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Investigating the Role of Cortical Inhibition in Tinnitus

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Abstract

Subjective tinnitus is characterized as the perception of a phantom sound with no external acoustic source, and is often described as a "ringing in the ears" sensation. While evidence supports a central origin for tinnitus, the underlying neural mechanisms for this condition remain elusive. The studies presented in this thesis offer significant contributions to understanding the neural basis of tinnitus by (1) validating a behavioural paradigm that can successfully screen rats for transient noise-induced tinnitus without any indications of false-positives, and (2) demonstrating that a local loss of inhibition is sufficient to induce gain enhancement in the primary auditory cortex, as well as tinnitus-positive behaviour—evidence that supports the central gain model, one of the leading hypotheses of tinnitus generation. Overall, these findings help provide more effective strategies to directly investigate putative mechanisms of tinnitus, and furthermore expand our current understanding of this distressing condition.

Keywords

Co-Authorship

Although the following co-authors made significant contributions to this body of work, I am the primary author and conducted the vast majority of the experimental data collection and analysis. Furthermore, this entire manuscript was written and prepared by me with consultation from the co-authors.

Dr. Brian L. Allman, PhD, provided extensive leadership throughout the course of this project and his ideas have helped to guide the project towards the end product. He also helped to critically review this body of work prior to its submission.

Ashley Schormans, PhD Candidate, provided invaluable leadership over the course of this project and offered guidance and support in both data collection and analysis. Custom MATLAB scripts used to analyze electrophysiological data were written by her, without which the interpretation of the results would not be possible. Furthermore, she provided extensive feedback and insight to this written body of work.

Dr. Marei Typlt, PhD, conducted many pilot experiments that led to the development of this project. Furthermore, custom MATLAB scripts used to run the behavioural paradigm employed in this project were programmed by her.

Dr. Daniel Stolzberg, PhD, developed the behavioural paradigm that is currently employed in our lab in collaboration with his colleagues and Dr. Brian L. Allman.

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List of Abbreviations

A1, Primary Auditory Cortex

ABR, Auditory Brainstem Response

aCSF, Artificial Cerebral Spinal Fluid

AM, Amplitude-Modulated

ANF, Auditory Nerve Fiber

ANOVA, Analysis of Variance

CAS, Central Auditory System

CSD, Current Source Density

DCN, Dorsal Cochlear Nucleus

EEG, Electroencephalography

fMRI, Functional Magnetic Resonance Imaging

GABA, Gamma-Aminobutyric Acid

GPIAS, Gap Prepulse Inhibition of the Acoustic Startle Response

IO, Input-Output

IR, Infrared

LED, Light-Emitting Diode

LFP, Local Field Potential

MEG, Magnetoencephalography

MGB, Medial Geniculate Body

MU, Multi-Unit

NBN, Narrow-Band Noise

PET, Positron Emission Tomography

PSTH, Peri-Stimulus Time Histogram

SEM, Standard Error of the Mean

SD, Standard Deviation

SPL, Sound Pressure Level

SS, Sodium Salicylate

TDT, Tucker-Davis Technologies

Chapter 1

1   Literature Review

1.1   Tinnitus

Tinnitus is a condition in which a person perceives a sound in the absence of an external auditory source. Two types of tinnitus exist amongst patients: objective and subjective. Objective tinnitus refers to the perception of a *real* sound generated by an internal acoustic source found within the body, such as vasculature or musculature surrounding the ear (Henry et al., 2005). By far, the more common form of tinnitus is subjective, which refers to the perception of a *phantom* sound without an identifiable acoustic source (Møller, 2011). The focus of this review will be on the subjective form of tinnitus, and as such, subjective tinnitus will henceforth be referred to only as "tinnitus".

Patients suffering from tinnitus often describe a "ringing in the ears" sensation when asked to characterize the phantom sound. Most adults at some point in their life will experience tinnitus transiently, with the phantom sound fading within a few hours or days (Henry et al., 2005). This type of tinnitus, frequently triggered by reversible causes such as listening to loud music for long durations of time, or consuming high doses of aspirin, is often of minimal concern to individuals who tend to be able to ignore the phantom sound until it resolves itself. However, it is estimated that as many as 10 to 15% of the general population suffer from persistent tinnitus, which is experienced for the most part, continuously (Heller, 2003). Patients affected by persistent tinnitus generally have a decreased overall quality of life, and often seek medical attention once the bothersome "ringing" starts to have negative impacts on their sleep patterns and daily activities (Shargorodsky et al., 2010). Approximately 1% of the general population suffers from an extreme case of persistent tinnitus, in which the individual finds his/her tinnitus debilitating, even leading to severe episodes of depression (Dobie, 2003). Unfortunately, despite decades of research, there is still no widely-accepted treatment available that can reliably eliminate the phantom perception. Instead, a majority of the currently available

therapies (e.g., sound therapy; cognitive behavioural therapy) are focused on helping patients to cope with their tinnitus, and alleviate the associated distress (Cima et al., 2014; Hoare et al., 2014). A number of drugs approved for treatment of other medical conditions have been prescribed in an effort to help patients manage their tinnitus (Allman et al., 2016). That said, these "off label" drugs often present with unwanted side effects, making these temporary solutions less than ideal. A more effective strategy would be to target the direct underlying mechanisms of tinnitus to abolish the phantom perception at its source. However, this has proven difficult due to the large variability in etiology, perceptual characteristics, and associated symptoms amongst patients. Ultimately, an improved understanding of the mechanisms that generate tinnitus is essential for the future development of effective treatments and pharmacotherapies.

1.2   Etiology of Tinnitus

It is well-established that tinnitus typically arises from exposure to noise, ototoxic drugs, and/or aging (Eggermont & Roberts, 2004). Noise-induced tinnitus typically develops from exposure to either recreational, occupational, or firearm noise, and as such, is becoming a growing concern in the population (Shargorodsky et al., 2010). In fact, one study conducted on university students found that 89.5% of the students interviewed had experienced transient tinnitus following loud music exposure (Gilles et al., 2012). While transient tinnitus is not of the utmost concern, repeated exposure to such high intensity sound levels may serve as a precursor for persistent tinnitus and other related symptoms in the future (Kujawa & Liberman, 2006, 2009; Weisz et al., 2006). For example, one study found that 33% of surveyed patients were exposed to occupational noise for years prior to tinnitus onset (Axelsson & Barrenas, 1991). Beyond the general population, military personnel are frequently exposed to loud firearm noise, increasing their risk of developing persistent tinnitus as a study found that 49% of returning war veterans went on to develop tinnitus (Cave et al., 2007; Theodoroff et al., 2015). While noise exposure is more commonly encountered in an everyday setting, ototoxic drugs, such as salicylate (the active ingredient in Aspirin), can also induce tinnitus (Cazals, 2000). However, early studies found that salicylate-induced tinnitus was reversible upon cessation of the treatment, leading one to wonder if noise- and salicylate-induced tinnitus are generated

by the same mechanisms (Falbe-Hansen, 1941; Graham & Parker, 1947). Finally, numerous studies have noted that the prevalence of tinnitus increases with age (Eggermont & Roberts, 2004; Møller, 2011; Shargorodsky et al., 2010). This is likely because the incidence of hearing loss also increases as you grow older due to an accumulation of physiological deterioration, noise exposure effects, and medical conditions (Huang & Tang, 2010).

There has been much debate over whether or not the mechanisms used to generate tinnitus are the same across the various etiologies, as each apparent trigger for tinnitus development (i.e., noise exposure, salicylate, aging) is related in some way to hearing loss. Indeed, a vast majority of tinnitus patients present with some level of detectible hearing loss (Axelsson & Ringdahl, 1989; Davis & Refaie, 2000; Henry & Wilson, 2001). Even those who suffer from tinnitus but have clinically unaffected hearing, may have threshold shifts or auditory damage in regions outside of the typical audiogram (Roberts et al., 2008; Weisz et al., 2006). Interestingly, studies asking patients to match their tinnitus pitch to various sound frequencies have revealed that the frequency of tinnitus tends to reflect the individual's region of hearing loss (Langers et al., 2012; Noreña et al., 2002; Roberts et al., 2008). Thus, some degree of hearing impairment, regardless of etiology, likely plays a crucial role in the development of phantom sound perception.

1.3 Approaches to Uncover the Neural Basis of Tinnitus

Original theories on the potential mechanisms of tinnitus suggested a peripheral origin from the cochlea, since patients suffering from tinnitus would perceive the phantom sound from within their ears, and moreover because tinnitus correlated strongly with hearing loss (Jastreboff, 1990; Kiang et al., 1970). In this peripheral model of tinnitus, it was believed that noise- or age-induced cochlear damage and hearing loss, resulted in hyperactivity of auditory nerve fibers (ANFs), which ultimately manifested as the aberrant phantom perceptions of tinnitus (Møller, 2011). However, support for this original theory is limited, as many studies have found the opposite to be true. For example, studies modelling cochlear pathology in animals found *reduced* spontaneous

firing rates from ANFs following aminoglycoside administrations (Harrison, 1978; Kiang et al., 1970), not the suggested increase in activity proposed by the peripheral model of tinnitus. Furthermore, House and Brackmann (1981) performed surgical transections of ANFs during the removal of acoustic neuromas and found that tinnitus persisted in a majority of patients following the procedure. If tinnitus was truly generated from within the cochlea, then severing the ANFs, which are solely responsible for transmitting the acoustic signals to the central auditory system (CAS), should abolish these phantom perceptions. These studies have led to the idea that the actual manifestation of tinnitus, although likely triggered by cochlear damage, may in fact be generated from changes within the CAS.

Since the advent of a potential central origin of tinnitus, human and animal studies have attempted to investigate the underlying neural mechanisms of this condition in the hopes of eventually developing a treatment targeted to the source of the phantom ringing. The investigation of tinnitus in humans often involves the use of various neuroimaging techniques to observe how neural activation differs in various regions of the brain between tinnitus and control populations (Eggermont & Roberts, 2015). However, the results of such imaging studies only provide correlations between neural activation and the presence of tinnitus, without any direct indication of where or how the tinnitus percept is generated.

Animal models provide several advantages to the investigation of the neural basis of tinnitus as they allow for the use of more invasive techniques and manipulations. Many animal studies rely on the use of microelectrodes to record neural activity in specific auditory structures following the induction of tinnitus (Kaltenbach, 2011). Using these approaches, single-unit (i.e., single neuron) and multi-unit (i.e., clusters of neurons) responses can be recorded to determine if the neurons fire differently between tinnitus and control conditions. Often neural activity is recorded either at rest (i.e., spontaneous activity) or in response to a sound stimulus (i.e., auditory-evoked) to fully understand how tinnitus affects the auditory pathway (Kaltenbach, 2011). Of course, the problem with the use of animal models is that tinnitus is a subjective phenomenon, and as of yet, no objective measures exist to determine whether or not a person (or animal) is

experiencing tinnitus. Humans are able to verbally explain what they are perceiving to others, making it easier to determine the presence/absence of tinnitus, as well as its perceptual characteristics; clearly, animals do not have the same capacity to do so. Thus, a large focus of the field has been to develop an effective way to screen animals for the presence of tinnitus.

1.3.1   Animal Models & Behavioural Evidence of Tinnitus

In order to use animal models to study the underlying neural mechanisms of tinnitus, it is necessary to induce tinnitus in animals similar to the way humans acquire it. Noise exposure and high doses of salicylate are the most convenient ways to study tinnitus in animals as these approaches can be conducted in relatively short timeframes. As such, the field has collectively focused on these two methods of tinnitus induction as a means of studying underlying neural mechanisms. However, following noise- or salicylate exposure, not all humans develop tinnitus, thus it is still necessary to screen animals following these manipulations to determine if they perceive phantom auditory sounds or not (Atherley et al., 1968; Cazals, 2000; Loeb & Smith, 1967). All of the currently available paradigms involve a change in an animal's behavioural performance during tinnitus when compared to a non-tinnitus state. Furthermore, a truly effective model should be able to (1) reliably screen for both transient and persistent tinnitus, (2) closely reflect the human condition, (3) be resistant to the confounding influence of hearing loss that often accompanies tinnitus, and (4) allow for individual, rather than group, comparisons to account for slight variabilities amongst those who suffer from tinnitus (Hayes et al., 2014). Only after reliably screening animals for tinnitus, can we then investigate the possible changes in neural activity that may be responsible for these phantom perceptions. The following sections first describe the key features of previous methods used to screen animals for tinnitus, with an emphasis on their important shortcomings, and then present the case for further validating one of the recently-developed behavioural paradigms to screen animals for not only drug-induced tinnitus, but also tinnitus following loud noise exposure.

1.3.1.1   Shock Avoidance Behaviour

The first behavioural evidence that animals could perceive phantom auditory sounds came from Jastreboff and colleagues (1988), who showed that subcutaneous injections of salicylate in rats could induce tinnitus-positive behaviour using their novel conditioned lick-suppression paradigm. They utilized a dose of salicylate that would result in serum levels within the range of salicylate-treated humans (Mongan et al., 1973). Their paradigm was based on training rodents to lick a spout for water when they were presented with a steady background noise, and to suppress their licking during quiet conditions. Failure to stop licking during quiet was matched with a mild foot shock during training sessions until they became proficient at the task. Rodents were then given an injection of salicylate and run on a testing session during which the foot shocks were turned off. If rats developed tinnitus, they were expected to demonstrate behavioural extinction (i.e., licking during quiet conditions) faster than rats who were given saline injections, because they would presumably perceive phantom sounds during quiet conditions. These phantom sounds were expected to sound similar to the background noise, which would instruct the rats to commence licking behaviour. While Jastreboff and colleagues elegantly demonstrated that the observed changes in behavioural performance were not due to the confounding influences of hearing loss or non-auditory salicylate effects, one of the main drawbacks of this paradigm is that the behaviour extinguishes over time, preventing a long-term study of tinnitus (Hayes et al., 2014). Furthermore, although the screening can be done in a relatively short time period, separate groups of animals are needed for control and experimental treatments, meaning that comparisons within the same animal are not possible (Hayes et al., 2014).

Since the development of this first animal model, several follow-up shock avoidance paradigms have been established to try and improve upon the drawbacks of Jastreboff's work (Bauer & Brozoski, 2001; Bauer et al., 1999; Guitton et al., 2003; Heffner & Harrington, 2002; Lobarinas et al., 2004; Rüttiger et al., 2003). While some models attempted to modify the model to screen for persistent tinnitus (Bauer & Brozoski, 2001; Bauer et al., 1999), behavioural extinction remained a large problem for a majority of the developed paradigms, regardless of the modifications that were made (Guitton et al.,

2003; Heffner & Harrington, 2002; Rüttiger et al., 2003). Furthermore, as noted in a review by Hayes et al. (2014), not all shock avoidance models were resistant to the confounding effects of hearing loss induced by noise exposure and salicylate. Ultimately, the downfall of shock avoidance models lies in the very nature of the behavioural measure. Indications of tinnitus are based on whether an animal licks a water spout, presses a lever, or jumps onto a pole more or less frequently when compared to controls. Ideally, it would be preferable for animals experiencing tinnitus to make a qualitatively different behavioural choice compared to controls, to avoid the confounding influences of hearing loss, hyperacusis, motivation, and stress (Hayes et al., 2014).

1.3.1.2   Gap Prepulse Inhibition of the Acoustic Startle Response

To date, the most common behavioural model used in tinnitus research has been the gap prepulse inhibition of the acoustic startle response (GPIAS) paradigm developed by Turner et al. (2006). The main reason for the popularity of this model is its high throughput nature. Whereas classical and operant conditioning models often require lengthy training periods, the GPIAS paradigm requires no training, and no food or water restriction, as it utilizes an animal's natural acoustic startle response as a metric for the presence/absence of tinnitus. The acoustic startle response refers to an animal's motoric reaction (e.g., full body "flinch") to a very loud and unexpected sound (startle stimulus). This startle response can be suppressed using a method called gap prepulse inhibition, whereby a silent gap in an otherwise continuous acoustic background sound presented 100 ms preceding the startle stimulus, decreases the amplitude of an animal's startle response. According to proponents of the GPIAS model, if the background sound closely matches an animal's tinnitus percept, then its tinnitus is expected to "fill in the gap," causing the animal to be unable to detect the actual gap in sound. Consequently, an animal experiencing tinnitus is expected to fail to demonstrate prepulse inhibition (i.e., its startle magnitude is equivalent between trials when a gap is present or not). In addition to its high throughput nature, the GPIAS paradigm has been championed because it can be used to identify tinnitus at the level of the individual, including investigating the perceptual characteristics of each animal's tinnitus pitch (simply by varying the background sound in which the gap is placed).

Despite the suggested advantages of the GPIAS paradigm, several discrepancies have been noted by a number of follow-up studies. For example, the startle reflex has been found to be strongly influenced by the presence of hearing loss following unilateral noise exposure (Lobarinas et al., 2013). Rats given unilateral noise exposures were found to have decreased baseline startle amplitude in response to startle-only (no-gap prepulse) trials. Because the main measure of gap prepulse inhibition is the gap:no-gap ratio, if the no-gap amplitude is decreased, it still gives the overall impression that tinnitus "filled in the gap" as the overall ratio would be larger. To further emphasize this point, Lobarinas and colleagues gave rats a unilateral conductive hearing loss with a foam earplug (which would not be expected to cause tinnitus), yet these rats showed false-positives for tinnitus simply due to the unwanted effect the hearing loss had on startle amplitude. Similar decreases in no-gap startle amplitudes were also observed in studies on noise-exposed mice (Longnecker & Galazyuk, 2011). Research on human tinnitus patients have also provided some challenges to the GPIAS paradigm. In a study by Campolo et al. (2013), tinnitus patients with some degree of hearing loss were asked if they could perceive a 50 ms gap in a continuous background narrow-band noise either below, above, or at their tinnitus tone. All subjects, including controls with normal hearing thresholds, were able to perceive the gap, suggesting that the basis of the GPIAS model that tinnitus "fills in the gap" is likely flawed. Furthermore, a study by Fournier and Hébert (2013), found that tinnitus patients had greater startle amplitudes in response to the startle stimulus than controls did, suggesting a confounding role of hyperacusis in the GPIAS paradigm. While in this study, tinnitus patients did show gap detection deficits, their inability to identify the silent gaps was not frequency-specific as would be suggested by the "filling in the gap" hypothesis. Ultimately, although the GPIAS paradigm has a number of beneficial aspects that make it an attractive model to study tinnitus, researchers should be extremely cautious in the interpretation of their results due to the strong confounding influences of hearing loss, hyperacusis, and the inconsistencies found in human studies.

1.3.1.3   Two-Choice Operant Conditioning Behaviour

To move beyond reliance on the GPIAS paradigm and to overcome a few of the inherent drawbacks of shock avoidance paradigms, some researchers have recently endeavored to

design two-choice operant conditioning models to screen animals for tinnitus. Models developed by Sederholm and Swedberg (2013), and Stolzberg et al. (2013) were both predicated on training rats to distinguish between auditory stimuli and quiet conditions. Behavioural responses to auditory stimuli were represented by rats choosing one lever or feeder trough, while responses to quiet were represented by choosing a secondary lever or feeder trough. Tinnitus-positive behaviour was believed to be indicated by a shift from the quiet lever/trough to the auditory lever/trough in responses to quiet stimuli, presumably because rats were experiencing phantom auditory sounds during quiet. More specifically, Stolzberg et al. trained rats to associate narrow-band noise (NBN) stimuli centered at 5 different frequencies to the left feeder trough to help generalize the tinnitus percept to that side, whereas Sederholm and Swedberg's paradigm introduced a single pure tone stimulus for the auditory lever. In the case of the two-alternative forced-choice paradigm designed by Stolzberg and colleagues, a reduced reward rate was also introduced during behavioural training, such that during future testing sessions the rats would not notice an absence of rewards for quiet stimuli; an approach implemented to prevent the potential extinction of behaviour. Furthermore, to confirm that shifts in behavioural responses were not merely a result from a bias introduced by tinnitus induction, the feeder trough for quiet trials (right side) was also associated with a separate acoustic stimulus—amplitude-modulated noise—which was not expected to sound like the tinnitus percept. Thus, rats experiencing tinnitus would be expected to select the NBN (left) trough during NBN and quiet stimuli (thus providing evidence of tinnitus), but would still correctly respond to the other (right) trough during amplitude-modulate trials; results that would confirm that rats screened positive for tinnitus, but not because of a confound associated with a developed bias to only the left feeder trough.

It is important to note that the aforementioned behavioural paradigm designed by Stolzberg et al. has thus far only been validated with acute salicylate exposure in rats immediately preceding behavioural testing and has not yet been used to screen rats for behavioural evidence of tinnitus following loud noise exposure. Given the suggested advantages of this operant conditioning paradigm (Hayes et al., 2014), it would be prudent to evaluate its efficacy and resilience for screening noise-exposed rats for transient as well as persistent tinnitus. As described in Chapter 2, we conducted a

comprehensive investigation of how rats performed on this two-alternative forced-choice paradigm following specific noise exposures that were designed to induce either transient or persistent tinnitus. Importantly, these noise exposure experiments were carried out to serve as a validation of the paradigm, and thus, provide a behavioural platform to then screen for evidence of tinnitus following novel experimental interventions that directly targeted a putative mechanism of tinnitus generation (i.e., central gain increase; see Section 1.4.5 below and Chapter 3 for details).

1.4 Neural Correlates of Tinnitus Derived from Human and Animal Studies

Studies conducted on humans using audiometric testing can provide insight into the psychoacoustic characteristics of each patient's tinnitus percept. For instance, it is possible to characterize the pitch, loudness, and spectral and temporal qualities of tinnitus based on questionnaires and tinnitus pitch matching procedures (Henry et al., 2014). From such studies, it was revealed that the tinnitus pitch tends to fall within the region of hearing loss (Langers et al., 2012; Noreña et al., 2002; Roberts et al., 2008). However, it should be noted that these clinical procedures offer minimal insight into the actual mechanisms that may underlie the phantom perception. To that end, researchers have compared the brains of tinnitus subjects versus healthy controls using neuroimaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). Collectively, these studies have provided support for the central theory of tinnitus, as tinnitus sufferers show enhanced neural activation in the primary auditory cortex (A1), as well as other brain regions outside of A1 (e.g., basal ganglia, cerebellum, prefrontal cortex, parietal cortex, and sensorimotor areas), when compared to control subjects (Giraud et al., 1999; Gu et al., 2010; Lockwood et al., 2001; Maudoux et al., 2012).

Separate from neuroimaging procedures, electro- and magnetoencephalography (EEG, MEG) techniques have also been used to investigate brain activity, and particularly neural synchrony, in tinnitus patients versus healthy controls. Rhythmic synaptic inputs on groups of neurons cause them to fire synchronously, producing an oscillating neural

signal at specific frequencies (Buzsáki et al., 2012). EEG and MEG methods are capable of recording these neural oscillations, and the cumulative neural trace can subsequently be separated into the different frequency bands that comprised the signal. The oscillatory power of each range is believed to correlate with the proportion of recorded neurons synchronously firing at that frequency. Briefly, there are several physiologically relevant frequency bins that have typically been used to categorize neural oscillations: delta (0 to 3 Hz), theta (4 to 7 Hz), alpha (8 to 12 Hz), and gamma (30 to 200 Hz) (Uhlhaas et al., 2008). Studies have shown that tinnitus patients tend to present with a specific oscillatory profile, such that the relative power is increased in both the low (delta) and high (gamma) frequency bins, and decreased in the alpha bin relative to control subjects (Adjamian et al., 2012; Ashton et al., 2007; Balkenhol et al., 2013; van der Loo et al., 2009; Weisz et al., 2007; Weisz et al., 2005). Attempts to interpret these power changes have led to suggestions that aberrant oscillations may be responsible for generating phantom sound perceptions. However, further investigation from animal studies are needed to confirm a causative relationship between cortical oscillations and tinnitus.

Provided animals can be accurately screened as having tinnitus, animal models offer the possibility of investigating the nature and extent of neuroplasticity that takes place in the auditory pathway. As described in detail below, numerous studies have used microelectrodes to record electrophysiological activity in various auditory structures, including the dorsal cochlear nucleus, inferior colliculus, medial geniculate body, and auditory cortex of animals experiencing drug- or noise-induced tinnitus (Kaltenbach, 2011). Based on the assortment of neural changes observed in the auditory pathway of animals, as well as humans, several putative mechanisms of tinnitus have been developed to try and explain how the tinnitus percept is generated.

At present, the proposed models of tinnitus include: (1) aberrant neural synchrony, (2) disrupted networks, (3) tonotopic map reorganization, (4) dorsal cochlear nucleus hyperactivity, and (5) central gain enhancement. The following sections will briefly describe each putative mechanism of tinnitus, with a focus on the experimental results that support (and perhaps refute) the model. Emphasis will be given to the central gain

enhancement model of tinnitus, as the neural plasticity associated with this model shaped the experiments conducted in Chapter 3.

1.4.1   Aberrant Neural Synchrony

In 1999, Llinás et al. first proposed that neural synchrony, as a result of dysrhythmic activity in the thalamocortical circuit, could be the mechanism underlying tinnitus. This model, which was later updated by De Ridder et al. (2015), is based on findings from EEG and MEG studies in humans. According to the neural synchrony model, deafferentation of the auditory nerve deprives the rest of the central auditory system of sensory inputs. As a result, the medial geniculate body (MGB) of the thalamus switches to a state of tonic inhibition or hyperpolarization, in which neurons of the MGB begin to synchronously burst fire at a theta frequency (4 to 7 Hz). Neurons of the MGB project up to the auditory cortex, and as such, cause aberrant synchronized firing of cortical neurons as well, typically at a gamma frequency (30 to 200 Hz). Increases in theta oscillations have been suggested to represent long-range synchrony, allowing for the retrieval of missing thalamocortical information (due to sensory deprivation) from parahippocampal memory. Alternatively, increases in gamma oscillations are typically ascribed to the conscious perception of stimuli. Ultimately, this aberrant theta-gamma coupling is believed to underlie the tinnitus percept, and has been observed in the EEG and MEG profiles of tinnitus patients (Ashton et al., 2007; Balkenhol et al., 2013; van der Loo et al., 2009; Weisz et al., 2007, 2005). Additional support for the mechanism of thalamocortical dysrhythmia was derived from an *in vitro* study by Sametsky et al. (2015), in which the authors were able to induce burst firing in MGB cells when brain slices were immersed in hyperpolarizing conditions. Furthermore, recordings in animals with behavioural evidence of noise-induced tinnitus found that MGB neurons showed both elevated spontaneous activity and altered burst firing (Kalappa et al., 2014). Moreover, electrophysiological studies in noise-exposed animals have found increases in neural synchrony at the level of the auditory cortex (Noreña & Eggermont, 2003; Seki & Eggermont, 2003). Thus, increases in burst firing within the MGB, could result in synchronized neural firing in the auditory cortex. However, it has yet to be shown that altered burst firing in the MGB directly causes the oscillatory profile of tinnitus that is

observed in humans. Moreover, it is unclear whether theta-gamma coupling is responsible for generating the tinnitus percept, or if it is merely an epiphenomenon of tinnitus pathology.

1.4.2   Disrupted Network Models

The network model of tinnitus suggests that areas of the brain outside of the auditory pathway are involved in the conscious perception of tinnitus (Elgoyhen et al., 2012; Leaver et al., 2011). The basis of this model arises from various neuroimaging studies that have observed modifications to connectivity networks not only involved in auditory processing, but in attention, stress, emotion, and memory as well (Burton et al., 2012; Husain & Schmidt, 2014; Kim et al., 2017; Maudoux et al., 2012; Schlee et al., 2008, 2009; Schmidt et al., 2013). Although the changes in functional connectivity of these networks could explain for the emotional aspects of tinnitus, such as annoyance and stress, further investigation is needed to determine if these non-auditory structures are necessary for the generation of the tinnitus percept, or if their aberrant activation is subsequent to the altered neural activity within the central auditory pathway (Eggermont & Roberts, 2015).

1.4.3   Tonotopic Map Reorganization

The tonotopic map reorganization model of tinnitus was first proposed by Rauschecker (1999). It is well-established that following damage to selective regions of the cochlea (e.g., high frequency area in the basal turn of the cochlea), the cortical consequences extend beyond just a hearing loss associated with the region (e.g., impaired high frequency hearing). Indeed, cortical neurons located in the high-frequency area of the cortical tonotopic map lose their afferent input and instead become more sensitive to the lower frequencies that were unaffected by the cochlear trauma. Ultimately, the amount of cortical area now responsive to the spared lower frequencies expands, and it is this reorganization of the normal tonotopic map that has been suggested to manifest as tinnitus. Evidence of map reorganization has been observed in several studies following noise exposure and salicylate treatment (Eggermont & Komiya, 2000; Muhlnickel et al.,

1998; Noreña & Eggermont, 2005; Noreña et al., 2003; Stolzberg et al., 2011). That said, there are problems associated with the tonotopic reorganization model of tinnitus. Most importantly, if the tinnitus pitch is caused by expansion of unaffected (edge) frequencies, then the tinnitus pitch itself should match the frequency at the lower edge of hearing loss. As noted by Henry et al. (2014), while some studies have found the tinnitus percept to be localized to the edge of hearing loss, others have shown the percept to be in the higher frequency region where maximal hearing loss occurred; findings that undermine the tonotopic reorganization model of tinnitus (Pan et al., 2009; Sereda et al., 2011). Based on the results available, it is reasonable to suggest that tonotopic map reorganization appears to be an epiphenomenon of tinnitus, rather than the central mechanism that generates the phantom perception.

1.4.4   Dorsal Cochlear Nucleus Hyperactivity

The basis of the dorsal cochlear nucleus (DCN) hyperactivity model comes entirely from animal studies that observed an increase in spontaneous and auditory-evoked activity, as well as enhanced neural synchrony and burst firing in the DCN following noise exposure or treatment with ototoxic drugs (Brozoski et al., 2002; Dehmel et al., 2012; Kaltenbach et al., 1998, 2002, 2004; Melamed et al., 2000; Wu et al., 2016). Proponents of this putative model of tinnitus suggest hyperactivity in the DCN is ultimately propagated throughout the rest of the auditory pathway, to be consciously perceived as tinnitus (Dehmel et al., 2012; Kaltenbach et al., 2005). However, if the DCN was indeed the site of tinnitus generation, then it reasons that disruption of DCN function should abolish indications of tinnitus. This was not the case; two studies that severed afferent and efferent inputs to the DCN failed to disrupt elevated spontaneous firing rates within this structure (Zacharek et al., 2002; Zhang et al., 2006). Moreover, a series of studies demonstrated that bilateral lesions of the DCN failed to remove behavioural evidence of chronic tinnitus, but did prevent the development of tinnitus in naive animals (Brozoski et al., 2002; Brozoski & Bauer, 2005). These studies undermine the DCN hyperactivity model as the mechanism that generates the tinnitus percept, as this structure continues to demonstrate correlates of tinnitus after transection. However, the DCN still likely plays a crucial role in tinnitus induction, such that it is needed to propagate neural changes to

higher order areas, allowing tinnitus to develop in other structures of the auditory pathway (Henry et al., 2014; Kaltenbach, 2011).

1.4.5   Central Gain Enhancement

Arguably, one of the leading proposals for the neural basis of tinnitus is the central gain model, which was first hypothesized by Jastreboff (1990), and further expanded upon by Schaette and Kempter (2006), and Noreña (2011). The central gain model of tinnitus suggests that following deafferentation of the auditory nerve, the central auditory system (CAS) becomes deprived of sensory inputs. Next, in an attempt to homeostatically preserve mean firing rates in the CAS around a set point, each component of the CAS experiences aberrant hyperactivity, resulting in the amplification of "neural noise", supposedly encoding for tinnitus. These homeostatic mechanisms likely alter the balance between excitatory and inhibitory inputs, thereby causing neural enhancement in the auditory pathway (Auerbach et al., 2014). Many studies have found evidence of central gain enhancement following tinnitus induction in various structures of the auditory pathway, as indicated through increases in both spontaneous and auditory-evoked activity.

Increases in spontaneous activity have been observed at many levels of the CAS following exposure to various tinnitus-inducers in animal models (Bauer et al., 2008; Brozoski et al., 2002; Dong et al., 2010; Eggermont & Kenmochi, 1998; Jastreboff & Sasaki, 1986; Kaltenbach & McCaslin, 1996; Kaltenbach & Afman, 2000; Kimura & Eggermont, 1999; Komiya & Eggermont, 2000; Manabe et al., 1997; Melamed et al., 2000; Mulders & Robertson, 2011; Mulheran & Evans, 1999; Noreña & Eggermont, 2005; Seki & Eggermont, 2003; Zhang & Kaltenbach, 1998). Proponents of the central gain model of tinnitus suggest that the increase in spontaneous activity that occurs throughout the CAS, culminating in the auditory cortex, ultimately causes the phantom perception of tinnitus. Support for this proposal comes from work in humans in which sound percepts were found to occur concurrently with increases in spontaneous activity following sound- and electrical-stimulation of peripheral or central auditory pathways (Clark, 2008; Colletti et al., 2009; Kaltenbach, 2011).

In addition to the increased firing rates that occur during quiet conditions, the central gain model of tinnitus also considers the increased activity that occurs in response to auditory stimulation. In this case, an increase in auditory-evoked activity would indicate that neurons have a stronger response to a given sound stimulus than they did prior to tinnitus induction. Typically, this is measured through increases in local field potential amplitude and auditory-driven neuronal firing rates. In tinnitus subjects, there appears to be a paradoxical difference in the amount of hyperactivity that occurs throughout the CAS. For example, at the level of the auditory nerve, there is a decrease in afferent activity following cochlear damage, whereas the auditory responses from the inferior colliculus show minimal changes, and yet responses in the auditory cortex show hyperactivity (Popelar et al., 1987; Qiu & Salvi, 2000; Salvi et al., 1992; Sun et al., 2009; Syka et al., 1994). Recently, Chambers et al. (2016) conducted an elegant study in which they unilaterally lesioned a large proportion of cochlear nerve synapses and subsequently monitored recovery of auditory-evoked responses in the auditory nerve, inferior colliculus and auditory cortex in the week and month following lesioning. Despite indications of hearing impairment at the level of the brainstem, lesioned mice could still behaviourally detect tonal stimuli. Furthermore, while responses from the auditory nerve failed to recover to control levels, responses from the inferior colliculus showed modest recovery 30 days post-lesioning, and responses from the auditory cortex surpassed control levels after 30 days. The investigators concluded that this neural plasticity (i.e., gain enhancement) that occurred in the inferior colliculus and auditory cortex likely explained the lack of behavioural deficits in these animals. Together, these studies provide direct evidence of increased central gain as a result of sensory deprivation, as auditory responses progressively get stronger as you ascend the pathway, indicating an accumulation of hyperactivity despite a lack of auditory input.

One criticism of the central gain model is that it has yet to be determined if gain enhancement must occur at a particular auditory structure to generate the tinnitus percept, or if it is the accumulation of hyperactivity throughout the entire auditory pathway that is responsible for tinnitus. Of all the central auditory structures, the auditory cortex seems to be a likely candidate for tinnitus generation, as it not only experiences the strongest indications of central gain, but it also demonstrates the fastest enhancement of neural

activity, often occurring within hours of tinnitus induction rather than several days post-tinnitus induction as has been observed in subcortical auditory structures (Noreña et al., 2010; Salvi et al., 1990, 2000; Sun et al., 2008, 2012; Syka et al., 1994; Syka & Rybalko, 2000). Offering further support for the role of the auditory cortex in central gain enhancement and tinnitus, an fMRI study reported that increased auditory-evoked activity in the primary auditory cortex was specific to tinnitus patients with normal hearing thresholds, as opposed to patients with both tinnitus and hyperacusis (Gu et al., 2010).

1.5   Overview of Thesis

Rationale: While the field has developed several putative models of tinnitus to explain how these phantom auditory perceptions are generated, there is an insufficient amount of direct evidence to support any of these theories. A majority of the studies that have been referenced to support each of these neural models are based largely on observational work, such that tinnitus is induced in animals, or tinnitus patients are recruited, and neural changes in these subjects are simply compared to control conditions. The drawback of these approaches is that the detected neural changes are merely correlated with the presence of tinnitus, and there is no direct evidence of a causal relationship. Furthermore, many animal studies fail to show behavioural evidence of tinnitus prior to electrophysiological recordings, and those that do, use behavioural paradigms that tend to be confounded by the effects of hearing loss. Thus, the observed neural changes are likely not specific to tinnitus alone, and conclusions on the mechanisms that generate tinnitus cannot be drawn. To that end, we suggest that a comprehensive study of the neural basis of tinnitus must (1) develop a valid behavioural paradigm to screen animals for the presence/absence of tinnitus, and (2) demonstrate that induction of a putative model of tinnitus can directly cause both the established neural correlates of tinnitus, and tinnitus-positive behaviour.

Objective: To investigate the central gain model as a potential mechanism to generate (1) the neural correlates of tinnitus (i.e., increased spontaneous and auditory-evoked activity), and (2) tinnitus-positive behaviour as assessed by a novel operant conditioning behavioural paradigm.

Hypothesis: A local loss of inhibition can induce central gain enhancement in the primary auditory cortex. Subsequently, this local increase in central gain is responsible for generating the phantom auditory perceptions of tinnitus.

1.5.1 Chapter 2: Validation of an Appetitive Operant Conditioning Paradigm to Assess Transient and Persistent Noise-Induced Tinnitus in Rats

Rationale: In order to comprehensively investigate the underlying neural mechanisms responsible for tinnitus generation, it is necessary to be able to accurately screen animals for the presence/absence of tinnitus. The behavioural paradigm designed by Stolzberg and colleagues (2013) was previously established to be able to screen rats for transient salicylate-induced tinnitus. Given the many advantages of this model, it would be prudent to confirm that it can effectively assess the presence/absence of transient noise-induced tinnitus as well. Moreover, persistent tinnitus is of greater concern in the population than transient tinnitus, thus it would be beneficial if this behavioural paradigm were able to assess for tinnitus that continues to persist after loud noise exposure. A complete validation of this paradigm to successfully screen rats for salicylate- and noise-induced tinnitus would provide sufficient evidence to support its use in investigations targeting the putative mechanisms of tinnitus.

Objective: To validate the behavioural paradigm previously established by Stolzberg et al. (2013) in its ability to screen for both transient and persistent tinnitus following 15- and 60-minute loud noise exposures, respectively.

1.5.2   Chapter 3: Central Gain Enhancement and Tinnitus-Positive Behaviour Induced by a Loss of Inhibition in the Auditory Cortex

Rationale: An abundance of studies provide support for central gain enhancement as a putative mechanism that underlies tinnitus generation. Indeed, examples of increased central gain have been observed in several auditory structures in animals following tinnitus induction (Auerbach et al., 2014). However, there has yet to be a study to directly show that increasing central gain *causes* tinnitus, and furthermore, *where* this gain enhancement must ultimately occur for these phantom auditory perceptions to manifest. Based on previous studies, we suggest that the primary auditory cortex (A1) is responsible for tinnitus generation, as it exhibits the greatest and fastest indications of gain enhancement in the central auditory system. To that end, a comprehensive investigation to test this theory must demonstrate that increased gain, specifically in A1, is sufficient to induce both neural and behavioural indications of tinnitus.

Objective: To determine for the first time if a direct impairment of inhibitory neurotransmission in the primary auditory cortex is sufficient to induce (1) neural indications of central gain enhancement, and (2) tinnitus-positive behaviour.

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Chapter 2

2 Validation of an Appetitive Operant Conditioning Paradigm to Assess Transient and Persistent Noise-Induced Tinnitus in Rats

2.1 Introduction

Tinnitus is the subjective perception of a phantom sound that is often described as a "ringing in the ears" sensation. In a majority of cases, tinnitus is experienced temporarily, with the phantom auditory perception fading within a few minutes or hours (Henry et al., 2005). However, for as many as 10 to 15% of the general population, tinnitus is experienced chronically, with 1% of the population having severe debilitating forms of tinnitus that negatively impact their daily lives (Heller, 2003). Despite decades of research, there is still no widely-effective treatment available that can readily suppress tinnitus, and this is largely because the underlying neural mechanisms responsible for this phantom perception remain elusive. Additional insight into the mechanisms generating tinnitus is essential for the development of successful pharmacotherapies.

Although there is no clear consensus over how tinnitus is generated, a few notable theories have been proposed over the past few decades. Based on the collective work using both human and animal models, researchers have suggested that aberrant neural synchrony (De Ridder, 2015; Llinás et al., 1999), disrupted neural networks (Elgoyhen et al., 2012; Leaver et al., 2011), tonotopic map reorganization (Rauschecker, 1999), dorsal cochlear nucleus hyperactivity (Kaltenbach et al., 2005), and/or central gain enhancement (Jastreboff, 1990; Noreña, 2011; Schaette & Kempter, 2006) are responsible for tinnitus generation. Further validation (or refutation) of these putative theories of tinnitus is expected to rely heavily on animal studies and advanced neurophysiological experiments,

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which first requires that researchers are able to effectively screen animals for the presence/absence of tinnitus. Related to this, it has been suggested that for a behavioural paradigm to be most effective, it should be able to (1) reliably screen for both transient and persistent tinnitus, (2) closely reflect the human condition, (3) be resistant to the confounding influence of hearing loss that often accompanies tinnitus, and (4) allow for individual comparisons to account for any variability amongst tinnitus sufferers (Hayes et al., 2014).

Many of the existing behavioural paradigms to screen animals for tinnitus are based on one of three general methods: shock avoidance (Bauer & Brozoski, 2001; Bauer et al., 1999; Guitton et al., 2003; Heffner & Harrington, 2002; Jastreboff et al., 1988; Lobarinas et al., 2004; Rüttiger et al., 2003), two-choice operant conditioning (Sederholm & Swedberg, 2013; Stolzberg et al., 2013), and gap prepulse inhibition of the acoustic startle response (GPIAS; Turner et al., 2006). Although each of these paradigms has its advantages, there are also notable limitations that can detract from their effectiveness as a screening tool for tinnitus. For example, traditional shock avoidance paradigms encountered the issue of behavioural extinction, which prevented the ability to study persistent forms of tinnitus, whereas two-choice operant conditioning models can be limited by the extensive period required to train the animals prior to actual behavioural testing. Consequently, the GPIAS paradigm—which does not require overt training—has become the most popular behavioural method used to screen animals for the presence/absence of tinnitus due to its high throughput nature. The basis of the GPIAS paradigm relies on two key features: (1) an animal's reflexive response to a loud stimulus (i.e., its startle reflex), and (2) the attenuation of the magnitude of this startle reflex if the animal is able to detect a short silent gap in an otherwise continuous background noise that precedes the loud startle stimulus (i.e., its gap prepulse inhibition). With respect to tinnitus screening, proponents of the GPIAS paradigm suggest that if the continuous background sound is the same pitch as the animal's tinnitus, then the animal should not be able to detect the silent gap because it is "filled in" by tinnitus. Ultimately, animals are screened positive for tinnitus if they fail to show attenuated startle responses during trials that include a silent gap (i.e., tinnitus-positive animals are believed to lack gap prepulse inhibition). Despite the benefit of the high throughput nature of the GPIAS paradigm,

recent studies have shown that the results are very sensitive to the effects of hearing loss (Lobarinas et al., 2013; Longnecker & Galazyuk, 2011). Furthermore, studies replicating the GPIAS paradigm in humans found that decreased gap prepulse inhibition was not specific to a given patient's tinnitus pitch, as would be expected with the tinnitus "filling in the gap" hypothesis (Fournier & Hébert, 2013). Additionally, work by Campolo and colleagues (2013) found that human subjects with- or without tinnitus could perceive 50 ms silent gaps in steady narrow-band noises, implying that decreased gap prepulse inhibition observed in the GPIAS paradigm is unlikely due to tinnitus "filling in the gap".

Recently, our lab developed a novel two-alternative forced-choice operant conditioning paradigm based on training rats to actively discriminate whether they were hearing (1) steady narrow-band noise (NBN) stimuli, (2) broad-band amplitude-modulated (AM) stimuli, or (3) quiet (Stolzberg et al., 2013). Rats demonstrating tinnitus-positive behaviour were expected to report hearing high frequency NBNs during quiet conditions more frequently than control rats who were not experiencing tinnitus; findings that would mimic the conditions under which humans report perceiving tinnitus. To validate the effectiveness of the paradigm to screen for transient tinnitus, Stolzberg and colleagues (2013) tested behavioural performance of rats exposed to a high dose of sodium salicylate (SS), which is a well-established tinnitus-inducer (Cazals, 2000; Jastreboff et al., 1988). As predicted, following systemic injections of SS, trained rats exhibited behavioural responses consistent with them "hearing" sounds similar to NBNs during ~60% of quiet trials, indicative of tinnitus-positive behaviour. In contrast, during a separate experimental session, these same rats correctly identified nearly all quiet trials following a vehicle injection of saline (Stolzberg et al. 2013). These comparisons were made at the level of the individual, allowing for the optimal behavioural control experiment.

In the present study, we sought to further validate the efficacy of our behavioural paradigm in its ability to screen for transient and/or persistent tinnitus in trained rats following noise exposures of varying durations and intensities. Because the two main tinnitus-inducers used in the field are SS and noise exposure, it is necessary to

demonstrate that our model is capable of accurately assessing both drug- and noise-induced tinnitus. Here, we show that our paradigm is capable of screening rats for transient noise-induced tinnitus-positive behaviour, as trained rats actively reported perceiving sounds reminiscent of NBNs during quiet conditions immediately following, but not the day after, 15-minute noise exposures. Furthermore, although we observed a confounding influence of hearing loss on rats' behavioural performance in the week following 60-minute noise exposures, the robust nature of our control sham exposures suggests a strong potential for this paradigm to be able to screen for persistent tinnitus upon some minor modifications to noise exposure parameters.

2.2   Materials and Methods

A total of 20 adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), separated into two experimental cohorts (n=10 in each), were used in the present study. All rats (60 days old at the onset of training), were housed in a 12-hour light-dark cycle with water *ad libitum*. Rats were maintained on a food restricted diet throughout the duration of the training and experimental periods such that rats reached 85% of free-feeding body weight. All experimental procedures were approved by the University of Western Ontario Animal Care and Use Committee and were in accordance with guidelines established by the Canadian Council of Animal Care.

2.2.1   Behavioural Apparatus and Sensory Stimuli

The behavioural apparatus consisted of a standard modular test chamber (ENV-008CT; Med Associates Inc., St. Albans, VT, USA) housed in a sound-attenuating box (29" W by 23.5" H by 23.5" D; Med Associates Inc.). The front wall of the behavioural chamber included a center port with two stainless steel feeder troughs positioned on either side; each fitted with an infrared (IR) beam used to detect nose-pokes. Each feeder trough was attached to a food pellet dispenser located behind the behavioural chamber. A house light was located on the back wall to illuminate the chamber, and a white light-emitting diode (LED) was located directly above the center nose-poke, which served as a GO cue during behavioural training. Auditory stimulus delivery, nose-poke responses, and

positive/negative reinforcement were controlled using custom-made MATLAB behavioural protocols (EPsych Toolbox, dstolz.github.io/epsych/) running in MATLAB (Mathworks, Nattick, MA, USA), and interfaced with real-time processing hardware (RZ6; Tucker-Davis Technologies (TDT), Alachua, FL, USA).

Three different types of acoustic stimuli were programmed to play from a speaker (FT28D; Fostex, Tokyo, Japan) mounted on the roof of the behavioural chamber. Acoustic stimuli were either quiet (speaker off), amplitude-modulated (AM; broad-band noise, 100% modulation, 5 Hz), or one of five narrow-band noises (NBN; 1/8th octave band, center frequencies at 8, 12, 16, 20, or 24 kHz). One of the three acoustic stimuli conditions was always presented in the behavioural box regardless of trial initiation by the rat. AM and NBN stimuli were calibrated using TDT software and hardware (RPvdsEx, RZ6 module; TDT) to ~75 dB sound pressure level (SPL) using a ¼" microphone (2530, Larson-Davis, Depew, NY, USA) and pre-amplifier (2221, Larson-Davis).

2.2.2   Behavioural Training

Prior to initiating behavioural training, the rats were food restricted to 85% of free-feeding weight to encourage exploration in the behavioural boxes. Rats were trained 30 minutes per day, and 6 days per week. Initial training sessions (Phase 1) required rats to nose-poke a center port (detected by interruption of the center IR beam) to trigger a GO cue (flash of LED). Upon removing its nose from the center port, the rat was immediately reinforced with a food pellet (Bio-Serv, Frenchtown, NJ, USA) dropped into the appropriate feeder trough associated with the acoustic stimulus playing from the overhead speaker; i.e., left feeder trough for 16 kHz NBN, and right feeder trough for quiet. If the rat then nose-poked the correct feeder trough within 5-seconds of the initial pellet delivery (detected by the interruption of the trough IR beam), the rat was given a second food pellet reward to further reinforce the stimulus association. During a 30-minute training session, trial type (16 kHz NBN or quiet) was distributed evenly and presented in a randomized order. As rats became more proficient at the task, the cue delay (time required to trigger the GO cue) was slowly increased from 500 to 2500 ms.
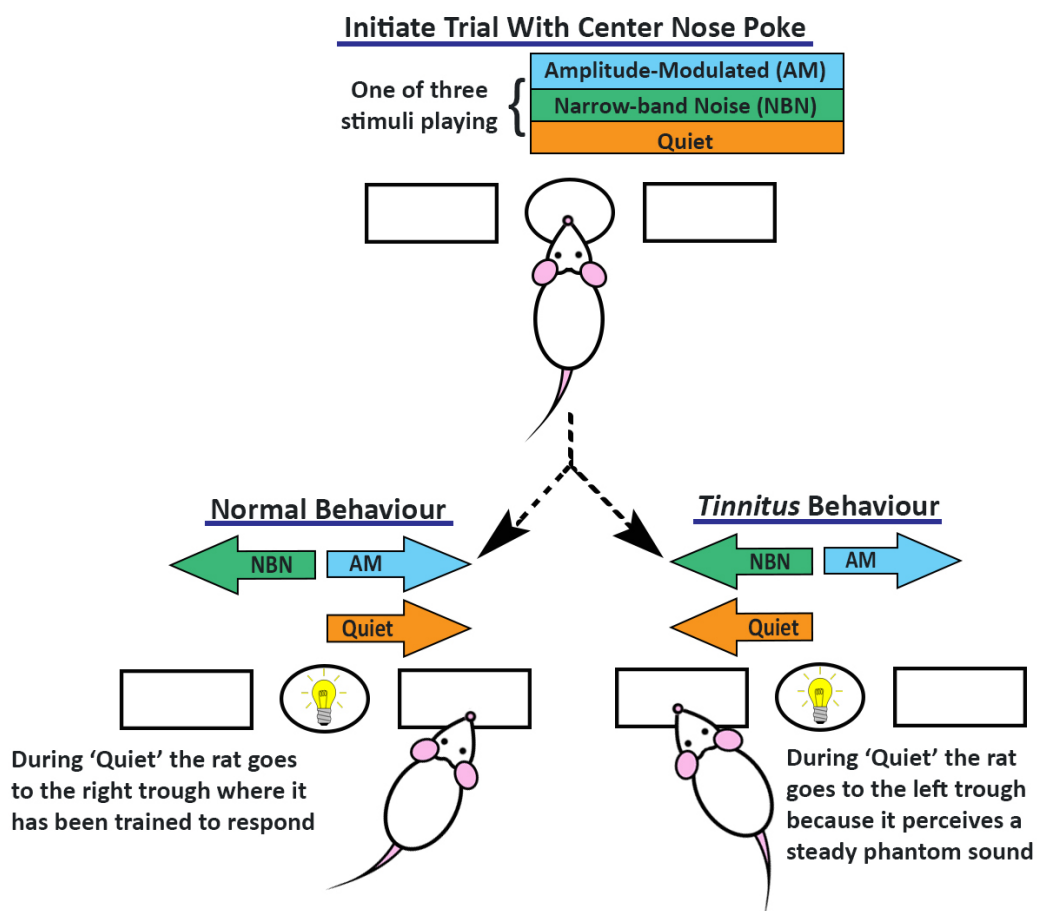
Figure 2-1. Overview of behavioural profiles

Rats were trained to respond to a specific feeder trough, depending on the auditory stimulus that was presented. During behavioural testing, rats were expected to respond correctly to all stimuli types if they did not have tinnitus. Furthermore, rats demonstrating tinnitus-positive behaviour were expected to respond to the narrow-band noise (NBN) feeder trough during quiet trials, implying they perceived a steady phantom sound in quiet conditions. Modified from Stolzberg et al. (2013).

Upon learning to frequently nose poke the center port (typically after 2 to 3 days), rats were then trained on a new protocol (Phase 2A) where the initial pellet reinforcement was removed and pellet delivery was provided only if the rat poked its nose in the correct feeder trough in response to the given auditory stimulus. Rats received 100% reward rates, and incorrect feeder trough responses were punished with a 15-second timeout during which time the next trial could not be initiated. Rats remained on Phase 2A until they could correctly associate feeder troughs with the given auditory stimuli with >92% accuracy for at least three consecutive days (typically after two weeks).

Once rats could correctly distinguish quiet trials from 16 kHz NBN trials, a new protocol (Phase 2B) was introduced where rats were trained to nose poke the right trough for quiet trials, and the left trough for all NBNs (8, 12, 16, 20, or 24 kHz). Rats continued to receive 100% reward rates for correct responses, and 15-second timeouts for incorrect responses. Trial type (NBN or quiet) was distributed evenly and presented in a randomized order. Upon learning the correct feeder trough associations for at least five consecutive days at >92% accuracy (typically after two weeks), rats were trained on a new protocol (Phase 2C) where the left feeder trough represented all NBN trials, and the right feeder trough represented quiet and AM trials (See Figure 2-1). During a 30-minute training session, 50% of trials were NBN, 30% of trials were AM, and 20% of trials were quiet; trials were presented in a randomized order according to criteria provided by Gellermann (1933). Rats continued to receive 100% reward rates for correct responses, and 15-second timeouts for incorrect responses. Once rats learned the correct feeder trough associations for all three stimuli types (typically after 1 month), reward rates were slowly lowered to 70% until the rats were able to consistently achieve a >92% hit-rate during each training session. See Table 2-1 for an overview of training protocols.

2.2.3   Behavioural Testing and Analysis

To screen for behavioural evidence of tinnitus, trained rats were run on a *testing protocol* in which the previously described training protocol was modified such that responses during quiet trials were no longer rewarded nor punished, in an effort to avoid biasing test day results. Rats experiencing tinnitus were expected to perceive a steady phantom sound

Protocol	Left Trough Stimuli	Right Trough Stimuli	Reward Rate	Approximate Time Spent Learning Protocol
Phase 1	NBN 16	Quiet	100% (plus automatic reward)	2-3 days
Phase 2A	NBN 16	Quiet	100%	2 weeks
Phase 2B	All NBN (8, 12, 16, 20, 24)	Quiet	100%	2 weeks
Phase 2C	All NBN (8, 12, 16, 20, 24)	Quiet, AM	100%	1-2 months
Final Training	All NBN (8, 12, 16, 20, 24)	Quiet, AM	70%	1 week

Table 2-1. Overview of behavioural training procedures

Rats were trained using successive protocols to slowly introduce them to each type of stimulus. Typically, 3 to 4-months were required for rats to complete training, maintaining a >92% hit rate over consecutive training days.

during quiet conditions, and as such would more frequently respond to the left (NBN) feeder trough (previously an incorrect response) during quiet trials, rather than the right (quiet and AM) feeder trough (previously a correct response; See Figure 2-1). During testing, reward rates were increased to 90% for NBN and AM trials to compensate for the lack of food pellets delivered during quiet trials. As a result, the overall reward rate would be similar to that of the normal training protocol (Stolzberg et al., 2013).

Raw hit-rates for quiet, AM, and NBN trials were compared between sham- and noise-exposure conditions. Baseline performance (normal training one day prior to testing), exposure day performance (test day), and one-day post-exposure performance (test day) was averaged across rats.

2.2.4   Fifteen-Minute Noise Exposure Paradigm

Following three consecutive days of normal behavioural training at hit-rates of >92% accuracy, a subset of trained rats (n=10) were placed in a sound-attenuating chamber and given either a 15-minute sham exposure (quiet, speaker off), or a 15-minute noise exposure (bilateral, 12 kHz tone, 110 dB SPL) from a super tweeter (T90A; Fostex) positioned over the home cage. Immediately after the exposure, rats were placed in the behavioural box and run on the aforementioned testing protocol for 120 to 130 trials. On the following day, rats again performed the testing protocol to determine if any effects of the 15-minute noise exposure persisted. Between exposures, rats were given a minimum of two normal training days, during which time they had to consistently perform with >92% accuracy.

2.2.5   Sixty-Minute Noise Exposure Paradigm

2.2.5.1   Exposures and Behavioural Testing

A separate cohort of rats (n=10) were assigned to the 60-minute noise exposure paradigm. Following three consecutive days of training in which these rats demonstrated hit-rates of >92% accuracy, they were anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Once the rat's pedal reflex was absent, it

was placed on a homeothermic heating pad (maintained core temperature at ~37ᵒC) in a sound-attenuating chamber (29" W by 23.5" H by 23.5" D; Med Associates Inc.) and given a 60-minute sham exposure (quiet, speaker off). Supplemental doses of ketamine/xylazine were administered intramuscularly as needed. Following the 60-minute exposure, anaesthesia was reversed using an intraperitoneal injection of atipamezole hydrochloride (1 mg/kg), and the rat was returned to its home cage for recovery. Rats were not trained for the six days following the sham exposure. One week after the initial sham exposure, rats were run on the testing protocol (previously described), and again tested on the following day as well. Rats were given a minimum of five normal training days following the 60-minute sham exposure test session before being prepped for the 60-minute noise exposure.

Once each rat had demonstrated another three consecutive days of normal training at >92% accuracy, it was again anaesthetized and placed in the sound-attenuating chamber. Rats were given a 60-minute noise exposure (bilateral, 12 kHz tone, 120 dB SPL) from a super tweeter (T90A; Fostex) placed directly in front of their head, 5 cm from the pinna of the ears. The tone exposure was generated with TDT software and hardware (RPvdsEx, RZ6 module; TDT). Following the exposure, the rat was administered an intraperitoneal injection of atipamezole hydrochloride (1 mg/kg) and returned to its home cage. Similar to the 60-minute sham exposure paradigm, rats were not trained for the six days following the noise exposure. One week post-exposure, rats performed the testing protocol, and were again tested on the next day.

2.2.5.2   Detection of Hearing Thresholds Using Auditory Brainstem Responses

At the conclusion of behavioural testing, hearing thresholds of rats were determined using the auditory brainstem response (ABR) to verify the presence/absence of hearing loss in the week following the 60-minute noise exposure. Rats were again anaesthetized with intraperitoneal injections of ketamine/xylazine and placed on a homeothermic heating pad (maintained core temperature at ~37ᵒC; 507220F; Harvard Apparatus) in a sound-attenuating chamber (29" W by 23.5" H by 23.5" D; Med Associates Inc.). Once

pedal reflex was absent, subdermal electrodes (27G; Rochester Electro-Medical, Lutz, FL, USA) were placed at the vertex (active electrode), over the right mastoid bone (reference electrode), and on the mid-back (ground electrode). Electrodes were connected to a low-impedance headstage (RA4L1; TDT), and auditory-evoked activity was preamplified and digitized (RA16SD Medusa preamplifier; TDT) prior to being sent to an RZ6 module (TDT) via a fiber optic cable. Signals were bandpass filtered (300 to 3000 Hz) and averaged using BioSig software (TDT).

Briefly, auditory stimuli consisted of a click (0.1 ms), 4 kHz tone, and 20 kHz tone (5 ms duration, 1 ms rise/fall time) presented from a speaker positioned 10 cm from the rat's exposed right ear (the left ear was blocked with a foam ear plug). Stimuli were each presented 1000 times (21 times per second) at decreasing sound intensities from 90 to 10 dB SPL in 5 to 10 dB steps. Close to ABR threshold, stimuli were repeated in order to confirm an accurate threshold judgement using the criteria of just noticeable deflection of the averaged electrical activity within the 10 ms window (Popelar et al., 2008). Sound intensity at the ABR threshold was presented a second time to confirm accurate threshold judgement. All auditory stimuli were calibrated using a ¼" microphone (2530; Larson-Davis), a pre-amplifier (2221; Larson-Davis), and custom MATLAB software (EPsych Toolbox, dstolz.github.io/epsych/) running in MATLAB (Mathworks).

2.2.6   Statistical Analysis and Data Presentation

Statistical analyses consisted of two-way repeated measures analysis of variance (ANOVA), and post-hoc paired t-tests depending on the comparison of interest (see Results section for details on each comparison). The level of statistical significance was set at $\alpha = 0.05$, and where appropriate, Bonferroni post-hoc corrections were used to adjust the significance level for potential "family-wise" error (Armstrong, 2014). Statistical calculations were conducted using SPSS Software, (Version 20, IBM Corporation, Armonk, NY, USA), and data was plotted using GraphPad Prism (Version 7.00 for Mac, GraphPad Software Inc., La Jolla, CA). Data are presented as the mean values ± standard error of the mean (SEM).

2.3 Results

2.3.1 Fifteen-Minute Noise Exposure and Transient Tinnitus

A cohort of 10 rats underwent behavioural training to distinguish between quiet, AM, and NBN stimuli. Once trained, rats were given 15-minute sham and noise exposures immediately prior to behavioural testing to determine if either exposure resulted in behavioural performance consistent with the presence/absence of tinnitus. Tinnitus-positive behaviour was scored as a shift in the response to quiet stimuli from the right trough (previously trained to be a correct response), to the left trough (previously trained to be associated with NBNs). Performance on AM and lower frequency NBN trials (8 and 12 kHz) were used to confirm that rats could still accurately perform the behavioural task above a 70% criterion threshold. Following 15-minute exposures, rats were still able to correctly identify >70% of AM trials, regardless of exposure type (sham: $90.8 \pm 1.5\%$ correct; noise: $86.0 \pm 3.5\%$ correct). Similarly, Figure 2-2A shows that rats were still able to correctly identify >70% of lower frequency NBN trials immediately following 15-minute sham and noise exposures (sham: $96.9 \pm 1.6\%$ correct; noise: $92.9 \pm 2.4\%$ correct). A two-way repeated measures ANOVA revealed no significant interaction between time (baseline, post-exposure, 1-day post) and exposure (sham or noise) for either AM or NBN trials. However, a main effect of time was observed for NBN trials ($F_{2, 18} = 4.049$, $p < 0.05$). Subsequent Bonferroni-corrected post-hoc paired t-tests revealed no significant difference between post-exposure NBN performance for sham and noise conditions. Because the noise-exposed rats maintained good performance on the NBN trials, it was then possible to interpret the behavioural responses made during the quiet conditions.

As expected, all rats were still able to correctly identify quiet trials following 15-minute sham exposures; findings which confirm that the behavioural testing did not result in a false-positive screening of tinnitus following a control condition (Figure 2-2B, left panel). In contrast, after the 15-minute noise exposure, all rats demonstrated tinnitus-positive behaviour by shifting their responses for quiet stimuli to the left (NBN) trough

A. Narrow-band Noise Stimuli
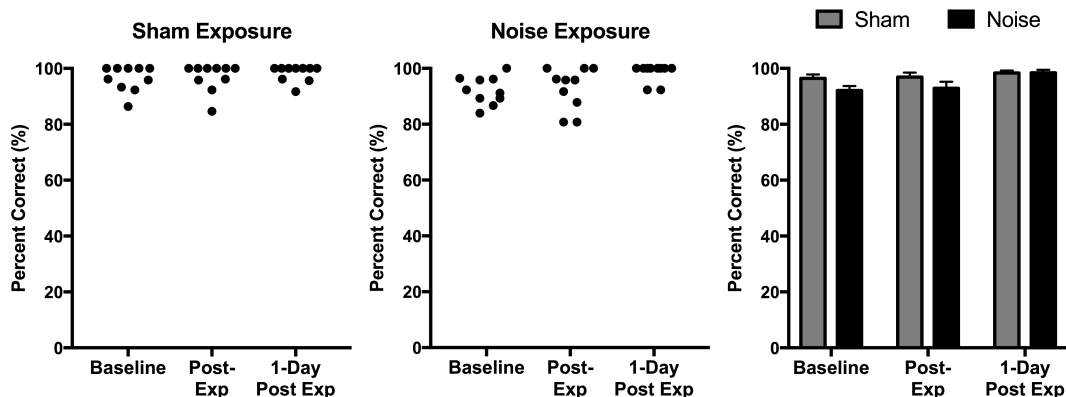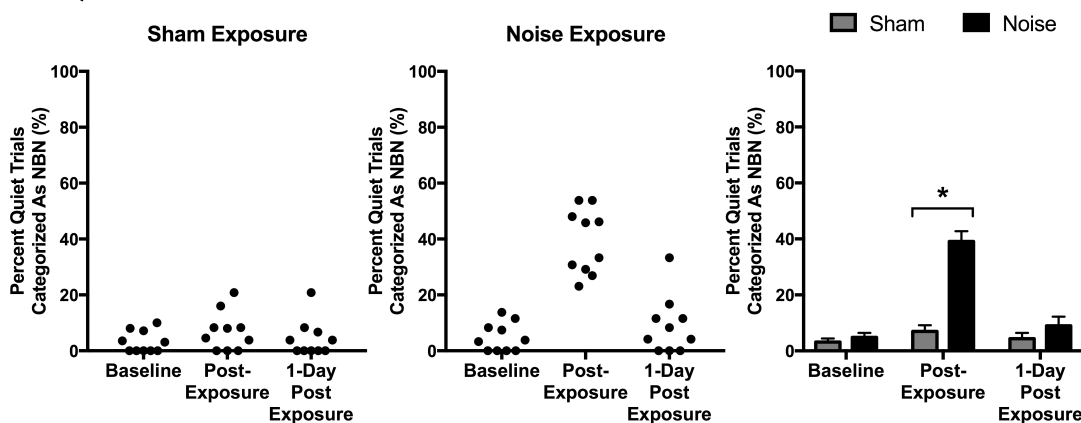
B. Quiet Stimuli

Figure 2-2. Performance on quiet and narrow-band noise trials following 15-minute noise exposure

(A) Following 15-minute sham and noise exposures, all rats could still accurately identify lower frequency narrow-band noise (NBN) stimuli (i.e., 8 and 12 kHz). **(B)** Following 15-minute sham exposures, all rats could still correctly identify all quiet stimuli. However, following 15-minute noise exposures, all rats mistakenly identified >20% of quiet trials as NBN, indicative of tinnitus-positive behaviour. On average, rats mistakenly identified significantly more quiet trials as NBN following noise exposure than they did following sham exposure. Statistical analyses included a two-way repeated measures ANOVA (time × exposure), followed by post-hoc paired t-tests with Bonferroni corrections. Comparisons were made between sham and noise exposure performance at each time point. * $p < 0.00001$

(Figure 2-2B, center panel). On average, noise-exposed rats mistakenly identified 39.1 ± 3.7% of quiet trials as NBN during behavioural testing, whereas the same rats given sham exposures only misidentified 7.0 ± 2.2% of quiet trials (Figure 2-2B, right panel). A two-way repeated measures ANOVA revealed a significant interaction between time (baseline, post-exposure, 1-day post) and exposure (sham or noise; $F_{2, 18} = 23.88$, $p < 0.00001$). Bonferroni-corrected post-hoc paired t-tests showed that rats misidentified significantly more quiet trials following the 15-minute noise exposure than they did following 15-minute sham exposures ($p < 0.00001$). This effect was not present on the subsequent test day, suggesting that rats only experienced transient tinnitus following the brief 15-minute noise exposure. Based on these collective results, rats were categorized as experiencing *transient* tinnitus if they (1) could still accurately identify AM and NBN stimuli, (2) misidentified >20% of quiet trials as NBN, and (3) did not demonstrate behavioural indications of tinnitus on the subsequent test day. This threshold of tinnitus-positive behaviour (i.e., >20% misidentified quiet trials) was used for the remainder of the experiment.

2.3.2   Sixty-Minute Noise Exposure and Persistent Tinnitus

A separate cohort of rats (n=10) were trained on the behavioural paradigm to distinguish between quiet, AM, and NBN stimuli, as described above. Upon completion of training, rats were given 60-minute sham and noise exposures, and were tested for disruption of their overall behavioural performance one week later. Tinnitus-positive behaviour was again scored as a shift in a rat's response to quiet stimuli from the right (quiet and AM) trough, to the left (NBN) trough. As with the 15-minute noise exposure paradigm, performance on AM and lower frequency NBN trials (i.e., 8 and 12 kHz) was analyzed to ensure rats could still correctly perform the behavioural task.

In the week following 60-minute sham exposures, rats on average correctly identified 94.3 ± 1.4% of AM trials, and 96.9 ± 1.3% of NBN trials (Figure 2-3A, left panel), providing strong evidence for an ability to accurately recall the task despite a week without behavioural training. However, the same rats later given 60-minute noise exposures only correctly identified 80.9 ± 3.9% of AM trials, and 68.8 ± 8.9% of NBN

trials 1-week post-exposure (Figure 2-3A, center panel). Moreover, separate two-way repeated measures ANOVAs revealed a significant interaction between time (baseline, 7-days post, 8-days post) and exposure (sham, noise) for both AM performance ($F_{2,18} =$ 4.087, $p < 0.05$) and NBN performance ($F_{2,18} = 10.043$, $p < 0.01$; Figure 2-3A, right panel). Subsequently, post-hoc paired t-tests with Bonferroni-corrected p-values found that rats made significantly more mistakes on AM and NBN trials 7 days ($p < 0.01$) and 8 days ($p < 0.017$) post-noise exposure. Although 9 of 10 rats correctly identified >70% of AM trials, only 5 of 10 noise-exposed rats could accurately identify >70% of NBN trials (Figure 2-3A, center panel), suggesting that interpretations of behavioural performance on quiet trials should perhaps only be made for half of the rats tested.

Consistent with performance on AM and NBN trials, one week after the 60-minute sham exposure, all rats were still able to correctly identify quiet trials during testing, thus confirming that performance was preserved despite a week without training (average: 5.1 ± 0.9% of quiet trials misidentified as NBN; Figure 2-3B, left panel). The robustness of the sham results represents a strong control condition for the 60-minute exposure paradigm, as no rats demonstrated false-positive indications of tinnitus. With respect to performance following the actual 60-minute noise exposure, the proportion of quiet trials misidentified as NBN 7-days post-noise exposure was variable (Figure 2-3B, center panel), similar to observations made from NBN performance (Figure 2-3A, center panel). On average, rats mistakenly identified 36.1 ± 6.2% of quiet trials as NBN. Similar effects were observed on the following test day as well. A two-way repeated measures ANOVA found a significant interaction between time (baseline, 7-days post, 8-days post) and exposure condition (sham or noise; $F_{2, 18} = 18.435$, $p < 0.0001$). Subsequent Bonferroni-corrected post-hoc paired t-tests showed that rats mistakenly identified significantly more quiet trials following the 60-minute noise exposure than they did following the 60-minute sham exposure both 7- and 8 days post ($p < 0.001$; Figure 2-3B, right panel). Using the 20% threshold for tinnitus-positive behaviour established from the 15-minute exposure paradigm, it appeared that 6 of 10 rats presented with indications of tinnitus (Figure 2-3B, center panel). However, this proportion includes rats (5 of 10) that were no longer able to

A. Narrow-band Noise Stimuli
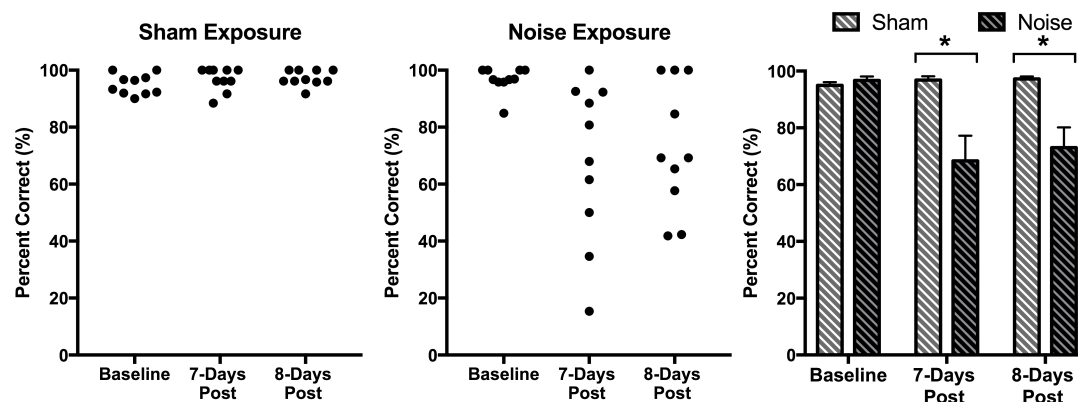
B. Quiet Stimuli
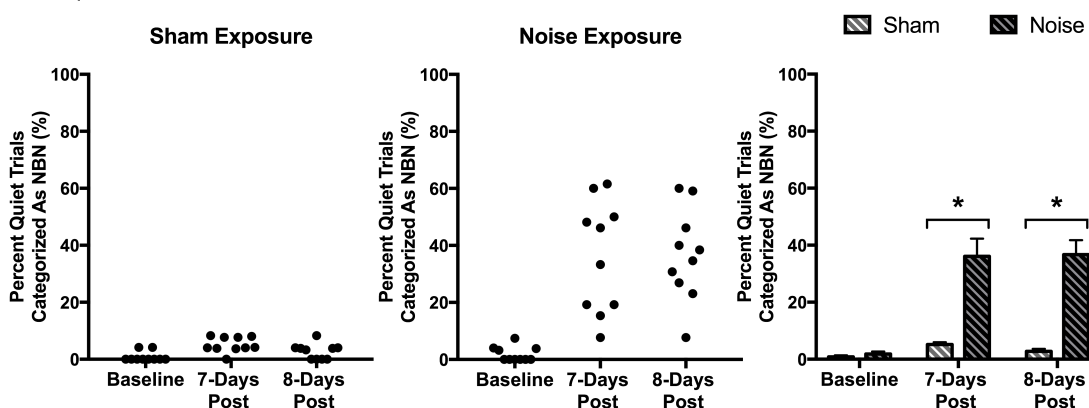
Figure 2-3. Performance on quiet and narrow-band noise trials in the week following 60-minute noise exposure

(A) One week following the 60-minute sham exposure, all rats could still accurately identify lower frequency narrow-band noise (NBN) stimuli (i.e., 8 and 12 kHz). However, 50% of rats were no longer able to identify lower frequency NBN stimuli above a 70% criterion threshold following the 60-minute noise exposure. On average, rats mistakenly identified significantly more NBN trials following the noise exposure than they did following sham exposure. This effect was observed on the subsequent test day * p < 0.017 **(B)** One week following the 60-minute sham exposure, all rats could still correctly identify the quiet stimuli. However, following the 60-minute noise exposure, a majority of rats demonstrated an increase in the proportion of quiet trials misidentified as NBN. On average, rats mistakenly identified significantly more quiet trials as NBN following noise exposure than they did following sham exposure. This effect was

observed on the subsequent test day. Statistical analyses included a two-way repeated measures ANOVA (time × exposure), followed by post-hoc paired t-tests with Bonferroni corrections. Comparisons were made between sham and noise exposure performance at each time point. * $p < 0.001$

correctly identify NBN trials during behavioural testing. As such, rats were subsequently categorized based on the 70% criterion threshold for correctly identified NBN trials (Figure 2-4).

It was postulated that the variability in NBN performance was due to a potential severe hearing loss that persisted in the week following the 60-minute noise exposure, such that rats could no longer perceive steady NBNs during the behavioural task. Thus, rats that could correctly identify >70% of NBNs could be expected to have maintained relatively low thresholds for their auditory brainstem response (ABR) for 20 kHz tonal stimuli, indicative of limited hearing impairment. However, this group of rats showed highly variable ABR thresholds, with some rats displaying minimal hearing loss, and others showing severe hearing deficits with thresholds ≥80 dB SPL (Figure 2-4A). Moreover, there appeared to be no clear relationship between hearing thresholds and the presence of tinnitus. Based on a tinnitus threshold of 20% mistakenly identified quiet trials (grey dashed line in Figure 2-4A), rats that behaviourally showed no evidence of tinnitus (i.e., <20% mistakes) could have low or high ABR thresholds, and rats that screened positive for tinnitus (i.e., >20% mistakes) had ABR thresholds within a variable range. It is important to note that, although rats with <70% correct NBN performance all presented with elevated ABR thresholds, interpretations on the presence/absence of tinnitus for this group cannot be made due to the strong confounding influence of hearing loss on behaviour (Figure 2-4B).

Collectively, these results indicate that rats did not demonstrate false-indications of tinnitus in the week following the 60-minute sham exposures, as rats could correctly identify AM, NBN, and quiet trials; findings that provide a strong control condition for the behavioural paradigm. Ultimately, only 5 of 10 rats given the 60-minute noise exposures could correctly identify NBN trials above a 70% criterion threshold, and these rats showed no clear relationship between ABR threshold and the presence of tinnitus.

2.4   Discussion

Two cohorts of rats were trained using operant conditioning to report whether they perceived quiet, AM, or NBN stimuli by probing the appropriate feeder trough. Upon conclusion of training, cohorts of rats were given sham and noise exposures, and were subsequently tested either immediately, or in the week following exposure to determine if they perceived tinnitus during quiet stimuli. As expected, both 15- and 60-minute sham exposures did not affect the correct identification of quiet trials, thus confirming the lack of false-positive indications of tinnitus during control conditions. In contrast, immediately following the 15-minute noise exposure, all rats (n=10) actively reported perceiving sounds reminiscent of NBNs during quiet stimuli. As these effects were not observed on the subsequent test day, it is reasonable to conclude that the rats experienced only transient tinnitus following 15-minute noise exposure. Although a similar shift in behavioural response to quiet stimuli was demonstrated in the week following the 60-minute noise exposure, a severely impaired ability to identify NBN stimuli was also observed in several rats. Further investigation found no clear relationship between behavioural performance and ABR threshold, suggesting a potential confounding influence of hearing loss in the 60-minute exposure paradigm. Collectively, the present study provides support for the use of our previously established two-alternative forced-choice behavioural paradigm to effectively assess rodents for transient noise-induced tinnitus, with the potential to screen for persistent tinnitus lasting one week upon correcting for confounding influences of hearing loss.

2.4.1   A Robust Paradigm to Screen for Transient Tinnitus

In the present study, rats were exposed to 15-minute sham and noise exposures, and were then immediately subjected to behavioural testing to screen for the presence/absence of tinnitus-positive behaviour (i.e., shift in response to quiet stimuli from the right trough (trained association), to the left trough (NBN association)). Sham exposures were not expected to cause tinnitus in rats, and this was reflected behaviourally as all rats (n=10) were able to correctly identify quiet, AM, and NBN trials, despite an altered

A. Rats with >70% NBN Performance

B. Rats with <70% NBN Performance
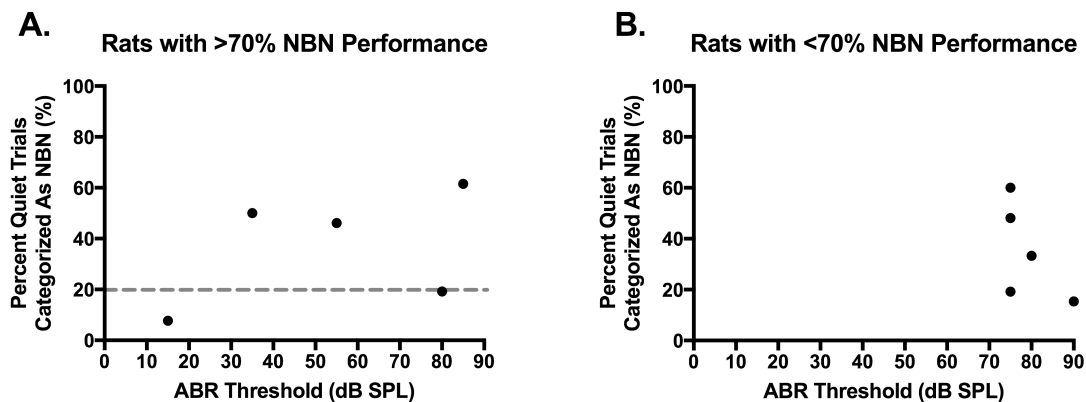
Figure 2-4. Relationship between performance on quiet trials and hearing threshold

Based on performance during narrow-band noise (NBN) trials, rats given the 60-minute noise exposure were separated based on an arbitrary 70% criterion threshold. Rats that could still accurately identify NBN trials **(A)** had no clear relationship between the percent of quiet trials misidentified as NBN, and their auditory brainstem response (ABR) threshold as determined by the 20 kHz pure tone stimulus. The grey dashed line indicates a threshold for tinnitus-positive behaviour derived from results of the transient tinnitus paradigm. Rats above this threshold are classified as having tinnitus, while rats below the threshold were not. Rats with performance on NBN trials < 70% **(B)** also present with high frequency hearing loss as indicated by ABR thresholds centered around 80 dB SPL. As such, conclusions on the presence/absence of tinnitus in this group cannot be drawn.

reinforcement rate. Similar behavioural profiles were observed in our previous study when rats were given systemic injections of saline (Stolzberg et al., 2013). The consistency of these control exposures emphasizes the robustness of our behavioural paradigm in its resistance to false indications of tinnitus; a criterion that is essential for successful behavioural models of tinnitus. Consistent with our recent work using salicylate as a method of inducing tinnitus, 15-minute noise exposures caused a noticeable shift in the behavioural responses to quiet stimuli (Stolzberg et al., 2013). All 10 rats reported perceiving >20% of quiet trials to be more similar to steady NBN. As a confirmation that this behavioural shift was not due to memory deficits induced by the brief abrasive noise exposure, all rats were still capable of correctly identifying AM and lower frequency NBN stimuli above a 70% criterion threshold. Importantly, behavioural shifts due to short duration noise exposure were not present on the following test day, confirming that our paradigm can effectively detect both the onset and offset of transient noise-induced tinnitus. Furthermore, these results demonstrate the reproducible nature of our paradigm, as tinnitus-positive behaviour was observed consistently across the entire cohort of rats following 15-minute noise, but not sham, exposures.

One of the notable benefits of our behavioural paradigm is its ability to make within-individual comparisons between sham and noise exposure conditions. Because rats are trained to expect reduced reward rates, they are unable to distinguish between periods of testing and periods of training, and as such, they can undergo recurrent testing with limited concern of behavioural extinction. Thus, separate control and experimental groups are unnecessary in our paradigm because the same rat can participate in both conditions without confounding behavioural results. The requirement of different animal cohorts for control and experimental series has been a considerable drawback of previously established shock avoidance tinnitus models as it is well-known that tinnitus in humans is highly variable at the level of the individual (Bauer & Brozoski, 2001; Bauer et al., 1999; Guitton et al., 2003; Hayes et al., 2014; Heffner & Harrington, 2002; Lobarinas et al., 2004; Rüttiger et al., 2003). Our adjusted reward rates implemented during behavioural training allow for within-subject comparisons to be made during behavioural testing. That said, this approach does cause a substantial increase to the

amount of time required to train animals to learn the initial behaviour, and as such may deter future investigators from using such a paradigm.

To our knowledge, this is the first study to demonstrate that traumatic bilateral noise exposures as short as 15-minutes can cause rats to develop short-duration tinnitus lasting no longer than one day. These findings are consistent with human studies showing that 5-minute exposures to loud noise were sufficient to induce transient tinnitus in subjects, therefore confirming that our model accurately reflects the human condition (Atherley et al., 1968; Loeb & Smith, 1967). It was previously suggested that noise exposures ≥60-minutes in duration would consistently induce tinnitus in animals, and as such, longer duration exposures have been used in numerous behavioural studies (Bauer & Brozoski, 2001; Heffner & Harrington, 2002; Sederholm & Swedberg, 2013; Turner et al., 2006). Here, we propose that our brief noise exposure could potentially be more efficient in the testing of protective tinnitus therapies, such that 15-minute exposures could be used as an alternative to 60-minute exposures, creating a higher throughput scenario. Further investigation would be needed, however, to demonstrate that mechanisms responsible for immediate-onset tinnitus are similar to those that cause persistent tinnitus, as the latter condition is often associated with a decreased quality of life and depression and is therefore of greater concern to the tinnitus population (Dobie, 2003; Shargorodsky et al., 2010).

Taken together, the findings from the present study emphasize the effectiveness of our behavioural paradigm as a model for transient noise-induced tinnitus as it (1) is resistant to false-positive indicators of tinnitus-positive behaviour, (2) allows for individual comparisons amongst rats to control for the variabilities in tinnitus development following noise exposure, and (3) successfully assesses short duration tinnitus that closely mirrors the human condition.

2.4.2　A Potential to Screen for Persistent Noise-Induced Tinnitus

Although we had not previously established our paradigm as a model for persistent tinnitus, here we show that our behavioural task has the capability of assessing tinnitus one week after loud noise exposure. Similar to our 15-minute exposure results, rats that

received the 60-minute sham exposure, followed by a week without behavioural training, did not show altered behavioural performance as all rats could still correctly identify quiet trials. Moreover, performance on AM and NBN trials was also unaffected, suggesting that rats were able to accurately remember the behavioural task, despite an entire week without training. These results further highlight the resilient nature of our behavioural paradigm to false-indications of tinnitus.

One week following the 60-minute noise exposure, 5 of 10 rats were unable to accurately identify >70% of NBN trials, despite an ability to still correctly perceive AM stimuli. It was postulated that these rats likely developed a high frequency hearing loss that prevented them from perceiving NBNs, and as such, they mistakenly probed the quiet trough during NBN trials. In agreement with this hypothesis, ABR thresholds confirmed that this subset of rats indeed had a high frequency hearing loss with thresholds centered around 80 dB SPL. Because these rats suffered from such an extensive hearing loss, we were less inclined to trust their behavioural performance, and conclusions on whether these rats had persistent noise-induced tinnitus could not be reliably drawn from their responses to quiet trials.

For the five rats that were still able to correctly identify NBN trials above the 70% criterion threshold, behavioural performance during quiet trials was used to determine if they were experiencing persistent tinnitus one week after the 60-minute noise exposure. The threshold for tinnitus-positive behaviour was set at 20% in accordance with results from the 15-minute exposure experimental series. Three rats mistakenly identified >20% of quiet trials as NBN, and as such were categorized as having persistent noise-induced tinnitus. The remaining two rats misidentified <20% of quiet trials and thus were classified as not having tinnitus. Consistent with our results, it is well-established that not all subjects exposed to the same level of excessive noise will develop tinnitus. For example, previous behavioural work by Brozoski and colleagues (2007) showed that one hour exposure to 120 dB SPL band-limited noise did not induce tinnitus-positive behaviour equally in all rodents. Variable behavioural profiles were also observed in individual rats following 4-hour noise exposures in work by Sederholm and Swedberg (2013). Moreover, human studies have revealed that of the number of returning war

veterans surveyed who were exposed to blast trauma (a severe form of noise exposure), only 49% of them went on to develop tinnitus (Cave et al., 2007). Thus, it was not surprising that only a subset of rats showed behavioural indications of tinnitus in the week following the 60-minute noise exposure used in the present study.

What was somewhat unexpected, however, was that there was no clear relationship between behavioural indications of tinnitus and hearing thresholds within the group of rats who could accurately identify NBN trials. Indeed, of the two rats that were classified as "no tinnitus", one rat had no hearing impairment (ABR threshold: 15 dB SPL), and one rat had a severe hearing loss (ABR threshold: 80 dB SPL). Likewise, the three rats that exhibited behavioural evidence of tinnitus had variable ABR thresholds (Figure 2-4A). It is often suggested that a strong connection exists between hearing loss and the presence of tinnitus, as a vast majority of patients who suffer from tinnitus have some degree of measurable hearing impairment (Axelsson & Ringdahl, 1989; Davis & Refaie, 2000; Henry & Wilson, 2001). As such, it would be expected that the presence of tinnitus increases as hearing thresholds worsen. However, the results of the present study suggest that the relationship between tinnitus and hearing loss is not straightforward, and it is possible that extensive hearing impairment confounds behavioural evidence of persistent tinnitus in the proposed paradigm.

Interestingly, of the five rats who could accurately identify NBN stimuli, two of the rats had ABR thresholds that were ≥80 dB SPL. As auditory stimuli are presented at ~75 dB SPL, these two rats would not have been expected to correctly identify as many NBN trials as they did. One potential explanation for the differences in behavioural performance during NBN trials and the hearing thresholds, is that hearing loss at the level of the brainstem, as is measured by the ABR, may be compensated for at the level of the auditory cortex. Evidence of such a phenomenon has been observed in a recent study by Chambers et al. (2016) who found that mice with near-complete cochlear denervation had elevated ABR thresholds, yet could still behaviourally detect tonal stimuli. The authors reasoned that this result occurred due to gain enhancement at the level of the auditory cortex; a mechanism that has been suggested to underlie the neural basis of tinnitus (Jastreboff, 1990; Noreña, 2011; Schaette & Kempter, 2006). Unfortunately, we did not

conduct electrophysiological recordings in the present study to determine if indications of increased central gain (i.e., elevated spontaneous and auditory-evoked firing rates; increased neural synchrony), were present in the two rats that demonstrated elevated hearing thresholds with normal NBN performance. As such, we cannot conclude that gain enhancement is the cause for these somewhat unexpected results. An alternative explanation for the divergent behavioural and ABR findings is that the presence of tinnitus masked any hearing loss that the rats had, such that they still perceived a steady sound during NBN trials and responded to the NBN trough accordingly. However, while one of the rats showed strong behavioural evidence of tinnitus, the other only mistakenly identified 19% of quiet trials as NBN and as such did not surpass the 20% tinnitus threshold, undermining the previous claim. It is worth noting that this same rat only misidentified 4% of quiet trials following the 60-minute sham exposure. The large separation between this rat's sham and noise exposure performance could suggest that it may have still have had tinnitus, albeit a weaker form of the condition; but due to the highly conservative nature of our 20% tinnitus threshold, this rat was excluded from the "tinnitus present" group. If the rat did indeed have tinnitus in the week following noise exposure, then an explanation of tinnitus masking the presence of hearing loss would be sufficient to explain why this rat was still able to correctly respond to NBN trials.

Despite the complex relationship between the behavioural results and the hearing thresholds assessed by ABRs, it is important to note that our experimental paradigm still has the potential to successfully screen for persistent tinnitus in rats. Because all rats were able to correctly identify all three stimuli types in the week following the 60-minute sham exposure, we are confident that we have established a rigorous control condition that is resistant to false-indications of tinnitus. Thus, if adjustments were made to the noise exposure parameters, such that the confounding effects of hearing loss are reduced or abolished entirely, then interpretations of behavioural results would be expected to become more reliable. For instance, the sound intensity of the exposure could be lowered to reduce the severity of hearing impairment, or researchers could opt for a unilateral rather than a bilateral exposure, as has been done in several studies to preserve hearing in the unaffected ear (Dehmel et al., 2012; Kraus et al., 2011; Lobarinas et al., 2013; Longnecker & Galazyuk, 2011; Middleton et al., 2011; Turner et al., 2006; Wang et al.,

2009; Zhang et al., 2011). Further investigation is required to confirm that adjustments to the 60-minute noise exposure would be sufficient to control for the effects of hearing loss and/or induce persistent tinnitus; however, we are confident that the robustness of our control condition (i.e., sham exposure) will be maintained.

2.5 Conclusion

A reliable behavioural paradigm is essential for investigating the mechanisms of tinnitus using animal models. Here, we provide further validation of our previously established two-alternative forced-choice paradigm in its effectiveness at assessing rats for transient noise-induced tinnitus. Moreover, upon some minor adjustments to exposure conditions, we are confident that this paradigm would allow for the successful screening of rats for persistent tinnitus lasting one week at the level of the individual. Such advantages, particularly for the transient tinnitus paradigm, will greatly benefit future studies looking at the putative neural mechanisms of tinnitus, as our paradigm is resilient against false-positives of tinnitus.

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Preface for Chapter 3

Animal models are essential to studying the putative underlying neural mechanisms of tinnitus, as they allow for the use of more invasive techniques and manipulations. However, this reliance on animal models has increased the need to develop behavioural paradigms that can effectively detect the presence/absence of tinnitus. The results of Chapter 2 demonstrated that our two-alternative forced-choice behavioural paradigm can reliably screen rats for transient tinnitus. Indeed, immediately following 15-minute noise exposures, all rats mistakenly identified a significant proportion of quiet trials as narrow-band noise (NBN); findings that suggest the rats perceived a steady sound during quiet (i.e., tinnitus). This effect was not observed on the subsequent test day; thus, our paradigm can effectively detect both the onset and offset of transient tinnitus. Furthermore, we demonstrated that our behavioural paradigm was resistant to false-positive indications of tinnitus, as all rats were able to correctly identify quiet trials following 15-minute sham exposures. Thus, because our behavioural paradigm has proven to be a reliable method for detecting transient tinnitus in rats, it can now be applied to investigations of the neural basis of tinnitus. Based on the findings from our 15-minute exposure paradigm, we established a 20% tinnitus threshold that can be used to determine if any of the putative models of tinnitus (see Section 1.4) can indeed generate phantom auditory perceptions. The aim of Chapter 3 was to investigate the central gain model of tinnitus established at the level of the primary auditory cortex (A1). We predicted that if local gain enhancement in A1 was indeed responsible for generating these phantom auditory perceptions, then this manipulation would be expected to cause rats to mistakenly identify >20% of quiet trials as NBN, similar to the 15-minute noise exposure results in Chapter 2. Indications of tinnitus-positive behaviour following a local increase in central gain would provide direct support to the central gain model of tinnitus as a mechanism responsible for generating tinnitus.

Chapter 3

3 Central Gain Enhancement and Tinnitus-Positive Behaviour Induced by a Loss of Inhibition in the Auditory Cortex

3.1 Introduction

Subjective tinnitus is the perception of a phantom sound in the absence of an identifiable auditory source, often described as a "ringing in the ears" (Eggermont & Roberts, 2004; Henry et al., 2014). A recent study performed by Statistics Canada noted that as many as 41% of Canadians have experienced tinnitus in some capacity during their lifetime (Statistics Canada, 2015). Such high prevalence rates have increased the need for effective treatments, particularly for those who experience tinnitus in its most debilitating forms. Currently available therapies, are largely based on helping patients increase their tolerance to their tinnitus-related distress or attempting to help them modulate the pitch or loudness of the phantom sound (Cima et al., 2014; Hoare et al., 2014). At present, there are no widely-accepted therapies that directly target the source of tinnitus by mediating the underlying mechanisms that generate the phantom perception. Unfortunately, despite decades of research, these mechanisms have yet to be fully elucidated, thus hindering the development of successful treatments.

Initial theories of tinnitus suggested that the aberrant signals were generated from within the cochlea, as those who experienced tinnitus often had some degree of hearing loss (Axelsson & Ringdahl, 1989; Davis & Refaie, 2000; Henry & Wilson, 2001; Jastreboff, 1990; Kiang et al., 1970). It was proposed that cochlear insults resulted in aberrant hyperactivity of auditory nerve fibers in the inner ear, and this hyperactivity was then propagated throughout the central auditory system to create phantom auditory perceptions (Møller, 2011). However, numerous studies found that treatment with ototoxic drugs and

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noise exposure—two well-established tinnitus inducers—actually resulted in *decreased* activity of auditory nerve fibers (Harrison, 1978; Kiang et al., 1970). Furthermore, a more recent study conducted by Schaette et al., (2012) found that phantom auditory perceptions emerged in human subjects who wore an earplug unilaterally for one week; their tinnitus subsequently disappeared upon removal of the earplug. These results suggest that cochlear damage is not necessary to generate tinnitus, as the earplug did not cause any physical insult to the structure; rather it is the absence of auditory input into the central auditory system (CAS) that appears to be the driving force for tinnitus. Together, these studies support the suggestion of a central origin of tinnitus.

One of the current leading hypotheses for tinnitus generation is the central gain model, which suggests that following a lack of auditory input, the brain attempts to homeostatically maintain mean firing rates at a set point value by altering levels of excitation and inhibition in the CAS (Henry et al., 2014; Jastreboff, 1990; Noreña, 2011; Schaette & Kempter, 2006). This imbalance in excitation and inhibition is suggested to cause abnormal amplification of "neural noise" in the CAS, which subsequently encodes the phantom auditory perceptions of tinnitus. In support of this hypothesis, various auditory structures, including the dorsal cochlear nucleus, inferior colliculus, and auditory cortex, have been found to exhibit aberrant hyperactivity following tinnitus-induction, characterized by an increase in spontaneous firing rates, as well as auditory-evoked activity (Bauer et al., 2008; Brozoski et al., 2002; Dong et al., 2010; Eggermont & Kenmochi, 1998; Jastreboff & Sasaki, 1986; Kaltenbach & McCaslin, 1996; Kaltenbach & Afman, 2000; Kimura & Eggermont, 1999; Komiya & Eggermont, 2000; Lu et al., 2011; Manabe et al., 1997; Melamed et al., 2000; Mulders & Robertson, 2011; Mulheran & Evans, 1999; Noreña, 2011; Noreña & Eggermont, 2005; Qiu & Salvi, 2000; Seki & Eggermont, 2003; Zhang & Kaltenbach, 1998; Zhang et al., 2011). Further investigations are needed to determine if one central auditory structure in particular is responsible for generating the tinnitus percept, or if phantom auditory perceptions are a result of widespread hyperactivity throughout the ascending pathway.

In considering central gain enhancement as a putative mechanism of tinnitus, it is important to note the changes in the auditory pathway that could contribute to its

manifestation. Auerbach et al. (2014) suggested that central gain increases could develop either following a loss of inhibition, increase in excitation, or an increase in the intrinsic excitability of neurons. Interestingly, studies have shown that upon local (auditory cortex) and systemic administration of various gamma-aminobutyric acid (GABA) agonists, which presumably increased the level of cortical inhibition, previously observed indications of tinnitus were abolished (Brozoski et al., 2007; Lu et al., 2011; Sun et al., 2009). These studies provide strong support for the suggestion that a loss of inhibition contributes to increased central gain, as well as the generation of tinnitus; however, this working hypothesis has yet to be tested comprehensively.

In the present study, we investigated whether an increase in central gain via loss of inhibition at the level of the primary auditory cortex (A1) was sufficient to induce tinnitus-positive behaviour in rats. To accomplish this, we first performed *in vivo* extracellular electrophysiological recordings in A1 of anaesthetized rats to determine if local infusion of the potent GABA$_A$-receptor antagonist, Gabazine, resulted in neural changes consistent with central gain enhancement as characterized by Noreña (2011). Subsequently, we used our two-alternative forced-choice operant conditioning paradigm (Stolzberg et al., 2013; see Chapter 2) to screen for the presence/absence of tinnitus-positive behaviour in rats following the same intra-A1 micro-infusion of Gabazine. Consistent with the central gain model of tinnitus, we predicted that a loss of cortical inhibition via central infusions of Gabazine would not only cause an increase in spontaneous and auditory-evoked firing rates of neurons in A1, but ultimately lead to behavioural evidence of tinnitus.

3.2   Materials and Methods

The present study involved two experimental series that each used a separate cohort of adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA, USA). Rats (n=27) were housed in a 12-hour light-dark cycle with food and water *ad libitum*. All experimental procedures were approved by the University of Western Ontario Animal Care and Use Committee and were in accordance with guidelines established by the Canadian Council of Animal Care.

3.2.1 Experiment 1: Electrophysiological Recordings in the Primary Auditory Cortex (A1)

3.2.1.1 Surgical Procedure

Fifteen adult male Sprague-Dawley rats (age: 103 ± 2 days; body mass: 395 ± 6 g) underwent surgical procedures in preparation for electrophysiological recordings. Surgeries and recordings took place within a double-walled, sound-attenuating booth (MDL 6060 ENV; WhisperRoom Inc., Knoxville, TN, USA). Anaesthesia was induced with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (5 mg/kg), and supplemental doses were administered intramuscularly as needed. Once pedal reflex was absent, rats were placed in a stereotaxic frame with blunt ear bars. Throughout the surgery and electrophysiological experiment, a homeothermic heating pad was used to maintain body temperature at ~37°C (507220F; Harvard Apparatus, Kent, UK). A midline incision was made in the skin of the scalp, and the tissue was reflected from the skull. A headpost was fastened to the skull over the right frontal bone using dental acrylic, and a stainless steel screw was inserted into the left frontal bone serving as an electrical ground as well as an anchor for the headpost. A stereotaxic micromanipulator was used to measure 4.5 mm caudal to bregma—the approximate rostral/caudal location of the primary auditory cortex (A1; Paxinos and Watson, 2007; Polley et al., 2007)—and a mark was made on the skull for later drilling. A craniotomy (3 mm x 2.5 mm) was performed over the left temporal bone (3 to 6 mm posterior to bregma, 2.5 to 5 mm ventral to the sagittal suture) to expose the left auditory cortex. To allow for pharmacological manipulation of the auditory cortex, a second craniotomy (3 mm x 3 mm) was made over the left parietal bone (3 to 6 mm posterior to bregma) that would allow access for the insertion of a drug infusion glass pipette (see below for details). Once the surgical procedures were complete, the right ear bar was carefully removed to allow for free-field auditory stimulation of the right ear as electrophysiological recordings took place in the contralateral A1. Throughout the duration of the experiment, rats were secured within the stereotaxic frame using the headpost and left ear bar.
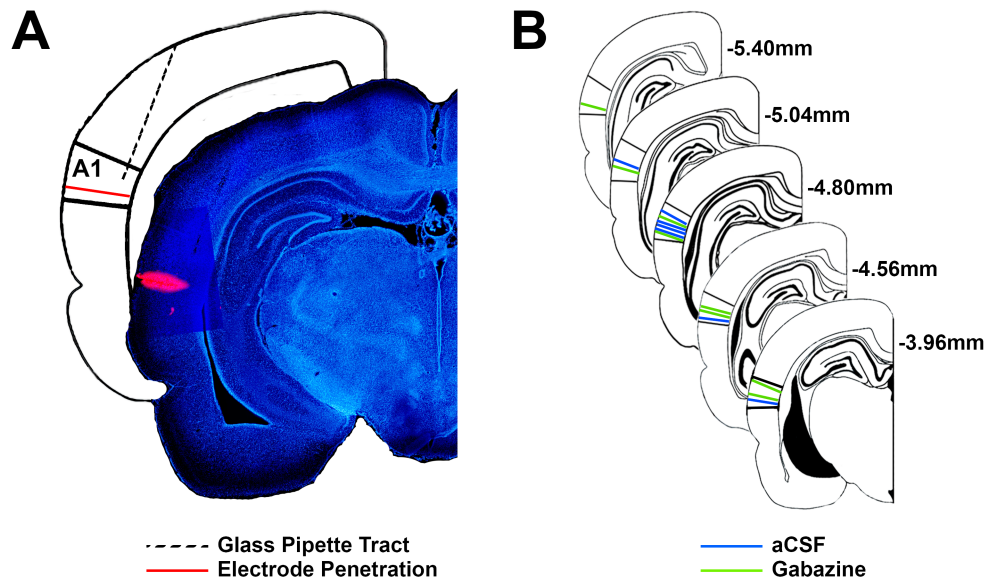
Figure 3-1. Extracellular electrophysiological recording penetrations in the primary auditory cortex

(A) DAPI-stained coronal section from a representative rat showing the electrode penetration and the tract left by the glass pipette. **(B)** Location of the 32-channel electrode array in the primary auditory cortex (A1) for rats that received aCSF (blue lines) or 50 μM Gabazine (green lines) infusion. The electrode array was inserted perpendicular to the pial surface, and spanned the full thickness of the cortex. Numbers indicate the distance from bregma in millimetres (Paxinos and Watson, 2007).

3.2.1.2   Electrophysiological Recordings and Central Infusions

Recordings were performed in a double-walled, sound-attenuating booth (MDL 6060 ENV; WhisperRoom Inc.). Extracellular electrophysiological signals were acquired from the auditory cortex using a 32-channel microelectrode array consisting of a single shank with 32 equally-spaced recording sites spanning 1.55 mm (A1x32-10mm-50-177-A32; NeuroNexus Technologies, Ann Arbor, MI, USA). Electrophysiological signals were preamplified and digitized (two RA16SD Medusa preamplifiers; Tucker-Davis Technologies (TDT), Alachua, FL, USA) prior to being sent to an RZ2 processing module (TDT) via a high-impedance head stage (NN32AC; TDT) and fiber optic cables. Neural activity was digitally sampled at 25 kHz and bandpass filtered online at 300 to 3000 Hz using voltage threshold for spike detection at three standard deviations above the noise floor. Spike detection thresholds were maintained throughout the duration of the experiment.

For each experiment, a single electrode penetration was completed, whereby the electrode was inserted perpendicular to the cortical surface through a small slit in the dura using a hydraulic microdrive (FHC, Bowdoinham, MA, USA). First, using a high-precision stereotaxic manipulator, the electrode array was slowly advanced into the cortex (4.5 mm posterior to bregma, 4 mm ventral to the sagittal suture on a 70° angle) in order to just penetrate the pia, and then withdrawn so that the tip of the electrode array was at the cortical surface. Next, the hydraulic microdrive was used to slowly advance the array to a depth of -1500 μm, such that the 32 recording sites spanned the entire cortical thickness (Paxinos and Watson, 2007). To confirm electrode depth and location of A1, local field potentials (LFP) and multi-unit (MU) activity were recorded in response to noise burst and pure tone stimuli. Current-source density (CSD) analysis was used to derive an LFP profile of activity recorded along the length of the electrode in response to noise burst stimuli. Electrode depth was verified if neural activation patterns across cortical layers matched previously established laminar profiles of A1 in the rat (Stolzberg et al., 2012). Criteria used to confirm the location of A1 included: (1) evidence of frequency-specific tuning (Polley et al., 2007; Rutkowski et al., 2003), (2) short response latencies and characteristic auditory response profiles (Polley et al., 2007), and

(3) sharp initial negative LFP peak (Di & Barth, 1992). Once the location was verified, the electrode was allowed to settle in the brain for one hour prior to initiating the electrophysiological recordings designed to assess the presence/absence of central gain enhancement.

Upon completion of the baseline recording protocol (described below), a glass pipette was slowly lowered 3 mm into the cortex using a high-precision stereotaxic manipulator at 4.5 mm caudal to bregma and 1.5 mm medial to the temporal ridge on a 30° angle using a dorsomedial-to-ventrolateral approach (see Figure 3-1A for a representative example of location of both the electrode and glass pipette). A Nanoliter 2010 Injector (World Precision Instruments, Sarasota, FL, USA) was used to inject 0.5 µL of artificial cerebral spinal fluid (aCSF; n=7; Harvard Apparatus Canada, St. Laurent, QC, Canada) or 50 µM Gabazine (n=8; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at a rate of 0.1 µL per minute. Following the injection, the glass pipette remained within the cortex throughout the rest of the recording protocol. Electrophysiological recordings began 7 minutes after the injection of aCSF or Gabazine.

3.2.1.3   Auditory Stimulation Paradigm

Sound stimuli were generated with an RZ6 processing module (TDT; 100 kHz sampling rate) and delivered through a magnetic speaker (MF1; TDT) positioned 10 cm from the rat's right ear. Auditory stimuli consisted of noise bursts (1 to 32 kHz; 50 ms duration) presented at 19 intensity levels ranging from 0 to 90 dB sound pressure level (SPL) in 5 dB steps. Additionally, a condition in which no auditory stimuli were presented was completed in order to observe spontaneous neural activity. Overall, the stimulus conditions were presented 50 times each in a randomized order, and separated by an inter-stimulus interval of 1 to 2 seconds. Noise burst stimuli were calibrated using a ¼" microphone (2530; Larson-Davis, Depew, NY, USA) and a pre-amplifier (2221; Larson-Davis) using custom MATLAB software.

3.2.1.4   Multi-Unit Analysis

To assess the consequences of a loss of inhibition on auditory processing, noise burst input-output (IO) functions were generated for multi-unit (MU) activity pooled by channels within the supragranular (depth ≥ -350 µm), granular (-650 µm $\leq$ depth < -350 µm), upper-infragranular (-950 µm $\leq$ depth < -650 µm), and lower-infragranular layers (depth < -950 µm). These cortical layer designations were allocated based on previous studies of the rat auditory cortex (Kaur et al., 2005; Stolzberg et al., 2012; Szymanski et al., 2009, 2011).

Prior to being allocated into each cortical layer, IO functions were generated for each channel using custom MATLAB scripts to produce rasters and peri-stimulus time histograms (PSTHs). MU IO functions were constructed for (1) the duration of the auditory response, (2) the number of spikes within the auditory response (spike count), (3) the peak firing rate, and (4) the mean firing rate as a percent of the maximum. For each MU cluster, the level of spontaneous activity was calculated from the last 500 ms of each trial and then calculated by averaging across all 50 trials. Response onset was defined as the first time that the firing rate within a 2 ms bin surpassed two standard deviations (SD) above spontaneous (Xu et al., 2016) and remained above for at least 8 ms. Response offset was defined as the time point at which the firing rate returned to the level of the spontaneous activity for at least 6 ms. Together, the response onset and offset were used to generate a response window for each auditory stimulus intensity (i.e. narrow grey shading on PSTHs in Figure 3-2). In the case where no response onset could be found (e.g. at lower sound levels), a fixed 40 ms response window was automatically placed at 20 ms from trial onset, and all remaining calculations (described below) were based off the spiking activity within this window.

Based on the response window, the duration of the auditory response was defined as the length of time during which the spiking activity surpassed the threshold criterion of 2SDs and is equivalent to the response window (Figure 3-2D). To assess the suprathreshold spiking output, *spike count* was first calculated by tallying the number of spikes within the response window for each of the 50 trials, and then calculating the average number of
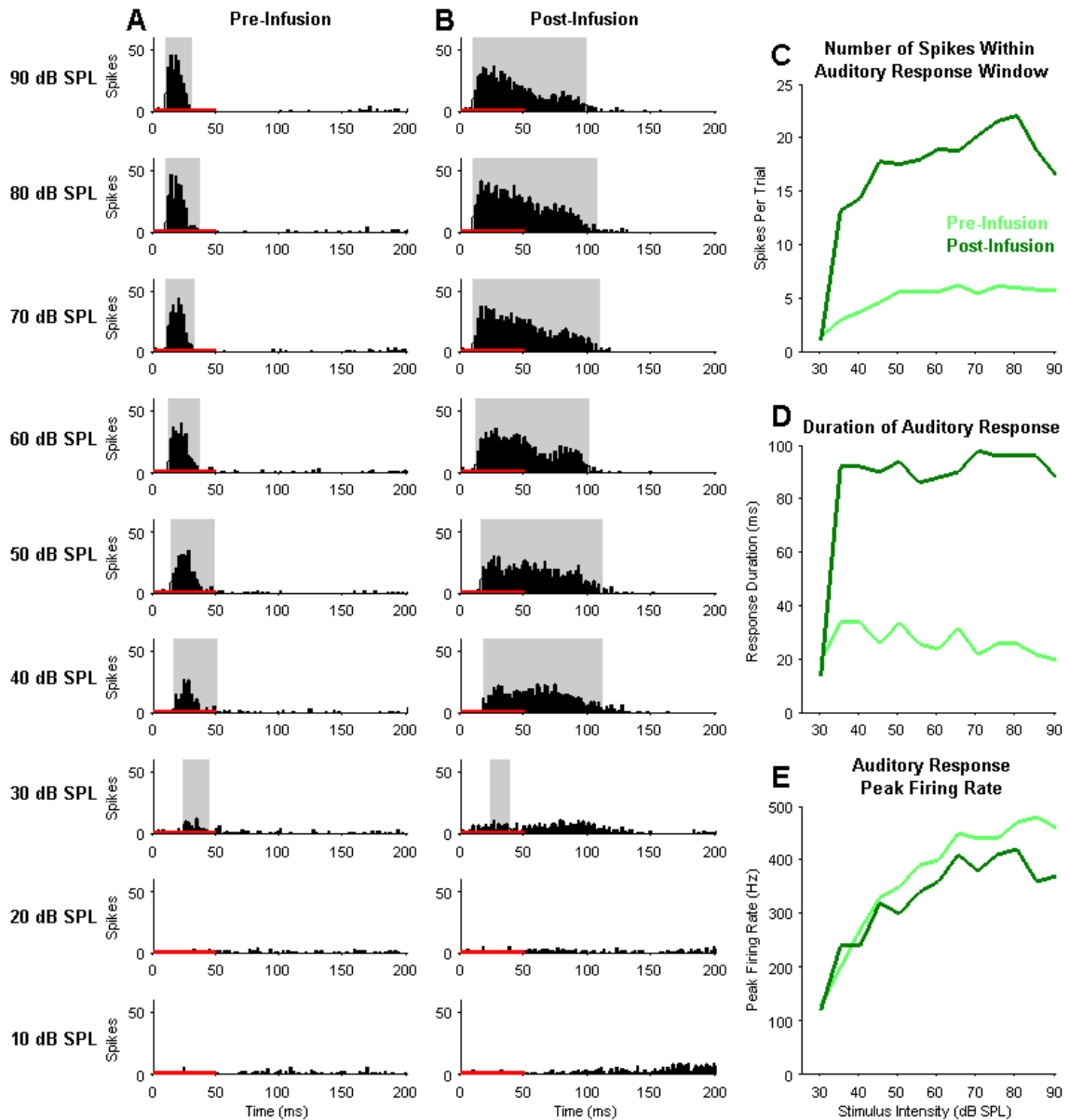
Figure 3-2. Representative auditory-evoked activity from a multi-unit cluster recorded before and after an infusion of 50 µM Gabazine

Panel (**A**) and (**B**) display peri-stimulus time histograms (PSTH; 2 ms time bins) recorded from one multi-unit cluster in response to a 50 ms noise burst stimulus (denoted by the red line) presented at various intensity levels pre- and post-infusion of 50 µM Gabazine, respectively. Auditory responses (outlined by grey shaded bar) were classified as activity that surpassed two standard deviations above spontaneous activity. Calculated from the multi-unit activity were (**C**) the number of spikes within the auditory response window, (**D**) the response duration, and (**E**) peak firing rates at each intensity level.

spikes per trial (Figure 3-2C). Contrary to spike count, the response magnitude (i.e., mean firing rate) was based on the average firing rate per trial, which was calculated by totaling the number of spikes within the response window and then dividing by the duration of the response for each trial, which was then averaged across trials (Hz/trial; Schormans et al., 2017b). Peak firing rate was determined as the maximum firing rate within a 2 ms bin that was located within the response window (Figure 3-2E). Finally, consistent with Polley et al. (2004, 2006, 2007), mean firing rate was converted into a percentage of the maximum by dividing the mean firing rate at each intensity level by the maximum firing rate observed across all intensity levels. In addition to the aforementioned calculations, an auditory response threshold was determined for each of the 32 electrode channels as the minimum intensity level at which the mean firing rate was significantly greater than spontaneous activity ($\alpha = 0.05$, paired t-test; Allman et al., 2008; Allman & Meredith, 2007; Schormans et al., 2017a, 2017b).

For all calculations, each animal provided an average metric (i.e. response duration, spike count, etc.) within each cortical layer. An overall cortical layer average was then generated for groups of animals according to their respective categorization (aCSF Pre-Infusion, aCSF Post-Infusion, Gabazine Pre-Infusion, or Gabazine Post-Infusion).

3.2.1.5   Histological Confirmation

To allow for confirmation of electrode penetrations, the electrode array was coated with DiI cell-labelling solution (V22885; Molecular Probes, Inc., Eugene, OR, USA) prior to being inserted into the auditory cortex. At the completion of each electrophysiological experiment, rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were subsequently extracted and stored in additional 4% paraformaldehyde for 24-hours, and 30% sucrose for another 24-hours. A microtome (HM 430/34; Thermo Scientific, Waltham, MA, USA) was used to section frozen brains into 40 μm coronal slices that were then mounted and stained with fluorescent DAPI mounting medium to label DNA (F6057 Fluoroshield ™ with DAPI; Sigma, St. Louis, MO, USA). An Axio Vert A1 inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) was used to image brain slices, and the electrode penetrations were

reconstructed using Adobe Photoshop CS6 (See Figure 3-1A for representative image). Experiments were removed if electrode penetrations were not located in the primary auditory cortex according to Paxinos and Watson (2007).

3.2.2 Experiment 2: Screening for Tinnitus-Positive Behaviour Following Central Infusions and Noise Exposure

3.2.2.1 Behavioural Training

Twelve adult male Sprague-Dawley rats (60 days old at the onset of training; body mass: 262 ± 4 g) were food restricted to 85 to 95% of free-feeding weight and trained for 2 to 3 months using a two-alternative forced-choice operant conditioning paradigm to differentiate between various auditory stimuli. Consistent with Stolzberg et al. (2013), rats were trained to nose-poke the left feeder trough if they perceived steady narrow-band noise (NBN) stimuli and the right feeder trough if they perceived an amplitude-modulated (AM) stimulus or quiet, to detect the presence of tinnitus. Correct feeder responses were positively reinforced with a sucrose pellet, while incorrect responses were negatively reinforced with a 15-second timeout during which rats could not initiate the next trial. Training took place 6 days per week and consisted of 30-minute sessions. Initial training was considered complete once a criterion of 90% was reached, after which the rate of reinforcement was progressively reduced from 100% to 70% (Stolzberg et al., 2013). Training continued until rats could achieve >92% hit rates for at least 15 consecutive training days. For a detailed description of the behavioural apparatus and training procedures, see Section 2.2.1 and Section 2.2.2.

3.2.2.2 Surgical Procedure

Once the rats had achieved the performance criterion (age: 262 ± 8 days; body mass: 439 ± 9 grams), they underwent a surgical procedure to unilaterally implant a drug delivery cannula into the auditory cortex to investigate the implications of a loss of inhibition on the emergence of tinnitus-positive behaviour. A stainless steel guide cannula (26G; 4.5 mm length; Plastics One, Roanoke, VA, USA) was permanently implanted in

behaviourally trained rats to target the left A1. Rats were anesthetized for surgery with isoflurane (induction: 4%; maintenance: 2%), and body temperature was maintained at 37°C using a homeothermic heating pad throughout the duration of the procedure. Once a surgical plane of anesthesia had been achieved, rats were placed in a stereotaxic frame with blunt ear bars, a midline incision was made along the scalp, and the top of the skull was cleaned with a scalpel blade to remove any remaining tissue. To minimize trauma, the cannula was inserted into the cortex on a dorsal-medial to ventral-lateral approach leaving the left temporalis muscle intact. As such, a burr hole was drilled into the parietal bone (4.5 mm caudal to bregma; 0.5 mm medial to the temporal ridge; 30° angle; see Figure 3A) and the guide cannula was carefully lowered into the approximate location of the left A1 (Paxinos and Watson, 2007; Polley et al., 2007). Furthermore, four additional bone screws were fixed in the skull (three in the right parietal bone, and one in the left frontal bone) and dental acrylic was used to adhere the cannula and bone screws to the surface of the skull. The skin surrounding the surgical implant was sutured, and the rat was allowed to recover for three days following the procedure. Rats were re-trained on the behavioural paradigm for one week post-surgery until they could once again achieve >92% hit rates for at least three consecutive training sessions.

3.2.2.3   Central Infusions

Once rats had successfully reached the performance criterion following the surgical procedure, a *test day* was performed in which their responses to quiet trials were no longer positively or negatively reinforced to avoid biasing test day results (for full details on behavioural testing protocols see Section 2.2.3). Prior to being placed in the behavioural chamber, rats received a unilateral infusion of either aCSF or Gabazine (50 µM) into the auditory cortex through a stainless steel infusion cannula (30G; 6.5 mm length; Plastics One). The tip of the infusion cannula extended 2 mm below the end of the guide cannula to reduce trauma within the auditory cortex (see Figure 3-3B for a diagram of all infusion locations). A 1 µL Hamilton syringe connection to a micro-syringe pump (Model 22 Syringe Pump Series; Harvard Apparatus Canada) was used to deliver 0.5 µL of aCSF or Gabazine over a five minute period. The infusion cannula was left in place for an additional two minutes to limit backflow of the drug. Following the infusion, rats

underwent a behavioural testing session until they completed 120 to 130 trials. The order in which the rats (n=12) received the infusion of aCSF and Gabazine was randomized. The day after infusion, each rat performed the same test day paradigm to ensure that any potential drug-induced effects were no longer present. On the following days, baseline performance was then ensured by having rats train on the original protocol for at least two consecutive days prior to the next infusion session.

3.2.2.4   Noise Exposure

To confirm that each rat could indeed demonstrate a tinnitus-positive behavioural profile, all rats also underwent a 15-minute noise exposure—a procedure which was previously confirmed to induce tinnitus in behaving rats (see Chapter 2). Following the completion of all infusion experiments, rats returned to normal training for three days to re-establish baseline performance. Once rats reached criterion (i.e., >92% correct), they were bilaterally exposed for 15 minutes to a 12 kHz tone (122 dB SPL) using a super tweeter (T90A; Fostex) placed above their cage. Tone exposure was completed within a sound-attenuating chamber and generated using an RZ6 processing module paired with RPvdsEx software (TDT). The stimulus was calibrated using a ¼" microphone (2530, Larson-Davis) and a pre-amplifier (2221, Larson-Davis) using custom MATLAB software. Immediately following the noise exposure, rats were placed in the behavioural chamber and run on the testing protocol to screen for tinnitus-positive behaviour. Consistent with infusion experiments, rats were also tested on the day after the noise exposure to determine if tinnitus persisted.

3.2.2.5   Histological Confirmation

At the completion of all behavioural experiments, rats were injected intraperitoneally with sodium pentobarbital (100 mg/kg) in preparation for transcardial perfusion of 0.9% saline followed by 4% paraformaldehyde. The brain was then extracted and post-fixed in 4% paraformaldehyde for an additional 24 hours before being placed in a 30% sucrose solution for cryoprotection. Using a microtome (HM 430/34; Thermo Scientific), frozen brains were sectioned coronally in 40 μm slices and collected. Sections were mounted
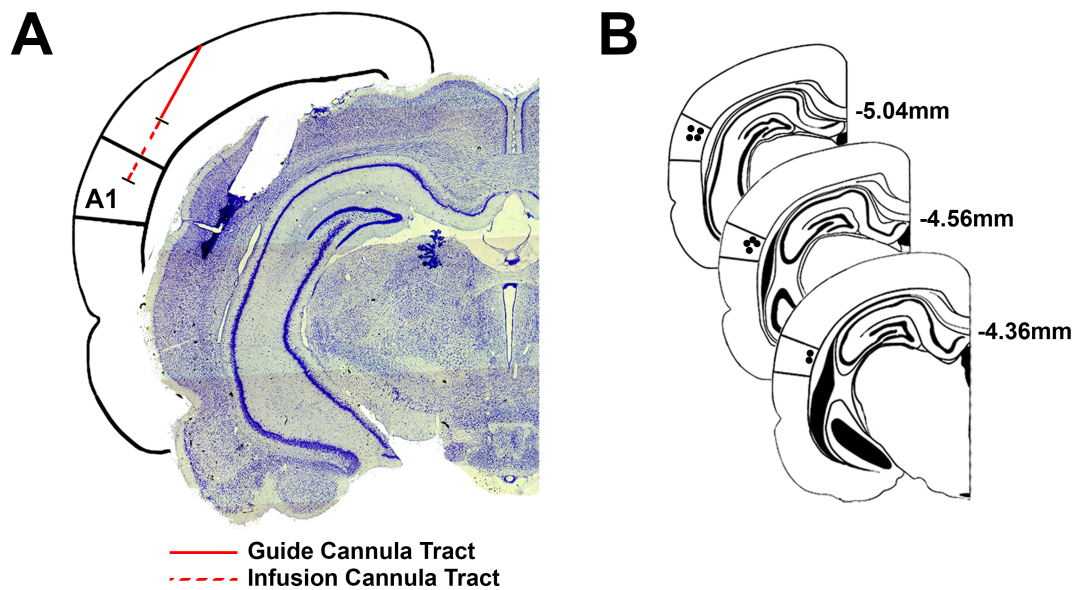
Figure 3-3. Infusion cannulae placement in the primary auditory cortex.

(**A**) Thionin-stained coronal section from a representative rat showing the guide cannula tract (solid red line in schematic) and the infusion cannula tract (dotted red line in schematic). The tip of the infusion cannula extended 2 mm below the end of the guide cannula, such that drugs were targeted to the primary auditory cortex (A1). (**B**) Each black dot represents the location of the most ventral point of the infusion cannulae within A1 from all experimental rats included in this study (n=10). Coronal sections are based on the atlas of Paxinos and Watson (2007). Numbers indicate the distance from bregma in millimetres.

onto microscope slides, stained with thionin, and cover-slipped for imaging. An Axio Vert A1 inverted microscope (Carl Zeiss) was used to image brain slices, and Adobe Photoshop CS6 was used to reconstruct brain sections to determine the location of cannulae tracts (See Figure 3-3A for representative image).

3.2.3　Statistical Analysis and Data Presentation

Statistical analysis involved two- or three-way repeated measures analysis of variance (ANOVA) followed by post-hoc paired samples t-tests, depending on the comparison of interest (see Results section for details on specific comparisons). Statistical significance was set at $\alpha = 0.05$, and when necessary, Bonferroni post-hoc corrections were applied to avoid "family-wise" error (Armstrong, 2014). SPSS software (Version 20; IBM corporation, Armonk, NY, USA) was used for statistical analyses, and MATLAB (Mathworks, Natick, MA, USA) and GraphPad Prism (Version 7.00 for Mac, GraphPad Software Inc., La Jolla, CA) were used to generate data figures. Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

3.3　Results

3.3.1　Experiment 1: Electrophysiological Recordings in the Primary Auditory Cortex (A1)

All rats (n=15) included in this experimental series underwent electrophysiological recordings before (pre) and after (post) an infusion of aCSF (n=7) or 50 μM Gabazine (n=8), which consisted of a single penetration of a 32-channel microelectrode array into A1. In rats that received an infusion of aCSF, a total of 224 multi-unit (MU) clusters were sampled, of which 214 (96%) were classified as being responsive to auditory stimuli. A total of 256 MU clusters were sampled in rats that received an infusion of Gabazine, of which 244 (95%) were classified as being responsive to auditory stimuli. As described in Section 3.2.1.4, an input-output (IO) function was generated for each MU in response to auditory stimuli ranging from 0 to 90 dB SPL, and the level of spontaneous activity was also determined. All analyses were done within the same treatment groups (i.e., aCSF and

Gabazine), and comparisons were made between pre-infusion and post-infusion time points. Data was collected 15- and 30 minutes following either infusion for spontaneous and auditory-evoked activity, respectively. Auditory-evoked MU activity was plotted for intensities ranging from 30 to 90 dB SPL, as this was the average response threshold that was observed in all rats.

3.3.1.1 Local Infusion of Gabazine Causes an Increase in Spontaneous Activity

A well-known characteristic of central gain enhancement is an increase in the level of spontaneous activity within the central auditory system (Auerbach et al.,2014; Noreña, 2011). To investigate whether this occurred within the primary auditory cortex (A1) following a loss of local inhibition, spontaneous MU activity was recorded before and 15-minutes after infusion of either aCSF or 50 µM Gabazine. As shown in Figure 3-4A, Gabazine caused an increase in the level of spontaneous activity of MU clusters across the majority of the cortical layers, while aCSF caused no change in spontaneous firing rates (SFR). More specifically, a loss of inhibition within A1, caused clusters within the supragranular, granular, and lower infragranular layers to increase their SFRs, as the majority of the clusters fall above the line of unity following an infusion of Gabazine (see green data points in Figure 3-4A). To control for similar firing rates among MU clusters, spontaneous firing rate was analyzed using a cumulative distribution function (CDF; see Polley et al., 2004) for each cortical layer. As expected, the distribution of MU spontaneous activity did not change following the infusion of aCSF. Consistent with multi-unit SFR, the CDF showed a qualitative rightward shift towards higher SFRs following the infusion of Gabazine within the supragranular, granular, and lower infragranular layers, suggesting that the majority of the MUs had higher SFRs post-infusion (see Figure 3-4B).

To determine the effect of aCSF and Gabazine infusion on spontaneous firing rates, separate two-way repeated measures ANOVAs (cortical layer × time) were performed. Within the Gabazine group, a main effect of layer ($F_{1.42, 9.95} = 19.465$, $p < 0.01$) and time ($F_{1,7} = 8.765$, $p < 0.05$) were revealed, indicating that the drug infusion (i.e., pre- and
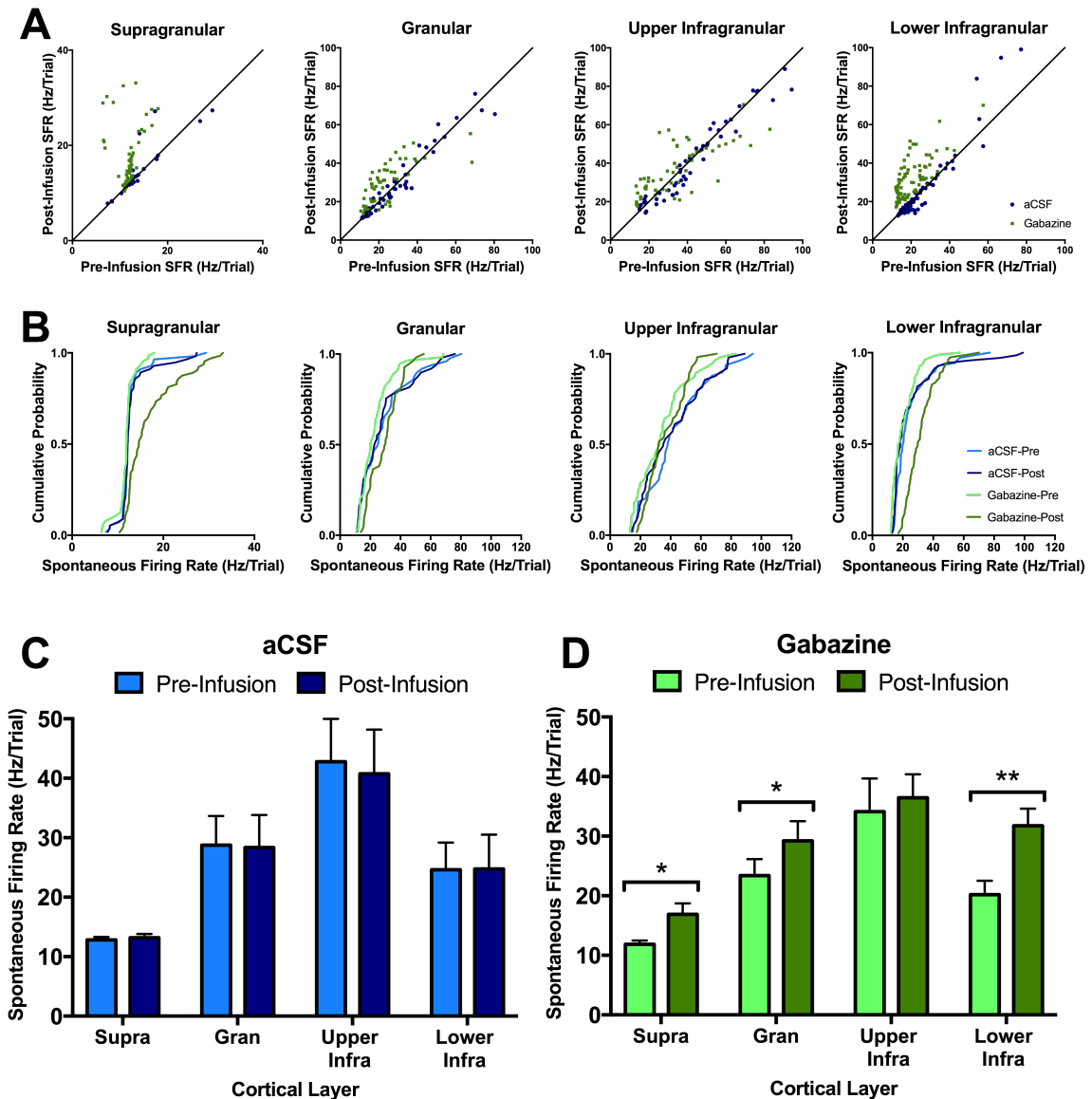
Figure 3-4. Infusion of Gabazine into A1 increased spontaneous firing rates

(**A**) Population data from all recorded multi-unit clusters depicting spontaneous firing rates pre- and post-infusion in the supragranular (aCSF: n=56, Gabazine n=64), granular (aCSF: n=49, Gabazine: n=56), upper-infragranular (aCSF: n=49, Gabazine: n=56), and lower-infragranular layers (aCSF: n=70, Gabazine n=80). The solid black line represents the line of unity, in which the pre- and post-infusion values are equivalent. (**B**) Cumulative distribution functions comparing the distribution of spontaneous firing rates pre- and post-infusion of aCSF and 50 μM Gabazine among multi-unit clusters. Panels (**C**) and (**D**) show the spontaneous firing rates averaged across animals that received aCSF infusion (n=7), and 50 μM Gabazine infusion (n=8), respectively. Data are plotted

as the mean ± SEM. Statistical analyses included separate two-way repeated measures ANOVAs (cortical layer × time) for each treatment group. Bonferroni-corrected post-hoc paired t-tests were then used to compare averaged pre- and post-infusion spontaneous firing rates within each group. * $p < 0.05$, ** $p < 0.0125$

post-infusion) affected spontaneous firing rates. As can be seen in Figure 3-4D, Bonferroni-corrected post-hoc analyses showed that an increase in SFR was observed within the supragranular (p = 0.048), and granular layers (p = 0.047), and a significant increase within the lower infragranular layer (p < 0.01) was observed compared to baseline recordings (i.e., pre-infusion). As expected, the aCSF group only showed a main effect of cortical layer ($F_{3,18}$ = 11.388, p < 0.001), as the degree of neuronal activity differed between the cortical layers, but there was no effect of aCSF infusion on spontaneous firing rates (Figure 4C). Overall, a loss of inhibition within the auditory cortex caused by the antagonism of $GABA_A$-receptors via Gabazine, resulted in an increase in the level of spontaneous activity across the majority of the cortical layers, indicative of central gain enhancement.

3.3.1.2 Local Infusion of Gabazine Causes an Increase in Auditory-Evoked Activity

Another characteristic of the central gain model of tinnitus is an increased responsiveness to suprathreshold stimuli (Auerbach et al., 2014). To assess this possibility following Gabazine infusion, an input-output (IO) function was generated in response to noise bursts (1 to 32 kHz) presented from 0 to 90 dB SPL, and analyses were completed on multiple auditory response metrics (i.e., spike count; response duration; peak firing rate; normalized mean firing rate; all described in Section 3.2.1.4). For each auditory response metric, a three-way repeated measures ANOVA (cortical layer × time × auditory intensity) was performed for each treatment group (i.e., aCSF and Gabazine). If a significant interaction or main effect of layer was observed within either treatment group, additional two-way repeated measures ANOVAs (time × auditory intensity) were completed within each individual cortical layer, allowing for post-hoc comparisons between pre- and post-infusion responses at each intensity level.

An infusion of Gabazine caused pronounced effects on auditory responsiveness within A1, which were assessed using (1) spike count (i.e., the number of spikes within the response window), (2) duration of responses, (3) peak firing rate, and (4) normalized
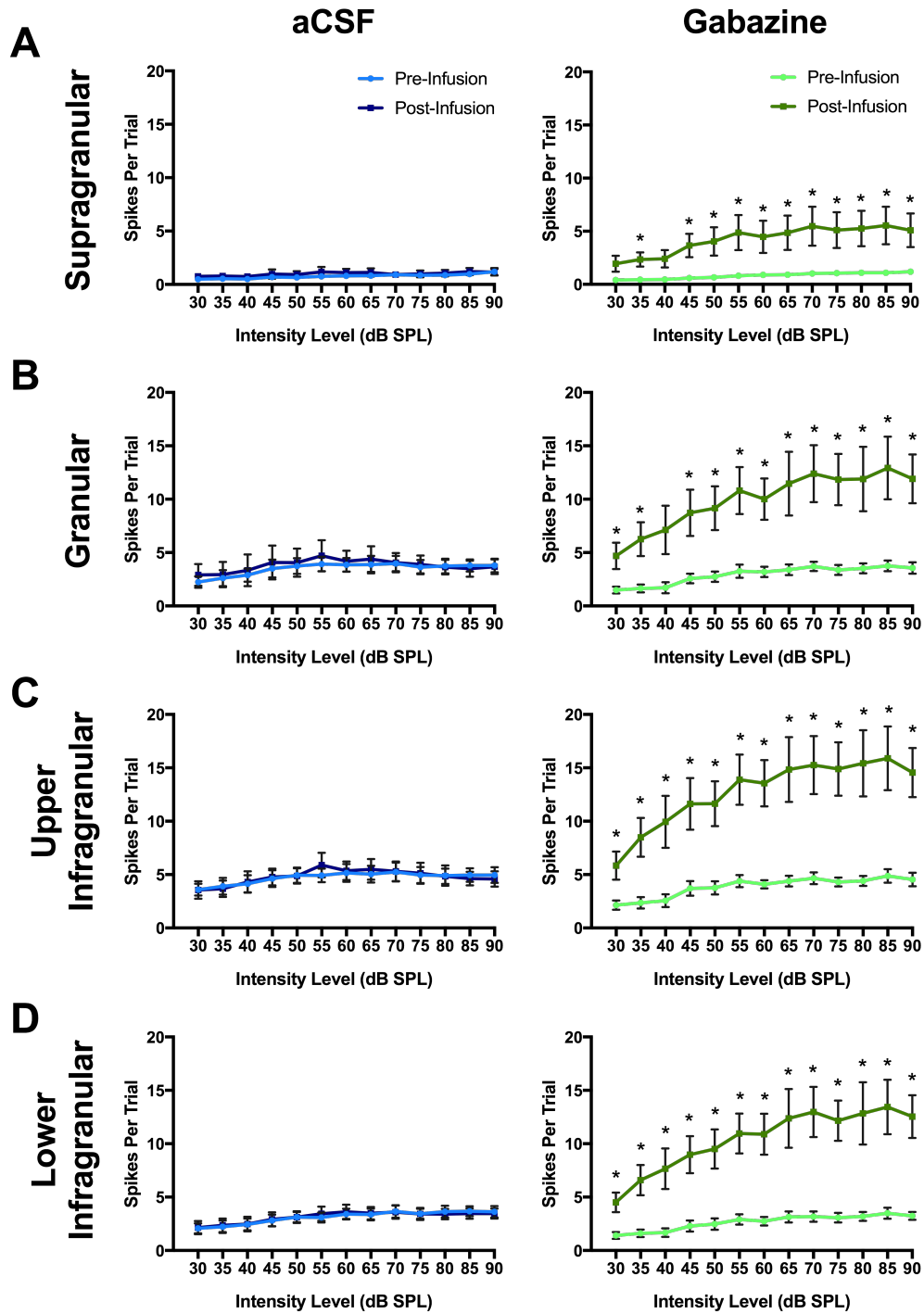
Figure 3-5. Infusion of Gabazine into A1 increased the number of spikes within the auditory response window

Panels (**A-D**) show the number of spikes per trial averaged across rats (i.e., spike count) observed within the auditory response window at various auditory intensity levels pre-

and post-infusion of aCSF (n=7) or 50 µM Gabazine (n=8) for each cortical layer. Gabazine caused an increase in the number of spikes per trial for each cortical layer at nearly all intensity levels. Conversely, infusion of aCSF did not alter spike count. Data are plotted as the mean ± SEM. Separate three-way repeated measures ANOVAs were conducted to compare cortical layer × time × auditory intensity within each treatment group, and where appropriate, subsequent two-way repeated measures ANOVAs (time × auditory intensity) were performed within cortical layers to determine if drug infusion affected pre- and post-infusion spike count at each intensity level (paired t-tests).
* $p < 0.05$

mean firing rate. As described in detail in the following sections, spike count, duration of the response, as well as normalized mean firing rate (represented as a percentage of the maximum) showed effects across multiple intensities and cortical layers following the infusion of Gabazine, but not aCSF.

3.3.1.2.1   Spike Count

Spike count, which is defined as the number of spikes within the auditory response window, demonstrated dramatic changes across the cortical layers (see Figure 3-2 for a representative example). A three-way repeated measures ANOVA revealed a significant interaction between layer × time × intensity ($F_{35,252} = 7.355$, $p < 0.00001$) within the Gabazine group. Additional two-way repeated measures ANOVAs within each cortical layer revealed significant interactions (time × intensity) within the supragranular ($F_{12,84} = 3.861$, $p < 0.001$), granular ($F_{12,84} = 4.422$, $p < 0.0001$), upper-infragranular ($F_{12,84} = 5.878$, $p < 0.0001$), and lower-infragranular ($F_{12,84} = 5.943$, $p < 0.01$) layers. As can be seen in Figure 3-5A-D (right panels), paired post-hoc t-tests comparing pre- and post-infusion within each of the cortical layers showed an increase in the number of spikes observed across the majority of the intensities. For example, at 60 dB SPL, the supragranular layer shows a $368 \pm 136\%$ increase in the number of spikes, while the upper infragranular layer shows a $267 \pm 75\%$ increase following the infusion of Gabazine. While a three-way repeated measures ANOVA revealed a main effect of layer ($F_{3,18} = 14.996$, $p < 0.0001$) within the aCSF group (which was expected), further analyses in each cortical layer revealed no interactions or main effects of time (Figure 3-5A-D, left panels). To summarize, Gabazine-induced loss of cortical inhibition within A1 caused large changes in auditory responsiveness as measured with spike count, irrespective of the stimulus intensity and cortical layer.

3.3.1.2.2   Response Duration

Consistent with spike count, duration of the auditory response showed pronounced changes across the cortical layers. Because a three-way repeated measures ANOVA revealed a significant interaction of cortical layer × time ($F_{1.367, 9.572} = 6.171$, $p < 0.05$)
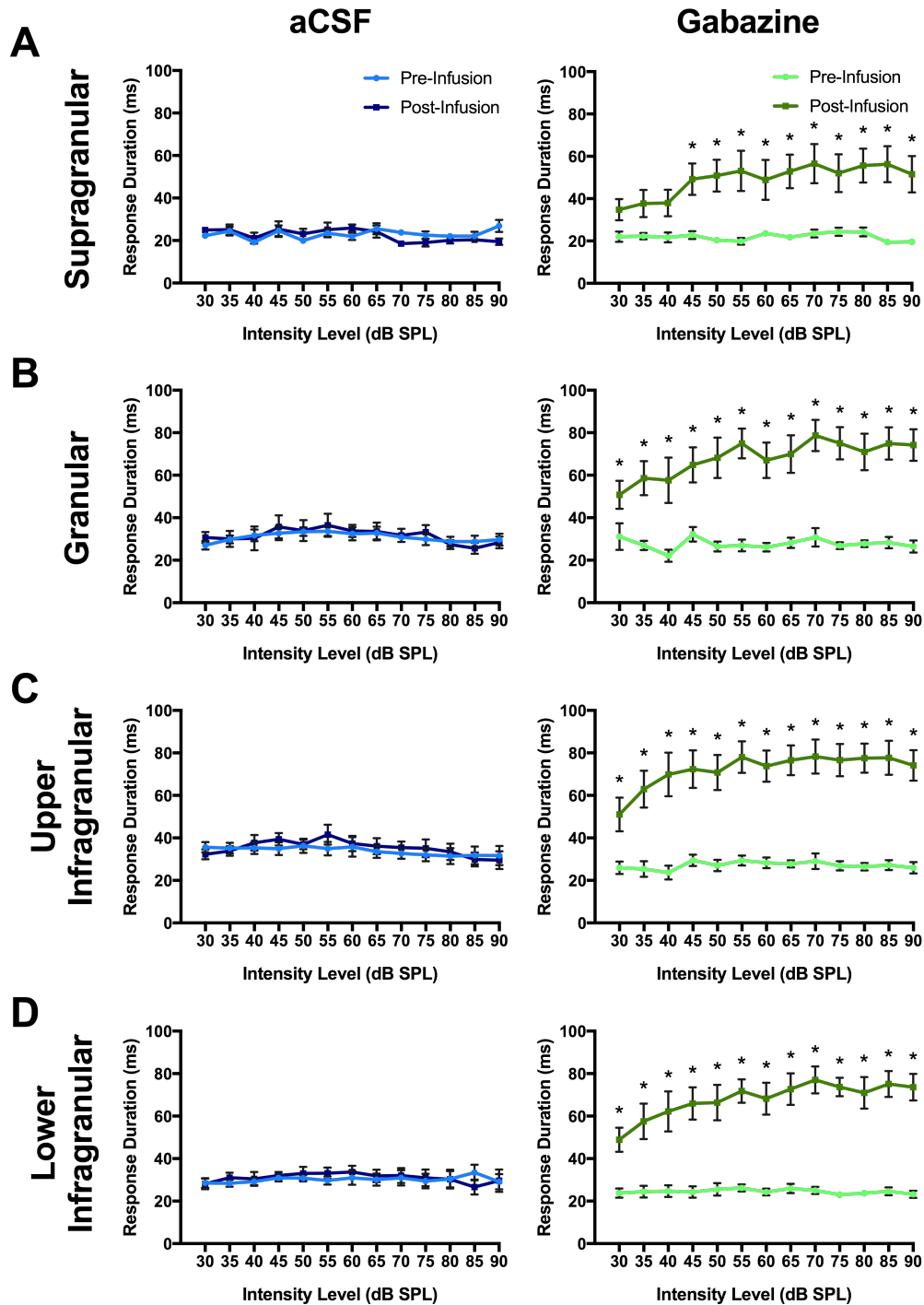
Figure 3-6. Infusion of Gabazine into A1 increased the duration of auditory responses

Panels (**A-D**) show the duration of auditory responses (averaged across rats) observed at various intensity levels pre- and post-infusion of aCSF (n=7) or 50 μM Gabazine (n=8)

for each cortical layer. Gabazine caused an increase in response duration for each cortical layer at nearly all intensity levels. Infusion of aCSF did not alter spike count. Data are plotted as the mean ± SEM. Separate three-way repeated measures ANOVAs were conducted to compare cortical layer × time × auditory intensity within each treatment group, and where appropriate, subsequent two-way repeated measures ANOVAs (time × auditory intensity) were performed within cortical layers to determine if drug infusion affected pre- and post-infusion response duration at each intensity level (paired t-tests).

* $p < 0.05$

within the Gabazine group, individual two-way repeated measures ANOVAs were completed within each cortical layer. As expected, an interaction of time × auditory intensity was observed within the supragranular ($F_{12,84} = 5.819$, $p < 0.0001$), granular ($F_{12,84} = 3.233$, $p = 0.001$), upper-infragranular ($F_{12,84} = 2.336$, $p < 0.05$), and lower-infragranular ($F_{12,84} = 3.770$, $p < 0.001$) layers. Within the aCSF group, no interaction or main effect was observed, suggesting that the duration of auditory responses did not change following the infusion of the vehicle (see Figure 3-6A-D, left panel). Most strikingly, Gabazine caused an increase in response duration across all intensities, with the exception of the supragranular layer where lower intensities did not show a significant change (see Figure 3-6A-D, right panel). Across all cortical layers and auditory intensities, there was a near tripling in the response duration, which would suggest that MU clusters continued to respond to a noise burst well-after the stimulus had ended (see Figure 2B for representative example; note the duration of the response relative to the duration of the auditory stimulus—red line).

3.3.1.2.3   Peak Firing Rate

Contrary to the two metrics discussed above, peak firing rate showed no change following the infusion of Gabazine. The generated IO function showed an increase in peak firing rate as the auditory intensity level increased in both treatment groups (i.e., aCSF and Gabazine). While a three-way repeated measures ANOVA revealed a significant interaction between layer × time × auditory intensity ($F_{36,252} = 1.909$, $p < 0.01$), post-hoc analyses only revealed an increase in peak firing rate at 30 dB SPL within the lower infragranular layer for the Gabazine group ($p < 0.05$, paired t-tests; see Figure 3-7D). Similar to the Gabazine group, rats that received an infusion of aCSF, showed a significant interaction of layer × auditory intensity ($F_{36,216} = 10.779$, $p < 0.0001$) following a three-way repeated measures ANOVA. However, as expected, further analysis into each cortical layer suggested that aCSF did not change peak firing rates following the infusion (see Figure 3-7A-D, left panel). Taken together, this indicates that the phasic or onset phase of the response was not enhanced following the infusion of Gabazine.
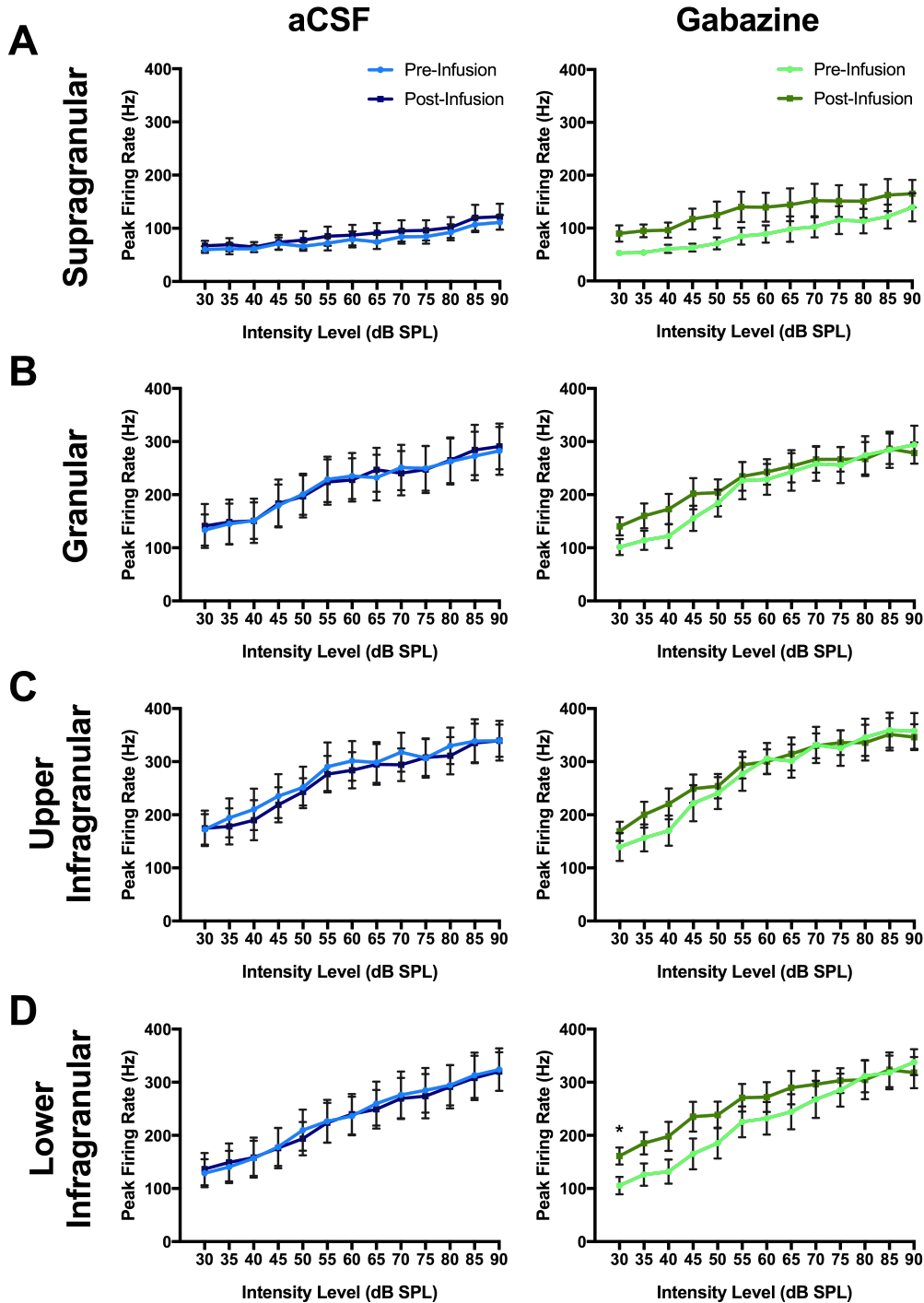
Figure 3-7. Infusion of Gabazine into A1 did not change peak firing rate

Panels (**A-D**) show the peak firing rate averaged across rats observed at various intensity levels pre- and post-infusion of aCSF (n=7) or 50 μM Gabazine (n=8) for each cortical layer. Neither Gabazine nor aCSF caused a notable change in peak firing rate. Data are

plotted as the mean ± SEM. Separate three-way repeated measures ANOVAs were conducted to compare cortical layer × time × auditory intensity within each treatment group, and where appropriate, subsequent two-way repeated measures ANOVAs (time × auditory intensity) were performed within cortical layers to determine if drug infusion affected pre- and post-infusion peak firing rate at each intensity level (paired t-tests).

* $p < 0.05$

3.3.1.2.4   Normalized Mean Firing Rate

An additional characteristic used to assess the auditory response profile is the sustained phase of the response, which can be observed by measuring the mean firing rate during the response window (Stolzberg et al., 2012). Mean firing rates were calculated from MU activity found within placed response duration windows (see Figure 3-2), and firing rates were subsequently averaged across animals within each group and cortical layer. To reduce variability between animals, the mean firing rate was represented as a percentage of the maximum firing rate (described in Section 3.2.1.4), hereafter referred to as normalized mean firing rates. Gabazine selectively increased the normalized mean firing rates, as changes were only observed within two cortical layers (see Figure 3-8A-D, right panel). A three-way repeated measures ANOVA revealed a significant interaction of layer $\times$ time ($F_{3,21} = 3.916$, $p < 0.05$), allowing for post-hoc analyses within each cortical layer. As can be seen in Figure 3-8, only the supragranular and lower infragranular layers showed enhanced firing rates. Within the supragranular layer, a main effect of time ($F_{1,7} = 15.805$, $p < 0.01$) and intensity ($F_{12,84} = 25.217$, $p < 0.0001$) were observed. Gabazine infusions preferentially increased normalized mean firing rates of MU clusters at lower intensity levels ($p < 0.05$, paired t-tests; 35 to 65 dB SPL, and 80 dB SPL; see Figure 3-8A). Similarly, the lower infragranular layer showed a significant interaction of time $\times$ auditory intensity ($F_{12,84} = 2.384$, $p < 0.05$), and post-hoc comparisons found an increase in normalized mean firing rates at lower intensity levels ($p < 0.05$; 30 to 40 dB SPL, and 50 to 70 dB SPL; see Figure 3-8D). This indicates that there was a selective increase in the number of action potentials in response to lower intensity levels generated by the supragranular and lower infragranular layers following an infusion of Gabazine, consistent with our previous results showing a near tripling in the spike count (see Figure 3-5). Whereas Gabazine caused preferential changes to the lower stimulus intensities, aCSF did not cause any significant changes from baseline in normalized mean firing rates.

Overall, the collective results from the electrophysiological recordings revealed that Gabazine exhibited the following effects on MU clusters within A1: (1) increased spontaneous firing rates in the supragranular, granular, and lower-infragranular layers,
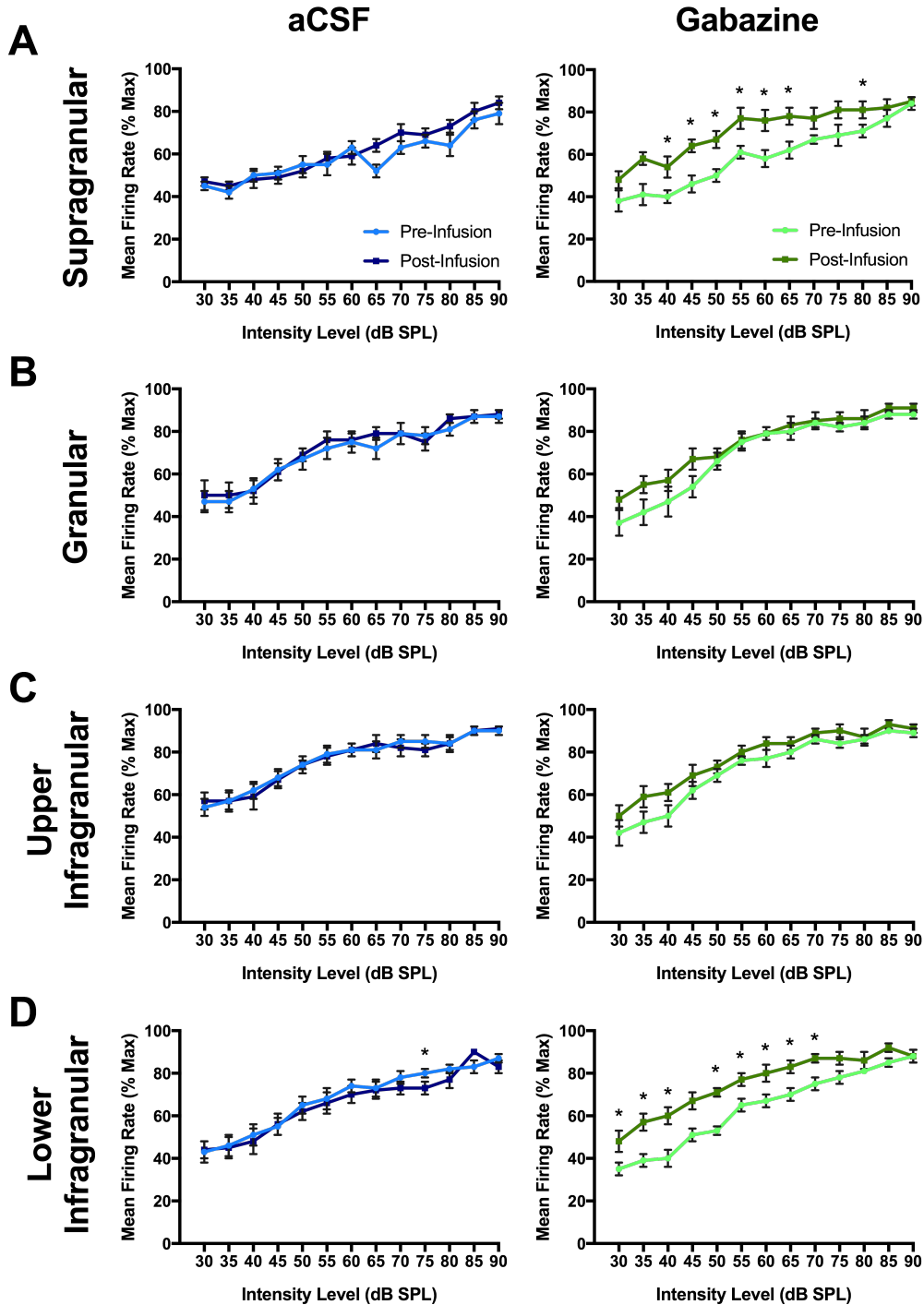
Figure 3-8. Infusion of Gabazine in A1 caused a selective increase in normalized mean firing rate for the supragranular and lower infragranular layers

Panels (**A-D**) show the mean firing rate normalized to the maximum firing rate averaged across rats observed at various intensity levels pre- and post-infusion of aCSF (n=7) or

50 μM Gabazine (n=8) for each cortical layer. Gabazine caused an increase in normalized mean firing rates for lower intensity levels measured in the supragranular and lower infragranular layers. Data are plotted as the mean ± SEM. Separate three-way repeated measures ANOVAs were conducted to compare cortical layer × time × auditory intensity within each treatment group, and where appropriate, subsequent two-way repeated measures ANOVAs (time × auditory intensity) were performed within cortical layers to determine if drug infusion affected pre- and post-infusion normalized mean firing rate at each intensity level (paired t-tests). * $p < 0.05$

(2) increased the number of spikes within a given auditory response in all cortical layers, (3) increased the response duration in all cortical layers, (4) did not affect the peak firing rates across all cortical layers, and (5) increased normalized mean firing rates in the supragranular and lower-infragranular layers at lower intensity levels. Importantly, none of these effects were observed following infusions of aCSF. Furthermore, these dramatic effects of Gabazine on neuronal activity cannot be attributed to hearing impairments at the level of the cortex, as Gabazine did not cause response threshold levels to increase from those recorded at baseline. In fact, the supragranular layer exhibited a modest *decrease* in response threshold following infusion of Gabazine, suggesting a hyper-sensitivity to auditory stimuli (see Figure 3-9).

3.3.2   Experiment 2: Screening for Tinnitus-Positive Behaviour Following Central Infusions and Noise Exposures

3.3.2.1   Local Infusion of Gabazine Induces Tinnitus-Positive Behaviour

A separate cohort of rats (n=12) were trained to distinguish between quiet, amplitude-modulated (AM), and narrow-band noise (NBN) stimuli using our novel two-alternative forced-choice behavioural paradigm. Following training and surgical implantation of cannulae, rats were given local infusions of aCSF and 50 µM Gabazine into the left A1 prior to behavioural testing to determine if either infusion resulted in behavioural performance indicative of the presence/absence of tinnitus. In this paradigm, tinnitus-positive behaviour was scored as a shift in behavioural response to quiet stimuli from the right trough (previously trained to be a correct response) to the left trough (trained to be associated with NBN). Upon conclusion of all experiments, histology revealed that cannulae locations for two rats were located outside of A1, and as such, their data points have been removed.

Following infusions of aCSF into A1, all rats were able to correctly identify AM (94.6 ± 1.3% correct) and NBN trials (96.9 ± 0.7% correct; Figure 3-10A left panel), suggesting
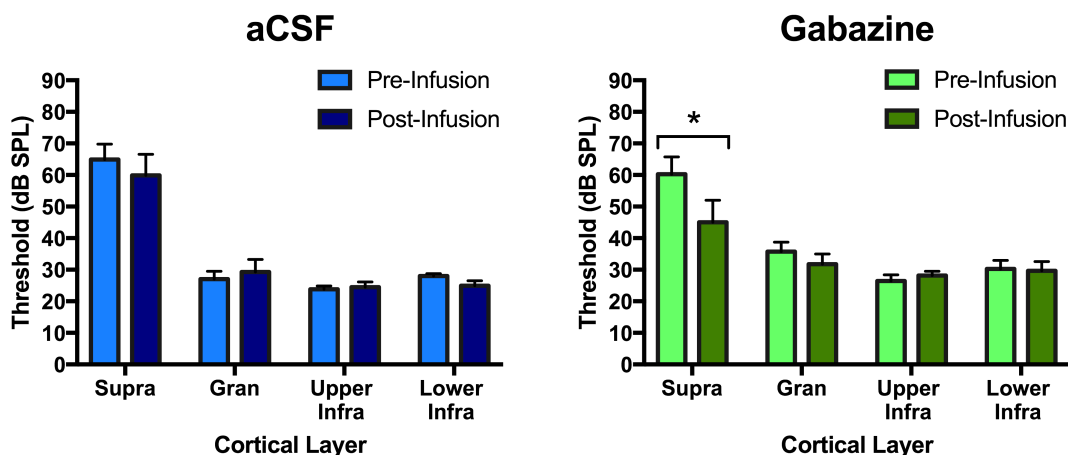
Figure 3-9. Infusion of Gabazine in A1 did not increase auditory response threshold levels

Threshold levels were determined to be the minimum intensity level that produced a significant auditory response. The response threshold for each cortical layer was averaged across animals (aCSF: n=7, Gabazine: n=8). Gabazine did not alter the response thresholds in the granular, upper infragranular, and lower infragranular layers of A1. Moreover, Gabazine caused a decrease in response threshold in the supragranular layer, suggesting an increased sensitization to auditory stimuli. Data are plotted as the mean ± SEM. Statistical analyses include separate two-way repeated measures ANOVAs (cortical layer × time) for each treatment group. Bonferroni-corrected post-hoc paired t-tests were then used to compare averaged pre- and post-infusion response thresholds within each group. * $p < 0.05$

that the infusion process alone did not affect rats' ability to perform the task. A two-way repeated measures ANOVA revealed no interaction between group (aCSF or Gabazine), and time (baseline, post-infusion, 1-day post-infusion) for NBN performance (NBN Gabazine rats: $92.4 \pm 2.1\%$ correct; Figure 3-10A center panel). In contrast, a two-way repeated measures ANOVA showed a significant interaction between group $\times$ time for AM performance ($F_{1.214, 10.922} = 8.761$, $p < 0.05$). Post-hoc paired t-tests with Bonferroni corrections revealed a significant decrease in the percent of correctly identified AM trials following Gabazine infusion when compared to aCSF infusion (AM Gabazine: $70.6 \pm 6.8\%$ correct vs. AM aCSF: $94.6 \pm 1.3\%$ correct; $p < 0.01$). Because rats receiving Gabazine infusions could still correctly identify NBN stimuli above threshold criterion, behavioural responses to quiet stimuli could be interpreted.

As predicted, all rats were still able to correctly identify quiet trials following infusions of aCSF ($3.4 \pm 0.9\%$ quiet trials misidentified as NBN); findings which confirm that the behavioural task was resilient to falsely-screening rats for tinnitus during control conditions (Figure 3-10B, left panel). In comparison, the proportion of quiet trials misidentified as NBN varied following infusions of Gabazine into A1 (Figure 3-10B, center panel). On average, following Gabazine infusion, rats mistakenly identified $29.7 \pm 7.3\%$ of quiet trials as NBN during behavioural testing (see Figure 3-10B, right panel). This result was not observed on the subsequent test day, suggesting that the drug effects had washed out. With respect to quiet performance, a two-way repeated measures ANOVA revealed a significant interaction between group $\times$ time ($F_{1.23,9.00} = 9.271$, $p < 0.01$). Bonferroni-corrected post-hoc paired t-tests showed that rats misidentified significantly more quiet trials following the Gabazine infusion than they did following the aCSF infusion ($p < 0.01$; Figure 3-10B). In fact, based on the >20% threshold for tinnitus-positive behaviour that was previously established in our 15-minute noise exposure paradigm (see Section 2.3.1), five of the ten rats demonstrated behavioural performance indicative of tinnitus.
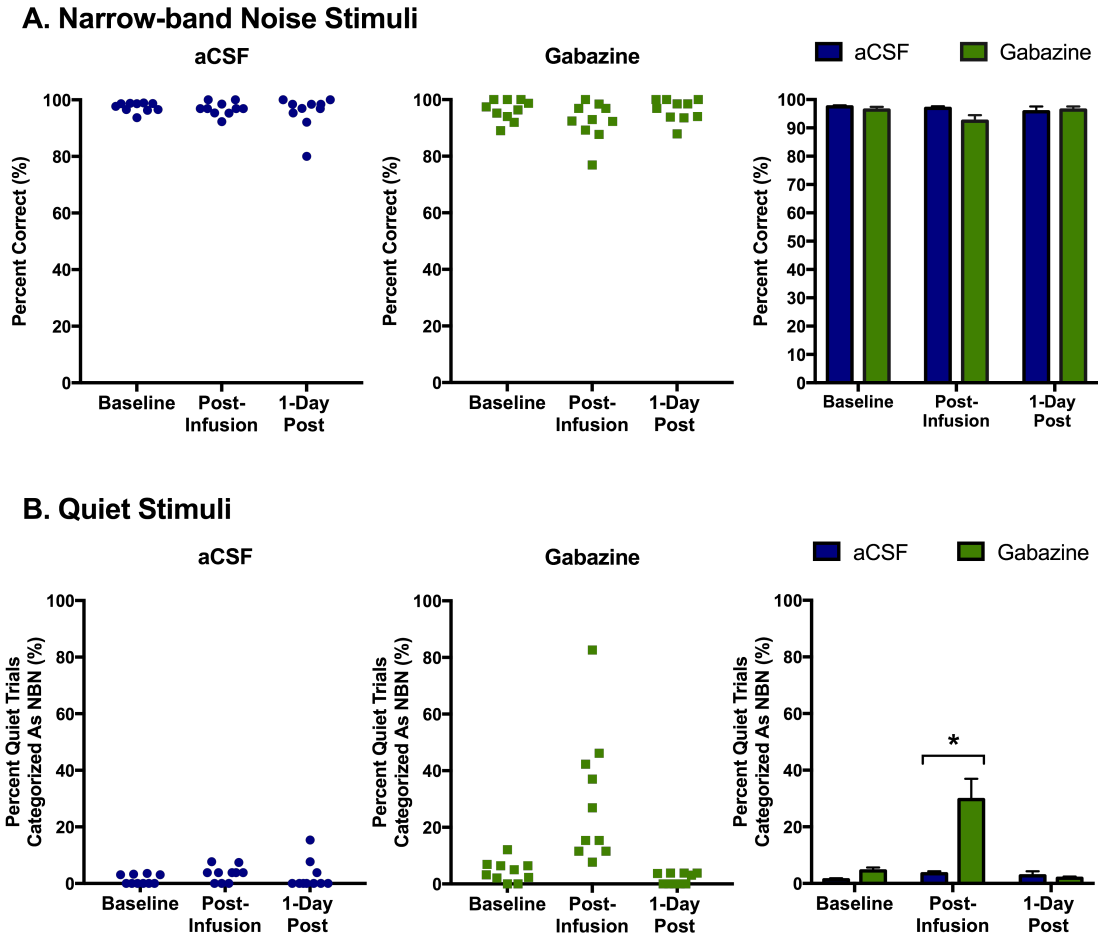
Figure 3-10. Performance on quiet and narrow-band noise trials following infusions into A1

(A) Following infusions of either aCSF or 50 µM Gabazine into A1, all rats could still accurately identify narrow-band noise (NBN) stimuli. **(B)** Following an infusion of aCSF, all rats could still correctly identify all quiet stimuli. However, following an infusion of Gabazine, 5 of 10 rats mistakenly identified >20% of quiet trials as NBN, indicative of tinnitus-positive behaviour. On average, rats mistakenly identified significantly more quiet trials as narrow-band noise following Gabazine infusion than they did following aCSF infusion. Statistical analyses included a two-way repeated measures ANOVA (time × exposure), followed by post-hoc paired t-tests with Bonferroni corrections. Comparisons were made between aCSF and Gabazine performance at each time point. * $p < 0.01$
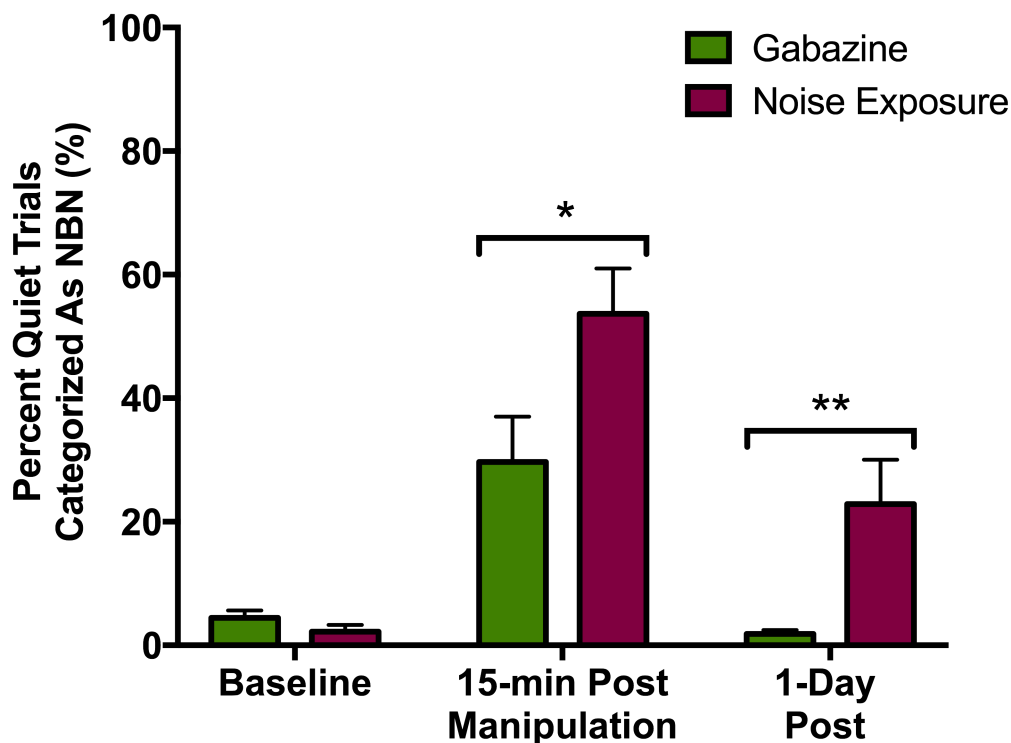
Figure 3-11. Infusion of Gabazine into A1 as well as noise exposure both caused behavioural evidence of tinnitus

Both Gabazine and noise exposure caused an increase in the percent of quiet trials misidentified as narrow-band noise (NBN) when compared to baseline levels established the day before. Subsequent test days revealed persistent tinnitus-positive behaviour following noise exposure, but not after Gabazine infusion. Statistical analyses included a two-way repeated measures ANOVA (group × time) followed by post-hoc paired t-tests comparing Gabazine to noise exposure performance at each time point. Bonferroni corrections were used to adjust the p-value. * $p < 0.05$ and ** $p < 0.017$

3.3.2.2   Confirmation of Tinnitus-Positive Behaviour with Noise Exposure

A few days following the conclusion of all infusion experiments, rats were given a brief 15-minute noise exposure (12 kHz tone, 122 dB SPL) immediately prior to behavioural testing to ensure that all rats could indeed show behavioural performance consistent with tinnitus. Similar to their performance following Gabazine infusions, rats demonstrated a shift in behavioural responses to quiet, indicating they perceived a steady NBN during $53.7 \pm 7.3\%$ of quiet trials (see Figure 3-11). A two-way repeated measures ANOVA revealed a significant interaction between group (Gabazine or noise exposure) and time (baseline, post-manipulation, 1 day post-manipulation) ($F_{2, 18} = 5.247$, $p < 0.05$). Post-hoc paired t-tests with Bonferroni corrections were used to compare performance on quiet trials following Gabazine infusion and noise exposure at each of the three time points. It was found that noise exposure resulted in a greater percentage of quiet trials misidentified as NBN during the initial test day (15-minutes post-manipulation; $p = 0.023$), as well as the next day (1-day post-exposure; $p < 0.017$). Collectively, these results suggest that Gabazine infusion caused transient tinnitus that lasted less than 24-hours, whereas noise exposure caused tinnitus to persist in some rats.

3.4   Discussion

To our knowledge, the present study represents the first direct investigation into the effect of impaired inhibitory neurotransmission on central gain enhancement and the associated emergence of tinnitus-positive behaviour. Here, we show that the hallmarks of central gain enhancement (i.e., increased spontaneous and auditory-evoked activity; Auerbach et al., 2014; Noreña, 2011), can be directly induced via infusion of 50 μM Gabazine into the primary auditory cortex (A1). By comparing spiking activity pre- and post-infusion, it was found that application of Gabazine caused a layer-specific increase in spontaneous firing rates (SFRs) in the supragranular, granular, and lower-infragranular layers. Moreover, although spike count and response duration were increased in all layers and at all intensities, no significant increase in peak firing rate was found, and normalized mean firing rates were only increased in the supragranular and lower-infragranular layers for

lower intensity levels. In a second experimental series, we demonstrate for the first time that awake rats infused with Gabazine in their A1 exhibited tinnitus-positive behaviour, which was characterized by an increase in the proportion of quiet trials misidentified as narrow-band noise (NBN). Collectively, these findings suggest that central gain enhancement, specifically in A1, is sufficient to generate a phantom auditory perception that is consistent with tinnitus-positive behaviour.

3.4.1 Loss of Inhibition as a Mechanism for Central Gain Enhancement

Although it is well-established that tinnitus inducers, such as exposure to salicylate and excessive noise, can cause central gain enhancement, the mechanism(s) through which these changes occur have remained elusive. Auerbach et al. (2014) suggested that central gain increases related to tinnitus could develop either through losses of inhibition, increases in excitation, or changes in the intrinsic excitability of cells. Results from the present study provide support for the suggestion that a loss of local inhibition, via antagonism of the $GABA_A$-receptor, can indeed lead to central gain enhancement at the level of the auditory cortex. More specifically, we demonstrated that local infusion of 50 µM Gabazine, a potent $GABA_A$-receptor antagonist (Ueno et al., 1997), into A1 caused neural changes indicative of central gain enhancement, including increases in spontaneous firing rates (SFRs), as well as auditory-evoked spike count, response duration, and normalized mean firing rates. Similar to our findings involving the pressure injection of Gabazine, Kurt et al. (2006) showed that iontophoretic application of Gabazine onto single neurons in the A1 of anaesthetized and unanaesthetized gerbils caused increases in SFRs and pure tone-evoked firing rates, as well as broadening of auditory responses. Although Kurt and colleagues only recorded from the middle layers of A1, their findings support an ability of Gabazine to cause neural enhancement reminiscent of increases in central gain. It is worth noting that the effect of Gabazine on central gain enhancement is not restricted to the auditory cortex, as comparable altered neural activity was also observed in mouse inferior colliculi, whereby increases in spike count and broadening of auditory responses were recorded in some neurons following microiontophoretic application of Gabazine (Ayala et al., 2016).

Further support for loss of inhibition as a putative mechanism contributing to central gain enhancement comes from studies using the known tinnitus-inducer, salicylate (Cazals, 2000). For example, *in vitro* patch-clamp studies on rat auditory brain slices demonstrate a selective depression of fast-spiking interneurons and inhibitory postsynaptic currents upon application of salicylate (Su et al., 2009; Wang et al., 2006). Moreover, the use of isoflurane, and locally administered baclofen and vigabatrin, substances that potentiate $GABA_A$-mediated inhibition, have been shown to restore salicylate-induced gain enhancement in A1 to baseline levels (Lu et al., 2011; Sun et al., 2009). Collectively, these results suggest that a loss of inhibition in A1 can contribute to central gain enhancement.

Furthermore, results from our second experiment also support this putative mechanism. Our behavioural testing revealed a significant decrease in the proportion of correctly-identified amplitude-modulated (AM) trials upon local administration of Gabazine into A1. Because the neurotransmitter GABA plays a significant role in the temporal processing of auditory stimuli (Grothe & Klump, 2000), modifying cortical $GABA_A$-mediated inhibition levels may have affected the rats' ability to accurately identify AM stimuli, which requires more precise neuronal firing. Indeed, in a study by Chambers and colleagues (2016), mice with near-complete cochlear denervation experienced gain enhancement in A1 over the course of 7 to 30 days. While these mice regained the ability to neurally encode more simple sound stimuli, their ability to encode complex auditory stimuli, such as modulated noise or speech tokens did not recover. Moreover, Kurt et al. (2006) found that application of Gabazine to gerbil A1 impaired the ability of the cortex to phase-lock with sound stimuli of high frequency amplitude modulation. This was suggested to occur because a loss of inhibition results in increases in auditory response duration, such that the duration of one auditory response extends beyond the duration of one modulation cycle. In the present study, we observed increases in auditory response duration measured electrophysiologically, and an inability to process amplitude-modulated stimuli measured behaviourally. Together, these findings support the notion that a loss of inhibition can induce gain enhancement in A1.

However, criticism of this claim comes from a study by Brozoski et al. (2012), who used high resolution point-resolved magnetic resonance spectroscopy to compare levels of GABA and glutamate in the central auditory system of noise-exposed rats with persistent tinnitus. They observed that levels of GABA were slightly increased in A1, while glutamate levels were modestly increased, when compared to the brains of unexposed rats. This would suggest that after tinnitus induction, A1 experiences a small increase in inhibition, with an even greater increase in excitation, as GABA and glutamate are the main inhibitory and excitatory neurotransmitters of the central nervous system, respectively. As such, increasing inhibition levels in A1 by potentiating $GABA_A$-receptor function would still act to ameliorate tinnitus-induced central gain increases. While these findings diverge from our previous suggestion, it is possible that the generation of the tinnitus percept ultimately depends on gain enhancement in A1, regardless of the mechanism through which it is produced, and thus losses of inhibition and increases in excitation could co-exist together to induce the central gain model of tinnitus.

3.4.2   Increased Central Gain in the Primary Auditory Cortex as a Mechanism for Tinnitus?

The hallmarks of the central gain model of tinnitus (i.e., increased spontaneous and auditory-evoked activity) have been observed in past studies following both salicylate and noise exposure in a variety of animal species (Auerbach et al., 2014). SFRs have been found to be elevated in anaesthetized animals, and similarly auditory-evoked activity, either measured through auditory firing rates or amplitude of sound-evoked local field potentials, has also been shown to be increased (Lobarinas et al., 2006; Lu et al., 2011; Noreña & Eggermont, 2003; Noreña et al., 2003, 2010; Ochi & Eggermont, 1996; Qiu & Salvi, 2000; Seki & Eggermont, 2003; Sun et al., 2009; Yang et al., 2007; Zhang et al., 2011). Although only some of these studies confirmed the presence of tinnitus with behavioural measures prior to or during electrophysiological recording, both salicylate and noise exposure are well-established tinnitus inducers that have been used by numerous studies in the field. However, one of the main disadvantages of studying tinnitus with these two approaches is their widespread influence on the entire auditory pathway. These systemic methods of tinnitus induction tend to cause varying degrees of

hearing loss, making it difficult to determine if changes in neural activity are specific to tinnitus or not. Moreover, their widespread effects confound the ability to verify where gain enhancement must occur (i.e., in which central auditory structure) in order to generate the tinnitus percept.

Our cortex-specific gain enhancement did not increase hearing thresholds measured at the level of the cortex, but did induce tinnitus-positive behaviour in rats. This would suggest that indications of hearing loss may develop from subcortical structures, while the tinnitus percept is generated at the level of the cortex. This notion is supported by previous studies that found that local application of salicylate onto the auditory cortex resulted in enhanced auditory-evoked activity without changes in hearing thresholds, while application of salicylate onto the round window caused threshold shifts with depressed auditory-evoked activity in the inferior colliculus and the auditory cortex (Sheppard et al., 2014; Sun et al., 2009).

The results of Experiment 2 show that gain enhancement, specifically in A1, likely plays a role in generating the tinnitus percept. In the current study, manipulations to inhibitory neurotransmission were limited to A1, and this was sufficient to cause rats to misidentify a significant proportion of quiet stimuli as a steady NBN. Because subcortical structures were not directly affected by our manipulation, we propose that increases in central gain must ultimately occur in A1 in order to develop phantom auditory perceptions consistent with tinnitus. Support for this comes from an imaging study which showed that increases in auditory-evoked activity in A1 were specific to patients with tinnitus only, as opposed to the widespread increases throughout the CAS that were observed in patients with both tinnitus and hyperacusis (Gu et al., 2010). Furthermore, A1 has been shown to demonstrate the greatest degree of gain enhancement relative to other central auditory structures, such as the dorsal cochlear nucleus, and the inferior colliculus (Chambers et al., 2016; Qiu & Salvi, 2000; Sun et al., 2009). Together, these findings suggest that gain increases in A1 are highly important for the manifestation of tinnitus. However, future studies should aim to determine if gain enhancement in subcortical auditory structures can also induce tinnitus-positive behaviour.

In contradiction to the aforementioned claim that gain enhancement in A1 is important for tinnitus generation, is the fact that neural changes associated with increased central gain typically occur more than one hour after tinnitus induction via salicylate or noise exposure, whereas the percept itself can be perceived almost immediately (Salvi et al., 1992; Noreña & Eggermont, 2003; Noreña et al., 2010; Sun, Zhang, Lu, & Yang, 2008, 2012; Syka et al., 1994; Syka & Rybalko, 2000). Indeed, in recent work conducted by Chambers et al. (2016), gain enhancement did not occur in mice A1 until more than 1-week following cochlear denervation. While neural changes in the present study took place after several minutes, we argue that our manipulation to A1 neurotransmission likely simulates the aberrant homeostatic plasticity that occurs in patients with persistent tinnitus. As such, the current results would represent a snapshot of the neuroplastic changes that would have occurred over the hours or days following the deprivation of auditory input. However, as mentioned previously, while persistent tinnitus develops over long durations of time, transient tinnitus has been found to occur immediately after traumatic noise exposure (see Chapter 2). Further investigation would be required to verify if gain enhancement in A1 could be the mechanism that underlies both transient and persistent tinnitus.

3.4.3   A Potential Role of Intracortical Connections in the Generation of Tinnitus

In the current study, a loss of inhibition in A1 caused a layer-specific increase in spontaneous and auditory-evoked activity. Typically the supragranular and lower infragranular layers are associated with intracortical connections, whereas the granular and upper infragranular layers are known to receive inputs from the thalamus (Stolzberg et al., 2012; Szymanski et al., 2009). We found significant increases in normalized auditory mean firing rates in the supragranular and lower infragranular layers following local administration of Gabazine. This observed layer specificity would suggest a strong role of intracortical connections in the generation of the tinnitus percept, as thalamo-recipient layers (i.e., granular and upper infragranular layers) were not strongly affected by Gabazine. Although this would be explained by the top-down approach of the infusion method, such that granular and upper infragranular layers should not be affected by direct

manipulations to the cortex, work by Stolzberg et al. (2012) suggests otherwise. In their study, an auditory-driven profile was created for the layers of the auditory cortex using multi-unit (MU) activity following systemic injections of salicylate. Similar to the present study, they observed an enhancement in auditory-evoked mean firing rates within the supragranular layer, and no significant change in mean firing rates within the granular or upper infragranular layer. In contrast to our study however, they observed a slight decrease to the mean firing rates within the lower infragranular layer. Despite this slight divergence from the current results, Stolzberg's study suggests a layer specific enhancement in auditory-evoked activity, even following systemic approaches to tinnitus induction, which would presumably affect both intracortical and thalamocortical connections. Together with the current study, this finding provides support for a role of A1 intracortical circuits, specifically those in the supragranular layer, for the generation of the tinnitus percept.

Interestingly however, whereas we showed significant increases in spontaneous firing rates for the lower infragranular layer with local Gabazine administration, Stolzberg et al. (2012) found a significant decrease with systemic salicylate. These divergent observations are not uncommon at the level of the auditory cortex, as conflicting studies have found both increases and decreases in SFRs (Eggermont & Kenmochi, 1998; Kimura & Eggermont, 1999; Komiya & Eggermont, 2000; Lu et al., 2011; Noreña & Eggermont, 2003; Noreña et al., 2010; Seki & Eggermont, 2003; Yang et al., 2007; Zhang et al., 2011). This opposition could be derived from the method of tinnitus induction used in the mentioned studies, as a majority of studies that used salicylate observed decreases in SFRs, and a majority of those using noise exposure found increases in SFRs. Thus, it is possible that a loss of inhibition in the auditory cortex induced by local Gabazine administration causes layer specific increases in spontaneous activity that are more reflective of noise-induced, rather than salicylate-induced, tinnitus.

One criticism for our claim that intracortical connections may be responsible for generating the tinnitus percept, comes from the fact that significant increases in spike count and response duration were observed in all cortical layers. While changes in the supragranular and lower infragranular layer were expected, enhancements to the granular

and upper infragranular layer were not. In recording MU activity, responses are generated from the spiking output of small clusters of neurons located near the electrode, however there is no way to determine what type of neuron is being recorded without using additional electrophysiological approaches (Stark & Abeles, 2007). While thalamic inputs onto pyramidal neurons located in these layers should not be affected by a cortical infusion of Gabazine, it is difficult to verify that the observed neural enhancement is due solely to the altered activity of interneurons. Importantly however, increases in spike count and response duration did not translate to enhancements in normalized mean firing rates, thus this layer specificity still suggests a potential role of intracortical connections in tinnitus.

3.5 Conclusion

To our knowledge, this study is the first to directly demonstrate that a loss of inhibition in the primary auditory cortex (A1) leads to tinnitus-positive behaviour through local gain enhancement. We found that infusion of Gabazine into A1 led to layer-specific increases in spontaneous and auditory-evoked activity, the two main hallmarks of increased central gain. Furthermore, this same infusion caused rats to mistakenly identify quiet conditions as narrow-band noise stimuli, presumably because they perceived a steady phantom sound. Collectively, these results provide strong support for the notion that central gain enhancement, induced by a loss of inhibition specifically in A1, are sufficient to generate the tinnitus percept. Importantly, the current study provides direct support for the central gain model as a plausible mechanism that underlies the neural basis of tinnitus.

3.6 References

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Chapter 4

4   General Discussion and Summary

4.1   General Discussion

This thesis provides substantial contributions to research investigating the neural basis of tinnitus. The first half of this thesis focused on the validation of a novel two-alternative forced-choice operant conditioning behavioural paradigm in its ability to screen rats for noise-induced tinnitus. Our behavioural paradigm was then subsequently used in the second half of this thesis where we investigated one of the leading hypotheses of tinnitus generation—the central gain model. We demonstrated that a direct loss of inhibition in the primary auditory cortex (A1) is sufficient to generate both the neural indications of gain enhancement, and tinnitus-positive behaviour. Collectively, the findings in this thesis (1) help to further establish a behavioural paradigm that can be reliably used to screen rats for tinnitus, and (2) provide extensive insight into a putative mechanism that underlies the generation of tinnitus. These contributions will likely prove useful in future animal studies aiming to develop viable tinnitus treatments based on targeting the direct source of these phantom auditory perceptions.

The collective results from this thesis, in agreement with several previously conducted studies, help to extend our current understanding of tinnitus and the approaches that should be used to investigate its underlying neural mechanisms. Since the advent of a potential central origin of tinnitus, it has become apparent that it is necessary to use animal models to allow for more invasive investigation into the source of phantom auditory perceptions; procedures which cannot be conducted in humans. Numerous animal studies have used microelectrodes to record neural activity in various central auditory structures following induction of tinnitus with salicylate- or noise exposure (Auerbach et al., 2014). While these findings have provided significant contributions to the development of proposed hypotheses of tinnitus generation, this approach has several considerable drawbacks. Firstly, salicylate and noise exposure cause widespread changes to the entire central auditory system (CAS), making it difficult to determine which auditory structure(s) generate tinnitus. Secondly, currently available behavioural

paradigms used to screen animals for tinnitus have several shortcomings. For example, traditional shock avoidance models often encounter an issue of behavioural extinction (Bauer & Brozoski, 2001; Bauer et al., 1999; Guitton et al., 2003; Heffner & Harrington, 2002; Jastreboff et al., 1988; Lobarinas et al., 2004; Rüttiger et al., 2003), while the frequently used GPIAS paradigm is strongly confounded by the effects of hearing loss (Campolo et al., 2013; Fournier & Hébert, 2013; Lobarinas et al., 2013; Longnecker & Galazyuk, 2011; Turner et al., 2006). As such, interpretations on the presence/absence of tinnitus using these paradigms must be approached with caution. Finally, even if behavioural indications of tinnitus are reliable, results from electrophysiological recordings only provide correlations between neural activation and the presence of tinnitus without insight into which structure is ultimately responsible for generating phantom auditory perceptions. The studies presented in this thesis address the aforementioned issues of animal models that have been used in the past to study tinnitus, and offer novel approaches to better investigate the underlying neural mechanisms of tinnitus.

We successfully validated our transient tinnitus paradigm as an effective tool to screen rats for noise-induced tinnitus. Previous work from our lab also confirmed that salicylate-induced tinnitus could be reliably assessed using our behavioural model (Stolzberg et al., 2013). In both situations, robust control conditions were established to confirm that our paradigm is resistant to false-positive indications of transient tinnitus. Moreover, the current results demonstrate that this behavioural task meets several criteria that are necessary to produce an effective animal model of tinnitus: (1) allow for individual comparisons amongst rats to control for the variabilities in tinnitus development following noise exposure, (2) be resistant to the confounding influences of hearing loss, and (3) successfully assess short duration tinnitus that closely mirrors the human condition (Hayes et al., 2014). The many advantages of this reliable behavioural paradigm make it a prime candidate for future investigations into the neural basis of tinnitus. As such, because we were able to successfully detect the onset and offset of transient tinnitus without any indications of false-positives, we proceeded to use this behavioural paradigm to study the central gain model of tinnitus.

This thesis provides a comprehensive investigation into the underlying neural mechanisms of tinnitus using a combination of electrophysiological recordings and a reliable behavioural model of tinnitus. While indications of tinnitus-related gain enhancement have been observed previously in several auditory structures (Bauer et al., 2008; Brozoski et al., 2002; Dong et al., 2010; Eggermont & Kenmochi, 1998; Jastreboff & Sasaki, 1986; Kaltenbach & McCaslin, 1996; Kaltenbach & Afman, 2000; Kimura & Eggermont, 1999; Komiya & Eggermont, 2000; Lu et al., 2011; Manabe et al., 1997; Melamed et al., 2000; Mulders & Robertson, 2011; Mulheran & Evans, 1999; Noreña, 2011; Noreña & Eggermont, 2005; Qiu & Salvi, 2000; Seki & Eggermont, 2003; Zhang & Kaltenbach, 1998; Zhang et al., 2011), our results suggest that gain enhancement within A1 is sufficient to induce tinnitus-positive behaviour. Our cortex-specific manipulation did not directly affect inhibition levels in subcortical structures, and as such a loss of inhibition in A1 alone was sufficient to induce both neural activity and behaviour indicative of central gain increases and tinnitus. In agreement with previous studies, our results support a role of inhibitory neurotransmission in the induction of central gain enhancement (Lu et al., 2011; Su et al., 2009; Sun et al., 2009; Wang et al., 2006). Our finding that impairment of local GABAergic neurotransmission directly caused tinnitus-positive behaviour and gain enhancement is in line with several studies that observed a recovery of neural and behavioural indications of tinnitus following potentiation of GABA neurotransmission (Brozoski et al., 2007; Lu et al., 2011; Sun et al., 2009). Interestingly, our results suggest a role of intracortical connections within A1 in the generation of tinnitus, as increases in spontaneous and auditory-evoked activity appeared to be specific to the supragranular and lower infragranular layer. Similar layer-specificity has been previously observed in another study using salicylate (Stolzberg et al., 2012). Together, the results in Chapter 3 provide strong support for the central gain model of tinnitus. It is worth noting that this study is the first to directly show that a putative mechanism of tinnitus can *cause* neural and behavioural indications of tinnitus. As such, we recommend that this approach be used in future investigations to elucidate other suggested models of tinnitus, rather than previously employed methods of inducing tinnitus and subsequently recording the neural changes that are simply correlated with the presence of these phantom auditory perceptions.

4.2 Limitations

As previously discussed in Chapter 2, while we successfully validated our transient tinnitus paradigm as an effective model of noise-induced tinnitus, results from our 60-minute noise exposure revealed that 50% of rats suffered from an extensive hearing loss that may have confounded behavioural performance during quiet trials. As such, we suggest that adjustments to the noise exposure parameters, such as using unilateral rather than bilateral exposures, could ameliorate the influence of hearing loss in this paradigm to make it a reliable model of persistent tinnitus in future studies. Both unilateral and bilateral tinnitus are clinically relevant in the patient population. An epidemiological study found that of the >500 tinnitus patients surveyed, 22% had bilateral tinnitus, 56% had lateralized tinnitus, and 34% had unilateral tinnitus (Lockwood et al., 2003), thus it would be necessary to eventually be able to screen for tinnitus of both forms. That being said, infusions conducted in Chapter 3 were unilateral in nature, targeting only the left auditory cortex. It is possible that electrophysiological and behavioural results would diverge from the current findings if bilateral infusions were performed instead. Indeed, this could explain why only 50% of rats showed tinnitus-positive behaviour above a 20% criterion threshold following a unilateral infusion of Gabazine. Tinnitus generated from the left auditory cortex alone may not be as severe as tinnitus generated bilaterally, thus these rats did not mistaken a sufficient amount of quiet trials as NBN to be included in the "tinnitus-positive" group. It is also possible that our criterion threshold of 20% mistakenly identified quiet trials is too conservative, thus resulting in false-negative indications of tinnitus. As such, rats that truly developed tinnitus following unilateral loss of inhibition may have been falsely categorized in the "tinnitus-negative" group. Indeed, while several rats did not surpass the tinnitus threshold, their proportion of misidentified quiet trials was increased following local Gabazine infusion relative to their baseline performance the day before. Future studies may need to revise how the presence of tinnitus is characterized, perhaps by making comparisons to baseline performance, rather than using a threshold value. However, we stand by our 20% criterion threshold as a means to consistently prevent false-positive indications of tinnitus.

Furthermore, the electrophysiological approach used in Chapter 3 to analyze multi-unit activity in A1 is limited by an inability to determine the type of neural activity that is being recorded. While changes in spontaneous and auditory-evoked firing rates can be detected, it is not possible to verify what type of neurons produced these extracellular signals. Indeed, an electrophysiological technique with greater resolution, such as intracellular patch-clamp recordings, would allow for the distinction between types of neurons (e.g., interneuron or pyramidal neuron). Although it would be helpful to know which neurons contributed to the overall indications of gain enhancement, the aim of this experiment was to confirm that a loss of inhibition can cause local central gain increases, regardless of which neurons were responsible.

4.3   Future Directions

While the results of this thesis provide considerable insight into the underlying neural mechanisms of tinnitus, they also provide several viable avenues for future research to further strengthen the claims made by our two studies. For example, in accordance with our earlier discussion, our suggested paradigm for persistent tinnitus (Chapter 2) could be improved by controlling for the confounding influences of hearing loss. We demonstrated that rats were able to correctly identify quiet, AM, and NBN stimuli one week following 60-minute sham exposures, confirming that our paradigm is resistant to false-indications of tinnitus, even without an entire week of behavioural training. If efforts were made to either decrease the intensity level of the noise exposure, or perhaps reduce the exposure duration, the resulting hearing loss may be less severe. As such, rats would presumably still be able to perceive and accurately identify NBN stimuli, allowing for a reliable interpretation of behavioural performance during quiet trials. Alternatively, unilateral, instead of bilateral, noise exposures could be used to preserve hearing in the unaffected ear as has been shown in several studies (Dehmel et al., 2012; Kraus et al., 2011; Lobarinas et al., 2013; Longnecker & Galazyuk, 2011; Middleton et al., 2011; Turner et al., 2006; Wang et al., 2009; Zhang et al., 2011). Furthermore, it would be of interest to extend the time period between the 60-minute sham exposure and behavioural testing. While we demonstrate that rats can recall the task despite a week without training, persistent tinnitus in humans is extensive, often lasting for several weeks or months.

Future studies could attempt to investigate if rats are capable of recalling our complex behavioural task after more than one week post-sham and noise exposure, to help make the paradigm more clinically relevant.

Although the collective work in this thesis demonstrates that a loss of inhibition in A1 causes electrophysiological indications of gain enhancement, and subsequently evidence of tinnitus-positive behaviour, these two results were observed separately. As such, it would be beneficial for future studies to use awake behaving neural recordings to confirm that a local loss of inhibition in A1 can cause neural indications of increased central gain in awake rats while they actively report perceiving tinnitus. Additionally, bilateral infusions of Gabazine could be introduced to investigate the effects of impaired inhibitory neurotransmission in both auditory cortices on gain enhancement and tinnitus behaviour.

Lastly, while the results of this thesis suggest that gain enhancement in A1 is capable of generating tinnitus, further confirmatory studies could help support this claim. For example, future studies could noise expose trained rats using the 60-minute exposure paradigm established in Chapter 2, and then locally infuse a GABA agonist directly into A1 prior to behavioural testing one week after the noise exposure. If the action of the GABA agonist is sufficient to suppress behavioural indications of tinnitus using our established paradigm (i.e., rats can still correctly identify quiet stimuli), then these results would provide additional support for the role of A1 in tinnitus generation. However, studies would be needed to explore the possible contributions of other auditory structures to the central gain model of tinnitus. Indeed, future investigations should attempt to directly induce gain increases in subcortical components of the auditory pathway, such as the inferior colliculus and dorsal cochlear nucleus, to determine if this results in behavioural indications of tinnitus as well. If enhanced neural activity in these structures results in tinnitus-positive behaviour without a matching observation of gain enhancement in A1, then it would be concluded that an increase in central gain at the level of A1 is sufficient, but not necessary, to generate tinnitus.

4.4 Summary

Despite decades of research, the underlying neural mechanisms of tinnitus have remained elusive, preventing the development of effective treatments and pharmacotherapies to abolish the phantom sound perceptions at their source. The findings in this thesis offer several novel contributions and insights into the neural basis of tinnitus. Specifically, it provides validation of our two-alternative forced-choice behavioural paradigm in its ability to effectively screen rats for transient noise-induced tinnitus, with the potential to assess for persistent tinnitus as well (Chapter 2). Additionally, the results of this thesis suggest a strong role of cortical inhibition in the induction of gain enhancement and tinnitus-positive behaviour in rats; findings which offer support to the central gain model of tinnitus (Chapter 3). Overall, these significant contributions may help influence future studies by providing more effective strategies to directly investigate putative mechanisms of tinnitus.

4.5 References

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