Targeting inhibitory neurotransmission in tinnitus

Ben D. Richardson[a], **Thomas J. Brozoski**[b], **Lynne L. Ling**[a], and **Donald M. Caspary**[a]

Ben D. Richardson: brichardson@siumed.edu; Thomas J. Brozoski: tbrozoski@siumed.edu; Lynne L. Ling: lling@siumed.edu; Donald M. Caspary: dcaspary@siumed.edu

[a]Department of Pharmacology, Southern Illinois University School of Medicine, 801 N Rutledge St, Rm. 3234, PO Box 19629 Springfield, IL 62794 USA

[b]Division of Otolaryngology, Head and Neck Surgery, Southern Illinois University School of Medicine, 801 N Rutledge St, Rm. 3205, PO Box 19629 Springfield, IL 62794 USA

Abstract

Tinnitus perception depends on the presence of its neural correlates within the auditory neuraxis and associated structures. Targeting specific circuits and receptors within the central nervous system in an effort to relieve the perception of tinnitus and its impact on one's emotional and mental state has become a focus of tinnitus research. One approach is to upregulate endogenous inhibitory neurotransmitter levels (e.g. glycine and GABA) and selectively target inhibitory receptors in key circuits to normalize tinnitus pathophysiology. Thus, the basic functional and molecular properties of two major ligand-gated inhibitory receptor systems, the $GABA_A$ receptor ($GABA_AR$) and glycine receptor (GlyR) are described. Also reviewed is the rationale for targeting inhibition which stems from reported tinnitus-related homeostatic plasticity of inhibitory neurotransmitter systems and associated enhanced neuronal excitability throughout most central auditory structures. However, the putative role of the medial geniculate body (MGB) in tinnitus has not been previously addressed, specifically in terms of its inhibitory afferents from inferior colliculus and thalamic reticular nucleus and its $GABA_AR$ functional heterogeneity. This heterogeneous population of $GABA_ARs$, which may be altered in tinnitus pathology, and its key anatomical position in the auditory CNS make the MGB a compelling structure for tinnitus research. Finally, some selective compounds, which enhance tonic inhibition, have successfully ameliorated tinnitus in animal studies, suggesting that the MGB and, to a lesser degree, the auditory cortex may be their primary locus of action. These pharmacological interventions are examined, in terms of their mechanism of action and why these agents, may be effective in tinnitus treatment.

Keywords

tinnitus; $GABA_AR$; GlyR; inhibition; medial geniculate body; homeostatic plasticity

Correspondence: BDR will handle pre-publication correspondence. DMC will handle post-publication correspondence and should be listed as the corresponding author.
BDR: Tel. (1)217-545-7009, Fax (1)217-545-0145
TJB: Tel. (1)217-545-6583, Fax (1)217-545-0145
LLL: Tel. (1)217-545-7009, Fax (1)217-545-0145
DMC: Tel. (1)217-545-2195, Fax (1)217-545-0145

1. Introduction

A number of studies over the past few years have focused on inhibitory neurotransmitter receptors, their related metabolic enzymes and trafficking/scaffolding proteins in effort to better understand the neurobiological underpinnings of tinnitus. As a result, tinnitus-related changes in inhibitory neurotransmission have been identified (Roberts et al., 2010; Wang et al., 2011). In an effort to counteract such maladaptive homeostatic plasticity of or compensatory changes in inhibitory mechanisms, recent animal studies have targeted GABAergic or glycinergic inhibitory function and have been moderately successful in reducing behavioral evidence of tinnitus (Brozoski et al., 2007; Brozoski et al., 2010; Yang et al., 2011). This review examines findings in these areas, focusing first on recently identified tinnitus-related changes in inhibitory mechanisms and ways in which these changes may impact neuronal activity in the DCN, IC and auditory cortex (see also Wang et al., 2011). Also considered is the putative role of the auditory thalamus (MGB) in tinnitus pathology and how targeting a specific subtype of $GABA_AR$ inhibition (tonic) may be advantageous in a therapeutic approach for tinnitus treatment. Finally, some successes of tinnitus pharmacotherapy in animal models and the compounds' mechanism of action are reviewed.

The idea that aberrations in inhibitory neurotransmission could underlie tinnitus pathology stems from the hypothesis supported by recent data that a partial peripheral deafferentation induced by an acoustic insult could trigger a series of maladaptive homeostatic plastic changes. Liberman and Kujawa (2009) demonstrated how a modest acoustic trauma results in permanent partial cochlear nerve deafferentation (i.e. SGC loss) despite normal or near normal acoustic thresholds. In fact, Bauer and colleagues (2007) showed SGC loss was more predictive of tinnitus than hearing loss in rats. Similarly, in a recent human study using electrophysiologic measures, Schaette and McAlpine (2011) found that individuals experiencing tinnitus displayed partial deafferentation of the cochlear nerve, even when hearing loss was undetected by standard audiologic measures. Since the loss of peripheral input appears to remain uncompensated, without a replacement of lost afferent neurons, pre and postsynaptic central plastic changes in neural inhibition (e.g. down-regulation of GABA/glycine release and altered postsynaptic receptor constructs) may represent an example of homeostatic plasticity, compensating for this loss of input. Such an altered balance between driving excitation and central inhibition may underlie and result in the percept of tinnitus.

2. Inhibitory Neurotransmitters, Receptors and Related Proteins

An accurate representation of the acoustic scene depends not only on the driving excitatory input, but on inhibitory mechanisms that serve to shape spontaneous activity, input/output functions, frequency tuning, and temporal response accuracy (Caspary et al., 2008). Two prominent ways neuronal responses may be inhibited are through activation of GlyRs and $GABA_ARs$ following release of the amino acid neurotransmitters glycine and GABA, respectively. GlyRs and $GABA_ARs$ are ligand-gated channels, made up of five separate proteins or subunits that, in adult animals, allow for Cl^- influx, resulting in hyperpolarization or clamping of a neuron's resting membrane potential below threshold. Nineteen $GABA_AR$ (α_{1-6}, β_{1-3}, γ_{1-3}, δ, π, ϵ, θ, and ρ_{1-3}) and five GlyR (α_{1-4} and β) subunits which assemble into pentamers with a limited number of stoichiometric combinations provide a range of functional receptors with variable levels of expression across cell types and locations in the CNS (Farrant and Nusser, 2005; Lynch, 2009; Walker and Semyanov, 2008).

It has become apparent that the presence of specific subunits in the composition of inhibitory receptors can confer dramatically different properties on these receptors, altering pharmacology, location of insertion in the membrane (synaptic vs. extrasynaptic) and kinetic/temporal properties (Belelli et al., 2009; Farrant and Nusser, 2005; Richardson et al., 2011; Walker and Semyanov, 2008; Figure 1). Moreover, data suggest that a relatively limited number of functional subunit combinations exist and are exclusively expressed in defined brain regions (Pirker et al., 2000; Sieghart and Sperk, 2002; Wisden et al., 1992).

One basic distinction in ligand-gated ionotropic inhibitory receptor types is between the tonic/prolonged form of inhibition (Figure 1A) mediated by a high affinity **extrasynaptic** receptor population and the classic phasic (30–50msec) inhibition (Figure 1B) mediated by a different low affinity **synaptic** GlyRs or GABA$_A$Rs population (Belelli et al., 2009; Farrant and Nusser, 2005; Xu and Gong, 2010; Zhang et al., 2008). The expression of receptors with differential time courses of inhibition in specific central auditory regions and cell types may be useful in establishing a specific/targeted tinnitus pharmacotherapy. Targeting receptors mediating tonic non-desensitizing inhibition with selective agonists or positive allosteric modulators would circumvent desensitization of phasic inhibitory receptors and could enhance responses to endogenous neurotransmitter release (see Section 4).

Neurons may regulate the diversity of inhibitory receptor constructs at the level of gene transcription or protein translation, but functionally, only receptors expressed on the cell membrane are relevant. The membrane insertion of functional GlyRs and GABA$_A$Rs is governed by specific trafficking and anchoring molecules which play an important role in regulating inhibitory synaptic strength/receptor density (for review see Kittler et al., 2002; Kneussel and Loebrich, 2007; Luscher and Keller, 2004; Luscher et al., 2011). Most notably, gephyrin serves two functions; trafficking receptors to and from membrane active zones and anchoring receptors to other scaffolding molecules like GABARAP and Radixin (Fritschy et al., 2008; Jacob et al., 2008; Vithlani et al., 2011). This group of molecules and their interaction with cytoskeletal components have the ability to dynamically regulate plasticity of GABA$_A$Rs and GlyRs by modulating receptor insertion into the cell membrane and controlling their lateral mobility in addition to their transcriptional/translational regulation (Calamai et al., 2009; Charrier et al., 2006; Danglot et al., 2003; Dumoulin et al., 2009; Jacob et al., 2005). However, regulatory mechanisms of inhibitory receptor plasticity are likely different for synaptic and tonically active extrasynaptic GABA$_A$R types as the two do not appear to interact with the same set of associated trafficking and scaffolding proteins (Peden et al., 2008; Vithlani et al., 2011). Understanding how protein expression and signaling pathways regulate participation of these molecules in normal synaptic function may be key to understanding the plastic inhibitory changes that occur in tinnitus (Wang et al., 2011 for review).

3. Tinnitus-Related Changes in Neuronal Activity and Inhibitory Mechanisms

One consistently noted tinnitus-related change in cellular function throughout the central auditory system is an increase in spontaneous/bursting activity and acoustically driven firing rates subsequent to peripheral insult, which are briefly reviewed here (see Kaltenbach and Godfrey, 2008; Roberts et al., 2010; Wang et al., 2011 for detailed review). In dorsal and/or ventral cochlear nuclei of guinea pigs, hamsters or chinchillas exposed to sounds capable of inducing behavioral evidence of tinnitus, spontaneous and driven activity were elevated (Brozoski et al., 2002; Kaltenbach et al., 2000; Kaltenbach et al., 2004; Vogler et al., 2011). For fusiform cells, the largest increases in tinnitus-related acoustic driven activity appear at higher intensities. The dependence of non-monotonic rate-intensity functions on glyicnergic inhibition points to altered glycinergic function and the GlyR as a candidate in the inhibitory

neuropathology that may occur with tinnitus (Brozoski et al., 2002; Caspary et al., 1987). Indeed, immunohistochemistry and strychnine binding indicate that the subunit make-up of GlyRs is altered and density of GlyRs is reduced whereas the trafficking protein, gephyrin, is elevated in the DCN of sound exposed rats (Wang et al., 2009). Other inhibitory mechanisms may also be altered and reflect tinnitus pathology at the level of the DCN. For example, enhanced neural synchrony is seen within the DCN of mice with behavioral evidence of tinnitus, which appears due to a loss of GABAergic inhibition (Middleton et al., 2011). This would also contribute to elevated single unit activity. Pilati and colleagues (2011) identified an increased incidence of burst responses in rat DCN fusiform cells following intense sound exposure, implicating a down-regulation of HVA K^+ channels. This is yet another example of homeostatic plasticity following noise exposure may comprise part of what underlies neuronal hyperexcitability in tinnitus, in addition to described losses/changes in ligand-gated inhibition. Significantly, the DCN appears necessary for establishment of tinnitus since ablation of the DCN prior to sound exposure prevents the establishment of behavioral evidence of tinnitus in a rat model (Brozoski et al., 2011).

In the mammalian IC, increases in spontaneous and evoked firing rates are found up to three months following sound exposure (Longenecker and Galazyuk, 2011; Ma et al., 2006; Mulders et al., 2011; Mulders and Robertson, 2011). Increases in specific patterns of neural firing (e.g. bursting) may be a necessary hallmark of the nervous system's physiological tinnitus signature, not simply increases in rate, and could be enhanced by a loss of inhibitory tone. In support of this hypothesis, Bauer and colleagues showed an increased intraburst rate in addition to increased spontaneous firing rates 8–9 months after noise exposure in the IC of chinchillas with behavioral evidence of tinnitus (Bauer et al., 2008). As in the DCN, changes in inhibitory neurotransmitter related mechanisms are also present in the IC following sound exposure. Levels of the presynaptic inhibitory synthetic protein, GAD, remain decreased up to a month following noise exposure suggesting that the level of GABA available for vesicular storage and release is likely decreased (Milbrandt et al., 2000). The work of Dong, Robertson and their colleagues (2010), used qRT-PCR to correlate plasticity of genes related to inhibitory neurotransmission with neuronal activity in the guinea pig IC. Following sound exposure, observed elevations in spontaneous firing rate were accompanied by a down-regulation of mRNA levels for the α_1 subunits of the GlyR and $GABA_A R$. These findings, in part, exemplify how homeostatic plasticity alters IC inhibitory receptors in tinnitus. However, as discussed previously, posttranslational modification of mature receptor constructs and receptor insertion and mobility in the plasma membrane may also contribute a significant component to plastic responses to an acoustic insult and will require further study at multiple levels of the auditory pathway.

Changes in neural activity and inhibitory mechanisms have also been characterized for auditory cortex. In addition to increased firing rates, re-organization of tonotopically aligned receptive fields of auditory cortical neurons seem characteristic of tinnitus (for reviews see Eggermont and Roberts, 2004; Roberts et al., 2010). Recently, Yang and colleagues (2011) addressed the role of GABAergic inhibition in cortical reorganization following sound exposure. Animals with behavioral evidence of tinnitus showed a loss of high frequency responding neurons (cortical remapping), elevations in firing rate, reductions in tonic and phasic $GABA_A R$ current amplitudes and frequency along with decreases in presynaptic GAD levels in high frequency regions (Yang et al., 2011). This direct, frequency specific loss of inhibition and increase in excitability, persisting for at least two weeks after exposure, convincingly demonstrating how tinnitus-related plasticity impacts not only molecular markers of inhibition, but inhibitory *function* as well. Similar approaches will be beneficial in further characterizing the nature of tinnitus related inhibitory plasticity that occurs throughout the auditory neuraxis.

In summary, evidence for decreased inhibitory neurotransmitter release may be accompanied by decreases in postsynaptic receptor density or the replacement of wild-type receptors by other receptor subtypes with different subunit combinations and functional/pharmacologic characteristics. One goal for pharmacotherapeutic treatment of tinnitus would be to enhance a specific system's remaining endogenous inhibitory mechanisms that may remain intact, but deficient.

4. The Auditory Thalamus and Tinnitus

While tinnitus and sound-exposure related brainstem, midbrain and cortical changes have received significant attention, few studies have examined the impact of tinnitus and sound exposure on auditory thalamic neurons (MGB). Traditionally thought of as only a conduit for neural signals representing the auditory scene, arising from midbrain ascending to the cortex, it is now apparent that additional processing occurs in the MGB (Antunes et al., 2010; Bartlett and Wang, 2007; Bartlett and Wang, 2011). The MGB is an obligatory nucleus of the auditory system, thus regardless of the site of genesis for the tinnitus signal; it is likely that the MGB is involved in tinnitus pathology. Leaver, Rauschecker and colleagues suggested that due to the prime position of the MGB in the ascending and descending auditory neuraxis and unique inhibitory mechanisms (see below), the MGB is a promising target for tinnitus research (Leaver et al., 2011; Rauschecker et al., 2010). The fact that many tinnitus sufferers have a severe emotional component to their tinnitus further points to the involvement of MGB in tinnitus pathology (Malouff et al., 2011). The MGB projection to the amygdala demonstrates LTP and is necessary for auditory fear conditioning (McKernan and Shinnick-Gallagher, 1997; Quirk et al., 1995; Rogan et al., 1997; Weinberger, 2011). This suggests that the MGB might be an important link in understanding the emotional aspect of tinnitus.

Inhibitory MGB inputs have been well characterized. In rodents, primary sources of inhibition are from GABAergic projections from the TRN and IC, with the addition of a substantial yet poorly characterized GABAergic interneuron population in the MGB of higher order species (Rouiller and de Ribaupierre, 1985; Shosaku and Sumitomo, 1983; Villa, 1990; Winer and Larue, 1996; Winer et al., 1996). Thus, GABAergic shaping of MGB neuron output is primarily through ascending and descending inhibition from the IC and TRN, respectively. Certain tinnitus sufferers are particularly affected by their tinnitus and others may pay little attention to the phantom sound. Evidence suggests that mechanisms which regulate attention may be impaired in tinnitus sufferers more bothered by their tinnitus (Cuny et al., 2004; Dornhoffer et al., 2006; Husain et al., 2011). One well characterized subcortical mechanism involved in the regulation of attention is the tonotopically aligned inhibitory (GABAergic) projection from the TRN to the MGB (Cotillon-Williams et al., 2008; Crick, 1984; Guillery et al., 1998; McAlonan et al., 2000; Weese et al., 1999; Yu et al., 2009). This interaction may also be important in understanding gating of the tinnitus signal that occurs at the level of the MGB (Rauschecker et al., 2010).

Opposite defined GABAergic inputs to the MGB are postsynaptic $GABA_ARs$ that are notably heterogeneous in MGB and other sensory thalamic regions. In addition to wild-type/classical synaptic $GABA_ARs$ (containing α_1 and γ_2 subunits) that are widely distributed throughout the brain, MGB neurons also express extrasynaptic receptors, defined by $GABA_AR$ constructs containing α_4 and δ subunits which mediate tonic inhibition (Figure 1 & 2A; Section 2; Richardson et al., 2011). Thalamic extrasynaptic $GABA_ARs$ are activated by low level increases in ambient GABA levels and mediate a tonic, long-lasting inhibitory current which has been suggested to account for up to 90% of a thalamocortical neuron's inhibitory conductance (Belelli et al., 2009; Cope et al., 2005; Farrant and Nusser, 2005; Walker and Semyanov, 2008). Gaboxadol, a "superagonist" at $GABA_ARs$, is capable of

binding and activating extrasynaptic $GABA_A$Rs selectively, when present at low (nM-μM) concentrations (Mortensen et al., 2010). Indeed, selective activation of extrasynaptic $GABA_A$Rs by low concentrations of gaboxadol or taurine is capable of reducing spontaneous and evoked firing rates of sensory thalamic neurons (Figure 3; Herd et al., 2009; Jia et al., 2008b). Such reductions in excitability of MGB neurons following activation of extrasynaptic $GABA_A$Rs, as exemplified in Figure 3, could prove advantageous in reducing the transmission of hyperexcitability to the auditory cortex.

One way to determine the presence of extrasynaptic $GABA_A$Rs and quantify conditional differences in receptor density is through quantitative receptor binding using [^{3}H]gaboxadol, as described previously (Friemel et al., 2007; Richardson et al., 2011). In a small cohort of animals, a nonlinear regression analysis and comparison of [^{3}H]gaboxadol binding between the MGB of FBN control rats and the contralateral MGB of rats with tinnitus was significantly different [F=4.709 (2,81), p=0.011], but not between control and the ipsilateral MGB of animals with behavioral evidence of tinnitus [F=2.526 (2,81), p=0.086] (Figure 2C&D). These preliminary findings suggest there may be a slight decrease (~20–30%) in extrasynaptic $GABA_A$R density as indicated by the tinnitus-related change in *Bmax* for [^{3}H]gaboxadol in the MGB of exposed animals (*Bmax*=174.4±36.5 (control), 117.1±25.6(contra), 137.2±30.2(ipsi). Despite significant differences, these changes are limited in magnitude, suggesting that while $GABA_A$Rs might be altered in tinnitus, extrasynaptic $GABA_A$Rs are still present and capable of mediating tonic inhibition in animals with tinnitus (Figure 2B). [^{3}H]Gaboxadol binding density is higher in the MGB relative to AI (Figure 2C). This increase corresponds to elevated $GABA_A$R extrasynaptic subunit protein concentrations and reflect the larger tonic current amplitudes (in response to 1μM gaboxadol using similar whole cell recording configurations) observed in sensory thalamocortical neurons (100–320pA) relative to sensory cortical neurons (50–60pA) (Chandra et al., 2006; Cope et al., 2005; Drasbek and Jensen, 2006; Herd et al., 2009; Peng et al., 2002; Pirker et al., 2000; Yang et al., 2011).

Pharmacologic targeting specific extrasynaptic $GABA_A$R constructs could prove more useful than targeting $GABA_A$Rs nonspecifically (Yang et al., 2011). In tinnitus treatment, the efficacy of agents targeting extrasynaptic $GABA_A$Rs would likely involve the MGB as the magnitude of tonic current and histological characterization of receptor density strongly indicates that extrasynaptic $GABA_A$R expression may be two or more fold greater than in the auditory cortex. In addition to gaboxadol, other agents capable of inducing $GABA_A$R mediated tonic inhibition have been effective at attenuating behavioral evidence of tinnitus (see Section 5).

5. Tinnitus Pharmacotherapy Targeting Inhibition and Underlying Mechanisms

A number of therapeutic strategies have been applied to tinnitus treatment, but presently there is no universally effective treatment or established standard of care. Non-pharmacologic interventions that combine acoustic stimulation and counseling reduce the intrusiveness of tinnitus, without altering the physical properties of the sensation, such as loudness or pitch. Cochlear implantation for aural rehabilitation of severe hearing loss significantly improves and in some cases eliminates tinnitus in many people (Baguley and Atlas, 2007; Kompis et al., 2012; Van de Heyning et al., 2008). Transcranial magnetic stimulation (TMS) is reported to provide relief to tinnitus sufferers and is proposed to do so through suppression of cortical activity by enhancing intracortical inhibition and inhibitory input from GABAergic TRN neurons onto MGB neurons (Langguth et al., 2007). Case reports and clinical trials suggest that limited success has been obtained with a variety of drug classes, including benzodiazepine derivatives, such as alprazolam (Johnson et al.,

1993), clonazapam (Bahmad et al., 2006), and the GABA homolog, gabapentin (Bauer and Brozoski, 2006); anti-epileptics, such as carbamazepine (Levine, 2006); and antidepressants (for detailed review see Salvi et al., 2009; Shulman et al., 2002; Sullivan et al., 1989). Due to the tinnitus-related changes observed with drugs which enhance inhibition and the importance of inhibition in regulating neuronal function, inhibitory receptors remain a primary target in this line of research.

Multiple compounds are now available as selective agonists or modulators of inhibitory receptors. With more refined pharmacological profiles, these compounds take advantage of the pharmacologic diversity, specificity and natural distribution of inhibitory receptor subunit combinations. One interesting class of agents acts to positively or negatively regulate/modulate activity of specific GABA$_A$R subtypes (i.e. with specific subunit configurations) which may be beneficial in treating tinnitus. For example, similar to the way in which benzodiazepines selectively allosterically modulate certain synaptic GABA$_A$R subtypes, other compounds can selectively activate or modulate the activity of extrasynaptic GABA$_A$R constructs like gaboxadol (Mortensen et al., 2010), taurine (Jia et al., 2008b), isoflurane (Jia et al., 2008a) and endogenous neurosteroids (Belelli et al., 2006; Herd et al., 2007; Lambert et al., 2009).

Since extrasynaptic GABA$_A$Rs are highly sensitive to changes in ambient GABA concentration., Artificial elevation of these levels, induced by blockade of GABA uptake transporters can enhance tonic inhibitory current amplitude (Figure 1; Cope et al., 2005). Recent studies using compounds that affect inhibitory neurotransmission have proven effective in alleviating or attenuating tinnitus in animal models. As suggested by Yang (Yang et al., 2011), based on their mechanism of action determined *in vitro*, the tinnitus-related benefits of these compounds may be via activation or amplification of a tonic inhibitory conductance mediated by extrasynaptic GABA$_A$Rs.

A recent study by Brozoski and colleagues (2010) used the partially selective GABA$_A$R agonist, taurine, to alleviate tinnitus in a rat model of tinnitus (Bauer and Brozoski, 2001; Jia et al., 2008b). Taurine is an endogenous sulfur containing β-amino acid with agonist activity at GlyRs (in IC) and extrasynaptic GABA$_A$Rs in the thalamus (Jia et al., 2008b; Xu et al., 2004; Xu et al., 2006). In this design, Long-Evans rats exposed to 116dB-16kHz narrowband noise for 60 minutes resulted in psychophysical evidence of tinnitus at 10, 16, and 20kHz in an operant conditioning paradigm (Brozoski et al., 2010). Following daily oral dosing of 4mg/ml of taurine dissolved in the animals' drinking water, evidence of tinnitus was significantly attenuated as indicated by the shift in the task suppression ratio function. This effect was most dramatic at the 16kHz test frequency, but also evident at 10 and 20kHz. The therapeutic administration of taurine in the drinking water over the course of weeks likely leads to a continuous up-regulation of taurine concentrations in the brain. Although CSF taurine increases are likely marginal, prolonged increases in taurine may achieve its physiological effect in the central nervous systems by mechanisms that fit this temporal profile. Synaptic GlyRs and GABA$_A$R demonstrate faster desensitization rates and require higher concentrations of taurine for activation than thalamic extrasynaptic GABA$_A$Rs agonists [and possibly uncharacterized tonically active extrasynaptic GlyRs (Wang et al., 2005; Xu et al., 2004; Xu et al., 2006)]. These extrasynaptic constructs would be sensitive to low level ambient increases in GABA [or glycine] and other selective agonists. Therefore, extrasynaptic GABA$_A$Rs in the MGB are likely to be a primary source of inhibition activated in systemic dosing of taurine responsible for the therapeutic effect on the animals' perception of tinnitus (Jia et al., 2008b; Richardson et al., 2011). As described in section 4, the elevated density of extrasynaptic GABA$_A$Rs in the thalamus most likely mediate taurine's therapeutic effect on the tinnitus percept through its ability to increase tonic inhibition of MGB neurons and to a lesser degree, auditory cortical neurons. Taurine's

activation of extrasynaptic $GABA_A$Rs in MGB which are sensitive to small changes in ambient agonist concentrations in the CSF (and to some degree GlyRs in IC) may reduce neuronal excitability and transmission of the neuronal tinnitus signal to cortex, suggesting a role for these ligand-gated ionotropic receptors in the future of tinnitus pharmacotherapy.

In addition to using selective agonists, blockers of endogenous neurotransmitter uptake and catabolism, which can enhance tonic inhibition and amplify inhibitory synaptic responses, have also been effective in ameliorating the tinnitus percept. Tonic inhibition mediated by extrasynaptic $GABA_A$Rs can be induced by elevation of ambient GABA levels following the blockade of GABA uptake by GAT-1 selective blockers (NNC-711 or NO-711) and blockade of GABA catabolism by a GABA-T inhibitor (vigabatrin) (Figure 1; Chandra et al., 2006; Jia et al., 2008b; Larsson et al., 1986; Wu et al., 2003; Yang et al., 2011). In animal models, systemic administration of NO-711 and vigabatrin have been shown to reduce behavioral evidence of tinnitus, presumably due to their induction of elevated ambient GABA levels *in vivo* (Brozoski et al., 2007; Richards and Bowery, 1996; Yang et al., 2011). Activation of extrasynaptic $GABA_A$Rs by this ambient increase in GABA could tonically hyperpolarize MGB and auditory cortical neurons further below threshold, reducing their probability of responding to lower level excitatory synaptic input with an action potential. Unfortunately, vigabatrin treatment has been shown to cause a restriction of the visual field which has only been attenuated following the up-regulation of taurine (Jammoul et al., 2009; Krauss et al., 1998).

6. Conclusions

An acoustic insult to the auditory periphery (i.e. loud sound-exposure) induces partial deafferentation and may alter the balance between driving excitation and plastic essential inhibitory mechanisms through the down-regulation or altering of ligand gated inhibitory receptors, their associated proteins and neurotransmitter metabolic enzymes. As a result, the central auditory system can become hyperexcitable, leading to the perception of tinnitus. Certain types of hyperactivity or altered temporal firing patterns (i.e. bursting) specific to regions of the central auditory system can result from 1) what is passed up from lower structures, 2) increases in intrinsic neuronal excitability or 3) unopposed excitatory drive that would normally be attenuated by inhibitory mechanisms. In order to develop tinnitus pharmacotherapy, specifically targeting inhibitory mechanisms may prove to be more effective than less-specific inhibitory mimetics or modulators due to the intricacy and heterogeneity of inhibitory neurotransmission. Some compounds have been found to be efficacious in attenuating the tinnitus percept and are linked to the activation of tonic inhibition through extrasynaptic $GABA_A$Rs or GlyRs which may also enhance synaptic inhibition as well. Due to the high expression of tonically active receptors in the auditory thalamus and its key position in the auditory neuraxis, targeting these receptors and the role of the thalamus in attention and sensory gating may result in more successful treatment. A tightly titred combination therapy which includes different classes of selective GABAergic compounds like benzodiazepines, vigabatrin and taurine may be beneficial in treating tinnitus with reduced negative side effects.

7. Experimental Procedure

7.1 Confocal Fluorescent Imaging

Fischer Brown Norway (FBN) rats were anesthetized with an intramuscular injection mixture of ketamine (105mg/kg, Aveco, Fort Dodge, IA, USA) and xylazine (7mg/kg, Lloyd Laboratories, Shennandoah, IA, USA) followed by transcardial perfusion with 100ml of saline and 750ml of fixative containing 4% paraformaldehyde in phosphate buffer saline (PBS, pH7.4). Sections (30μm) were cut through MGB and collected in PBS. Free-floating

sections were processed in specially designed tissue capsules and incubated 30min in blocking solution containing PBS with 1.5% of normal serum and 3% bovine serum albumin. Sections were then transferred to primary antibody solution (goat anti-GABA$_A$R α_4 and rabbit anti-GABA$_A$R δ (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), incubated for 1hr at room temperature and then overnight at 4°C on the shaker. After washing with PBS, sections were incubated with donkey anti-goat IgG (Dylight 488, 1:100, Jackson ImmunoResearch, West Grove, PA, USA) and donkey anti-rabbit IgG (Rhodamine Red-X, 1:100; Jackson ImmunoReseach Laboratory, West Grove, PA, USA) for 1hr at room temperature. Following PBS washing, the sections were mounted onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA), cover-slipped with VectorShield (Vector Laboratories, Burlingame, CA, USA). Fluorescent imaging was performed using a Leica TCS SP5 II Confocal Laser Microscope (Leica Microsystems CMS GmbH, Mannheim, Germany).

7.2 Acoustic Exposure and Tinnitus Induction

Methods are similar to Richardson et al. (2011) and Wang et al. (2009). For radioligand binding experiments, FBN rats (3–4 months old) from two groups (unexposed control [n=5] and exposed to acoustic trauma with behavioral evidence of tinnitus [n=5]) were prepared identically to those in Wang et al., 2009. Briefly, to induce behavioral evidence of tinnitus, rats were anesthetized with a mixture of ketamine (50mg/kg) and xylazine (9mg/kg) and exposed to 116dB SPL octave-band noise, centered at 17kHz peak intensity for 1hr and behavioral evidence was determined using the gap/startle method of Turner and colleagues (2006) (see Wang et al., 2009 for behavioral results). For whole cell recording experiments (see Richardson et al., 2011 for detailed methods), rats (19–24 months) were similarly exposed to those described above except under isoflurane anesthesia and exposure was adjusted to 122dB SPL octave-band noise, centered at 16kHz peak intensity for 2hrs and resulted in the expression of behavioral evidence of tinnitus as determined by the gap/startle method of Turner and colleagues (2006).

7.3 Radioligand Binding

Following the classification of rats into groups based on behavioral performance following sound exposure, they were decapitated and the brains were rapidly removed, rinsed in ice-cold phosphate buffer at 4°C (pH 7.4), frozen in powdered dry ice and stored at −80°C. Serial transverse sections were cut at 16μm using a Leica CM1850 cryostat at −18°C. Selected sections were thaw-mounted onto Superfrost/Plus slides and stored at −20°C. Anatomical locations of the MGB were verified to match neural structures with those previously described (Paxinos and Watson, 1998).

[³H]Gaboxadol (courtesy Merck & Co. Inc., Rahway, NJ, USA) was used with modified protocols from Milbrandt and Caspary (1995) and Dr. Bjarke Ebert (personal communication). In brief, tissue sections were subjected to pre-wash twice for 5 minutes in buffers, followed by incubating with [³H]gaboxadol: 10–400nM and post-wash with buffers for 4 quick dips. Buffer solutions used were 50mM Tris-citrate (pH7.1). Non-specific binding was determined in adjacent sections by the addition of cold excessive GABA to the ligand buffer and was subtracted from total binding.

Dried slides were apposed to [³H]-hypersensitive phosphor screens for 3–5 days at room temperature. The phosphor screens were scanned using a Cyclone Storage Phosphor System (PerkinElmer, Waltham, MA, USA). The MGB was outlined and analyzed using OptiQuant Image Analysis software (Canberra-Packard, Schwadorf, Austria) which provided tools for gray-scale quantification in digital light units (DLU). DLUs were then converted to nCi/mg protein using a standard curve generated from co-exposed ³H-embedded plastic standards

(ARC, St. Louis, MO, USA)(Pan et al., 1983). Values from the left and right MGB of control animals were averaged for each animal and treated as one group (n=5) while values from the MGB contralateral and ipsilateral to the exposed ear were treated as two separate groups (ipsilateral: n=5, contralateral: n=5) in statistical analysis. Statistical comparison of [^{3}H]gaboxadol binding saturation curves between groups was completed using non-linear regression analysis with a one-site binding model.

Acknowledgments

Abbreviations

CSF	cerebrospinal fluid
DCN	dorsal cochlear nucleus
FBN	Fischer Brown Norway
GABA	γ-aminobutyric acid
GABARAP	GABA$_A$ receptor associated protein
GAD	glutamic acid decarboxylase
GlyR	glycine receptor
GABA$_A$R	GABA$_A$ receptor
GABA-T	GABA transaminase
GAT	GABA transporter
HVA	high voltage activated
IC	inferior colliculus
MGB	medial geniculate body
qRT-PCR	quantitative real time – polymerase chain reaction
SGC	spiral ganglion cell
TRN	thalamic reticular nucleus

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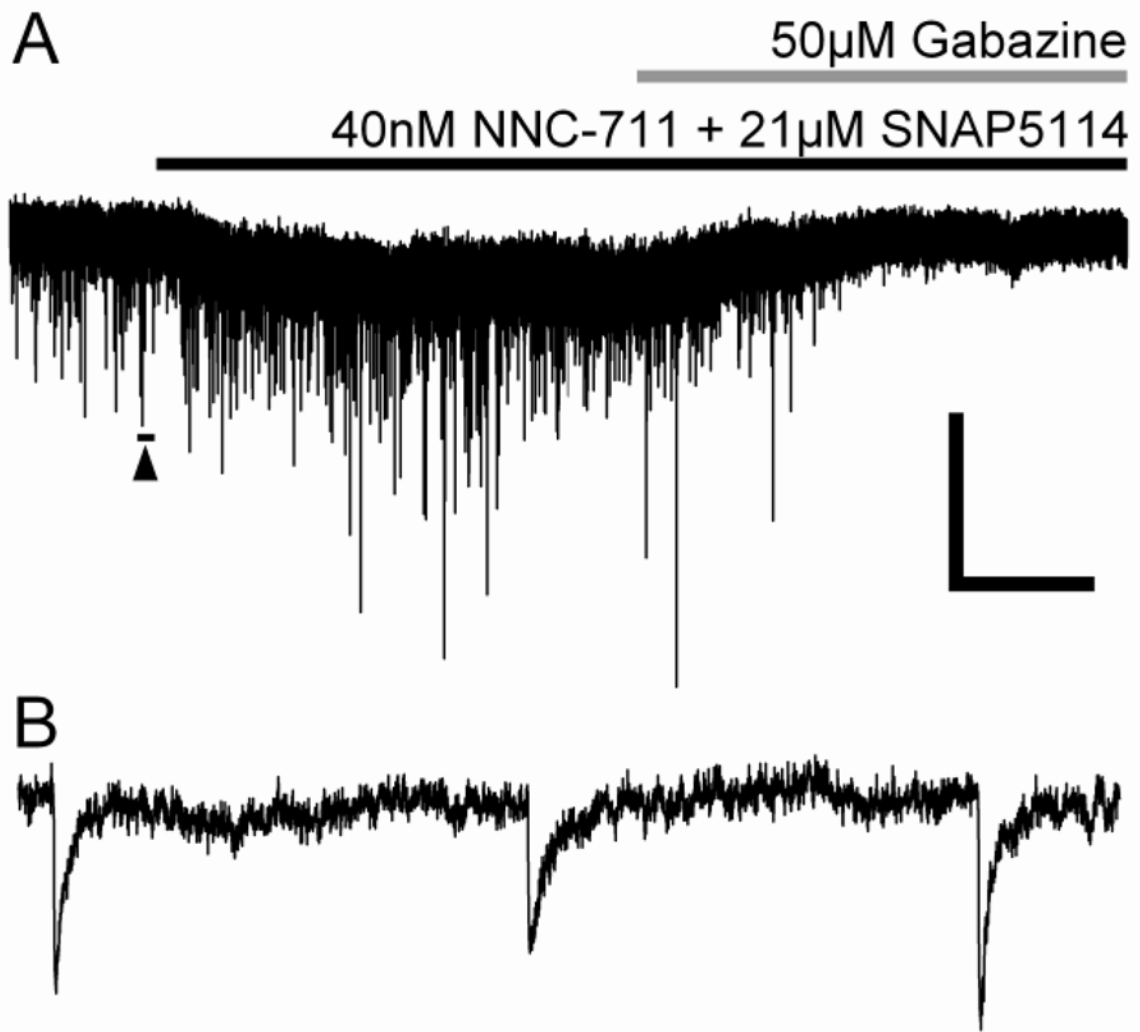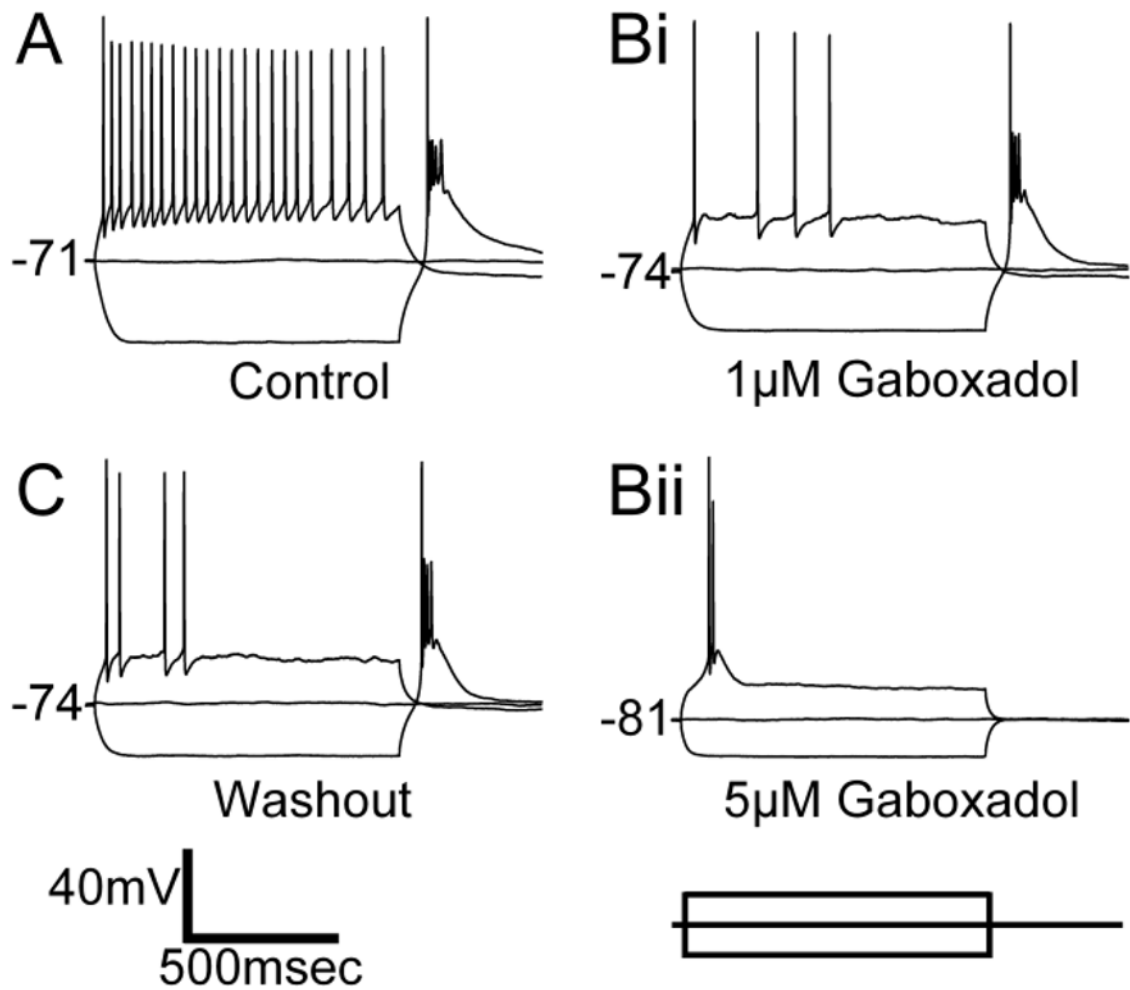
Figure 1.
Tonic and Phasic Inhibition in the Rat MGB. A) Voltage-clamp (CsCl based internal solution, $V_{hold} = -60mV$, $E_{Cl} \approx 0mV$) recording from an MGB neuron of a 7 month old FBN rat during the application of GABA uptake blockers (NNC-711 and SNAP5114, blockers of GAT-1,2 &3 mediated GABA uptake) [black bar] and subsequent application of the $GABA_AR$ selective antagonist 50μM gabazine (SR95531) [gray bar] reveal the presence of extrasynaptic $GABA_ARs$ mediating a tonic Cl⁻ current in the MGB which is blocked by gabazine. B) Expanded sIPSCs (underlined region in A with arrowhead) are also blocked by gabazine and therefore $GABA_AR$ mediated (A). Scale bar is 50sec and 50pA for A and 100msec and 40pA for B. Modified from Richardson et al., 2011.
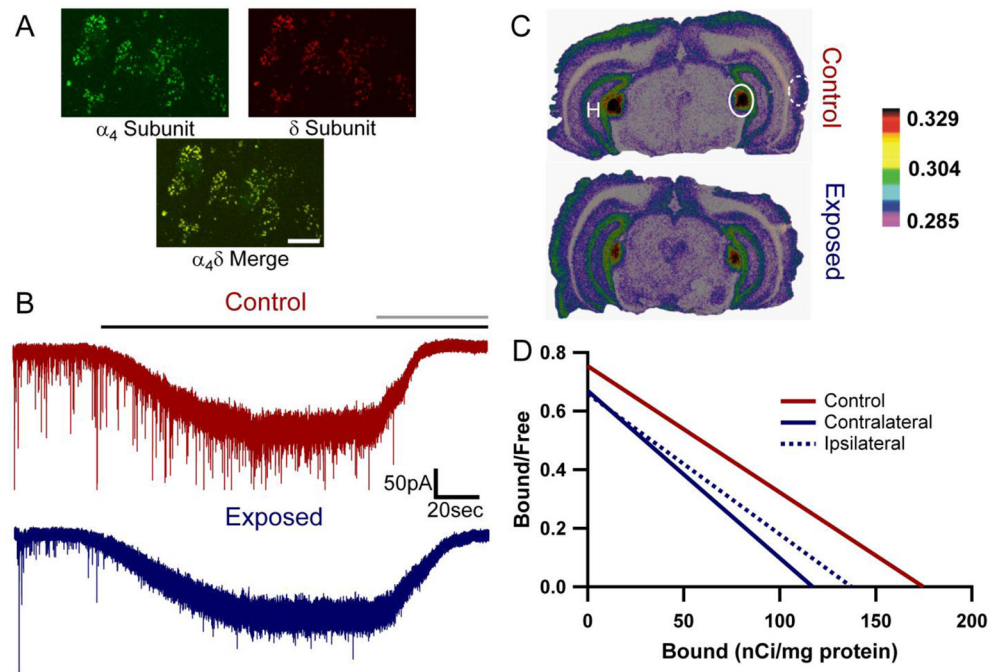
Figure 2.
Extrasynaptic GABA$_A$Rs and Tonic Inhibition in MGB. A) Confocal image of fluorescent immunolabeling of α_4 (green, top left), δ (red, top right) and colocalized $\alpha_4\delta$ (yellow, bottom) GABA$_A$R subunits in the MGB demonstrate the presence of the subunits necessary for functional tonically active extrasynaptic GABA$_A$R constructs. Scale bar = 10μm. B) Voltage-clamp recording (CsCl based internal solution, V$_{hold}$ = −60mV, E$_{Cl}$ ≈ 0mV) of tonic inhibitory current mediated by extrasynaptic GABA$_A$Rs in the MGB of a control 19 month old FBN rat (red, top) and from an MGB neuron contralateral to the sound exposed ear in a 24 month old FBN rat with behavioral evidence of tinnitus (blue, bottom) in response to 1μM gaboxadol (black bar) and blocked by 50μM gabazine (gray bar). C) Autoradiographs of 250nM [^{3}H]gaboxadol binding in adult FBN rats with and without behavioral evidence of tinnitus (modified from Richardson et al., 2011). Relative optical density spectrum is positioned at right. At these concentrations, [^{3}H]gaboxadol binds preferentially to extrasynaptic GABA$_A$Rs. Binding density is highest in the MGB (outlined with solid line) and lower in auditory cortex (dashed line) and hippocampus (H). D) Binding scatchards and saturation curves (not shown) indicate a significant difference in [^{3}H]gaboxadol binding between the MGB of control and animals with tinnitus and a 20–30% decrease in B_{max} (x-axis), corresponding to a decrease in extrasynaptic GABA$_A$R density in the MGB, while K_d (slope) (Kd=231.7±89.6 (control), 175.0±77.9 (contra), 208.3±87,95 (ipsi)) is relatively unaffected (n=5 control, 5 tinnitus).

Figure 3.
Regulation of MGB Neuron Excitability by Activation of Extrasynaptic GABA$_A$Rs. Whole cell recording of response from a MGB neuron of a 4 month FBN rat to a 1sec, ±100pA current injection in normal ACSF (A), the presence of 1μM gaboxadol (Bi), 5μM gaboxadol (Bii) and following a 10min washout period (C). As the concentration of gaboxadol is increased, the firing rate in response to the depolarizing pulse decreases and the resting membrane potential becomes more negative. The cell eventually responds with a single t-type Ca^{2+} dependent burst upon depolarization and remains inactive, consistent with increasing tonic inhibition and hyperpolarization. Internal solution is K-gluconate based.