Effects of pulsatile electrical stimulation of the round window on central hyperactivity after cochlear trauma in guinea pig

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Short title: Cochlear electrical stimulation effects on hyperactivity

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Abstract

Partial hearing loss induced by acoustic trauma has been shown in animal models to result in an increased spontaneous firing rate in central auditory structures. This so-called hyperactivity has been suggested to be involved in the generation of tinnitus, a phantom auditory sensation. Although there is no universal cure for tinnitus, electrical stimulation of the cochlea, as achieved by a cochlear implant, can result in significant reduction of the tinnitus percept. However, the mechanism by which this tinnitus suppression occurs is as yet unknown and furthermore cochlear implantation may not be an optimal treatment option for tinnitus sufferers who are not profoundly deaf. A better understanding of the mechanism of tinnitus suppression by electrical stimulation of the cochlea, may lead to the development of more specialised devices for those for whom a cochlear implant is not appropriate. This study aimed to investigate the effects of electrical stimulation in the form of brief biphasic shocks delivered to the round window of the cochlea on the spontaneous firing rates of hyperactive inferior colliculus neurons following acoustic trauma in guinea pigs. Effects during the stimulation itself included both inhibition and excitation but spontaneous firing was suppressed for up to hundreds of ms after the cessation of the shock train in all sampled hyperactive neurons. Pharmacological block of olivocochlear efferent action on outer hair cells did not eliminate the prolonged suppression observed in inferior colliculus neurons, and it is therefore likely that activation of the afferent pathways is responsible for the central effects observed.

1. Introduction

The phantom auditory sensation of tinnitus affects 10 to 20% of the human population. It is known to severely affect quality of life in about one-fifth of sufferers, causing sleep problems, anxiety, distress and even suicidal thoughts (Hoffman et al., 2004). Tinnitus is strongly correlated with hearing loss. There is still no cure for tinnitus, though there are treatments that show beneficial effects for some people affected by this condition (Jastreboff, 2007; Moffat et al., 2009; Smith et al., 2005). One intervention that has regularly shown beneficial effects is the cochlear implant. Though the primary function of the implant is to restore hearing to the profoundly deaf, it has been extensively documented that many, though not all, recipients report an improvement of their tinnitus when using the implant (Arts et al., 2012; Baguley et al., 2007; Olze et al., 2012).

Although the mechanism by which the cochlear implant reduces tinnitus is as yet unclear, this positive secondary effect of the implant has now led to its therapeutic use in severe tinnitus cases (Kleinjung et al., 2009; Van de Heyning et al., 2008; Zeng et al., 2011). This has sometimes been done at the expense of the patient's natural hearing ability, as the implant procedure commonly destroys much of the cochlea's delicate features (Van de Heyning et al., 2008; Zeng et al., 2011). Not all tinnitus patients have sufficient hearing loss or tinnitus to warrant receiving an implant.

A better understanding of the mechanism by which the implant modulates tinnitus may lead to more specialised devices allowing for a more inclusive and effective approach to tinnitus management. In this regard, it is interesting to note that extra-cochlear electrical stimulation of the round window has also been shown to be effective for reducing tinnitus (Hazell et al., 1993; Portmann et al., 1979; Rubinstein et al., 2003; Wenzel et al., 2014). Investigations into the mechanism by which electrical stimulation of the cochlea suppresses tinnitus are limited in human studies. It may be due to acoustic masking though this seems unlikely since it has been described that after a period of three months tinnitus can be reduced in some patients even when the cochlear implant is off (Quaranta et al., 2008). Other postulated mechanisms are that the restored input to the brain by the cochlear implant reverses some of the central plasticity evoked by the hearing loss, such as tonotopic reorganization of the cortex (Robertson et al., 1989) or that the implant evokes lateral inhibition in the auditory pathway which could attenuate the tinnitus percept (Pantev et al., 2012). It is also difficult in human

studies to rule out a placebo effect since patients are aware whether their implant is active or not.

Studies in animal models may be useful for investigating the effects of cochlear electrical stimulation in an endeavour to further elucidate the mechanisms responsible for tinnitus suppression. In a previous report we showed that direct current stimulation at the round window causes marked changes of hyperactivity in the inferior colliculus (IC) (Norena et al., 2015). Hyperactivity occurs throughout the central auditory pathway after hearing loss and is thought to be involved in the generation of tinnitus (Basura et al., 2015; Brozoski et al., 2002; Kaltenbach et al., 2005; Mulders et al., 2009; Mulders et al., 2014; Norena, 2011; Robertson et al., 2013; Vogler et al., 2011). In the present paper, in order to mimic more closely the physical properties of implant stimulation, we investigated the effect of pulsatile electrical stimulation on the round window of the cochlea on IC hyperactivity.

2. Methods

2.1. Animals

A total of 15 pigmented guinea pigs of either sex were used in this study. Twelve animals were subjected to acoustic trauma (270-590g at the time of trauma). Nine of these were used to investigate the effects of round window electrical stimulation on activity of IC neurons, and 3 were used to investigate whether strychnine modified the effects of electrical stimulation of the cochlea observed in IC neurons. A further 3 animals not exposed to acoustic trauma were used to assess the effect of strychnine on cochlear changes caused by medial olivocochlear efferent stimulation All procedures were approved by the Animal Ethics Committee of The University of Western Australia.

2.2 Acoustic trauma

Animals received a subcutaneous (s.c.) injection of 0.1 ml atropine (0.65 mg/ml atropine sulphate), then an intraperitoneal (i.p.) injection of 1 ml/kg of Pamlin (5 mg/ml Diazepam), followed 20 minutes later by an intramuscular (i.m.) injection of 1 ml/kg of Hypnorm (0.135 mg/ml Fentanyl citrate, 10 mg/ml Fluanisone). Lignocaine (20 mg/ml) was administered s.c. in the incision region. A third of the original dose of Hypnorm was administered halfway through the experiment.

Once the foot withdrawal reflex was absent, animals were placed on a heating pad in a sound-proof room and mounted in hollow ear bars. Peripheral auditory thresholds were determined by measurement of a compound action potential (CAP) audiogram. For this purpose, a small hole was made in the bulla and a silver wire recording electrode was placed on the round window (RW) of the cochlea. A compound action potential (CAP) audiogram was then constructed for frequencies ranging from 4 to 24 kHz (Johnstone et al., 1979). All sound stimuli were presented in a calibrated closed sound system through a ½" condenser microphone driven in reverse as a speaker (Bruel and Kjaer, type 4134). Pure tone stimuli (10 ms duration, 1 ms rise/fall times) were synthesized by a computer equipped with a DIGI 96 soundcard connected to an analog/digital interface (ADI-9 DS, RME Intelligent Audio Solution). Sample rate was 96 kHz. The interface was driven by a custom-made computer program (Neurosound, MI Lloyd), which was also used to collect single neuron data during the final experiments. CAP signals were amplified (1000x), filtered (100 Hz-3 kHz bandpass) and recorded with a second data acquisition system (Powerlab 4SP, AD Instruments).

After verifying that the audiogram was within the normal range (Johnstone et al., 1979), the left ear was exposed to a continuous pure tone of 10 kHz at 124 dB for 2 hours, while the

right ear was blocked. Then the audiogram was measured again to determine the extent of acute hearing loss. The incision was sutured and animal were allowed to recover from anaesthesia for 2 weeks before single neuron recordings were obtained.

2.3 Single neuron recordings

Animals were anaesthetised by s.c. administration of 0.1 ml atropine (0.65 mg/ml Atropine sulphate), followed by an i.p. injection of 30 mg/kg of sodium pentobarbitone. After 10 minutes, an i.m. injection of 0.15 ml of Hypnorm (0.135 mg/ml Fentanyl citrate, 10mg/ml Fluanisone) was administered. Lignocaine (20 mg/ml) was administered s.c. to the incision areas. Once full surgical anaesthesia was achieved a tracheostomy was performed and the animal was artificially ventilated with carbogen (95% oxygen and 5% carbon dioxide). A full dose of the original Hypnorm dose was administered each hour, and half the pentobarbitone dose was administered every two hours to maintain surgical anaesthesia. 0.1 ml of Pancuronium (2 mg/ml Pancuronium bromide) was administered prior to single neuron recordings to induce paralysis. An electrocardiogram was monitored continuously throughout the experiment. At the end of the experiment the animals were euthanized with an overdose of 0.3 ml of Lethabarb (325 mg/ml pentobarbitone sodium).

After the tracheotomy, animals were placed on a heating pad in a sound-proof room and mounted in hollow earbars. CAP audiograms are measured from both ears in the same manner as during the acoustic trauma procedure.

For electrical stimulation a chlorided silver wire that was Teflon insulated except at the tip, was placed on the round window (RW) and a current return wire was inserted in muscle close to the opened bulla. Biphasic electrical stimulation was applied via an isolated stimulator output (AM Systems Model 2100).

Then a small craniotomy was performed exposing the surface of the right occipital cortex and a tungsten-in-glass microelectrode was inserted into the cortex until the recorded electrical activity was indicative of the CNIC. The dorsal aspect of the CNIC was indicated by the presence of strong sound-driven activity with a short latency (cluster onset latencies <6.5 ms) and a systematic progression from low to high characteristic frequencies (CF) with increasing depth. Once proper placement was obtained, the exposed surface of the brain was covered with a 5% agar solution to ensure stability of recording.

When a CNIC neuron was isolated its CF and threshold at CF were determined audio-visually and depth from the cortical surface was recorded using methods described previously (Ingham et al., 2006; Mulders et al., 2010). Then spontaneous activity was measured during a

10 s sample period. The majority of neurons (approximately 93%) in the CNIC of control animals under our anaesthesia protocol exhibit a SFR < 5 spikes/second (Mulders et al., 2009; Mulders et al., 2011b), therefore neurons found with an SFR exceeding this value were classified as hyperactive. In some neurons it was investigated whether action potentials could be directly elicited in response to electrical stimulation of the RW. If this was observed, the lowest current to which the neuron would consistently fire was recorded. Then electrical stimuli of varying parameters were applied to the RW and firing rate was recorded as a peristimulus time histogram (PSTH repetition rate 1/s), in the absence of acoustic stimulation. Electrical pulses were biphasic (width of each polarity 0.1 or 1 ms). Pulse train duration was 100 ms or 200 ms, at a rate of either 25, 100, 200 or 500 Hz, with a repetition rate of 1s for all neurons unless otherwise specified.

2.4 Strychnine experiments

Three animals that underwent a 10 kHz acoustic trauma were used to assess the effect of i.p. strychnine injection on the response of IC neurons to electrical stimulation of the round window. Single neuron recordings were made as described above. Once a hyperactive neuron was found and shown to exhibit effects of round window stimulation, the animal was injected with strychnine (i.p. 4 mg/kg). Recordings were then taken every 10 minutes after strychnine administration from the same neuron for 50 to 80 minutes without and with electrical stimulation of the round window.

Three animals were used to establish the time course of the effect of an i.p. injection with strychnine on medial olivocochlear efferent action on cochlear responses to sound. For this purpose, animals (without acoustic trauma) were anaesthetized as for single neuron recordings and a CAP audiogram between 4 and 24 kHz was measured on the left side to verify normal hearing thresholds. Then a craniotomy was performed to expose the rostral aspect of the cerebellum and the caudal part of the visual cortex. The midline cerebellum was aspirated to expose the floor of the IVth ventricle. Stimulating electrodes (custom-made bipolar tungsten electrodes, glass and Araldite-insulated, connected to an isolated stimulator output: AM Systems Model 2100) were then placed on the olivocochlear bundle (OCB). To achieve correct placement, the stimulating electrodes were placed on the midline where the threshold current to evoke a facial twitch by single shocks was lowest (Mulders et al., 2010; Seluakumaran et al., 2008). Animals were then paralysed and proper placement of the stimulating electrodes on the OCB was confirmed by measuring the classical effects on CAP

189 and CM (Desmedt et al., 1975; Mulders et al., 2000) i.e. suppression of the CAP and increase
190 in cochlear microphonic (CM) receptor potential after electrical stimulation (trains of
191 biphasic current pulses 100 ms duration, 0.1 ms pulses, 300 Hz, repetition interval 1/s). CM
192 responses were measured using 1 kHz tones (10ms duration) at sound levels ranging from 94
193 to 100 dB SPL in order to obtain a smooth CM trace. After baseline effects were established,
194 strychnine was injected (i.p. 4 mg/kg in saline) and measurements were repeated every 10
195 minutes for approximately 2 hours after the injection. Strychnine is a potent blocker of the α9
196 nicotinic acetylcholine receptor and i.p. injection has been shown to reversibly block efferent
197 action on the cochlear outer hair cells without affecting afferent fibres (Maison et al., 2007;
198 Maison et al., 2013; Rajan, 1988; Rajan et al., 1988).
199

3. Results

3.1 Cochlear changes after acoustic trauma

The effect of the acoustic trauma on the CAP thresholds is shown in figure 1. As described in our earlier papers using the same animal model (Mulders et al., 2009; Mulders et al., 2014; Mulders et al., 2011b) the acoustic trauma resulted in a large immediate but temporary threshold loss in the exposed ear at all frequencies ≥ 8 kHz ($p<0.001$; paired two-tailed t-test), which at two weeks after acoustic trauma, had recovered substantially to a small permanent threshold loss which showed statistical significance at 12 and 16 kHz ($p<0.05$; paired two-tailed t-test). Also similar to what we have reported when using this model, CAP thresholds in the un-exposed ear were unaffected.

3.2 Neuronal recordings

A total of 134 neurons were collected from the CNIC from 9 animals at two weeks after acoustic trauma. CFs of these neurons ranged from 0.47 to 28.5 kHz (mean 9.9±0.54 kHz). Spontaneous firing rates varied from 0.0 to 106.5 spikes/sec (Fig. 2). Ninety-four of these neurons showed firing rate < 5 spikes/sec. The remaining 40 neurons were classified as hyperactive (mean firing rate 21.2 ±3.2 spikes/sec; CFs varying from 1.3 to 25.3 with a mean CF of 11.3±0.9 kHz).

In 79 of the 134 neurons it was systematically investigated whether direct excitation occurred as a result of the RW electrical stimulation. This was characterised by reliable action potentials being evoked at brief latencies after each electrical pulse using a train of 100 ms with a low stimulation rate of 25Hz and pulse duration either 0.1 or 1 ms) In 39 of these neurons (49% of total neurons tested) there was no evidence of direct excitation up to 1 mA using these stimulation parameters. The CFs of these neurons varied from 0.8 to 23.4 kHz (mean 9.5±1 kHz) and spontaneous firing rates varied from 0 to 24.3 spikes/sec (mean 2.8±1 spikes/sec; seven of these neurons could be classified as hyperactive with > 5spikes/s). In the other 40 neurons (51% of total neurons tested) there was evidence of direct excitatory effects of stimulation in the form of short latency action potentials with stimulation thresholds varying from 70 to 800 μA. The CFs of these neurons varied from 1.1 to 28.5 kHz (mean 9.9±1.1 kHz) and spontaneous firing rates varied from 0 to 106.5 spikes/sec (mean 2.9±1.1 spikes/sec; six of these neurons could be classified as hyperactive with > 5spikes/s). Unpaired t-tests revealed no statistically significant differences in CF or spontaneous firing rates between neurons that showed direct excitatory effects and those that did not (Fig. 3). The lack

of correlation with CF was rather surprising as the stimulating electrode was positioned at the RW at the high frequency end of the cochlea and this suggests a diffuse spread of current in the cochlea using electrical stimulation of the RW.

3.3 Hyperactive neurons

In 35 of the 40 hyperactive neurons (spontaneous firing rates >5 spikes/sec) recordings were sufficiently stable to enable collection of spontaneous firing rate data with and without RW electrical stimulation. All but 2 neurons showed inhibition of their spontaneous firing rate after a train of shocks applied to the RW. It should be noted that the 2 neurons that did not show an effect of electrical stimulation only had a limited set of shock parameters tested, which could explain the failure to find an effect. Examples of histograms revealing inhibition are shown in figure 4. This figure also shows the variation of firing patterns observed after the inhibition. The duration of inhibition after the shock train varied from 10 to 1300 ms depending on the electrical stimulus parameters. Increasing the stimulation frequency and pulse duration caused significantly longer duration of suppression as shown in figure 5. In 19 neurons effects were compared using either 100Hz or 500Hz stimulation using similar duration of shock train (Fig. 5a). Paired two-tailed t-tests showed significantly ($p<0.0003$) longer inhibition after 500Hz (205±64 ms) than after 100 Hz electrical stimulation (103.9±18 ms). In 21 neurons effects were compared between 25Hz or 100Hz stimulation using similar duration of shock train (Fig. 5b). Significantly longer inhibition was observed after 100Hz (124±17 ms) than after 25 Hz electrical stimulation (81±9 ms) (paired two-tailed t-test, $p<0.0003$). In 11 neurons the effects of pulse durations of 0.1 and 1 ms were compared (Fig. 5c). Paired two-tailed t-test showed significantly longer inhibition using 1 ms (mean 103±48 ms) compared to 0.1 ms pulse durations (mean 43±9 ms) ($p<0.0028$). Increasing train duration was only systematically tested in 4 neurons and though this did not seem to affect the duration of suppression after the end of the shock train ($p=0.09$) (Fig. 5d), group size may have been too small to reveal significance.

One possible cause of the suppression of SFR after the shock train may be direct excitation during the shock train, which could cause a temporary reduction in excitability thereafter. Indeed in 9 neurons (26%) there was consistent excitation throughout the shock train followed by inhibition (Fig. 6A). However, in the remaining 74% of neurons that still showed clear inhibition after the train, the effects on spike rate during electrical stimulation were different. In 9 neurons (26%) there was inhibition throughout the shock train followed by

further inhibition after the end of the shock train (Fig. 6B). In the remaining 17 neurons (48%) during the shock train there was an initial increase in firing rate, followed by inhibition which then continued after the shock train (figure 6C). These data suggest that there may be multiple mechanisms by which inhibition of the spike rate following electrical stimulation occurs.

Following the period of suppression caused by shock trains, 39% of neurons showed a temporary increase (rebound) in the level of firing before returning to baseline levels (Fig. 6A, C and see also Fig. 4A-C). In addition, in some instances more complex patterns were observed such as illustrated in Figure 6C where a brief increase in firing rate was followed by a second period of inhibition. The remaining 61% of neurons showed a slow or fast (Fig. 4D, 6B) recovery from inhibition and a return to baseline levels of firing without a temporary increase in firing rate.

3.4 Effects of strychnine

Shocks applied to the RW have the potential to excite not only the afferent fibres of the cochlea, but also the peripheral processes of efferent neurons (Rajan et al., 1983). Activation of the medial efferent neurons (MOCS) has been shown to reduce the spontaneous firing rates of a subset of primary afferents and hence this could contribute to changes in SFR seen in central neurons. In order to investigate whether activation of medial olivocochlear terminals in the cochlea by the RW electrical stimulation contributed to the suppression in firing rates observed, we aimed to block the medial olivocochlear efferent action in the cochlea with an i.p. injection of strychnine. First we verified that the strychnine dose and route successfully eliminated the classical medial olivocochlear effects in the cochlea caused by electrical stimulation of the olivocochlear bundle. Figure 7 shows the effect of an i.p. injection of strychnine on the CAP suppression (Fig. 7A) and CM enhancement (Fig. 7B) produced by electrical stimulation of the OCB in three different animals. Strychnine is known to block the receptors of the medial olivocochlear system (Maison et al., 2007; Maison et al., 2013; Rajan, 1988; Rajan et al., 1983), and in line with this, it markedly reduced both the CAP suppression and CM enhancement within 60 minutes after injection and this blockade was still present after 2 hours. These data show that i.p strychnine effectively blocks the peripheral action of medial olivocochlear efferent innervation and these experiments were used to specify the time period over which the effects of strychnine on changes in IC hyperactivity caused by RW electrical stimulation were investigated.

The results of an i.p. strychnine injection on the effects of RW electrical stimulation was tested in three IC neurons from three different animals subjected to acoustic trauma 2 weeks earlier. The inter-neuron variation in effects described earlier meant that it was crucial to record from the same neuron before and for some time after strychnine injection. In one animal effects were followed for 50 minutes after injection and in the other two animals effects were able to be monitored for 80 minutes after injection. Figure 8 shows the data from the latter two animals. The histograms show the effects of electrical stimulation before (Fig. 8A,C) and 80 min after strychnine injection (B,D). As the figure illustrates strychnine did not eliminate the suppression that was observed after the shock train. If anything, the inhibition after the shock train seemed to increase slightly in duration. However, during the shock train it was observed that the firing rate increased. The neuron shown in Figure 8A, B showed initially a brief increase in firing rate followed by inhibition during the shock train but after strychnine there was a continuous increase in firing rate. The neuron in Figure 8C,D showed excitation during the shock train which was further increased after strychnine. These data suggest that effects seen during the electrical stimulation may partly involve activation of medial olivocochlear terminals, but that effects after the shock train involve different mechanisms. Although we cannot rule out a central effect of strychnine on the changes seen during the shock train, the persistence of the suppression after the shock train clearly shows that this latter effect of RW stimulation is quite independent of any peripheral MOCS activation that may be occurring.

In addition, in one animal it was investigated whether current spread from the RW to the stapedius muscle was involved in the effects observed. In this animal changing the position of the stimulus electrode from the RW to the bony shelf close to the stapedius muscle significantly decreased the inhibition observed. This suggests that current spread to the stapedius muscle is not involved in the effects observed. This was confirmed by the observation that there was no difference in the amount of inhibition when stimulating the RW with the stapedius muscle intact or with the muscle severed at its attachment to the stapes.

4. Discussion

The present results show that biphasic pulsatile electrical stimulation of the RW of the cochlea results consistently, once the stimulation ceases, in inhibition of the increased spontaneous activity (hyperactivity) in IC that develops after hearing loss. However, the effects during stimulation were varied and included both inhibition and excitation. Blockade of the receptors of the medial olivocochlear system in the cochlea showed that the inhibition after the stimulation was not caused by activation of intracochlear efferent nerve endings though it may be involved in some of the effects seen during stimulation.

The inhibition of the hyperactivity observed in this study may be one of the mechanisms involved in the effectiveness of electrical stimulation of the cochlea in reducing tinnitus. In agreement with previously reported findings using the same acoustic trauma, two weeks after acoustic trauma guinea pigs showed a small frequency restricted hearing loss and about 30 to 40% of neurons showed a firing rate >5/s (Mulders et al., 2009; Mulders et al., 2013; Mulders et al., 2011b; Robertson et al., 2013). This is contrast to control animals under the same anaesthesia, where most neurons in IC (90-95%) show a spontaneous firing rate <5/s (Mulders et al., 2009; Mulders et al., 2011b). Such increases in spontaneous firing rates of neurons in the auditory pathway after acoustic trauma have been described in different animal models (Bauer et al., 2008; Kaltenbach et al., 2004; Norena et al., 2003) and this so-called hyperactivity has been suggested to be involved in the generation of tinnitus (Kalappa et al., 2014; Kaltenbach et al., 2000; Norena, 2011). This notion is supported by a recent paper showing that hyperactivity in auditory cortex is present in animals with behavioural signs of tinnitus but not in animals without this (Basura et al., 2015).

Electrical stimulation at the round window resulted in short latency excitatory effects as evidenced by action potentials being generated during the shock train in about 50% of IC neurons. Such excitation in central neurons is most likely a consequence of activation of excitatory ascending pathways caused by the direct depolarization of primary afferent fibres and/or inner hair cells in the cochlea (van den Honert et al., 1984) and is in line with previous reports showing high synchrony and short latency action potentials in single cochlear nerve fibres in response to biphasic electrical stimulation at the round window (Hartmann et al., 1984; van den Honert et al., 1987). We did not find a correlation between the CF of the neurons recorded and the presence of these excitatory effects, which seems surprising

because the stimulation was delivered to the RW, the high frequency end of the cochlea. However, our findings are in agreement with a previous recordings from single cochlear nerve fibres and suggest complex and diffuse spread of current in the cochlea using this stimulation approach (Hartmann et al., 1984; van den Honert et al., 1987). This would be in line with the position of the return electrode in the neck muscles near the opening in the bulla and the likely current return path being via the perilymph.

In the other half of the IC neurons the effects seen during stimulation were either a brief period of excitation followed by inhibition (during the shock train) or fast and lasting inhibition. The mechanism behind this inhibition within the shock train remains to be determined. It may be that it involves activation of the medial olivocochlear terminals in the cochlea, which synapse on the outer hair cells (Liberman et al., 1986; Warr et al., 1979). Round window stimulation has been shown to activate this efferent system (Rajan et al., 1983). In addition to suppression of sound evoked responses, efferent activation has been shown to reduce spontaneous activity of about 10% of the primary auditory nerve fibres (Wiederhold et al., 1970), probably as a consequence of a small reduction in the scala media endocochlear potential and the standing current through the inner hair cells (Guinan et al., 1988b). A reduction in spontaneous activity of the auditory nerve fibres could, in its turn, evoke a reduction of the hyperactivity in the IC that occurs after hearing loss as we have shown previously (Mulders et al., 2009; Mulders et al., 2010). A possible role for the medial olivocochlear system in the inhibition observed during the stimulation is supported by the strychnine experiments described in this paper. When strychnine was used to block the intracochlear effects of the medial olivocochlear system (Eybalin, 1993; Maison et al., 2007; Maison et al., 2013; Rajan et al., 1988), the inhibition during the shock train did decrease whereas the inhibition after the shock train did not. This latter observation strongly suggests that the prolonged inhibition observed in IC neurons when shocks are applied to the RW, is not due to inadvertent activation of the peripheral endings of MOC neurons. The observation that the inhibition during the shock train was decreased when strychnine was applied would be in line with a rapid activation of the peripheral elements of the medial olivocochlear system by the RW electrical stimulation, causing a reduction of spontaneous firing of the afferent fibres. However, because strychnine was administered systemically, a possible central action of strychnine cannot be excluded. In addition, one should note that only a limited number of neurons (n=3) were recorded before and after strychnine administration.

399 The explanation for why some neurons showed excitation during the shock train while others
400 showed inhibition is unclear. Despite the mechanisms discussed above it is also possible that
401 variations in charge balance during each stimulus pulse may have an influence as we have
402 shown previously that positive and negative polarities of d.c. current applied to the RW have
403 opposite effects on firing rate of IC neurons (Norena et al., 2015).
404
405 Despite the variety of effects seen during stimulation, there was always a period of complete
406 suppression of firing rate in the hyperactive IC neurons after stimulation of the RW. There
407 are several mechanisms that could be involved in this suppression. In the neurons that
408 showed excitation during the stimulation, it could be due to a temporary reduction in
409 excitability after activity (post-stimulatory adaptation). This phenomenon has been shown to
410 occur at different levels of the auditory pathway, including the IC (Harris et al., 1979; Nelson
411 et al., 2009; Wehr et al., 2005). Estimates of the time-course of recovery from such
412 adaptation in the central nucleus of IC are few. A study in chinchilla using forward masking
413 reports time-constants of less than 100 ms (Arehole et al., 1987). Hence it is possible that
414 some of the post-stimulatory suppression we see is due to adaptation. However, the fact that
415 suppression of spontaneous firing in IC occurred regardless of whether or not direct
416 activation during stimulation was observed, suggests that other mechanisms must be involved
417 as well.
418
419 An additional mechanism that may be involved is activation of central inhibitory circuitry
420 (Voytenko et al., 2008). GABA-ergic synapses in IC target somata and large dendrites of
421 neurons providing strong inhibitory inputs (Nakamoto et al., 2014). Decreased inhibition
422 from regions affected by hearing loss has been proposed as a possible cause of tinnitus
423 (Gerken, 1996; Vale et al., 2004) and conversely a return of lateral inhibition could lead to a
424 reduction of hyperactivity and tinnitus. Such a mechanism is in line with our data showing
425 that direct activation during stimulation is not necessary for suppression to occur in about half
426 of the IC neurons. In addition, it is in agreement with data obtained in a tinnitus patient with a
427 cochlear implant, in which low rate stimulation at the apical electrodes located at the low
428 frequency end of the cochlea, yielded the greatest suppression of the high frequency tinnitus
429 (Zeng et al., 2011). This suggests that stimulating specific regions of the cochlea that provide
430 input to central circuitry providing indirect inhibitory pathways to hyperactive neurons may
431 have greater suppressive effects. Finally, activation of medial cochlear efferents is unlikely to
432 be a contributing factor to the suppression observed after the shock train, since this inhibition

was unaffected by blockade of the cochlear receptors of the medial olivocochlear system. Another system that could potentially be involved is the lateral olivocochlear system which terminates on the primary afferent dendrites contacting the inner hair cells (Warr et al., 1979). However excitation of this system seems less likely in view of the fact that pulsatile electrical stimulation, as used in this paper, has never been shown to result in direct activation of the lateral olivocochlear axons even when applied at the floor of the IVth ventricle (Guinan et al., 1988a; Rajan et al., 1988), which is most likely due to the fact that these axons are thin and unmyelinated (Guinan et al., 1983; Warr et al., 1979).

Following the suppression after the stimulation about 40% of neurons showed a rebound firing pattern before a return to baseline levels of activity. Rebound excitation is a common occurrence following hyperpolarising inputs and one of the proposed mechanisms is the involvement of low-voltage-activated T-type calcium channels (Boehme et al., 2011). The rebounds observed are consistent with reports from tinnitus patients with cochlear implants who report a temporary increase in tinnitus level immediately after the devices are turned off (Baguley et al., 2007; Zeng et al., 2011).

Our results clearly show that pulsatile electrical stimulation of the RW has suppressive effects on the central hyperactivity observed after hearing loss. These data suggest that this reduction of hyperactivity may be involved in the mechanism by which cochlear implants or RW stimulating devices in humans reduce tinnitus (Baguley et al., 2007; Wenzel et al., 2014), although a number of factors should be considered. Firstly, in our model there is only limited loss of hair cells (Mulders et al., 2011b), whereas in most implantees there is a complete loss of hair cells. This means that any effects observed in our animals that may be due to stimulation of hair cells or medial olivocochlear pathway (since its targets are the outer hair cells (Warr et al., 1979)) would not be involved in the effects seen in many cochlear implant patients. An animal model of complete deafness, which can be chemically induced (Xu et al., 1993), could be used to investigate to what extent our results are modulated by hair cell or olivocochlear activation. Secondly, our experiments were conducted two weeks after acoustic trauma whereas recipients of a cochlear implant would have had a much longer duration of hearing loss. We have shown previously that the relationship between central hyperactivity and auditory nerve activity changes over time (Mulders et al., 2009; Mulders et al., 2011a; Robertson et al., 2013) and it would therefore be of interest to repeat the present experiment at a later time-point after acoustic trauma.

Figure legends

Figure 1: Peripheral threshold loss based on measurements of the compound action potential (CAP) of the auditory nerve immediately after (acute; black diamonds) and two weeks after acoustic trauma (2 weeks; white open circles). Data based on 12 animals. # = $p<0.001$; * = $p<0.05$. Mean ± SEM.

Figure 2: Scatterplot showing the CF versus the spontaneous firing rate of all neurons (n=134) recorded from the CNIC from 9 animals at two weeks after acoustic trauma.

Figure 3: Figure showing the lack of a relationship between direct excitation when using low frequency stimulation (25 Hz) and either the characteristic frequency (A) or spontaneous firing rate (B).

Figure 4: Histograms illustrating inhibitory effects of RW stimulation on spontaneous firing rate of hyperactive neurons. (A) RW stimulation 100Hz; CF 10.8 kHz, threshold 29 dB SPL. SFR 51.9 spikes/sec (84 sweeps). (B) RW stimulation 100Hz; CF 6.1 kHz, threshold 57 dB SPL. SFR 40.9 spikes/sec (100 sweeps). (C) RW stimulation 200Hz; CF 15.5 kHz, threshold 57 dB SPL SFR 7.9 spikes/sec (100 sweeps). and (D) RW stimulation 200Hz; CF 9.5 kHz, threshold 70 dB SPL. SFR 109.5 spikes/sec (100 sweeps).

Figure 5: Figure showing the statistically significant increase in duration of the inhibition measured after the shock train by increasing the frequency of stimulation (A,B) and increasing pulse duration (C). Increasing the shock train duration did cause a small increase in duration of the inhibition but this did not reach statistical significance (D).

Figure 6: Figure illustrating that, although inhibition was consistently observed after the shock train, effects during the shock train varied. A: histogram of a neuron (CF 20.7 kHz, threshold 86 dB SPL) showing consistent excitation throughout the shock train. B: histogram of a neuron (CF 15.5 kHz, threshold 57 dB SPL) showing consistent inhibition throughout the shock train and C: neuron (CF 19.6 kHz, threshold 67 dB SPL) showing initial excitation followed by inhibition through the shock train. All histograms bin size 1 ms, with 100 sweeps.

Figure 7: Effects of an i.p. injection with strychnine (time of injection indicated by arrow) on the percentage of CAP suppression (A) and CM enhancement (B) after electrical stimulation of the OCB. Effects shown from three different animals.

Figure 8: Histograms from 3 different neurons in three different animals showing effects of strychnine injection on firing rate after electrical RW stimulation. Left column before strychnine injection. Right column the same neurons 80 min (A-D) or 50 min (E,F) after strychnine injection. A,B: CF 19.2, threshold 41 dB SPL, SFR 14.8 spikes/sec. C,D: CF 19.7 kHz, threshold 56 dB SPL, SFR 57.1 spikes/sec. E,F: CF 10.6 kHz, threshold 24 dB SPL, SFR 20.0 spikes/sec.

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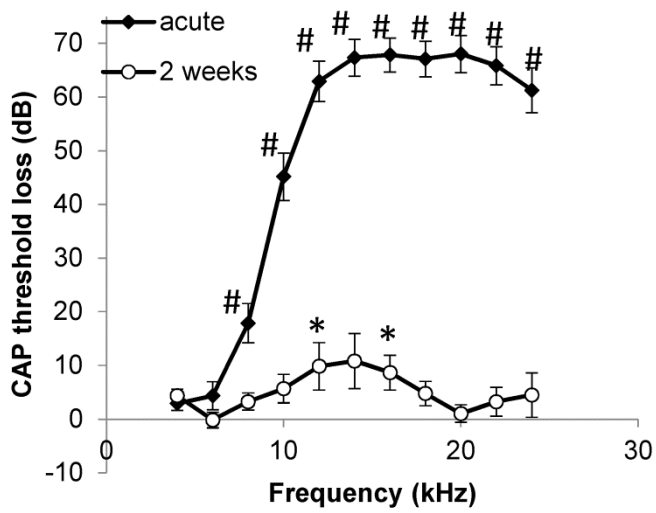

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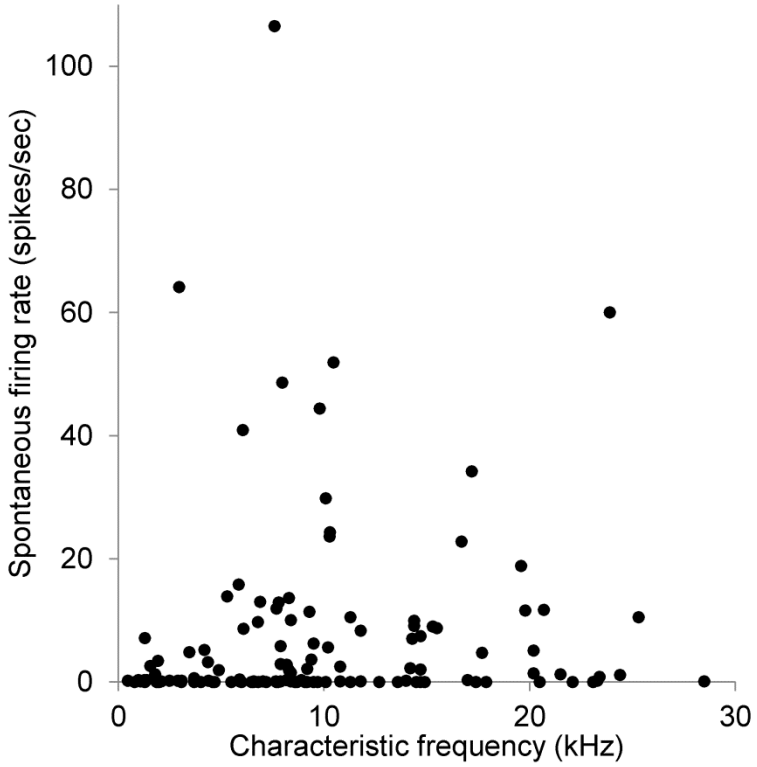

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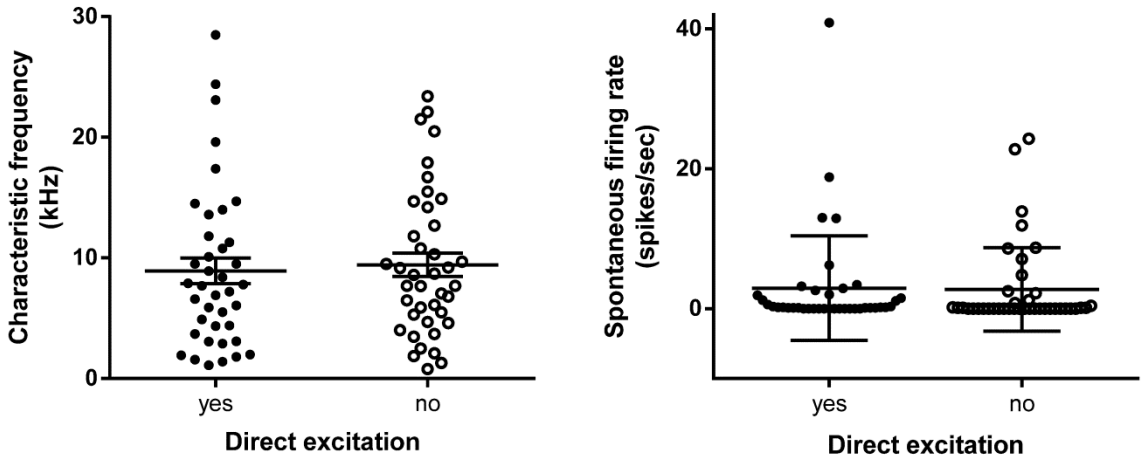
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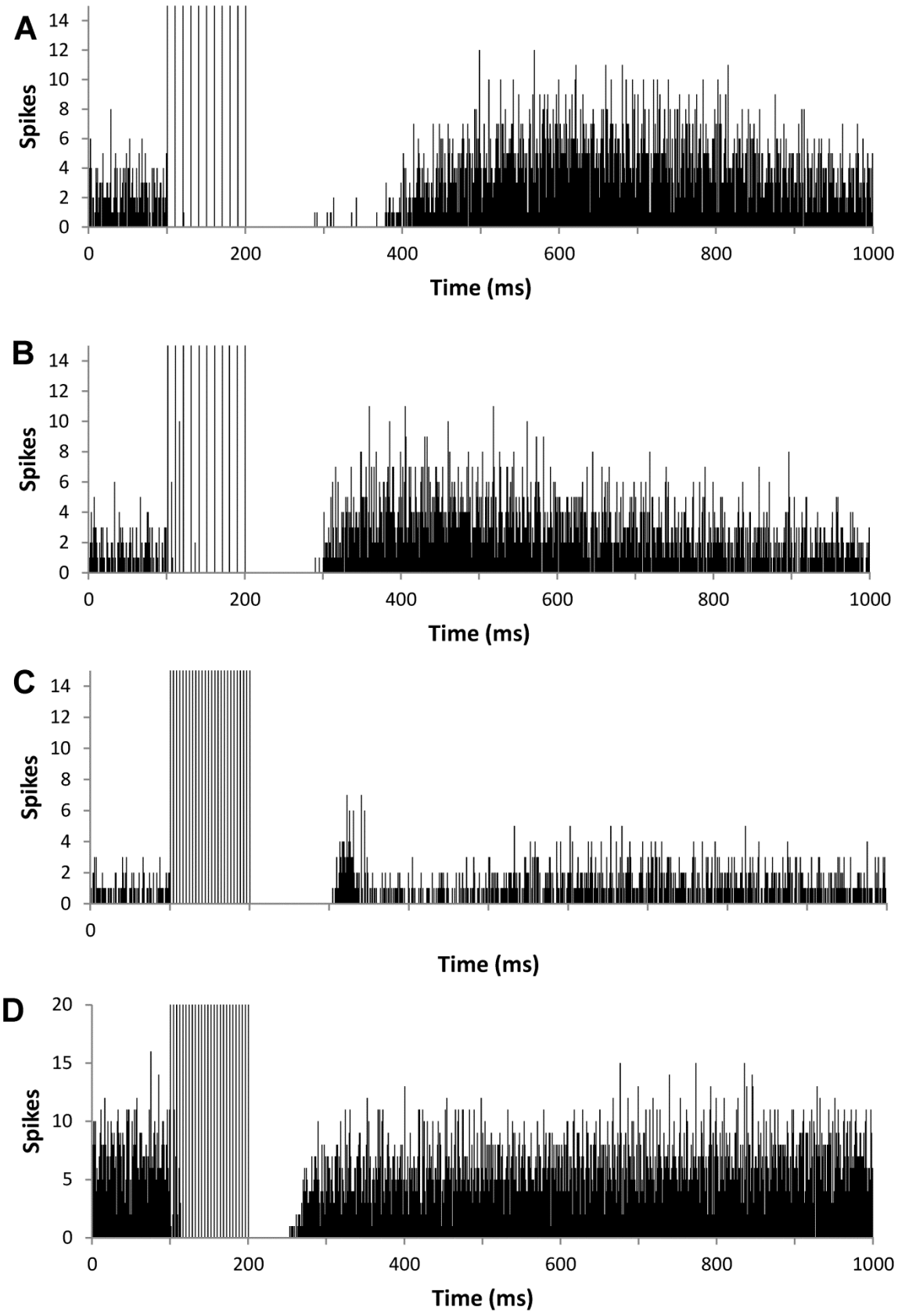
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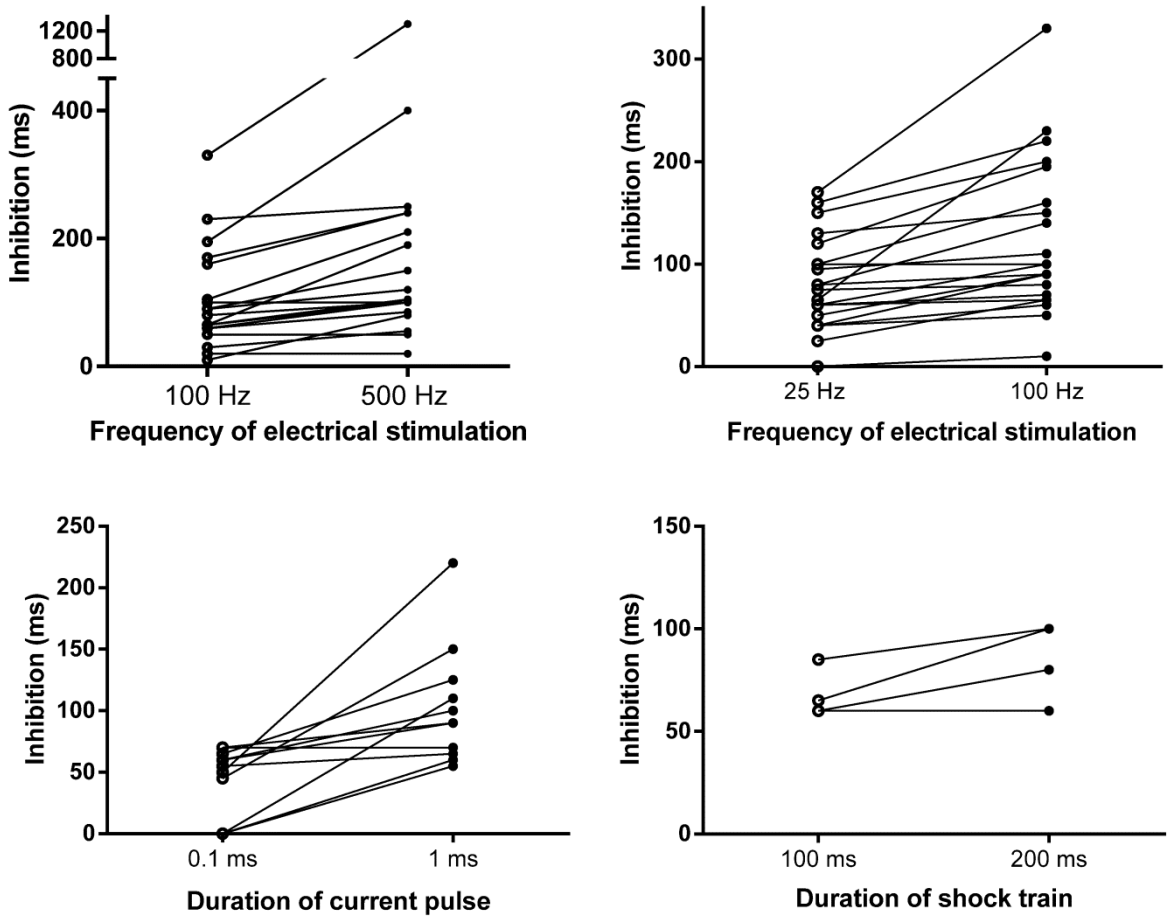
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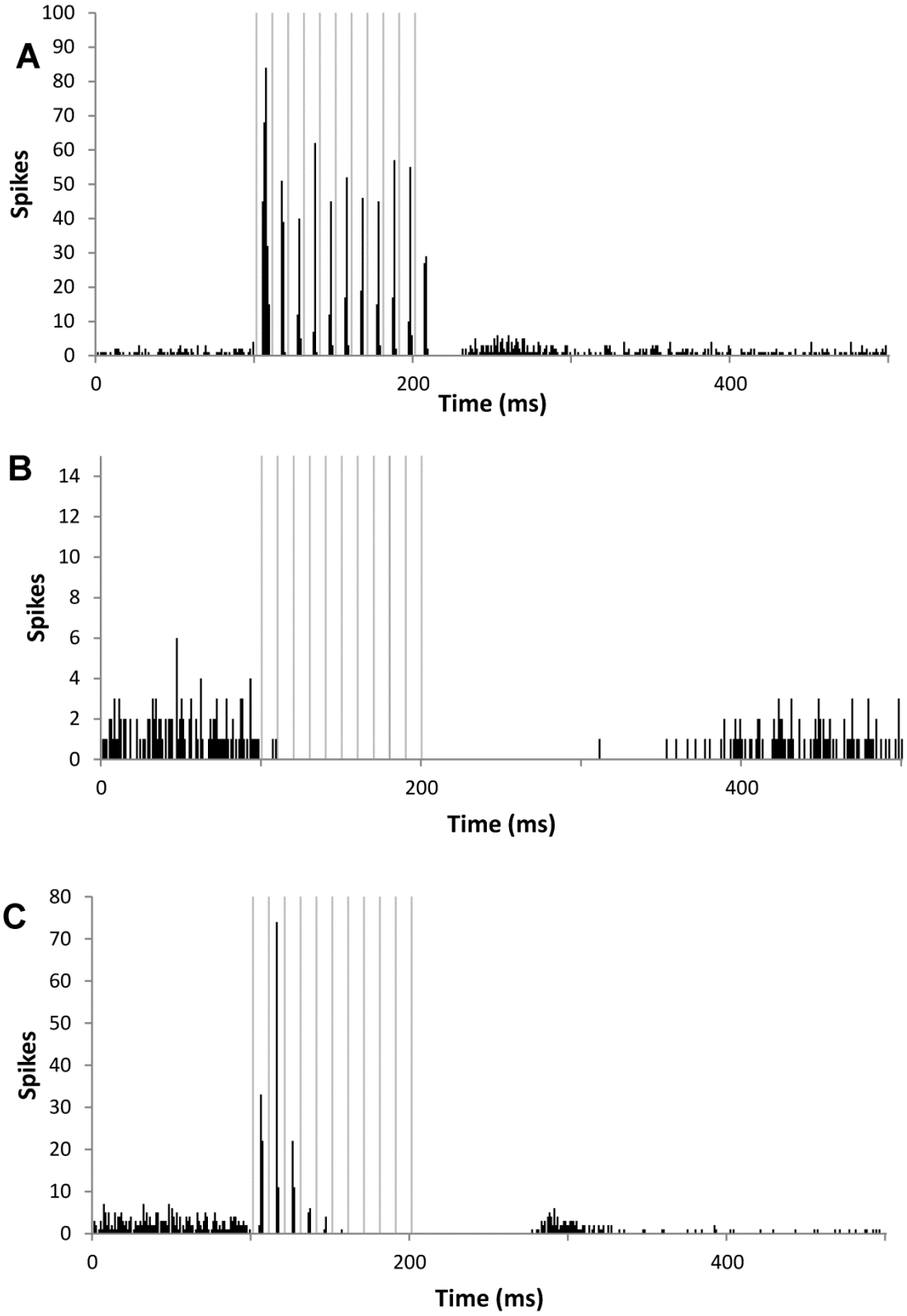

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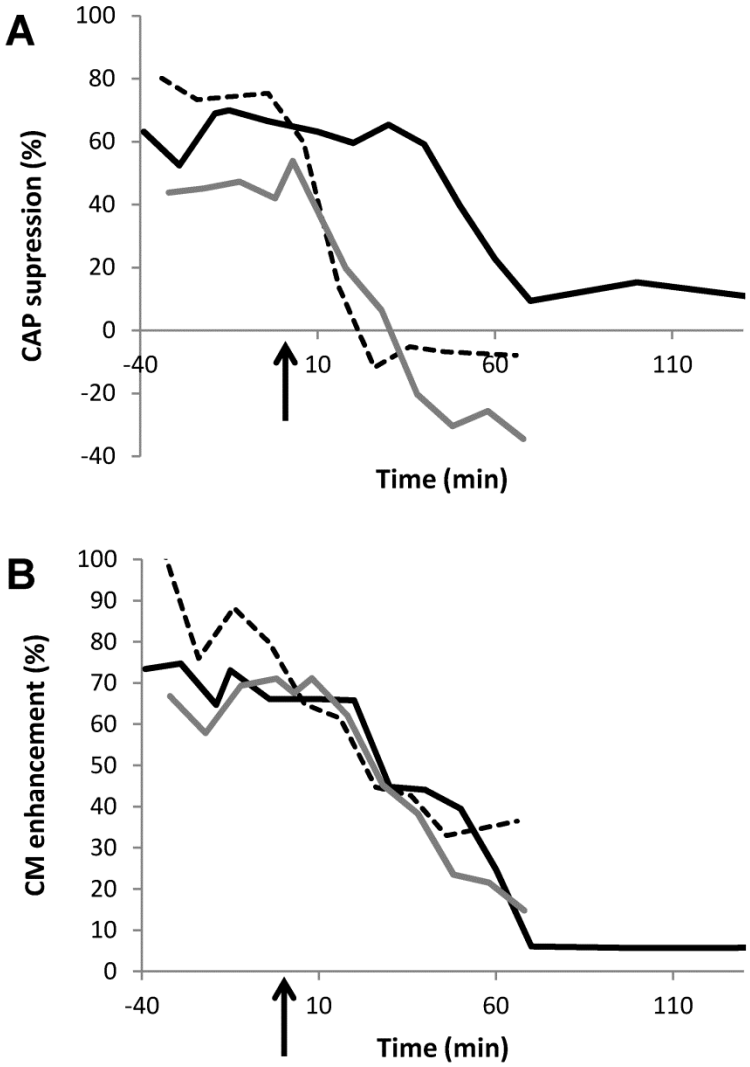
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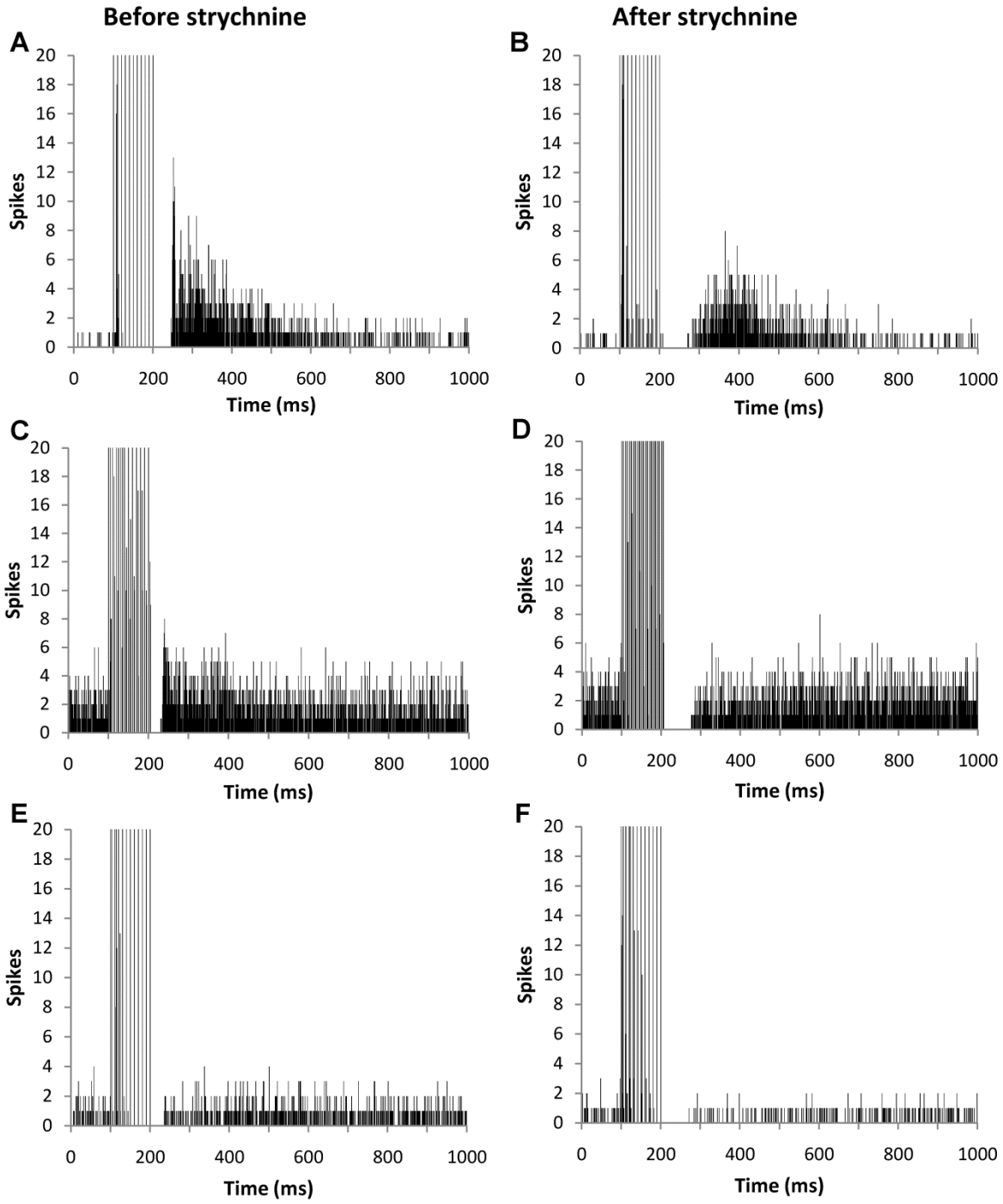

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