Endogenous mGluR Activity Suppresses GABAergic Transmission in Avian Cochlear Nucleus Magnocellularis Neurons

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ABSTRACT

GABAergic transmission in the avian cochlear nucleus magnocellularis (NM) of the chick is subject to modulation by GABA\textsubscript{B} autoreceptors (Lu et al. 2005). Here, I investigated modulation of GABAergic transmission in NM by metabotropic glutamate receptors (mGluRs) with whole-cell recordings in brain slice preparations. I found that tACPD, a non-specific mGluR agonist, exerted dose-dependent suppression on evoked inhibitory postsynaptic currents (eIPSCs) in NM neurons. At concentrations of 100 or 200 µM, tACPD increased the failure rate of GABAergic transmission. Agonists for group I (3,5-DHPG, 200 µM), II (DCG-IV, 2 µM), and III (L-AP4, 10 µM) mGluRs produced a significant reduction in the amplitude of eIPSCs, and a significant increase in failure rate, indicating the involvement of multiple mGluRs in this modulation. The frequency, but not the amplitude, of miniature IPSCs (mIPSCs) was decreased significantly by 3,5-DHPG or DCG-IV. Neither frequency nor amplitude of mIPSCs was affected by L-AP4. mGluR antagonists LY341495 (20 µM) plus CPPG (10 µM) significantly increased the amplitude of eIPSCs, indicating that endogenous mGluR activity suppresses GABA release to NM neurons. Furthermore, blockage of mGluRs increased GABA-evoked discharges recorded under physiological Cl\textsuperscript{-} concentrations, whereas tACPD (100 µM) eliminated them. The results indicate that mGluRs play important roles in achieving balanced excitation and inhibition in NM, and preserving fidelity of temporal information encoded by NM neurons.
INTRODUCTION

The majority of neurons in the central nervous system (CNS) receive both excitatory and inhibitory synaptic inputs. Glutamate and GABA are the two most abundant neurotransmitters mediating excitation and inhibition, respectively. Balanced activation of excitatory and inhibitory pathways to the same neuron is critical not only for normal neuronal function but also for cell maintenance and survival (reviewed in Moulder et al. 2006). One of the key mechanisms to achieve balanced excitation and inhibition is through modulation of synaptic transmission by endogenous neurotransmitters via autoreceptors or heteroreceptors.

Neurons in the avian cochlear nucleus magnocellularis (NM) receive glutamatergic excitatory inputs from the auditory nerve, and GABAergic inhibitory inputs from the ipsilateral superior olivary nucleus (SON) (Burger et al. 2005; Lachica et al. 1994; Parks and Rubel 1978; Yang et al. 1999). NM neurons are similar in both morphology and function to mammalian bushy cells in the ventral cochlear nucleus, and they code precise timing information of sound stimuli (reviewed in Oertel 1999; Trussell 1999). Both the glutamatergic and GABAergic transmission in NM possess some unusual features compared to the majority of CNS neurons. The excitatory inputs to an NM neuron are mediated by only a few (1-3) large synapses called endbulbs of Held (reviewed in Ryugo and Parks 2003), one of the features ensuring high fidelity of signal transmission. Glutamate released at these synapses elicits a fast synaptic response mediated by AMPA receptors that lack GluR2 (reviewed in Parks 2000), rendering the receptors permeable to not only Na⁺ and K⁺, but also Ca²⁺. Besides ionotropic glutamate receptors, glutamate also activates metabotropic glutamate receptors (mGluRs), a class of G-protein coupled
receptors. mGluR activation in NM is believed to play an important role in maintaining calcium homeostasis of NM neurons (reviewed in Rubel and Fritzsch 2002).

Interestingly, glutamatergic transmission in NM is not subject to modulation by metabotropic glutamate autoreceptors, but is modulated by GABA$_B$ heteroreceptors via a presynaptic mechanism (Brenowitz et al. 1998; Brenowitz and Trussell 2001; Otis and Trussell 1996). This modulation enhances synaptic efficacy of the glutamatergic inputs and improves the ability of NM neurons to respond with fidelity to high frequency inputs.

GABAergic transmission in NM is unusual in that it is depolarizing but potently inhibitory (Hyson et al. 1995; Lachica et al. 1994; Lu and Trussell 2001; Monsivais and Rubel 2001). It is depolarizing because of an unusually high intracellular Cl$^-$ concentration (35-60 mM) in NM neurons even after neuronal maturation, measured with perforated patch recording techniques (Lu and Trussell 2001; Monsivais and Rubel 2001). It is potently inhibitory because GABA-induced membrane depolarization not only produces shunting inhibition, and inactivates Na$^+$ channels, but also activates a low threshold K$^+$ conductance (Monsivais and Rubel 2001). The GABAergic inputs enhance phase-locking fidelity of NM neurons in response to auditory inputs and hence improve the ability of NM neurons to code timing information of sound stimuli (Monsivais et al. 2000). The GABAergic inputs are also proposed to function as a gain control for the excitatory inputs to NM (Burger et al. 2005; Dasika et al. 2005).

However, being depolarizing in nature and having a reversal potential more positive than spike threshold (Lu and Trussell 2001; Monsivais and Rubel 2001), strong activation of the GABAergic inputs to NM does generate action potentials in slice preparations (Lu and Trussell 2001; Lu et al. 2005; Monsivais and Rubel 2001). These
GABA-induced spikes are unlikely to phase-lock to the excitatory inputs from the auditory nerve should they occur in vivo, and hence are disadvantageous for coding timing information performed by NM neurons. Two mechanisms have been shown to regulate the synaptic strength of the GABAergic transmission to NM. The first is by the low threshold K⁺ conductance present in postsynaptic NM neurons (Monsivais and Rubel 2001), and the second is by GABA_B autoreceptors (GABA_BRs) via a presynaptic mechanism (Lu et al. 2005). Activation of GABA_BRs is sufficient to eliminate GABA-induced spikes in NM. However, endogenous GABA_B activity was not observed in vitro (Lu et al. 2005), rendering uncertain the extent of modulation of GABA release by GABA_BRs under physiological conditions. Here, I investigated mGluR-mediated modulation of the GABAergic inputs to NM neurons and show that: 1) activation of mGluRs suppresses GABAergic transmission in NM; 2) multiple mGluRs are involved; 3) endogenous mGluR activity exists; and 4) mGluR activation eliminates GABA-induced spikes in NM. I propose a model regarding the dual modulation of the GABAergic transmission in NM by both metabotropic glutamate heteroreceptors and GABA_B autoreceptors.
MATERIALS AND METHODS

Slice preparation and in vitro whole-cell recordings

Fertilized chicken eggs were purchased from Purdue University (West Lafayette, IN), and some eggs were purchased from Hyline (Warren, IN). Eggs were incubated using an RX2 Auto Turner (Lyon Electric Co., Chula Vista, CA) from Embryo day 1 (E1) to E18, and a Clearview Brooder (Lyon Electric Co., Chula Vista, CA) from E19 to E21. Brainstem slices (250-300 μm in thickness) were prepared from E18-E21 chicken embryos, as described previously (Monsivais et al. 2000; Reyes et al. 1994). All the procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) at NEOUCOM. Slices were incubated for at least 1 hr in normal artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 26 NaH₂CO₃, 3 KCl, 3 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄ and 10 dextrose. ACSF was constantly gassed with 95% O₂ and 5% CO₂ (pH 7.4). For recording, slices were transferred to a 0.5 ml chamber mounted on a Zeiss Axioskop 2 FS Plus (Zeiss, Germany) with a 40X- water-immersion objective and infrared, differential interference contrast (DIC) optics. The chamber was continuously superfused with ACSF (1-2 ml/min) by gravity. The microscope was positioned on the top center of an Isolator CleanTop II and housed inside a Type II Faraday cage (Technical Manufacturing Corporation, Peabody, MA). Voltage clamp experiments were performed with an Axopatch 200B amplifier while current clamp experiments were performed with an Axoclamp 2B amplifier (Molecular Devices, Union City, CA). Recordings were performed at a temperature of 34-36°C, controlled by a Single Channel Temperature Controller TC324B (Warner Instruments, Hamden, CT).
Patch pipettes were drawn on an Electrode Puller PP-830 (Narishige, Japan) to 1-2 μm tip diameter using borosilicate glass Micropipets (inner diameter of 0.86 mm, outer diameter of 1.60 mm) (VWR Scientific, Seattle, WA). The electrodes had resistances between 3 and 7 MΩ when filled with a solution containing (in mM): 105 K-gluconate, 35 KCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 4 ATP-Mg, and 0.3 GTP-Na, with pH of 7.2 (adjusted with KOH) and osmolarity between 280 and 290 mOsm. The Cl⁻ concentration (37 mM) in the internal solutions approximated the physiological Cl⁻ concentration in NM neurons (Monsivais and Rubel 2001). Placement of the electrodes was controlled by a motorized micromanipulator MP-225 (Sutter Instrument, Novato, CA). The liquid junction potential was 10 mV, and data were corrected accordingly. Data were low-pass filtered at 3 or 5 kHz for current clamp and voltage clamp experiments, respectively. Data were then digitized with a Data Acquisition Interface ITC-18 (Instrutech, Great Neck, NY) at 20 kHz. All recording protocols were written and run using the acquisition and analysis software Axograph, version 4.9 (Molecular Devices, Union City, CA), on a PowerMac G4 computer. Means and standard deviation (SD) are reported in the text, and means and standard errors of the mean (SEM) are shown in figures.

In each voltage clamp recording, series resistance was compensated by 80 to 90%, and cells were clamped at a membrane potential of -70 mV. Before each synaptic stimulation protocol was applied, a 5 mV hyperpolarizing command (5 ms of duration) was given to monitor series resistance and input resistance during the experiment.

All chemicals and drugs were obtained from Sigma (St Louis, MO) except 3,5-Dihydroxyphenylglycine (3,5-DHPG), (2S,2'R,3'R)-2-(2’,3’Dicarboxycyclopropyl)glycine (DCG-IV), L-(+)-2-Amino-4-phosphonobutyric acid
(L-AP4), (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), and (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG), which were obtained from Tocris (Ballwin, MO). Drugs were bath-applied.

**Synaptic stimulation and recordings**

Extracellular stimulation was performed using concentric bipolar electrodes (World Precision Instruments, Sarasota, FL) with tip core diameter of 127 µm. The electrode was placed using a Micromanipulator NMN-25 (Narishige, Japan) in the fiber tract dorsal and lateral to NM. Square electric pulses of 200 µs duration were delivered through a Stimulator A320RC (World Precision Instruments, Sarasota, FL). Stimulus was a train (10 Hz) with 5 pulses at the intensity of 0.1-2.5 mA (average 0.6 mA), or 5-35 V (average 11 V). Optimal stimulation parameters were selected for each cell to give maximal amplitude of postsynaptic currents or potentials and lowest failure rate unless indicated.

Evoked inhibitory postsynaptic currents (eIPSCs) were recorded in the presence of antagonists for ionotropic glutamate receptors (50 µM DNQX plus 100 µM AP5); and 1 µM tetrodotoxin (TTX) was added when recording miniature inhibitory postsynaptic currents (mIPSCs). It has been well established from previous reports that 1) the amplitude of eIPSCs in response to repeated single-pulse synaptic stimulation varies substantially; 2) in response to train stimulation with multiple pulses, eIPSCs show a mix of facilitation and depression; 3) eIPSCs and mIPSCs reverse at a potential (-35 mV to -25 mV) more positive than spike threshold (about -45 mV); 4) both eIPSCs and mIPSCs are completely eliminated by bicuculline, a GABA<sub>A</sub>R antagonist; and 5) failures in eIPSC
recordings are failures of transmitter release as opposed to propagation of the stimulated axons (Lu and Trussell 2000, 2001; Lu et al. 2005; Monsivais and Rubel 2001).

Therefore, I used a train stimulation (10 Hz, 5 pulses) as the standard protocol to elicit eIPSCs. The train stimulation was repeated 12 times (once every 10 seconds) under each experimental condition. The 12 raw traces were averaged off-line and the peak values of eIPSCs were measured. For each averaged current trace, I normalized the baseline (about 5-10 ms before the onset of the first stimulus pulse) to zero, and then measured the peak amplitude of the first eIPSC. The same procedures of normalization and measurements of the peak eIPSC amplitudes to the 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th}, and 5\textsuperscript{th} stimulus pulses were repeated. The average of the 5 peak values was considered as 1 data point, representing the averaged eIPSC under the experimental condition. I also included failures in the calculation of averaged responses. These methods have been well established in previous study (Lu et al. 2005).

\textit{Data analysis}

Percent inhibition is defined as $100 \times \frac{\text{mean eIPSC amplitude under control condition} - \text{mean eIPSC amplitude under drug}}{\text{mean eIPSC amplitude under control condition}}$. Failures of GABAergic transmission can be readily detected by visual inspection and failure rate is defined as $100 \times \frac{\text{number of failures}}{\text{total number of stimulation pulses}}$.

Events of mIPSCs were detected by a template function using a function for product of exponentials, $f(t) = [1 - \exp(-t/\text{rise time})] \times \exp(-t/\text{decay tau})$, in which “t” stands for time and tau for time constant. The values of the parameters for the template are:
amplitude of -150 pA, rise time of 0.6 ms, decay tau of 20 ms, with a template baseline of 40 ms and a template length of 40 ms. These parameters are determined based on an average of real events, as well as on previous reports in which mIPSCs were detected using a threshold protocol (Lu et al. 2005). The detection threshold is 3 times the noise standard deviation, which detects most of the events with the least number of false-positives. The average of detected events for each cell was obtained using AxoGraph in order to measure rise time, amplitude, and decay tau.

Statistics were performed using Excel (Microsoft, Redmond, WA) and Statview (Abacus Concepts, Berkeley, CA), and graphs were made in Igor (Wavemetrics, Lake Oswego, OR). Paired t-test was used unless indicated.
RESULTS

*mGluR activation suppresses eIPSCs*

In order to determine whether GABAergic signaling in nucleus magnocellularis (NM) is modulated by metabotropic glutamate receptors (mGluRs), I examined the dose-dependent effects of a non-specific mGluR agonist (±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (tACPD) on GABA_A-mediated eIPSCs in NM neurons. Figure 1A shows 10 superimposed original (A1) and averaged eIPSC traces (A2) from a representative NM neuron obtained under the following conditions: control, tACPD (200 μM), washout of tACPD. Failures of transmission (flat lines at stimulus pulses) can be seen clearly in the original traces. Under the conditions of control and washout of tACPD, a few failures were present. During tACPD (200 μM) application, many failures of transmission were observed, and eIPSCs were smaller than those under control. Pooled data show that the normalized amplitude of eIPSCs decreased as a function of tACPD concentration (Fig. 1B). In Figures 1, 2, and 7, I normalized the averaged amplitude of eIPSCs to that of the control because eIPSCs varied widely in amplitude among individual NM neurons. Such large variations in evoked GABAergic responses in NM neurons were also documented previously (Lu and Trussell 2000; Monsivais and Rubel 2001). During application of tACPD at concentrations of 1, 10, 100, and 200 μM, the amplitude of eIPSCs was 86±30% (n=7 cells), 68±28% (n=8 cells), 23±14% (n=8 cells), and 23±11% (n=6 cells) of the control.

The average failure rates during application of tACPD at concentrations of 1, 10, 100, and 200 μM were 15±21% (n=7 cells), 19±21% (n=8 cells), 66±24% (n=8 cells), and 73±15% (n=6 cells), respectively, whereas under control conditions the failure rates
were below 20% (Fig. 1C). At concentration of 1 μM, tACPD affected neither the amplitude of eIPSCs nor the failure rate, indicating that the concentration may be too low to activate sufficient mGluRs. At 10 μM, tACPD reduced the amplitude of eIPSCs significantly without affecting the failure rate. The average failure rate was increased significantly by tACPD at concentrations of 100 and 200 μM (p<0.001). Under control conditions, failures of GABAergic transmission occurred occasionally in response to any pulses in the train stimulation (Fig. 1D and E). No significant differences were detected in failure rates in response to different pulses (ANOVA p>0.05). Application of tACPD (100 or 200 μM) increased the failure rates reliably across the stimulus pulses. Although not significant (ANOVA p>0.05), the failure rate in response to the first pulse was the highest under both control and drug conditions (Fig. 1D and E). No noticeable changes in the kinetics of eIPSCs were seen during tACPD application.

To date, there are 8 members of mGluRs identified, and they are further divided into 3 groups (group I: mGluR1 and 5; II: mGluR2 and 3; and III: mGluR4, 6, 7, and 8) based on their homology, pharmacology, and signal transduction pathways (reviewed in Kew and Kemp 2005). Specific agonists for each group have been developed. For example, group I mGluRs can be activated by an agonist 3,5-DHPG at an EC$_{50}$ of 6-60 μM. DCG-IV selectively activates group II mGluRs at an EC$_{50}$ of 0.1-0.3 μM. L-AP4 activates mGluR4, 6, and 8 at an EC$_{50}$ less than 1 μM, but much higher concentration (>100 μM) is needed to activate mGluR7 (reviewed in Cartmell and Schoepp 2000). I chose these 3 agonists at the concentration of 200, 2, and 10 μM to activate group I, II, and III mGluRs, respectively. At the chosen concentrations, 3,5-DHPG and DCG-IV are likely to activate all members of mGluRs in group I, and II, respectively, with little or no
crosstalk with other mGluRs. In contrast, L-AP4 at 10 μM is likely to activate all but one of the group III mGluR members (mGluR7) (reviewed in Schoepp et al. 1999).

Multiple mGluRs (members in groups I, II, and III) are involved in modulating GABAergic transmission at NM. This conclusion is based on the observation that each of the group agonists for mGluRs produced a significant reduction in the amplitude of eIPSCs and a significant increase in failure rates (Fig. 2). Figure 2A-C shows effects of 3 different mGluR agonists on averaged eIPSCs of 3 NM neurons. Each of the agonists substantially reduced the amplitude of eIPSCs, and at least a partial recovery of eIPSCs was seen in all cases. During application of 3,5-DHPG (200 μM), DCG-IV (2 μM), and L-AP4 (10 μM), the normalized amplitude of eIPSCs was 42±26% (n=7 cells), 11±15% (n=5 cells), and 44±24% (n=6 cells) of the control (Fig. 2D). The failure rate was increased significantly from 19±15% to 43±28% (Fig. 2E, n=7 cells) by 3,5-DHPG (200 μM), 20±24% to 84±25% (Fig. 2F, n=5 cells) by DCG-IV (2 μM), and 12±13% to 39±23% (Fig. 2G, n=6 cells) by L-AP4 (10 μM). I also examined the effects of the three specific agonists (200 μM 3,5-DHPG, 2 μM DCG-IV, and 10 μM L-AP4) for different group mGluRs on eIPSCs of NM neurons in brain slices obtained from one hatchling (P1). Each agonist caused a reduction in the amplitude of eIPSCs similar to what was observed in late embryos (data not shown).

Loci for mGluR-mediated modulation of GABAergic transmission

The GABAergic synapses onto NM neurons are too small to allow direct electrophysiological recordings from the presynaptic terminals. I examined the effects of different mGluR agonists on mIPSCs, which indirectly indicate whether mGluR-mediated
modulation of GABAergic transmission in NM is due to a presynaptic, postsynaptic, or dual mechanism. Modulation of the frequency but not the amplitude of mIPSCs would imply a presynaptic mechanism, modulation of the amplitude but not of the frequency of mIPSCs would imply a postsynaptic mechanism, and modulation of both the frequency and the amplitude of mIPSCs would imply a mechanism involving both pre- and postsynaptic elements of the synapse (e.g. Chu and Moenter 2005; Piet et al. 2003; Valenti et al. 2003). A lack of changes in the frequency and the amplitude of mIPSCs by a drug, however, does not necessarily exclude presynaptic modulation, because some drugs may influence transmission by inhibiting voltage-gated Ca^{2+} channels on presynaptic terminals, without affecting Ca^{2+} and action potential-independent release of neurotransmitters (e.g. Gereau and Conn 1995).

As shown in Figure 3, group I agonist 3,5-DHPG (200 μM) may modulate GABAergic transmission in NM through a presynaptic mechanism. Figure 3A shows representative results from 1 NM neuron, and Figures 3B-E show group data. Application of 3,5-DHPG (200 μM) did not have significant effects on kinetics of mIPSCs. The 10-90% rise times of mIPSCs under control and 3,5-DHPG conditions were 0.50±0.28 and 0.69±0.67 ms, respectively (n=9 cells, p>0.05), and decay time constants (tau) were 15.9±4.3 and 17.5±3.6 ms, respectively (n=9 cells, p>0.05). Due to its large variation between cells, the mIPSC frequency of individual cells was normalized to the value under control conditions. Application of 3,5-DHPG (200 μM) significantly reduced the frequency of mIPSCs to 80% of the control (Fig. 3B, n=9 cells). In contrast, 3,5-DHPG did not have significant effects on the mean amplitude of mIPSCs (Fig. 3C). Cumulative distributions of inter-event interval (IEI) and amplitude of mIPSCs were consistent with
the averaged data; the agonist increased IEIs (hence reduced frequency) without affecting the amplitude (Fig. 3D, E).

Group II mGluR agonist DCG-IV (2 μM) significantly reduced the frequency of mIPSCs to 75% of the control (Fig. 4A, B, n=9 cells), and did not significantly influence the amplitude of mIPSCs (Fig. 4C, n=9 cells). The curve of cumulative fraction for IEI was shifted slightly to the right by application of DCG-IV, indicating that DCG-IV decreased mIPSC frequency (Fig. 4D). Figure 4E shows that the distribution of mIPSC amplitude during DCG-IV application was nearly identical to that of control. DCG-IV did not affect the 10-90% rise time of mIPSCs (0.56±0.27 and 0.48±0.12 ms for control and DCG-IV, respectively, p>0.05). However, a small but consistent increase in decay tau was seen (19.1±4.4 and 21.8±5.5 ms, respectively, p<0.05). In contrast, group III agonist L-AP4 (10 μM) did not have significant effects on any of the parameters of mIPSCs measured (Fig. 5).

Endogenous mGluR activity suppresses GABA release at NM

To determine whether there is endogenous mGluR activity modulating GABAergic transmission in NM in vitro, I examined the effects of a cocktail of mGluR antagonists (20 μM LY341495 plus 10 μM CPPG) on eIPSCs of NM neurons. LY341495 is a potent antagonist for group II mGluRs, with IC$_{50}$ of 1.3-2.3 nM. At high concentrations, LY341495 blocks all mGluRs; the IC$_{50}$ is below 5 μM for all but one mGluRs (25 μM for mGluR4) (reviewed in Schoepp et al. 1999). CPPG is a potent antagonist for group III and II mGluRs, with an IC$_{50}$ of 2.2 nM and 46.2 nM, respectively, with little effects on group I mGluRs (Toms et al. 1996). The cocktail of
mGluR antagonists (20 μM LY341495 plus 10 μM CPPG) is likely to block the majority of mGluRs. In addition, the synaptic stimulation I used in these experiments can activate both the GABAergic pathway and the glutamatergic pathway to NM due to the fact that the fibers of these two pathways are mixed at dorsal and lateral positions around NM where the stimulating electrode was placed. Concurrent activation of glutamatergic and GABAergic pathways to NM neurons was seen in response to the same stimulus in about half (47%) of the cells (Fig. 6). Therefore, synaptically released glutamate may activate mGluRs, exerting their modulatory effects on GABA release. If this is the case, enhancement of eIPSCs by mGluR antagonists was expected.

Indeed, I observed a significant increase in the amplitude of eIPSCs by application of the cocktail of mGluR antagonists in neurons where concurrent activation of the two pathways was observed (Fig. 7). Figure 7A shows ten superimposed original current traces (A1) and averaged eIPSCs (A2) obtained under conditions of control, a cocktail of mGluR antagonists (20 μM LY341495 plus 10 μM CPPG), and washout of the antagonists. Application of the antagonists increased the amplitude of eIPSCs, and a nearly complete recovery of the responses was seen after the washout. The eIPSC amplitude was -214±82, -327±152, and -217±106 pA for conditions of control, the cocktail of mGluR antagonists, and washout, respectively. Grouped data show that the cocktail of mGluR antagonists significantly increased the amplitude of eIPSCs; the antagonists increased the normalized amplitude of eIPSCs to 155±48% of the control, and after washout the eIPSCs returned to 102±37% of the control (Fig. 7B, ANOVA p<0.05, n=7 cells). Post hoc Fisher’s analyses revealed significant differences in eIPSC amplitude between control and antagonists, and between washout and antagonists (Fig. 7B).
The average failure rate of GABAergic transmission was not affected by the cocktail of mGluR antagonists (28±18, 15±15, and 26±21% for control, drugs, and washout, respectively) (Fig. 7C). Consistent with previous observations (Fig. 1D and E), failures of GABAergic transmission occurred in response to any pulses in the train stimulation, and the failure rate to the first pulse is the highest under the same experimental condition. No significant differences in failure rates at any pulses were detected among control, mGluR antagonists, and washout (Fig. 7D, ANOVA p>0.05).

Both individual eIPSC traces (Fig. 7A) and pooled data (Fig. 7E) seem to show a mix of facilitation and suppression in response to the train stimulation, which was also reported by Lu and Trussell (2000). However, statistical analyses did not reveal significant differences in amplitude of eIPSCs elicited by the five individual pulses, under conditions of either control or mGluR antagonists (Fig. 7E, ANOVA p>0.05, n=7 cells). To test whether averaging all IPSCs obscured a differential effect of mGluR antagonists on individual eIPSCs, I performed a statistical analysis to compare the relative changes (in percentage) in eIPSCs in response to the five individual pulses. Results showed no significant differences (Fig. 7F, ANOVA p>0.05). In addition, although failure rate tended to decline during the stimulus train, no significant differences in failure rate were detected across individual pulses under conditions of control, or mGluR antagonists, or washout (Fig. 7D, ANOVA p>0.05); this was also observed in Figure 1 (Fig. 1D, E).

*Effects of mGluR agonist and antagonist on GABA-evoked firing*
I used the current clamp configuration to study the effects of activating or blocking mGluRs on action potentials (APs) generated in NM neurons by stimulation of the GABAergic pathway. Glutamate spikes were blocked by antagonists for ionotropic glutamate receptors (iGluRs) (50 μM DNQX and 100 μM AP5). GABA_A-mediated APs were observed in 14 out of 26 (54%) NM neurons (55 mM Cl⁻ in recording pipettes). I used a high Cl⁻ concentration in the recording pipettes in order to enhance the probability of obtaining NM neurons that generate GABA-induced spikes. The Cl⁻ concentration of 55 mM is within the physiological range (35-60 mM) for NM neurons (Lu and Trussell 2001; Monsivais and Rubel 2001). The results of mGluR agonist and antagonists on APs in these cells are shown in Figure 8. Figure 8A shows representative membrane potential traces obtained under the conditions of control, mGluR agonist tACPD (100 μM), washout for 3 min, and washout for 8 min of the same NM neuron. Application of tACPD (100 μM) almost completely shut down the GABAergic transmission and eliminated all spikes in this NM neuron. In contrast, application of the cocktail of mGluR antagonists (20 μM LY341495 plus 10 μM CPPG) increased GABA-induced firing in another neuron (Fig. 8B). Figure 8C shows that tACPD (100 μM) eliminates GABA-induced APs completely in 8 out of 9 cells (open circles). In the remaining cell, tACPD largely reduced the AP probability (filled circles). This spike probability is an average over the whole stimulus train. On average, the application of mGluR agonist tACPD caused a significant decrease (98%) in the firing probability (paired t-test p<0.001, n=9 cells). In contrast, mGluR antagonists LY341495 (20 μM) plus CPPG (10 μM) increased AP probability in 5 out of 6 cells (Fig. 8D). In the remaining cell, the application of mGluR antagonists did not change the neuron’s AP probability. On average, the application of mGluR antagonists
caused a moderate but significant increase (16%) in the firing probability (paired t-test p<0.05, n=6 cells).

It is unlikely that the effects of tACPD on GABA-induced APs are due to a change in the excitability of NM neurons because mGluR activation by 1S,3R-ACPD (200 μM, the active isomer of tACPD) does not affect the excitability of NM neurons in animals of the same age (Lu and Rubel 2005). The effects of mGluR antagonists on GABA-induced APs are also not due to a change in NM excitability. This conclusion is based on experiments in which a series of brief current commands (with increments of 0.05 nA, and 2 ms in duration) were applied to NM neurons before and during application of mGluR antagonists (10 repetitions of the current injection protocol under each condition). Then I plotted AP probability against amplitude of injected currents in order to measure the threshold current. The threshold current, defined as the current needed to elicit APs at a probability of 50% and used as a measurement of neuronal excitability, was 1.24±0.39 nA and 1.22±0.37 nA for control and after application of mGluR antagonists, respectively (n=7 cells, p>0.05), and resting membrane potential (RMP) under these two conditions was -67.3±3.4 mV and –64.7±3.5 mV, respectively (p>0.05, Table 1).

I measured several basic parameters of GABA-induced APs in NM neurons prior to and after washout of tACPD, and prior to and after application of mGluR antagonists. The parameters include RMP, latency (the time period between the onset of the stimulus and the onset of the response), AP threshold, AP height (difference between the threshold and the peak value), and AP half-width (duration at half AP height). No significant differences were detected in any of these parameters (Table 1).
DISCUSSION

Compared to the conventional GABAergic inhibition in the majority of CNS neurons where activation of the GABAergic pathway leads to membrane hyperpolarization mediated by GABAA receptors, the GABAergic inputs to the avian cochlear nucleus magnocellularis (NM) exert more potent inhibitory effects by making the postsynaptic neuronal membrane depolarizing (Monsivais and Rubel 2001). This strong inhibitory input to NM neurons may better serve to counteract the fast, powerful, and highly active glutamatergic excitatory transmission to these cells. Given the importance of the GABAergic inhibitory input to NM neurons, modulation of this input by neurotransmitters is critical in setting the dynamic range of its synaptic strength. In the discussion below, I focus on three aspects of the results: 1) involvement of multiple mGluRs in modulation of GABA release in NM neurons; 2) possible mechanisms (action loci); and 3) possible functional roles of this heterosynaptic modulation of GABAergic transmission in the avian auditory brainstem.

Involvement of multiple mGluRs in modulation of GABA release in NM

Each of the agonists for mGluRs (3,5-DHPG, DCG-IV, and L-AP4 for group I, II, and III, respectively) significantly reduced eIPSCs of NM neurons, indicating involvement of multiple mGluRs in the modulation, similar to some previous studies (Drew and Vaughan 2004; Zheng and Johnson 2003). I chose the concentrations of 200, 2, and 10 μM for 3,5-DHPG, DCG-IV, and L-AP4, respectively. These concentrations are at least three times their EC50 (reviewed in Cartmell and Schoepp 2000; Schoepp et al. 1999). Group II mGluR agonist DCG-IV produced the largest reduction in the
amplitude of eIPSCs (Fig. 2D) and the highest failure rate of GABAergic transmission (Fig. 2F). I speculate that group II mGluRs may play a larger role than other mGluRs in modulating GABA release in NM neurons. Two scenarios may account for involving multiple mGluRs in modulating GABAergic transmission in these neurons. First, mGluRs of different groups may be clustered at different membrane loci, exerting modulation of GABA release via specific signaling pathways depending on the availability of neurotransmitter glutamate. Second, the density of each group mGluRs on the presynaptic membrane may be low so that multiple members from different groups need to be activated simultaneously in order to generate sufficient regulation of GABA release. It would be interesting to complement this work with immunohistochemical studies to show the expression pattern of multiple mGluRs on the inhibitory terminals impinging onto NM neurons.

One concern is about the specificity of the agonists I used for different group mGluRs. Eight mammalian mGluRs have been cloned. To my knowledge, there is no avian mGluR that has been cloned. Although at the molecular level it is unknown how similar avian mGluRs are to mammalian ones, some studies do suggest conservation and similarity in mGluRs between birds and mammals. Antibodies against mGluR1 or mGluR5 (both are group I members) raised in mammals recognize proteins of the appropriate molecular weight in chicken brain and retina, suggesting similarity of group I mGluRs in birds and mammals (Kreimborg et al. 2001). The three agonists used in the current study (3,5-DHPG, DCG-IV, and L-AP4) targeting specific group mGluRs have been used in birds, and showed selectivity on avian mGluRs (Caramelo et al. 1999; Dutar et al 1999; Gomes et al. 2004; Kreimborg et al. 2001; Lu and Rubel 2005; Sampaio and
Paes-de-Carvalho 1998; Tasca et al. 1999). The specificity of these drugs is well demonstrated in one study, which shows that mGluRs modulate acetylcholine (ACh) release from cultured amacrine-like neurons in the chick retina (Caramelo et al. 1999). Group III agonist L-AP4 exerted a dose-dependent modulation on ACh release, with an IC$_{50}$ of about 4 μM. In contrast, group I agonist 3,5-DHPG (50 μM) and group II agonist DCG-IV (1 μM) had no effects. The authors concluded that group III, not groups I or II mGluRs, mediate the modulatory effects. Interestingly, 3,5-DHPG or DCG-IV prevented the effects of L-AP4 on ACh release, indicating that group I and II mGluRs do exist in the same system and can be activated by 3,5-DHPG and DCG-IV, respectively (Caramelo et al. 1999). These results not only demonstrate crosstalks between groups I and III, and groups II and III mGluRs, but also suggest that the three agonists are specific for respective group mGluRs in the chick.

*Mechanisms for modulation of GABA release by multiple mGluRs in NM*

In NM, group I mGluR agonist 3,5-DHPG reduced the frequency but not the amplitude of mIPSCs, suggesting a presynaptic action. Given the known signal transduction pathway (phospholipase C pathway) for group I mGluRs and their expression loci (primarily in postsynaptic cells) (reviewed in Cartmell and Schoepp 2000; Schoepp 2001), this result was somewhat surprising. However, modulation of GABAergic transmission by group I mGluRs is indeed heterogeneous in that the modulation can occur at either presynaptic or postsynaptic loci, and the consequence of the modulation can be either suppression or enhancement of GABA release, depending on cell type (Chu and Hablitz 1998; Cozzi et al. 2002; Galante and Diana 2004; Gereau

In NM, group II agonist DCG-IV produced inhibition of eIPSCs, and a reduction in the frequency but not the amplitude of mIPSCs, indicating a presynaptic mechanism. This is consistent with previous reports that modulation of GABAergic transmission by group II mGluRs is relatively homogenous (reduction in transmitter release via presynaptic mechanisms) (Chu and Moenter 2005; Doi et al. 2002; Llano and Marty 1995), and consistent among different systems (reviewed in Cartmell and Schoepp 2000; Schoepp 2001).

Group III mGluR agonist L-AP4 also significantly reduced the amplitude of eIPSCs of NM neurons, and increased failure rate of GABAergic transmission, suggesting a presynaptic action. Such a modulation of GABAergic transmission by presynaptic group III mGluRs has been extensively studied in many systems such as hippocampus (Kogo et al. 2004; Semyanov and Kullmann; 2000), globus pallidus (Matsui and Kita 2003), and supraoptic nucleus (Panatier et al. 2004). However, L-AP4 had no significant effects on mIPSCs of NM neurons. This discrepancy, reported previously in dopamine neurons (Giustizieri et al. 2005; Zheng and Johnson 2003), might be interpreted by differential effects of different mGluRs on upstream and downstream events of Ca$^{2+}$ influx through voltage-gated calcium channels (VGCCs).

VGCCs are subject to modulation by mGluRs (reviewed in Catterall 2000; Stefani et al. 1996). Since evoked transmitter release is dependent on Ca$^{2+}$ influx through VGCCs in presynaptic terminals, modulation of VGCCs by mGluRs is highly likely to account for mGluR-mediated regulation of GABA release. In the postsynaptic NM
neurons, each of the mGluR agonists used in the present study leads to inhibition of VGCCs (Lu and Rubel 2005). If this is the case at the presynaptic GABAergic terminals, reduction in GABA release is expected by each of the mGluR agonists. Since group I and II mGluR agonists also affected mIPSCs which are believed to be independent of Ca$^{2+}$ entry via VGCCs, some additional mechanisms for modulation mediated by these receptors might exist. Group III agonist L-AP4 reduced eIPSCs and increased failure rate of GABAergic transmission at NM without affecting mIPSCs, suggesting a presynaptic action on VGCCs only.

Dual modulation of GABAergic transmission in NM: functional significance

Several questions can be raised regarding the dual modulation of GABAergic transmission by both autoreceptors and heteroreceptors in NM neurons: 1) how likely is it for synaptically released glutamate to reach and activate mGluRs on the inhibitory terminals? 2) GABA$_B$Rs modulate glutamate release and mGluRs modulate GABA release at NM, what is the role for such a reciprocal regulation of transmitter release? and 3) why the system needs a seemingly redundant dual control on its inhibitory inputs?

Figure 9 shows a highly simplified schematic diagram of the circuitry formed between the brainstem auditory nuclei in the chick, and a hypothetical model to account for dual modulation of the GABAergic transmission in NM. For heterosynaptic glutamate modulation of GABAergic transmission to occur, synaptically released glutamate needs to “spillover” to activate mGluRs on the inhibitory terminals. Since synaptically released glutamate is actively transported across the cell membrane, it is normally difficult for glutamate molecules to reach mGluRs at distance in order to initiate such crosstalk.
between two distinct types of synapses (Barbour and Häusser 1997). However, some synaptic geometry may favor “spillover” of glutamate to nearby synapses, such as those at NM. NM neurons have only a few short bushy-like dendrites, and each NM neuron receives only a few (1-3) large glutamatergic synapses from the auditory nerve (Reviewed in Ryugo and Parks 2003). The presynaptic terminals cover the majority (~60%) of the somata area (Parks 1981). GABAergic terminals form bouton-like puncta synapses which also impinge onto NM somata, presumably located nearby glutamatergic terminals (Lachica et al. 1994). These morphological features favor interactions between the glutamatergic and GABAergic pathways to NM (Brenowitz et al. 1998; Brenowitz and Trussell 2001; Otis and Trussell 1996; Otis et al. 1996; and present study). Moreover, glutamate released from other sources such as from glial cells may participate in keeping an ambient level of glutamate around the inhibitory terminals (reviewed in Belan and Kostyuk 2002).

Regulation of synaptic strength of both the glutamatergic and GABAergic inputs to NM is critical for NM neuronal function. GABA inhibits glutamate release at a presynaptic locus via metabotropic GABA\(_B\)Rs (Brenowitz et al. 1998; Brenowitz and Trussell 2001; Otis and Trussell 1996). Neurons in the superior olivary nucleus (SON), driven by excitatory inputs from nucleus laminaris (NL) and nucleus angularis (NA), probably fire in a sound level dependent manner (Burger et al. 2005; Dasika et al. 2005). That is, stronger excitatory glutamatergic inputs to the avian cochlear nucleus lead to stronger feedback inhibitory inputs. On the other hand, strong activation of the GABAergic pathway could disrupt phase-locking fidelity by generating GABA spikes. Activation of mGluRs on inhibitory terminals suppresses GABAergic transmission,
preventing excitatory action of GABA. Hence, even though they have functionally opposite ligands, the two synaptic pathways may achieve balanced excitatory and inhibitory inputs to NM through reciprocal inhibition of transmitter release via their metabotropic receptors.

Two scenarios are proposed here to account for dual modulation of GABAergic transmission in NM (Fig. 9B). In the first scenario, I consider an orderly temporal sequence for activation of mGluRs and GABA\(\text{B}_\text{Rs}\) in modulating GABAergic transmission. As illustrated in Figure 9A, following a sound stimulus, the feedback GABAergic input to an NM neuron lags in timing behind the glutamatergic excitatory input from the auditory nerve. However, spillover of synaptically released glutamate can be detected by mGluRs on the GABAergic inhibitory terminals. Activation of mGluRs may preset a limit for the amount of GABA release in response to the sound stimulus. After the release of synaptic vesicles containing GABA, activation of presynaptic GABA\(\text{B}_\text{Rs}\) further exerts regulation on GABA release.

The second scenario, in contrast, predicts tonic activation of presynaptic mGluRs on the GABAergic terminals. In a slice preparation, NM neurons do not fire action potentials spontaneously. However, they are highly active in vivo, with a spontaneous firing rate of 50-100 Hz, and even higher rates when sound stimuli are present (e.g. Fukui et al. 2006; Warchol and Dallos 1990). High-rate activity may well delay the clearance of glutamate (Scanziani et al. 1997). It is likely that the neurotransmitter glutamate is present relatively constantly in the synaptic cleft and surrounding areas at a level that is sufficient in activating mGluRs and exerting a tonic regulation on GABA release, as reported by other investigators (Chu and Moenter 2005; Kullmann and Semyanov 2002; Mitchell and
Silver 2000; Schrader and Tasker 1997; van den Pol et al. 1998). GABA$_B$ autoreceptors may be recruited as intensive GABA release occurs, functioning as a use-dependent, negative feedback mechanism. In other words, mGluRs provide a tonic control whereas GABA$_B$Rs provide a feedback control of GABA release. In either of these two scenarios, GABA$_B$Rs and mGluRs work in concert with each other to provide dual modulation of the mechanistically unusual and functionally important depolarizing GABAergic transmission in NM, ensuring high fidelity for the relay of temporal information of sound to higher order auditory neurons.
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LITERATURE CITED


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FIGURE LEGENDS

Figure 1. Effects of tACPD, a non-specific mGluR agonist, on the amplitude of evoked inhibitory postsynaptic currents (eIPSCs) and the failure rate of GABAergic transmission in nucleus magnocellularis (NM). A: ten superimposed original current traces (A1) and their averaged eIPSCs (A2) obtained under conditions of control, tACPD (200 μM), and washout of the agonist. The train stimulus (10 Hz, 5 pulses) is shown below the original traces. Note that many failures of transmission are present during tACPD (200 μM) application. The same stimulus protocol was used to generate data shown in Figures 2, 6, 7, and 8. Stimulus artifacts in these figures are blanked or truncated for clarity. Note different scales for the original traces and the averaged traces. B: normalized amplitude of eIPSCs decreases as a function of tACPD concentration (n=7, 8, 8, and 6 cells for 1, 10, 100, and 200 μM, respectively). C: the overall failure rate is significantly increased by 100 or 200 μM tACPD. At lower concentrations (1 or 10 μM), tACPD does not have significant effects on the failure rate. D and E: failure rates plotted against stimulus pulse numbers under conditions of control and tACPD (100 or 200 μM). No significant differences were detected in failure rates under conditions of control and tACPD (ANOVA p>0.05). In this and subsequent figures (Figs. 1-7), I show means ± 1 standard error. Note that means and standard deviations are reported in the text. Also in this and subsequent figures, *, **, and *** indicate p<0.05, p<0.01, and p<0.001, respectively (paired t-test unless indicated). The cells were held at a membrane potential of –70 mV for voltage clamp experiments (Figs. 1-7).
**Figure 2.** Multiple mGluRs (members in groups I, II, and III) are involved in modulating GABAergic transmission in NM. **A-C**: representative averaged eIPSCs obtained under conditions of control, group I mGluR agonist (3,5-DHPG, 200 μM), group II mGluR agonist (DCG-IV, 2 μM), group III mGluR agonist (L-AP4, 10 μM), and washout of the agonists (see Fig. 1 for recording conditions). At least partial recovery of the responses was seen after washout of each of the agonists. The scale bars apply to A, B, and C. Data were obtained from 3 different cells. **D**: normalized amplitude of eIPSCs under conditions of control and specific group mGluR agonists (n=7, 5, and 6 cells for 3,5-DHPG, DCG-IV, and L-AP4, respectively). Each of the agonists produces significant inhibition of eIPSCs. ctr: control; DHPG: 3,5-DHPG; DCG: DCG-IV. **E-G**: the failure rate of GABAergic transmission is increased significantly by application of each of the mGluR agonists.

**Figure 3.** Effects of group I mGluR agonist (3,5-DHPG, 200 μM) on miniature inhibitory postsynaptic currents (mIPSCs) of NM neurons. **A**: a representative example in which mIPSCs were recorded before and during 3,5-DHPG application. Shown on the right are the averaged traces of all detected events of the sample cell. Note different scale bars for the original and the averaged traces. **B**: application of 3,5-DHPG (200 μM) significantly reduces the frequency of mIPSCs (p<0.05, n=9 cells). **C**: in contrast, 3,5-DHPG does not have significant effects on the mean amplitude of mIPSCs (p>0.05). **D and E**: cumulative distribution of inter-event interval (IEI) and amplitude of mIPSCs (1145 and 952 events for control and 3,5-DHPG, respectively). For clarity, cumulative
fractions smaller than 0.90 are not shown, and the portions of the two curves that are not shown overlap each other closely.

**Figure 4.** Effects of group II mGluR agonist (DCG-IV, 2 μM) on mIPSCs of NM neurons. A-C: for figure legends, see Figure 3. Application of DCG-IV (2 μM) significantly reduces the frequency of mIPSCs (p<0.01, n=9 cells), without affecting the mean amplitude of mIPSCs. D and E: cumulative distribution of IEI and amplitude of mIPSCs (1081 and 809 events for control and DCG-IV, respectively). For clarity, cumulative fractions smaller than 0.90 are not shown, and the portions of the two curves that are not shown approximately parallel each other in D, and overlap each other closely in E.

**Figure 5.** Effects of group III mGluR agonist (L-AP4, 10 μM) on mIPSCs of NM neurons. A-C: for figure legends, see figure 3. Application of L-AP4 (10 μM) does not have significant effects on the frequency or the mean amplitude of mIPSCs (n=9 cells). D and E: cumulative distribution of IEI and amplitude of mIPSCs (1083 and 1093 events for control and L-AP4, respectively). For clarity, cumulative fractions smaller than 0.90 are not shown, and the portions of the two curves that are not shown overlap each other closely.

**Figure 6.** Concurrent activation of glutamatergic and GABAergic pathways to NM neurons. Stimulation electrode was placed dorsal and lateral to NM, a location where the auditory nerve and fibers originating from the superior olivary nucleus that project to NM
neurons are mixed. Under control conditions, a large and rapid EPSC and a smaller and slower IPSC were recorded in response to the same electrical stimulus. Antagonists for ionotropic glutamate receptors (50 μM DNQX plus 100 μM AP5) eliminated the EPSC. The stimulus artifacts are truncated. Note that a train stimulus (10 Hz, 5 pulses) was used to elicit the responses, but only the responses to one stimulus pulse are shown for clarity.

**Figure 7.** Blockage of mGluRs enhanced eIPSCs of NM neurons, indicating that synaptically released glutamate suppresses GABAergic transmission in NM.  

**A:** ten superimposed original current traces (A1) and averaged eIPSCs (A2) obtained under conditions of control, a cocktail of mGluR antagonists (20 μM LY341495 plus 10 μM CPPG), and washout of the antagonists. A nearly complete recovery of the responses is seen after the washout. Note that many stimulus-unlocked synaptic events, probably due to spontaneous and/or asynchronized GABA release, are present in the recordings of this cell under all three conditions. Noticeably more synaptic failures are seen under the conditions of control or wash than during application of the mGluR antagonists.  

**B:** the cocktail of mGluR antagonists (antag) significantly increases the normalized amplitude of eIPSCs (n=7 cells, ANOVA p<0.05). Post hoc Fisher’s analyses revealed significant differences in normalized eIPSC amplitude between control and antagonists, and between wash and antagonists.  

**C:** the mGluR antagonists did not significantly affect the overall failure rate of GABAergic transmission.  

**D:** failure rates plotted against stimulus pulse numbers under conditions of control, mGluR antagonists, and wash. No significant differences among the three groups are detected in failure rates in response to any stimulus pulses (ANOVA, p>0.05).  

**E:** Statistical analyses did not reveal significant differences in
amplitude of eIPSCs elicited by the five individual pulses, under conditions of either control or mGluR antagonists (ANOVA p>0.05). **F:** The relative changes (in percentage) in the amplitude of eIPSCs induced by the antagonists showed no significant differences across stimulus pulses (ANOVA p>0.05). Note that the relative changes were calculated based on eIPSC amplitude of individual cells, not the average eIPSC amplitudes shown in E.

**Figure 8.** Effects of mGluR agonist and antagonists on GABA-induced action potentials (APs) in NM neurons. Cells were recorded under current clamp mode and were held at their resting membrane potential (RMP). **A:** representative membrane potential traces obtained under the conditions of control, mGluR agonist tACPD (100 μM), washout of tACPD for 3 min, and washout for 8 min. The inset shows the first spike (indicated by the asterisk in the original trace under control condition) at an expanded time scale. Application of tACPD (100 μM) reduces the amplitude of evoked inhibitory postsynaptic potentials (eIPSPs) and eliminated all spikes in this neuron (RMP: -68 mV). Stimulus artifacts are truncated for clarity. **B:** representative membrane potential traces obtained under the conditions of control, a cocktail of mGluR antagonists LY341495 (20 μM) plus CPPG (10 μM), and washout of the antagonists. Application of mGluR antagonists increases GABA-induced firing in this neuron (RMP: -66 mV). **C:** tACPD (100 μM) completely eliminates GABA-induced APs in 8 out of 9 cells (open circles). In the remaining cell, tACPD largely reduces the AP probability (filled circles). **D:** mGluR antagonists LY341495 (20 μM) plus CPPG (10 μM) increase AP probability in 5 out of 6 cells (open circles). In the remaining cell, the application of mGluR antagonists did not
change the neuron’s AP probability (filled circles). The application causes a moderate but consistent increase in the firing probability (16% in average).

**Figure 9.** Schematic diagrams showing dual modulation of the GABAergic transmission in NM by heteroreceptors (mGluRs) and autoreceptors (GABA_BRs). **A:** a simplified neural circuitry among the brainstem auditory nuclei in the chick. Once entering the brainstem, the auditory nerve bifurcates. One branch innervates the nucleus angularis (NA), a nucleus important in coding intensity information of sound. The other branch of the auditory nerve innervates NM. NM neurons in turn send their axons ipsilaterally to innervate the dorsal dendrites of neurons in the nucleus laminaris (NL), the third order neurons in the avian central auditory system that compute interaural time differences and generate cues for sound localization. Axons of NM neurons also cross the midline of the brainstem and innervate the ventral dendrites of NL neurons (not shown). Neurons in the superior olivary nucleus (SON) are primarily GABAergic and are driven by excitatory inputs from NA and NL. SON cells send inhibitory inputs to NM, NL, and NA (for simplicity, only the projection to NM is shown here). **B:** dual modulation of GABAergic transmission by mGluRs and GABA_BRs. As illustrated in A, to a given sound stimulus, the GABAergic inhibitory input to an NM neuron lags behind the glutamatergic excitatory input which directly impinges onto the cell. Endogenous activation of mGluRs may set the upper limit for the amount of GABA release in response to sound stimulation. Activation of presynaptic GABA_BRs further exerts regulation on GABA release. Alternatively, a tonic activation of presynaptic mGluRs on the inhibitory terminals may exist due to the high-rate activity of glutamatergic pathway to NM, exerting a tonic
modulation of GABA release. GABA_BRs may be activated as intense GABA release occurs, functioning as a use-dependent, negative feedback mechanism. These two mechanisms (autoregulation by GABA_BRs, and heteroregulation by mGluRs) work in concert with each other, providing dual modulation on the mechanistically unusual and functionally important depolarizing GABAergic transmission in NM. Note that arrows in A and B indicate excitatory pathways and activation of receptors, respectively.
Table 1. Basic properties of GABA-induced action potentials (AP) in NM neurons under control condition, after washout of tACPD (100 μM), and during the application of a cocktail of mGluR antagonists

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Wash tACPD</th>
<th>t-test p value</th>
<th>Control</th>
<th>Antagonists</th>
<th>t-test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-66.1 ± 3.6</td>
<td>-63.3 ± 3.8</td>
<td>&gt; 0.05</td>
<td>-67.3 ± 3.4</td>
<td>-64.7 ± 3.5</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>1.32 ± 0.96</td>
<td>1.45 ± 0.91</td>
<td>&gt; 0.05</td>
<td>1.55 ± 1.00</td>
<td>1.46 ± 0.95</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>-43.7 ± 4.0</td>
<td>-40.6 ± 5.2</td>
<td>&gt; 0.05</td>
<td>-43.5 ± 3.9</td>
<td>-43.5 ± 2.9</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AP height (mV)</td>
<td>27.5 ± 9.9</td>
<td>28.1 ± 10.6</td>
<td>&gt; 0.05</td>
<td>28.2 ± 5.0</td>
<td>28.4 ± 5.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AP half width (ms)</td>
<td>0.29 ± 0.09</td>
<td>0.25 ± 0.06</td>
<td>&gt; 0.05</td>
<td>0.24 ± 0.03</td>
<td>0.26 ± 0.04</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
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Means and standard deviations are shown. No significant differences were detected in any of the parameters.

Note that tACPD (100 μM) was applied to 9 NM neurons, and washout data were obtained in only 7 cells.

Antagonists: LY341495 (20 μM) plus CPPG (10 μM); RMP: resting membrane potential.
Fig. 2
Fig. 3

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single column
Fig. 4

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single column
Fig. 5

Lu 2006
single column
Fig. 6

Lu 2006
single column
Fig. 7

Lu 2006
Single column
Fig. 8

Lu 2006

single column