that showed a GPIAS deficit at both frequencies tested, the mean percentage suppression at both frequencies was calculated) before acoustic trauma (BT), at the time-point of tinnitus development (tinnitus) and after treatment on the day of electrophysiological recordings (EOE) in animals allocated to sham treatment ($n=5$), 1 Hz rTMS ($n=7$) and 10 Hz rTMS treatment ($n=6$). *Significantly different ($p<0.05$) from before acoustic trauma. **Significantly different ($p<0.01$) from before acoustic trauma. Two-way ANOVA with repeated measures and Sidak's multiple comparison tests

following acoustic trauma guinea pigs showed a large hearing loss at all frequencies > 8 kHz (Fig. 5a). At the time of the final electrophysiological experiment, CAP audiograms showed recovery of thresholds with a permanent threshold loss evident in all acoustic trauma animals albeit with large individual variation (Fig. 5b). One-way ANOVAs at the different frequencies did not reveal any significant effects between groups.

Spontaneous firing rates MGN

We collected data from 40 neurons from each animal's MGN whilst EEG interval remained < 300 ms with 2 exceptions in the 10 Hz rTMS group. In this group we only collected 39 neurons from 1 of the animals and 35 in one other. In the sham rTMS group CF of neurons collected varied from 120 Hz to 34.8 kHz (7.7 ± 0.57 kHz; mean ± SEM), in the 1 Hz rTMS group from 100 Hz to 24.4 kHz (8.5 ± 0.48 kHz; mean ± SEM) and in the 10 Hz rTMS group from 600 Hz to 43 kHz (8.8 ± 0.65 kHz; mean ± SEM) and a Kruskal–Wallis test revealed no significant differences between groups suggesting similar populations were sampled.

Mean spontaneous firing rates were different between groups and are shown in Fig. 6. A Kruskal–Wallis test with post hoc Dunn's multiple comparison tests showed a significant increase of 61% in the 1 Hz rTMS group ($p=0.0022$) as well as a significant 25% increase in the 10 Hz rTMS group ($p=0.0371$) compared to the sham rTMS group.

Correlation analysis was used to investigate a possible correlation between mean spontaneous firing rate in MGN and hearing loss as well as GPIAS score. Overall hearing loss was calculated as the sum of the threshold losses at each

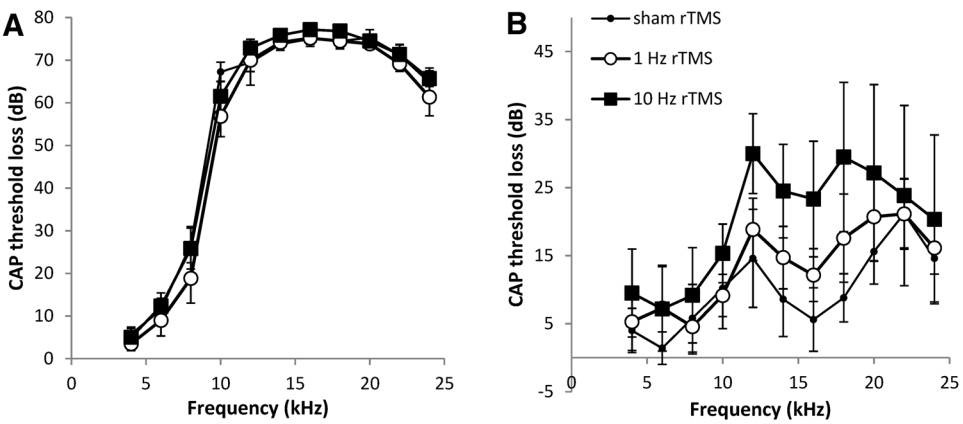

Fig. 5 Mean CAP threshold loss in the left ear immediately after acoustic trauma (**a**) and at the time-point of the final electrophysiological recordings in MGN (**b**) varying between 5 and 12 weeks after acoustic trauma. Sham rTMS ($n=5$), 1 Hz rTMS ($n=7$), 10 Hz rTMS ($n=6$). No significant differences were detected between groups at any of the frequencies. All data mean ± SEM. One-way ANOVAs at the different frequencies

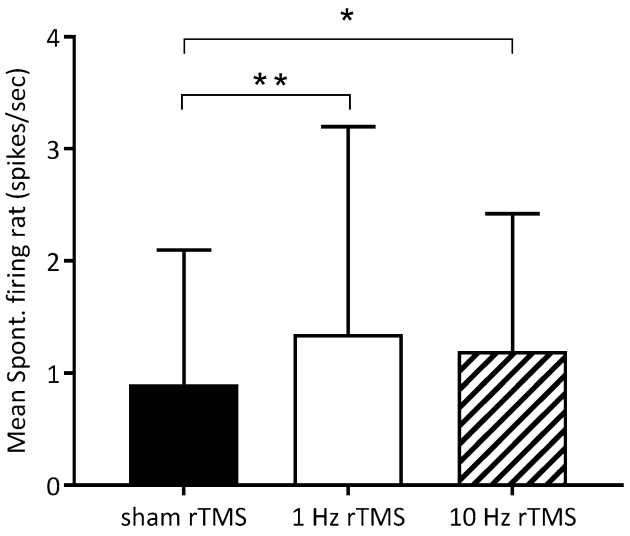

Fig. 6 Histogram showing median with interquartile range of spontaneous firing rates in sham rTMS (200 neurons from 5 animals), 1 Hz rTMS (240 neurons from 7 animals) and 10 Hz rTMS groups (154 neurons from 6 animals). *Significantly different ($p<0.05$) from before acoustic trauma. **Significantly different ($p<0.01$) from before acoustic trauma. Kruskal–Wallis test with post hoc Dunn's multiple comparison tests

of the CAP audiogram frequencies in each animal. There was no correlation between spontaneous firing rate and hearing loss (Pearson correlation coefficient; $r=-0.1274$, $n=15$, $p=0.65$) or between spontaneous firing rate and GPIAS score (Pearson correlation coefficient; $r=-0.02520$, $n=15$, $p=0.9290$).

Neuronal density of parvalbumin and calbindin

Densities of calbindin and parvalbumin immunopositive neurons in the different PFC regions are summarised in Table 1. Representative staining of calbindin and parvalbumin in PFC is shown in Fig. 7. No statistical significant differences were detected for calbindin for overall density, in dorsal or ventral regions or at individual ROI level, though a trend was observed for the calbindin density to be reduced in the dorsal regions (Fig. 8a, c, e, g). In agreement with this observation, the differences in calbindin density in ROI 1 approached significance (one-way ANOVA $F(2,13)=3.405$, $p=0.0647$).

The density of parvalbumin immunopositive neurons showed similar trends as observed for calbindin. In the dorsal region (combined ROI 1 and ROI 2; Fig. 8b) as well as in ROI 2 (Fig. 8h) one-way ANOVA results approached significance (Dorsal region: $F(2,13)=3.744$ $p=0.0520$ and ROI 2: $F(2,13)=3.213$ $p=0.0735$) though Sidak's multiple comparisons tests showed no differences between groups in either region. However, in ROI 1 (Fig. 8f) one-way ANOVA significance ($F(2,13)=4.098$ $p=0.0417$) and Sidak's multiple comparisons tests showed a significant difference

Table 1 Density of calbindin and parvalbumin immunopositive neurons in PFC (neurons/mm^2) in the different experimental groups and in different ROIs

	Calbindin			Parvalbumin		
	Sham rTMS	1 Hz rTMS	10 Hz rTMS	Sham rTMS	1 Hz rTMS	10 Hz rTMS
Overall	147 ± 30	120 ± 17	133 ± 5	61 ± 11	53 ± 6	73 ± 6
Dorsal (ROI 1 + 2)	132 ± 21	86 ± 17	120 ± 4	46 ± 12	32 ± 6	60 ± 6
Ventral (ROI 3 + 4)	163 ± 41	155 ± 19	146 ± 8	75 ± 11	74 ± 7	86 ± 6
ROI 1	66 ± 11	42 ± 8	65 ± 4	43 ± 11	35 ± 7	63 ± 5
ROI 2	198 ± 33	130 ± 28	174 ± 10	50 ± 14	29 ± 6	56 ± 7
ROI 3	122 ± 18	109 ± 8	124 ± 6	94 ± 13	100 ± 9	102 ± 6
ROI 4	203 ± 63	201 ± 33	168 ± 17	56 ± 11	49 ± 5	70 ± 7

Mean ± SEM

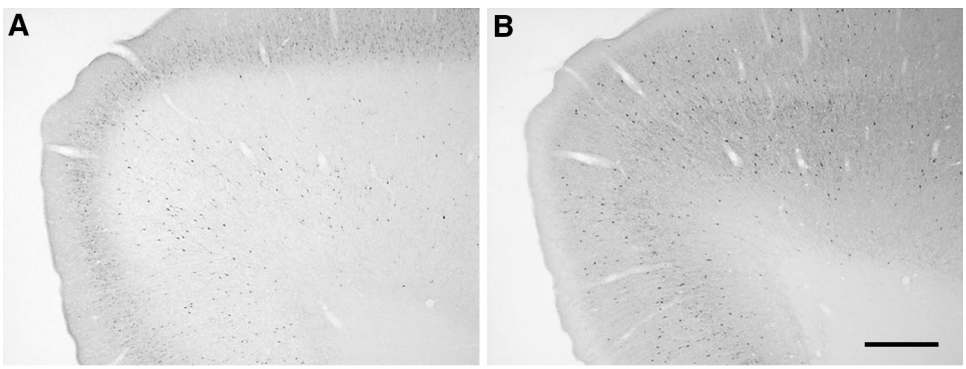

Fig. 7 Photomicrographs of calbindin (**a**) and parvalbumin (**b**) immunostaining in prefrontal cortex. Scale bar = 0.5 mm

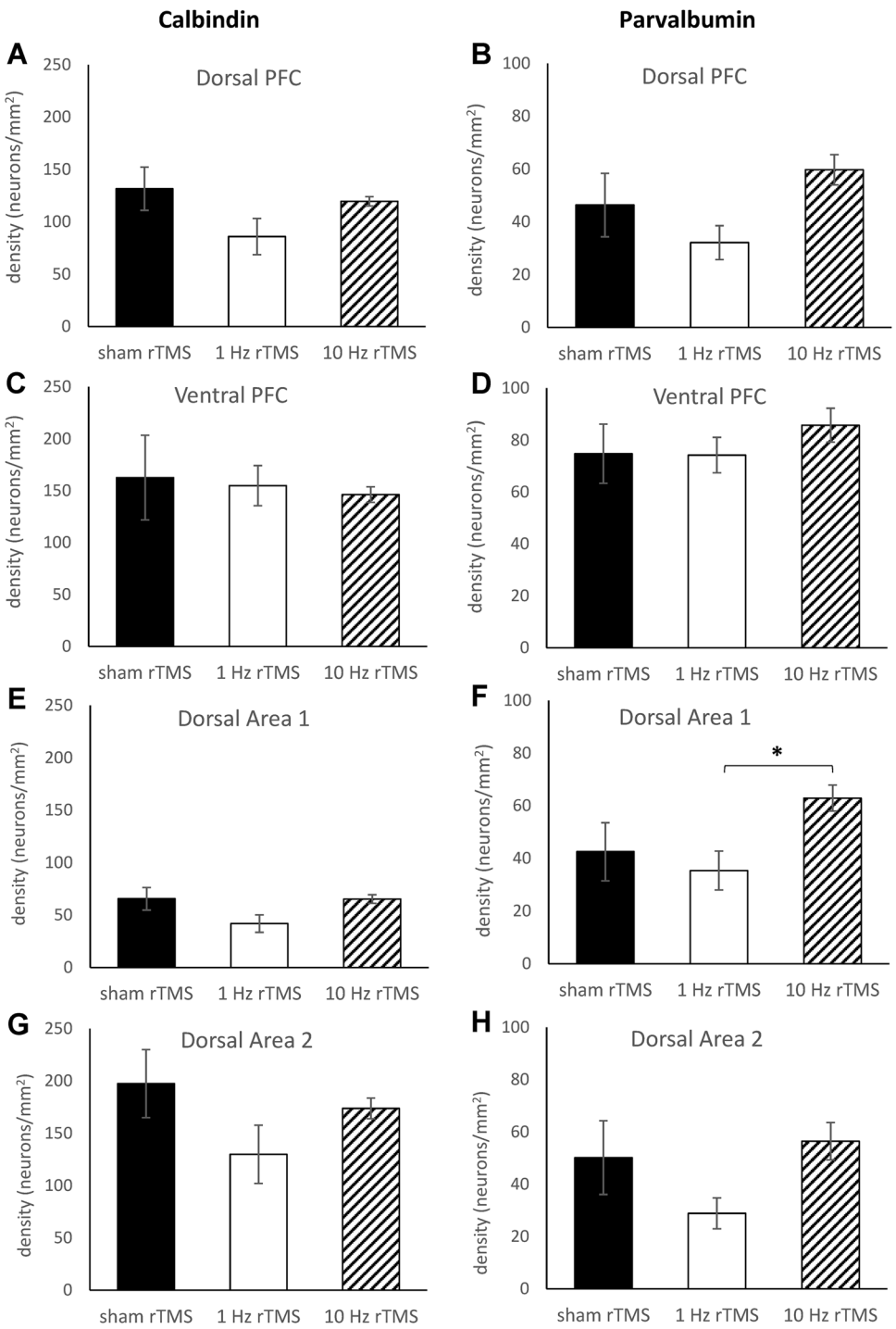

Fig. 8 Histograms showing calbindin (left column) and parvalbumin (right column) densities (mean ± SEM) in sham rTMS ($n = 5$), 1 Hz rTMS ($n = 7$) and 10 Hz rTMS ($n = 6$) groups in: **a, b** the dorsal PFC (ROI 1 and 2); **c, d** the ventral PFC (ROI 3 and 4); **e, f** ROI 1 and **g, h** ROI 2. *$p < 0.05$. One-way ANOVA and Sidak's multiple comparisons tests. For details see text

between the 1 Hz and 10 Hz rTMS group ($p = 0.0458$), revealing a 86% increase after 10 Hz treatment.

In view of the apparent trends in the data in the dorsal regions of PFC, correlation analysis was used to investigate possible correlations between mean spontaneous firing rate in MGN and calbindin and parvalbumin density in dorsal regions. No correlation between spontaneous firing rate and parvalbumin density was detected. However, a significant correlation was present between mean spontaneous firing rate in MGN and the density of calbindin immunopositive neurons in dorsal ROI 1 (Pearson correlation coefficient; $r = -0.5601$, $n = 14$, $p = 0.037$) (Fig. 9), showing that increased density of calbindin was significantly correlated with lower spontaneous firing rates in MGN.

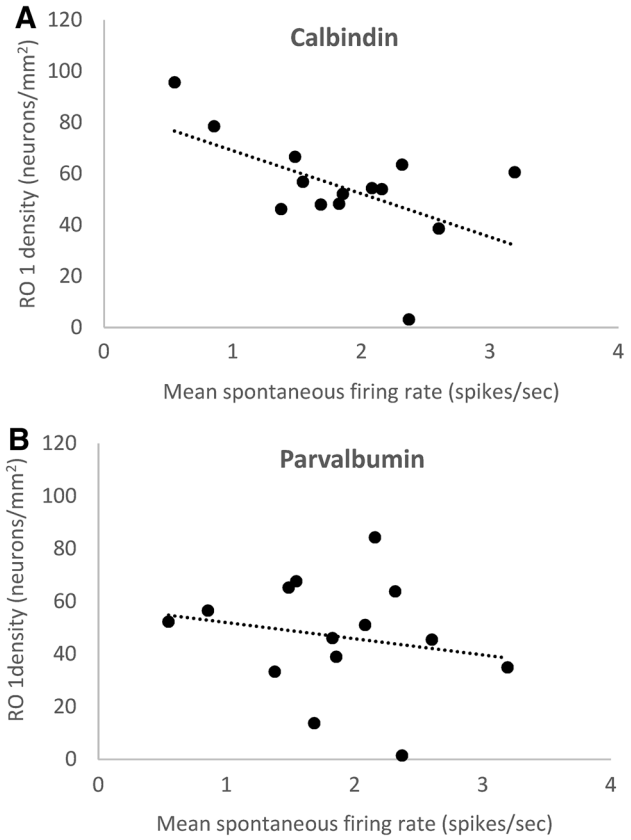

Fig. 9 Scatterplot showing relationship between mean spontaneous firing rates in MGN and density in ROI 1 of calbindin (**a**) and parvalbumin (**b**). Note that calbindin shows a significant correlation, whereas parvalbumin does not. Correlation analysis using Pearson correlation coefficient. Trendline as dotted line. For details see text

Discussion

In the present study, we used a guinea pig model to investigate the effects of non-invasive stimulation using LI-rTMS over prefrontal cortex on behavioural signs of tinnitus. In addition, we measured spontaneous activity in MGN and density of CBPs in PFC in order to shed light on possible mechanisms involved. Low- and high-frequency LI-rTMS were found to have no effect on behavioural signs of tinnitus compared to sham rTMS, but both treatments did increase spontaneous firing rates in MGN, with low-frequency LI-rTMS showing a larger effect. In addition, both high- and low-frequency LI-rTMS altered the density of the CBPs, parvalbumin and calbindin, in PFC.

We have previously shown that treatment with low-frequency LI-rTMS applied over auditory cortex can reduce the behavioural signs of tinnitus, possibly by decreasing excitability in the auditory cortex itself (Mulders et al. 2016). In the present study we used LI-rTMS in an attempt to modulate excitability in PFC, with the aim of modifying its input to the MGN (Barry et al. 2017) as this circuitry is suggested to be involved in tinnitus generation (Rauschecker et al. 2010, 2015). The lack of effect on behavioural signs of tinnitus is unlikely to be due to the absence of an effect of LI-rTMS in PFC because our data demonstrate that LI-rTMS modified CBP densities in PFC, albeit localised in the dorsal regions only. Effects on parvalbumin and calbindin were found only in the dorsal regions of the PFC, in line with the small and relatively focal magnetic field of our custom-built animal specific coils (Tang et al. 2015a, 2016b; Mulders et al. 2016), which also make it unlikely that MGN or auditory cortex were stimulated directly (see Fig. 1). Densities of both CBPs tended to decrease with low-frequency LI-rTMS, whereas densities remained unchanged or were slightly elevated with high-frequency LI-rTMS, resulting in a significant effect in the deeper layers of the dorsal area for parvalbumin only. Opposing effects of low- and high-frequency stimulation rTMS are in agreement with the proposed inhibitory and excitatory effects of these stimulation paradigms, respectively (Pell et al. 2011; Grehl et al. 2015; Tang et al. 2015b).

Previous studies on animal models also reported changes in the density of CBPs following TMS stimulation, but these appear to vary across species. Studies in rats consistently show suppressive effects on either parvalbumin or calbindin, independent of the rTMS paradigm (Benali et al. 2011; Mix et al. 2014; Castillo-Padilla and Funke 2016). These studies applied cTBS (continuous theta burst stimulation), an inhibitory protocol and iTBS (intermittent Theta Burst Stimulation), thought to be excitatory, and found decreases in numbers of parvalbumin neurons following iTBS and decreases in calbindin neurons following cTBS 2 h after a single stimulation session (Benali et al. 2011) or 90 min after a single stimulation session (Mix et al. 2014). Similarly, Labedi et al. (2014) showed reduced numbers of neurons expressing calbindin or parvalbumin 30 min following a single session of iTBS. A study of chronic rTMS stimulation in rats also showed reduced parvalbumin expression after 2 weeks of iTBS, and 24 h after the final stimulation (Castillo-Padilla and Funke 2016). By contrast, studies in mice have shown results in agreement with our present findings of increased parvalbumin after 2 weeks of rTMS stimulation. Makowiecki et al. (2018) showed that a single high-frequency stimulation increased parvalbumin expression in mouse cortex roughly 20 min after the end of stimulation. The reasons for the variety of results in the different studies is not clear and may be related to the treatment protocol itself, the delay between end of treatment and sacrifice, and/or the species being used. A systematic investigation of different stimulation protocols in different species would be required to fully understand the effect of TMS on CBPs expression. Parvalbumin expression in cortex differs between rats and mice based on its colocalisation with perineuronal net proteins (Lensjo et al. 2017), a pattern that

might underpin the species-specific response to plasticity suggested in the literature reviewed above. It is noteworthy that Mix and co-workers (2014) even showed significant variation dependent on rat strain and they suggested this was due to different basal expression levels of parvalbumin in the different strains. This is in line with the results of Makowiecki et al. (2018) showing that effects depended on the baseline activity of the cortex being stimulated.

The meaning is of the altered densities of CBPs in this study remains unclear. A reduction or increase in density may be the result of increased or reduced degradation of the proteins, respectively. Another explanation may be that gene expression or synthesis of the proteins has been modified. Alternatively it may be due to altered binding of Ca^{2+} to the proteins as this has been shown to alter immunoreactivity (Winsky and Kuznicki 1996). Previous in vitro studies have shown that LI-rTMS can alter the intracellular Ca^{2+} concentrations in cultured neurons (Grehl et al. 2014) and hence the altered densities may reflect altered Ca^{2+} content in the neurons. In addition, it is also unknown whether the altered densities of the parvalbumin and calbindin expressing neurons are a response to changes in excitability caused by rTMS or whether they are part of the mechanisms by which excitability is altered by rTMS. Both calbindin and parvalbumin often co-localise with GABA within cortex, but are thought to label two distinct subpopulations of interneurons in cortex (Hendry et al. 1989). Makowiecki et al. (2018) suggested that the increased parvalbumin density after high-frequency LI-rTMS, as also seen in the present study, was a compensatory response to the increased excitability caused by the high-frequency treatment, in view of the fact that parvalbumin neurons modulate cortical gain by their inhibitory effects on pyramidal cells (Atallah et al. 2012). A similar, opposite, mechanism could be responsible for the down regulation of calbindin and parvalbumin neurons following the low-frequency, inhibitory LI-rTMS.

Changes in the spontaneous firing rates in the MGN suggests that LI-rTMS affected the circuitry between PFC and MGN, most likely via a direct effect on PFC as the deeper located MGN itself is too far away from the small coil to be directly affected by the magnetic field (Tang et al. 2015a, 2016b). Alterations in the spontaneous firing rates are in line with a previous study in rats that showed changed spontaneous firing rates in MGN with electrical stimulation of PFC (Barry et al. 2017). The increase in spontaneous firing rates observed after both high and low-frequency LI-rTMS is also consistent with the lack of effect observed on the behavioural signs of tinnitus in our study. It remains unknown whether the increased spontaneous firing rate would have altered the perception of tinnitus in the animals. Currently no data are available to suggest that a louder tinnitus would cause a larger effect on GPIAS. In addition, it is unknown whether or how small changes in the characteristics of the perception, such as timbre or pitch would affect GPIAS. It therefore cannot be excluded that the animals experienced a change in their tinnitus. Nonetheless, tinnitus is thought to be correlated with increased spontaneous firing rates in the auditory pathway including the MGN (Kalappa et al. 2014; Basura et al. 2015; Eggermont and Roberts 2015) and hence potential treatments are generally aimed at reduction of this hyperactivity (Bauer and Brozoski 2006; Brozoski et al. 2007a, b; Vanneste and De Ridder 2011; Mulders et al. 2014, 2016).

The fact that both high- and low-frequency LI-rTMS increased spontaneous firing rates is surprising in view of the opposing effects of these treatments on CBPs reported here, and on cortical excitability in most rTMS studies (Wilson and St George 2016). However, a previous study showed a range of effects on spontaneous activity in MGN following electrical stimulation of the PFC in rat suggesting that complex multi-synaptic pathways may be involved (Barry et al. 2017). It is therefore possible that several different pathways were recruited by the different LI-rTMS frequencies, but that these produced similar outcomes on excitability in the remote target of the MGN. In that respect, it is of interest that the calbindin densities correlated with the firing rates in the MGN, whereas the parvalbumin did not, further supporting the possibility of different pathways. Future studies investigating expression in the MGN of calbindin and parvalbumin, or other markers of rTMS effects such as cfos, may provide more insight into the pathways involved in regulating spontaneous activity in this structure (Hoppenrath and Funke 2013). Our observations of altered firing rates in MGN also highlight the potential of rTMS to affect complex neural circuitry not only at the primary source of stimulation but throughout the brain. This is in agreement with recent MRI studies in rodents using low-intensity rTMS (Seewoo et al. 2018), and clinical observations of the efficacy of rTMS as a treatment of depression and its suggested use in the treatment of other neurological and psychiatric conditions (Lee et al. 2012; Fuggetta and Noh 2013). These findings also emphasise that any investigation of the effect of rTMS should not be limited to the area directly affected by the electromagnetic field but should extend throughout the brain. Finally, with regard to our tinnitus model the changes in CBP densities were only observed in the more superficial layers, whereas Barry and co-workers showed the largest inhibitory effects with stimulation of deeper areas of the PFC (Barry et al. 2017). It may be that the use of higher intensity rTMS would stimulate these deeper layers thereby causing inhibitory effects on MGN and potentially attenuating the signs of tinnitus.

Our results are in contrast with human studies that have shown beneficial effects of non-invasive stimulation of PFC, using either direct current stimulation or rTMS, on tinnitus in some patients (Kleinjung et al. 2008; Vanneste et al. 2010;

Vanneste and De Ridder 2011; De Ridder et al. 2013; Lehner et al. 2013). Human studies use high-intensity stimulation, whereas our custom designed animal coils, which do achieve the focality of human rTMS, provide only low-intensity stimulation (90 mT) (Rodger and Sherrard 2015; Tang et al. 2015a; Mulders et al. 2016; Heath et al. 2018). High-intensity stimulation reaches deeper regions of the brain and in addition, induces changes in excitability by evoking action potentials, thereby causing long-term depression (LTD) or potentiation (LTP), respectively (Thickbroom 2007; Vlachos et al. 2012; Wassermann and Zimmermann 2012). Low-intensity rTMS, on the other hand, does not elicit action potentials, but is thought to cause changes in excitability by modulating intracellular calcium levels (Capone et al. 2009; Grehl et al. 2015; Tang et al. 2016a). Thus, it cannot be concluded from our results whether stimulation of PFC was unsuccessful in reducing spontaneous firing rates in MGN and attenuating tinnitus due to an inability to stimulate the deeper layers of PFC or due to the involvement of different plasticity mechanisms. Future studies using high-intensity rTMS in an animal model may shed light on these issues.

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