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Is GABA Neurotransmission Enhanced in Auditory Thalamus Relative to Inferior Colliculus?

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Enhanced GABA neurotransmission in MGB

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Abstract

GABA is the major inhibitory neurotransmitter in the central auditory system. Sensory thalamic structures show high levels of non-desensitizing extrasynaptic GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) and a reduction in the redundancy of coded information. The present study compared the inhibitory potency of GABA acting at GABA<sub>A</sub>Rs between the inferior colliculus (IC) and the medial geniculate body (MGB) using quantitative *in vivo, in vitro, and ex vivo* experimental approaches. *In vivo* single unit studies compared the ability of half maximal inhibitory concentrations of GABA to inhibit sound-evoked temporal responses, and found that GABA was 2-3 times (*p* < 0.01) more potent at suppressing MGB single unit responses than IC unit responses. *In vitro* whole-cell patch-clamp slice recordings were used to demonstrate that gaboxadol (GBX), a δ subunit selective GABA<sub>A</sub>R agonist, was significantly more potent at evoking tonic inhibitory currents from MGB neurons than IC neurons (*p* < 0.01). These electrophysiological findings were supported by an *in vitro* receptor binding assay which used the picrotoxin analog, [³H]TBOB, to assess binding in the GABA<sub>A</sub>R chloride channel. MGB GABA<sub>A</sub>Rs had significantly greater total open chloride channel capacity relative to GABA<sub>A</sub>Rs in IC (*p* < 0.05) as shown by increased total [³H]TBOB binding. Finally, a comparative *ex vivo* measurement compared endogenous GABA levels and suggested a trend towards higher GABA concentrations in MGB than in IC. Collectively these studies suggest that, per unit GABA, high affinity extrasynaptic and synaptic GABA<sub>A</sub>Rs confer a significant inhibitory GABA<sub>A</sub>R advantage to MGB neurons relative to IC neurons. This increased GABA sensitivity likely underpins the vital filtering role of auditory thalamus.
Keywords: gamma-aminobutyric acid (GABA), GABA<sub>A</sub> receptor (GABA<sub>A</sub>R), inferior colliculus (IC), medial geniculate body (MGB)

Introduction

Gamma-aminobutyric acid (GABA) is considered the major inhibitory neurotransmitter of the mammalian central nervous system, including sensory systems, where it functions to control gain, improve signal-to-noise, localize environmental cues and, in general, shape the ascending acoustic message. The inferior colliculus (IC, auditory midbrain) and the medial geniculate body (MGB, auditory thalamus) are key structures of auditory neuraxis. Previous studies demonstrated that acoustic information about stimulus identity is further refined/reduced in single unit recordings from MGB relative to similar IC units recordings (Chechik et al. 2006).

The auditory midbrain (IC) is rich in sources of GABAergic neurotransmission. IC receives ascending GABAergic inputs from the dorsal, intermediate and ventral nucleus of the lateral lemniscus (DNLL, INLL, and VNLL), and the superior paraolivary nucleus (SPON) of the superior olivary complex (SOC) (Kulesza et al. 2003; Zhang et al. 1998). In addition, diverse GABAergic neurons form collaterals within IC (Oliver et al. 1994). From a functional perspective, studies in chinchilla cochlear nucleus and IC suggested that glycine and/or GABA could selectively alter near or below best modulation frequency (BMF) responses changing band-pass responses into more low-pass responders. A study by Koch and Grothe concluded that GABA inhibition sharpened frequency modulation tuning for the majority of neurons in IC of the big brown bat (Koch
In addition, GABA\textsubscript{A}R blockade alters responses to sinusoidal amplitude modulated stimuli (SAM) in IC of rats and bats by exerting a gain control effect on temporal and rate modulation (Burger and Pollak 1998; Caspary et al. 2002). Dynamic control of discharge rate near best frequency was found as the major role for GABA inhibition in IC of chinchilla, guinea pig, and bats (Le Beau et al. 1996; Palombi and Caspary 1996; Park and Pollak 1993). Furthermore, GABA\textsubscript{A}Rs have a role in controlling gain of stimulus specific adaptation in IC (Perez-Gonzalez et al. 2012). In general, the role of GABAergic inhibition in IC involves shaping and controlling gain of responses to a variety of simple and complex acoustic stimuli.

Compared to IC, rat MGB has few intrinsic inhibitory interneurons (~1%) (Bartlett and Smith 1999; Winer and Larue 1996). The two major GABAergic inputs to MGB are from IC (Ito and Oliver 2012; Peruzzi et al. 1997; Winer and Larue 1996) and the thalamic reticular nucleus (TRN) (Rouiller et al. 1985). Recently, Saldana and colleagues described unexpectedly large inhibitory projections from subcollicular sources to non-lemniscal auditory thalamus (Saldaña 2013). Descending auditory corticothalamic projections terminate on MGB neurons in a region-specific manner, with each auditory cortical region projecting to specific MGB subnuclei (Andersen et al. 1980; Bajo et al. 1995; Diamond et al. 1969; Kelly and Wong 1981; Lee et al. 2004; Lee and Winer 2005; Pandya et al. 1994; Pontes et al. 1975; Rouiller and de Ribaupierre 1985; Sousa-Pinto and Reis 1975; Winer et al. 2001; Wong and Kelly 1981). A recent series of detailed studies by Bartlett and Wang suggest that MGB neurons display unique, complex responses to modulated and click train stimuli when compared to neurons in IC (Bartlett and Wang 2011; 2007). Based on the disparate nature of inputs and known differences
in the nature of GABA<sub>A</sub>Rs between IC and MGB, it is reasonable to expect distinct
differences in functional GABAergic neurotransmission between these two auditory
structures. Few studies have directly compared the potency of GABAergic inhibition
between these two structures in response to acoustic stimuli in vivo. Based on
differences between these structures in the expression and distribution of GABA<sub>A</sub>R
subtypes (see below), a direct IC and MGB comparison of GABA efficacy could provide
insights into coding characteristics of these structures.

GABA<sub>A</sub>Rs are heteromeric pentamers made up of 19 possible subunits (α1–6, β1–3,
γ1–3, δ, ε, θ, π and ρ1–3). A limited number of GABA<sub>A</sub>R constructs are prevalent and
are regionally distributed in different proportions throughout different brain structures
where they display differences in ligand binding affinity, receptor kinetics, Cl<sup>-</sup>
conductance and subcellular location (Belelli et al. 2009; Brickley and Mody 2012; Bright
and Brickley 2008; Semyanov et al. 2004; Walker and Semyanov 2008). Wild-type (2α<sub>1</sub>,
2β<sub>2</sub>, and γ<sub>2</sub>) GABA<sub>A</sub>Rs makeup as much of 70% of the distribution of GABA<sub>A</sub>Rs
constructs and represents the overwhelming majority of GABA<sub>A</sub>R constructs in IC
(McKernan and Whiting 1996; Pirker et al. 2000). Immunocytochemical and quantitative
receptor binding studies showed that α<sub>4</sub> and δ subunits co-exist and are prevalent in
significant numbers in sensory thalamic nuclei, including MGB (Belelli et al. 2009; Cope
et al. 2005; Richardson et al. 2011). They have not been functionally described in IC.
Thalamocortical neurons show unique tonic inhibitory properties mediated by
extrasynaptic, α4 and δ subunit containing, non-desensitizing GABA<sub>A</sub>R constructs
(Cope et al. 2005; Richardson et al. 2011). Pirker’s survey study of GABA<sub>A</sub>R subunit
protein expression describes high levels of α<sub>4</sub> and δ GABA<sub>A</sub>R subunits in MGB, and also
found low levels of the GABA\(_\alpha\)R \(\delta\) subunit in IC (Pirker et al. 2000). The nature of the GABA\(_\alpha\)R subunit construct determines affinity and efficacy for a given ligand. GABA\(_\alpha\)Rs with a \(\gamma_2\) subunit mediating rapidly desensitizing inhibitory post-synaptic currents (IPSC) characterized by a relatively low affinity (EC\(_{50}\) \(\sim\) 6-14 \(\mu\)M) for the endogenous agonist GABA (Farrant and Nusser 2005). In contrast, \(\delta\) subunit containing GABA\(_\alpha\)Rs (\(\delta\)GABA\(_\alpha\)R) show slow desensitization kinetics and high affinity (EC\(_{50}\) \(\sim\) 0.3-0.7 \(\mu\)M) for ambient GABA and a higher affinity (EC\(_{50}\) \(\sim\) 30-50 nM) for the selective \(\delta\)GABA\(_\alpha\)R agonist gaboxadol (GBX) (Farrant and Nusser 2005; Meera et al. 2011).

Based on its critical role in sensory gating and the presence of high affinity extrasynaptic GABA\(_\alpha\)Rs, one might predict an enhanced sensitivity to GABA inhibitory neurotransmission in the sensory thalamus relative to other auditory structures. To test this hypothesis, four distinct sets of experiments were designed as follows: 1. \textit{In vivo} iontophoretic unit recordings comparing GABA potency in IC and MGB; 2&3. \textit{In vitro} whole-cell patch-clamp and receptor binding assessing agonist evoked Cl\(^-\) currents and channel activations in IC and MGB slices; and 4. \textit{Ex vivo} proton magnetic resonance spectra (\(1^H\)-MRS) comparing endogenous GABA levels.

**Materials and Methods**

All experiments were completed using Fischer Brown Norway (FBN) or Long-Evans (LE) male rats maintained on an \textit{ad libitum} diet and reversed light-dark cycle. Procedures were done in accordance to protocols (No. 41-10-002 and 41-09-024) approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine.
The FBN and LE rats were 4-10 months of age and considered adult rats, based on average life span (Schroeder et al. 1965; Turner and Caspary 2005).

**Iontophoresis**

Thirty-seven adult male FBN rats (4-6 mos) were initially anesthetized with I.M. injection (1.4 ml/kg) of a 3:1 mixture of ketamine-HCl (100 mg/ml) and xylazine (20 mg/ml). Anesthesia was maintained with i.p. injections of 100% urethane initially 1.3 ml/kg, and then maintained at one-third of the initial amount [booster doses]). Urethane was chosen as the anesthetic agent because its actions are on multiple neurotransmitter systems rather than simply potentiating the effects of inhibitory systems, thus it has less net effect on GABAergic neurotransmission than barbiturates or other anesthetic agents (Hara and Harris 2002). Rats were placed in a modified stereotaxic frame in an IAC sound-attenuating booth. For MGB, a 2 X 2 mm craniotomy was drilled, exposing the dorsal surface of the cortex (-5.5 mm from Bregma; 3.5 mm lateral from midline). The IC was approached dorsally by exposing the calvarium just rostral to the Lambda and lateral to the midline (2 mm) at a 15~20° angle (Caspary et al. 2002). A carbon fiber electrode attached to a five-barrel iontophoretic electrode, Carbostar-6 (Kation Scientific, Minneapolis, MN), was coupled to the headstage, then to a preamplifier and controlled by a PC based Multichannel Acquisition Processor (MAP) system running MAP software (Plexon Inc., Dallas, TX). Spikes were visualized using Sort Client (Plexon Inc., Dallas, TX) for real-time spike sorting. A piezoelectric driver (David Kopf Instruments, Tujunga, CA) advanced the electrode to the dorsal aspect of MGB or IC using a broadband noise (BBN) search signal. Single units (3:1 SNR) were
discriminated based on waveform morphology and/or principal component analysis. In a few cases small clusters were studied. Stimulus presentation, real-time data display and analysis used Auditory Neurophysiology Experiment Control Software (ANECS, Ken Hancock, Blue Hills Scientific, Boston, MA) coupled to TDT System III hardware. Acoustic signals were amplified (ED1), transduced (EC1), and juxtaposed to the right ear canal using polypropylene tubing. The sound system was calibrated offline using a quarter inch Brüel & Kjaer model 4938 microphone (Naerum, Denmark) into a simulated rat ear (2-46 kHz ± 2 dB) (Palombi and Caspary 1996). SAM carrier frequency was set at the unit’s characteristic frequency (CF) or BBN; rate modulation transfer functions (rMTFs) were determined for each unit at 30 dB above CF threshold in response to 2/sec SAM stimuli (4 ms raise-fall time, 100% depth) with modulated frequency ($f_m$) stepped between 2 Hz and 512/1024 Hz. Stimuli were 450 ms in duration (presented randomly across the trial among different $f_m$s) with spikes collected over a 500ms period following stimulus onset (10 stimuli/envelope frequency). Multi-barrel iontophoretic electrodes were coupled to a constant current system (BH-2 Neuro Phore System). The balancing barrel was filled with KAc (2 M), other barrels filled with GABA (500 mM, pH = 4.0, Sigma-Aldrich, St. Louis, MO) and GBX (10 mM, ibid). Retaining currents were set at -15 nA with ejection currents between 0 and 100 nA. A reversible change greater than 15 percent of control was considered a positive drug effect. Neurons reported here showed full baseline recovery following cessation of drug application. Repeated runs were frequently used to confirm small effects. Rats were cardiac perfused with phosphate buffered saline (0.1 M, pH 7.4) followed by paraformaldehyde (4%). Brains were removed, placed in paraformaldehyde (1-2 hrs.),
transferred to sucrose (20%) overnight, sectioned at 50 µm and stained with fast thionin for localization of recording sites (Palombi and Caspary 1996).

Patch clamp

Patch-clamp experiments were conducted using procedures and equipment previously described (Kalappa et al. 2010; Richardson et al. 2011). Briefly, adult male FBN rats (4-6 mos) were anesthetized with 2.5 - 3.0% isoflurane gas and decapitated. Their brains were rapidly removed and transferred to ice-cold sucrose-rich solution (in mM): sucrose 250, KCl 3, NaH2PO4 1.23, MgCl2 5, CaCl2 0.5, NaHCO3 26, glucose 10 (pH 7.4).

Horizontal sections, 200 – 300 µm containing either the central nucleus of IC or the ventral division of MGB were prepared using a Vibratome 1000 Plus (Leica Microsystems GmbH, Wetzlar, Germany). Post sectioning, slices were transferred to a storage chamber, perfused (30 min) at 30°C with artificial cerebrospinal fluid (ACSF) (in mM): NaCl 125, KCl 3, NaH2PO4 1.26, CaCl2 2, MgCl2 1, NaHCO3 26, glucose 10, and maintained at room temperature for up to ~8 hrs. For patch-clamp recordings, slices were transferred to the recording chamber and perfused with ACSF at a rate of 1 ml/min. A MultiClamp 700B amplifier, with a Digidata 1440A A/D converter (Molecular Devices, Sunnyvale, CA) was used to sample data (10 kHz) with a 2.2 kHz low-pass filter. Patch pipettes were pulled using Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA) with a resistance of ~ 4 – 6 MΩ when filled with the internal solution (see below). Following the formation of a stable gigahm seal (> 2 GΩ), whole-cell configuration was established. To measure the membrane capacitance ($C_m$), membrane
resistance (R_m), and access resistance (R_a), a 10 mV depolarizing step voltage command was applied using the membrane test function integrated in pClamp10 software (Molecular Devices, Sunnyvale, CA). Voltage-clamp recordings were made at room temperature with a -10 mV holding potential to enhance our ability to detect Cl^- mediated outward currents. To reduce noise induced by K^+ mediated currents, a Cs-methanesulfonate-based internal solution was used (in mM): CsMeSO3 140, NaCl 6, MgCl2 2, Mg-ATP 2, Na-GTP 0.3, HEPES 10, CsOH 0.3 (pH 7.4). GBX and gabazine (GBZ, Ibid) were bath applied. Membrane voltages were not corrected for the liquid junction potentials: V_LJ (CsMeSO3) = 9.8 mV (Kalappa et al. 2010). Access and series resistance was not compensated for in these voltage-clamp experiments. Analysis of data was conducted offline using Clampfit 10.2.

[^3H]TBOB binding

[^3H]t-butylbicycloorthobenzoate ([^3H]TBOB) binding used increasing concentrations of GABA (0 nM to 5 µM) to modulate picrotoxin sites within the GABA_A chloride channel in MGB and IC. Concentrations based on Milbrandt et al (1996) were centered near the K_d, which optimized the potential to quantify binding differences in these two structures. In brief: Adult male FBN rats (4-6 mos) were decapitated; brains rapidly removed and frozen. Brain slices (16 µm thick) were cut using a Leica CM1850 cryostat (Leica Microsystems, Buffalo Grove, IL) at -18°C, and stored at -20°C. Sections were prewashed in a buffer containing 50 mM Tris-HCl and 1 mM EDTA (pH 7.4), placed in the incubation buffer (50 mM Tris-HCl, and 120 mM NaCl, pH 7.4) with[^3H]TBOB and GABA (concentration from 0 nM to 5 µM) for 90 minutes at room temperature. Cold
picrotoxin (20 µM) was added as a displacer. Autoradiograms were generated by apposing the slides to a phosphor screen, subsequently scanned using a Cyclone phosphor system (Packard BioScience, PerkinElmer, MA). Images were collected at 600 DPI, and the area of MGB and IC was identified and outlined. Binding intensity was analyzed using OptiQuant image analysis software which provides tools for gray-scale quantification in digital light units (DLU). DLU were converted into fmol/mg protein using a standard curve generated from co-exposed 14C-embedded plastic standards (ARC, St. Louis, MO). (Detailed methods see Milbrandt et al. 1996).

Spectroscopy

Seven adult (mean age 10 mos) male LE rats (Harlan, Indianapolis, IN) were used in ex vivo spectroscopy studies. Volume limited 1H-MRS were obtained using a vertical bore Varian Unity/Inova 600 mHz NMR spectrometer with a 14.1 T magnet. Calibration spectra were determined for GABA standards (10 mM) dissolved in sterile normal saline. Volume of interest (VOI) GABA levels were determined using the integrated area of the spectral peak in closest approximation to 2.2 ppm (i.e., the calibration standard). Animals were treated 10 min prior to euthanasia with 10 mg/kg (i.p.) 3-mercaptopropionic acid (product M5801, Sigma-Aldrich, St. Louis, MO) to arrest post-mortem GABA inflation (van der Heyden and Korf 1978). Immediately before acquisition, animals were given a lethal dose of anesthetic (Euthasol, Virbac, Ft. Worth, TX), decapitated, the mandible removed and excess muscle tissue dissected away from the skull. The head was placed in a polyethylene holder along with a 1 mm diameter glass capillary filled with CuSO₄ (3 mM). The CuSO₄ image phantom unambiguously
indexed laterality. For each animal, an initial MRI brain scan was used to locate the VOI for $^1$H-MRS. Contiguous transverse (i.e., coronal) slices, 0.5 mm thick (26 µm planar resolution), were obtained, extending 13 mm caudally from Bregma (26 slices total). VOI for $^1$H-MRS were determined in ventral MGB (vMGB), dorsal MGB (dMGB) and IC. Spectra, as TIFF images, were imported into Image J (ver. 1.44p, http://imagej.nih.gov/ij). Peaks in closest approximation to the calibration peak for GABA were outlined, and the area under each curve (AUC) was determined. AUC were expressed in spectrum baseline units (i.e., curve lower bound) in order to correct for image gain. (Detailed methods see Brozoski et al. 2012).

Results

GABA$_A$R Endogenous and Selective Agonists Alter SAM Responses in MGB

SAM stimuli were used to elicit auditory responses in extracellular recordings from MGB units in anesthetized rats. Figure 1 displays examples of the iontophoretic paradigm used to assess potency of GABA$_A$Rs. A representative unit showing a bandpass response pattern (BP) (30% of MGB response types) displayed a 46% reduction of SAM evoked activity ((653-300)/653) $\times$100% with low dose GABA application (Fig. 1A). A significant decrease in spike rate was evident during drug application near BMF in both the dot raster and the rate modulation transfer function (rMTF, Figs. 1Ab&1B). Application of GABA onto 23 MGB BP units (average GABA dose 8.17 ± 1.57 nA) resulted in a 46.24 ± 2.97% decrease of SAM responses from rMTFs at 30 dB above threshold (Fig. 1C). GABA dose was calculated as the smallest dose which produced a greater than 15% change in response. Another MGB SAM response type termed
"Mixed" type (Fig. 2C, 35% of the MGB response types) showed a similar reduction in discharge rate of 48.15 ± 3.71% for 31 Mixed units at an average GABA dose of 10.97 ± 2.02 nA (data not shown here). Taken together, MGB neurons showed exquisite sensitivity to iontophoretic application of GABA.

INSERT FIGURE1 HERE

The selective δGABAAR agonist, GBX, was applied to MGB neurons to test whether more selective activation of extrasynaptic GABAARs would differ from responses to GABA, a less selective endogenous agonist. Responses to both GABA and GBX applications, with recovery to control discharge rates, were obtained from 15 MGB units responding to SAM stimuli. The suppression of SAM responses by GBX was similar but somewhat smaller and less selective than anticipated. The majority of units (11/15) showed greater reduction in total spikes with application of GABA than with application of GBX (Figs. 1D-F). Average total spikes were 32.59 ± 4.86% under GABA application and 67.15 ± 4.62% under GBX application (Fig. 1F). However, four units showed similar levels of inhibition to the two agonists (Figs. 1G-I). Due to limitations of the iontophoretic technique, relative sensitivity of GABAARs to different GABA and GBX dose levels are considered difficult to compare (Krogsgaard-Larsen et al. 2004; Mortensen et al. 2004). Based on the present data, both GABA and GBX could inhibit MGB auditory response to a certain extent, but GABA appeared more efficacious than GBX.

GABAAR Sensitivity: IC and MGB units, an in vivo comparison
Experience from prior IC iontophoretic studies (Caspary et al. 2002; Palombi and Caspary 1996), initial MGB iontophoretic studies, and the studies described above suggested that MGB neurons are more sensitive to GABA application than IC neurons. To quantify these suspected GABAAR sensitivity differences, we examined the impact of increasing GABA dose (dose response) using the same electrodes and GABA concentrations to minimize possible experimental design or methods differences in a comparative IC vs MGB single unit study. Figure 2 compares responses from representative IC and MGB units to GABA application in response to SAM stimuli.

Increasing GABA doses were applied to each unit resulting in increased suppression/inhibition of firing for both units (Figs. 2A&2C). Note that the MGB unit in Fig. 2C responds at very low doses (from leaking, “0” nA) of GABA with increasing suppression of driven activity. A clear decrease in responses at both lower and higher fM could be seen at the 4 nA dose (Fig. 2C). Only onset responses remained relatively unaffected at higher GABA doses. GABA also inhibited SAM responses of IC units but required higher doses of GABA (Fig. 2A). The patterns of suppression of rMTFs in IC units were similarly altered by GABA application with the greatest suppression observed at or near BMF for both structures (Figs. 2B&D). IC and MGB dose sensitivity to GABA application was quantified by plotting dose against total spikes for each run (normalized) (Fig. 2E). Regression lines showed a dose dependent response to GABA application. The 50% inhibition dosage (ID$_{50}$) was calculated based on the normalized spike rate. Higher iontophoretic GABA doses were needed to achieve a 50% reduction of discharge rate for IC units (Fig. 2E). The mean ID$_{50}$ was significantly lower (14.01 ± 3.00 nA) for MGB units compared to IC units (49.56 ± 6.75 nA) (**p < 0.01, independent t-
test) (Fig. 2F). We also compared ID$_{50}$ for GABA sensitivity between the two major MGB divisions for nine well localized dMGB and 15 vMGB units (Fig. 2G). No significant ID$_{50}$ differences in GABA$_A$R sensitivity were found ($p > 0.05$, independent $t$-test).

**Tonic currents mediated by extrasynaptic GABA$_A$Rs: IC and MGB, an *in vitro* comparison**

To compare the relative sensitivity of GBX sensitive extrasynaptic GABA$_A$Rs in IC and MGB, whole-cell voltage-clamp recordings from IC ($\pm$ SEM, $n = 5$; $C_m$, 75.5 $\pm$ 3.19 pF; $R_m$, 376.45 $\pm$ 59.18 M$\Omega$; $R_a$, 19.85 $\pm$ 1.27 M$\Omega$) and MGB ($\pm$ SEM, $n = 5$; $C_m$, 99.75 $\pm$ 8.83 pF; $R_m$, 349.25 $\pm$ 36.06 M$\Omega$; $R_a$, 17.5 $\pm$ 1.68 M$\Omega$) neurons from adult slices were conducted with CsMeSO$_4$ internal solution (see methods). Once a stable whole-cell configuration was established, neurons were held at -10 mV to maximize visualization of tonic Cl$^-$ currents upon activation of extrasynaptic $\delta$-GABA$_A$Rs. Bath application of increasing doses (0.1 µM-10 µM) of GBX, a $\delta$-GABA$_A$Rs subunit selective agonist, evoked tonic currents from both IC and MGB neurons in a dose-dependent manner. The amplitude of GBX evoked tonic currents were revealed by addition of 10 µM GBZ, a GABA$_A$R antagonist (Fig. 3B). Comparison of dose response curves revealed that GBX was significantly more potent in activating tonic currents in MGB neurons than in IC neurons ($EC_{50_{MGB}} = 2.25$ µM; $EC_{50_{IC}} = 4.45$ µM; $F$ (1, 54) = 30.17, $p < 0.0001$; $F$-test) (Fig. 3A). Maximal mean peak currents ($\pm$ SEM) for MGB and IC were 144.02 $\pm$ 8.15 pA and 97.80 $\pm$ 14.51 pA, respectively. These findings are consistent with predictions
based on immunocytochemical GABA<sub>A</sub>R subunit protein distribution studies by Pirker et al. which suggested higher relative densities of δGABA<sub>A</sub>Rs in MGB than in IC (Pirker et al. 2000).

One limitation of this approach is the difference in passive membrane properties of IC and MGB neurons. Although, IC and MGB neurons exhibited comparable membrane resistance, IC neurons demonstrated lower membrane capacitance than MGB neurons. However, comparison of EC<sub>50</sub> values from normalized dose response curves of GBX evoked tonic currents (i.e. response net amplitude per unit of membrane capacitance) from IC (EC<sub>50IC</sub> = 4.91 µM) and MGB (EC<sub>50MGB</sub> = 1.18 µM) neurons demonstrated that GBX was still significantly more potent (F (1, 64) = 76.68, p < 0.0001; F-test) in activating tonic currents in MGB neurons than in IC neurons (normalized curves not shown). A second limitation of this approach is that the voltage clamp experiments were conducted under the assumption that space clamp in both MGB and IC neurons were similar and adequate. Although the extent of space clamp may differ between these neurons, it would be safe to assume that IC neurons exhibit better space clamp due to lower membrane capacitance than MGB neurons. Hence, if any discrepancies in peak amplitude estimation were to occur due to inadequate space clamp, the tonic current amplitudes of MGB neurons may be somewhat underestimated in comparison to IC neurons which are likely to exhibit better space clamp. Overall, these observations suggested that the differences in adequacy of space clamp and membrane capacitance in IC and MGB neurons may have subtle influence on the absolute values but may not significantly affect the overall conclusion of this voltage clamp study.
Modulation of Binding at the GABA<sub>R</sub> Picrotoxin site: IC and MGB, in vitro comparison

An estimate of total GABA evoked chloride flux was assessed using a ligand that binds in the open GABA<sub>R</sub> chloride channel at the picrotoxin binding site. The ability of [³H]TBOB, a picrotoxin competitive analog (Lawrence et al. 1985), to bind at, and be modulated by increasing concentrations of GABA (0 nM - 5 µM) was used to compare the maximum number of available GABA<sub>R</sub>s in IC vs MGB. Figure 3C compared the [³H]TBOB modulation curves for MGB and IC with 0 nM GABA set as the normalized control condition. This represents the resting/control condition for the open/closed state of GABA<sub>R</sub> Cl⁻ channels. At low GABA concentrations, both structures showed increased binding indicative of increased activation/openings of GABA<sub>R</sub> Cl⁻ channels (Fig. 3C). Opened channels allow for increased binding at picrotoxin sites in the GABA<sub>R</sub> Cl⁻ channel of the originally closed GABA<sub>R</sub> Cl⁻ channels. Significant differences between IC GABA<sub>R</sub>s and MGB GABA<sub>R</sub>s were seen at 100 nM (*p < 0.05, two-way ANOVA). The larger area under the MGB curve (from 50 nM to 1000 nM) suggested that MGB neurons have a greater total chloride flux capacity relative to IC neurons (Fig. 3C). Both MGB and IC showed desensitization at higher GABA concentrations following peak values that reflected a high percentage of closed GABA<sub>R</sub> Cl⁻ channels at 1000 nM and 5000 nM.

GABA level by ¹H-MRS: IC and MGB, an ex vivo comparison
Improvements in magnetic resonance spectroscopy enabled the acquisition of well-resolved spectra from small tissue volumes, as documented in a recent study (Brozoski et al. 2012). Absolute GABA tissue levels were obtained from IC and MGB using high-resolution point-resolved proton magnetic resonance spectroscopy ($^1$H-MRS) (Fig. 4A). IC, vMGB, and dMGB were selected separately in each rat, and data from left and right hemispheres were combined. Mean GABA concentrations for each structure were dMGB = 3.72 ± 1.13, vMGB = 3.05 ± 0.86, and IC = 1.50 ± 0.29 (Fig. 4B, presented as mM, mean ± SE). A non-significant trend toward higher GABA levels was found for both vMGB and dMGB when compared to GABA levels in IC. Independent $t$-test showed a $p$ value of 0.05107 (IC vs dMGB) and 0.0809 (IC vs vMGB) between IC and MGB. A similar comparison (Brozoski et al. 2012), found GABA levels in MGB overall were significantly higher than in IC ($p = 0.011$). Both results suggest elevated GABA level in the auditory thalamus relative to the IC.

Discussion

The present series of studies sought to compare the pharmacology of GABA neurotransmission in IC and MGB and found: 1) Significantly increased ability of lower dose GABA to suppress SAM evoked unit responses of MGB units compared to IC units. 2) Significantly larger GABA$_{	ext{A}}$R mediated tonic currents evoked by the selective GABA$_{	ext{A}}$R agonist, GBX, in voltage-clamped adult MGB neurons compared to similarly examined IC neurons. 3) Significantly increased [$^3$H]TBOB binding following GABA
application, in MGB compared to IC. 4) A clear trend towards increased GABA tissue concentrations in MGB compared to IC using \(^1\)H-MRS imaging. Collectively, data from these four studies suggest that GABA levels in MGB may be higher than in IC and that activation of synaptic and extrasynaptic GABA\(_A\)Rs by GABA and GBX is enhanced in MGB compared to IC.

GABA\(_A\)R Composition and Efficacy in IC and MGB

In IC and MGB, wild type GABA\(_A\)Rs (2\(\alpha_1\), 2\(\beta_2\), and \(\gamma_2\)) are thought to mediate fast GABAergic inhibition and are activated by release of the inhibitory neurotransmitter GABA (McKernan and Whiting 1996; Milbrandt et al. 1997; Pirker et al. 2000). In addition, other heteromic GABA\(_A\)R constructs are highly expressed in sensory thalamic structures. \(\alpha_4\) and \(\delta\) subunit containing GABA\(_A\)R show high affinity for GABA and the subunit selective agonist GBX (Brown et al. 2002; Mortensen et al. 2010). They are localized to extrasynaptic postsynaptic sites and show tonic non-desensitizing, hyperpolarizing chloride currents (Cope et al. 2005; Richardson et al. 2011). The discussion below suggests that our findings of increased GABA sensitivity in MGB neurons compared to IC neurons may reflect the existence of higher levels of extrasynaptic GABA\(_A\)R in MGB. By contrast, these GABA\(_A\)R constructs are not prominent in IC, since \(\alpha_4\delta\) subunit protein levels were low and \(\alpha_4\delta\) message levels were below the level of detection (Pirker et al. 2000; Wisden et al. 1992). We probed this using the methodology of Richardson et al. (Richardson et al. 2011) adapted from Cope et al. (Cope et al. 2005), and found, for the first time, that tonic currents could be evoked
by GBX application in IC neurons, in a manner similar to what has been seen in thalamus. However, as predicted, the ability of the subunit selective agonist GBX to evoke tonic currents was reduced by 50% in IC neurons compared to MGB neurons.

A recent series of studies on marmoset suggest that MGB neuronal responses to modulated and click stimuli display unique, more complex responses than do neurons in IC (Bartlett and Wang 2011; 2007). In addition, sensory thalamic neurons are able to switch their discharge pattern from the so called “tonic” mode to “burst” mode, depending on thalamocortical rhythmicity regulating sleep and attention and depending on the strength and nature of the stimulus (Steriade et al. 1993). These transformations are likely critical to the way sensory pathways process information (see reviews: Sherman 2001; Sherman and Guillery 1996). We hypothesized that these thalamic attributes were due to unique GABA circuits and receptors within MGB. While examining GABA’s role in coding modulated signals in a separate study, it became evident that response suppression by GABA application onto MGB units responding to SAM stimuli was far more potent than observed previously in IC (Burger and Pollak 1998; Caspary et al. 2002; Koch and Grothe 1998). We initiated a parallel dose response comparison using the exact same methodology between structures (electrodes, anesthesia and animal) to compare GABA efficacy. GABA was 2-3 times \( (p < 0.01) \) more potent at suppressing SAM-evoked MGB unit responses than SAM-evoked IC unit responses, further supporting the enhanced GABA sensitivity of sensory thalamic neurons.
GABA Concentration in IC and MGB

GABA is the major inhibitory neurotransmitter in the central auditory system. The extrasynaptic GABA\(_\text{A}\)Rs in sensory thalamus suggest that their presence and regulation of this GABA\(_\text{A}\)R construct might reflect ambient GABA concentrations. In the present study, a trend toward higher GABA levels in MGB relative to IC was detected using \textit{ex vivo} \(^1\)H-MRS. These findings are consistent with our previous published findings showing significantly higher MGB GABA levels relative to IC, using an earlier MRS data set (Brozoski et al. 2012). \(^1\)H-MRS \textit{ex vivo} GABA levels include all GABA compartments, including astrocytic (Papp et al. 2004; Sperlagh et al. 2002) and other non-vesicular stores (Demarque et al. 2002). Reports of GABA levels/concentrations by others used different methods to quantify GABA in IC and MGB with varying results. A recent HPLC study in hamster found relatively lower GABA levels in MGB subdivisions (7.6 ~ 8.2 mmol/kg dry wt) than IC subdivisions (8.3 ~ 12.4 mmol/kg dry wt) (Godfrey et al. 2012). HPLC studies in human tissue, found lower GABA levels in MGB (3.31) than IC (5.20) (Banay-Schwartz et al. 1989), while these same authors working in rats, found similar GABA levels between these two structures (MGB: 85 vs IC: 83) (Banay-Schwartz et al. 1993). The above cited studies showing either similar GABA levels/concentration between the two structures or somewhat higher IC GABA levels were carefully conducted but differ significantly in methodology from the present study. The differences between these studies are likely due to use of indirect measures, using different sample/tissue treatments and species differences. The present \(^1\)H-MRS studies used direct measures of GABA concentration against a known standard and found a trend toward higher MGB GABA concentrations relative to IC GABA.
concentrations, in agreement with our previously published study (Brozoski et al., 2012). The present findings support the notion that elevated GABA levels in MGB could underpin the unique distribution of GABA$_A$R high affinity constructs found in thalamus which include extrasynaptic $\alpha_4\delta$ GABA$_A$R constructs that are thought to be regulated by endogenous local GABA concentrations (Belelli et al. 2009; Cope et al. 2005; Richardson et al. 2011).

**Acoustic Information Processing through IC and MGB**

The presence of extrasynaptic GABA$_A$R, elevated GABA concentrations, and unique response modes to inhibition make the thalamus a distinctive auditory structure. The present study used parallel methods in IC and MGB to reveal more potent GABAergic inhibition in MGB relative to IC. Previous study showed that acoustic representation in the IC differs from that in MGB and primary auditory cortex (A1) which suggests a change in coding strategy, from a mixed rate and temporal code to more sparse rate code in MGB (Joris et al. 2004; Liang et al. 2002). Studies in cat also demonstrated coding changes as acoustic information ascended from IC through MGB to A1. Information redundancy presented in IC was thought to be reduced in MGB (Chechik et al. 2006; Las et al. 2005). In support of unit studies in animals, fMRI studies in humans found trends showing a population based neural representation of the beginning and end of distinct perceptual events that is weak or absent in IC but emerge at the level of MGB (Harms and Melcher 2002). A recent review reported a more efficient temporal coding strategy at sensory thalamocortical levels relative to midbrain coding strategies.
(“multiplexed temporal processing scales”) (Panzeri et al. 2010), which operate multiple neural codes simultaneously at different temporal scales. Together with previous studies, the hypothesis that stimulus coding diverges in MGB compared to IC, is plausible and is supported by neurophysiological data. Thus, we assume that the existence of synaptic/extrasynaptic receptors, with properties of fast/slow decay time and low/high agonist sensitivity, may enable MGB to be a multiplexed router.

Summary

The present study shows enhanced GABA sensitivity in MGB compared to IC, and this enhancement is likely mediated by both high affinity extrasynaptic and synaptic components. This increased GABA sensitivity may serve as a basis of the vital filtering role of the auditory thalamus in processing ascending acoustic information.

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**Figure legends**

Fig. 1 *In vivo*: effects of the endogenous agonist, GABA, and the selective agonist, GBX, application onto MGB units. MGB unit is sensitive to application of the endogenous agonist GABA (A-C, Db, Gb). 5 nA GABA resulted in a near 50% decrease in spike rate, as shown in dot raster display (Ab). GABA application showed a selective suppression at or near rate best modulated frequency (rBMF) (B). Average total spike change by low dose GABA (less than 10 nA) for 23 units was 46.24 ± 2.97% (C). Application of the δ subunit selective agonist GBX onto MGB units showed varying responses. Average total spikes were 32.59 ± 4.86% under GABA application and 67.15 ± 4.62% under GBX application. GABA showed greater reduction of spike rate than GBX for majority units (11/15) (F). The representative unit in D showed profound spike suppression with 0 nA (leaking) GABA and a smaller effect with 20 nA GBX onto the same unit. Group data indicated that applied GABA was almost twice as potent as GBX (**p < 0.01, independent t-test) (F). There were a small number of units (4/15) that had similar levels of suppression with applied GABA and GBX (G-I). In the representative mixed type unit (G), GABA and GBX show a similar pattern of reduction in discharge rate (15.91% vs 17.42%) across different modulation frequencies (H). (I) Group data for this response type indicated the same effects for GABA and GBX (p > 0.5, independent t-test). Data shown as Mean ± SE. N: number of spikes; n: number of units in all figures.
Fig. 2 *In vivo*: ability of half maximal inhibitory concentrations of GABA to inhibit sound evoked responses in IC and MGB. Dot raster displays sound evoked responses inhibited by increasing iontophoretic doses of GABA for representative IC (A) and MGB (C) units. Response rates were plotted against \( f_m \) (B & D) for each unit. (E) Total spikes for each run were calculated as percent of control. Gray dash-line indicates a 50% reduction from the control condition. Higher dosages of GABA were needed to suppress 50% of the responses for IC units (red line). Group ID50s are shown in F, the mean values are 49.56 ± 6.75 nA for IC and 14.01 ± 3.00 nA for MGB, suggested that MGB units were significantly more sensitive than IC units to GABA application (\( **p < 0.01 \), independent \( t \)-test). No sensitivity difference was found between dorsal and ventral subdivisions of the MGB (F). Data shown as Mean ± SE.

Fig. 3 *In vitro*: GBX induced tonic inhibition and [\(^3\)H]TBOB binding in brain slices. Whole-cell patch-clamp recordings from 5 IC and 5 MGB neurons from adult slices were used to compare the relative sensitivity of extrasynaptic GABA\(_A\)Rs in IC and MGB neurons. (A) Tonic currents were plotted against increasing dosages of GBX. GBX was significantly more potent in activating tonic currents in MGB units than in IC units (EC50\(_{MGB}\) = 2.25 µM; EC50\(_{IC}\) = 4.45 µM; F (1, 54) = 30.17, \( p < 0.0001 \); F-test). (B) Bath application of increasing doses (0.1 µM-10 µM) of the \( \delta \)- GABA\(_A\)R subunit selective agonist, GBX, evoked tonic currents in both IC and MGB neurons. Amplitudes of GBX evoked tonic currents were revealed by addition of 10 µM GBZ. (C) Modulation of [\(^3\)H]TBOB channel (picrotoxin) binding with increasing concentrations of GABA (0 nM to 5 µM) was performed on IC and MGB slices. 0 nM GABA was set as the control (100%)
condition and represented the resting/control openings of GABA<sub>A</sub>R Cl<sup>-</sup> channels. At low concentrations, both structures showed increased binding indicative of increased GABA<sub>A</sub>R, Cl<sup>-</sup> channels openings. Peak percent increase in binding occurred at 100 nM for MGB (*<i>p</i> < 0.05, two-way ANOVA) and 50 nM for IC, with a significantly larger area (shadow) under the MGB curve (black) suggesting a greater MGB neuronal total chloride flux capacity relative to IC neurons. Both MGB and IC showed desensitization (1000 nM and 5000 nM) reflecting a greater percentage of closed GABA<sub>A</sub>R Cl<sup>-</sup> channels than in the control condition.

**Fig. 4** *Ex vivo* GABA concentrations of IC and MGB. Absolute GABA levels were obtained from IC and MGB using <sup>1</sup>H-MRS. IC, vMGB and dMGB were carefully selected separately in each rat (A). (B) Combined values of left and right hemispheres were shown in average group data. Mean GABA concentrations for each structure were IC = 1.50 ± 0.29, dMGB = 3.72 ± 1.13 and vMGB = 3.05 ± 0.86. A non-significant but clear trend toward higher GABA levels was found for both vMGB and dMGB when compared to GABA levels in IC. Independent <i>t</i>-test showed a <i>p</i> value of 0.05107 (IC vs dMGB) and 0.0809 (IC vs vMGB) between IC and MGB. Scale bar = 2 mm.