Published in final edited form as:

Hear Res. 2017 March ; 346: 14–24. doi:10.1016/j.heares.2017.01.011.

Metabotropic glutamate and GABA receptors modulate cellular excitability and glutamatergic transmission in chicken cochlear nucleus angularis neurons

Wei Shi^{1,2,3,*} and Yong Lu^{1,2,*}

¹Department of Anatomy and Neurobiology, College of Medicine, Northeast Ohio Medical University Rootstown, OH 44272, USA

²School of Biomedical Sciences, Kent State University Kent, OH 44240, USA

³School of Life Science, Tsinghua University Beijing, 100084, P.R China

Abstract

Neurons in the avian cochlear nucleus angularis (NA) receive glutamatergic input from the auditory nerve, and GABAergic input from the superior olivary nucleus. Physiologically heterogeneous, NA neurons perform multiple functions including encoding sound intensity information. Using *in vitro* whole-cell patch recordings from acute brain slices and immunohistochemistry staining, we investigated neuromodulation mediated by metabotropic glutamate and GABA receptors (mGluRs and GABA_BRs) in NA neurons. Based on their intrinsic firing patterns in response to somatic current injections, NA neurons were classified into one-spike (onset), damped, and tonic cells. Pharmacological activation of group II mGluRs, group III mGluRs, and GABA_BRs, by their respective agonists, suppressed the cellular excitability of non-onset firing NA neurons. Each of these agonists inhibited the glutamatergic transmission in NA neurons, in a cell type-independent manner. The frequency but not the amplitude of spontaneous release of glutamate was reduced by each of these agonists, suggesting that the modulation of the glutamatergic transmission was via presynaptic actions. Interestingly, activation of group I mGluRs increased cellular excitability and suppressed glutamatergic transmission in non-onset neurons. These results elaborate that auditory processing in NA neurons is subject to neuromodulation mediated by metabotropic receptors activated by native neurotransmitters.

*Correspondence to: Wei Shi, shiwei@mail.tsinghua.edu.cn, Yong Lu, Telephone: 330-325-6656, Facsimile: 330-325-5916, ylu@neomed.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Compliance with Ethical Standards

The experimental procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) at Northeast Ohio Medical University, and are in accordance to NIH policies on animal use.

Conflict of Interest Statement

The authors declare no competing financial interests.

Author Contributions

Y.L. conceived and supervised the study; W.S. and Y.L. designed the experiments; W.S. performed the research and analyzed data; W.S. and Y.L. wrote the paper.

Keywords

mGluR; GABA_BR; nucleus angularis; neuromodulation; cellular excitability; synaptic transmission

1. Introduction

The avian cochlear nucleus has two anatomically and physiologically distinct components, the nucleus magnocellularis (NM) and nucleus angularis (NA), forming separate central auditory pathways encoding timing and intensity information of sound, respectively (Sullivan and Konishi, 1984). NM neurons send excitatory inputs bilaterally to the nucleus laminaris (NL) where interaural time difference (ITD) is first computed, whereas NA neurons send excitatory inputs to the contralateral dorsal nucleus of lateral lemniscus (LLD, formerly VLVp, the nucleus ventralis lemnisci lateralis pars posterior) where interaural level difference (ILD) is first encoded (review in MacLeod and Carr, 2007). NA neurons receive both excitatory and inhibitory inputs. The excitatory inputs from the auditory nerve use glutamate as the neurotransmitter. Excitatory postsynaptic currents (EPSCs) of NA neurons are graded, small in amplitude, and are mediated by both AMPA and NMDA receptors (MacLeod and Carr, 2005). The inhibitory inputs to NA originate primarily from the superior olivary nucleus (SON), using both GABA and glycine as the neurotransmitters. Activation of ionotropic GABA and glycine receptors gives rise to depolarizing inhibition due to a relatively high intracellular chloride concentration in NA neurons (Kuo et al., 2009). Besides activating ionotropic receptors that mediate fast neural responses, glutamate and GABA also activate metabotropic receptors that exert neuromodulation on intrinsic and synaptic neuronal properties, regulating cellular excitability, spike timing, and signal processing (review in Niswender and Conn, 2010; Ulrich and Bettler, 2007). Neuromodulatory function of metabotropic glutamate and GABA receptors (mGluRs and GABA_BRs) in the avian timing-coding pathway (NM and NL) has been extensively elucidated (review in Lu, 2014). In contrast, the roles of neuromodulation in the intensity-coding pathway (NA and LLD) remain completely unknown.

The majority of NA neurons are believed to be multi-functional including encoding intensity of sounds (Köppl and Carr, 2003). While some NA neurons possess monotonic rate-intensity functions, in which spike discharge rate increases with increasing sound levels, others have non-monotonic functions (Warchol and Dallos, 1990; Sato et al., 2010). This is in marked contrast with NM neurons, all of which have monotonic rate-intensity functions (Warchol and Dallos, 1990). NA neurons also have a larger dynamic range (the range of sound intensity within which a neuron fires spikes between 5% and 95% of its maximal spike rate) than NM neurons, suggesting high sensitivity of NA neurons to changes in sound levels (Warchol and Dallos, 1990; but also see Köppl and Carr, 2003). This property of NA neurons points to the possibility of mGluR autoreceptor-mediated modulation of synaptic excitation in NA, while such auto-regulation is not detected in NM neurons (Otis and Trussell, 1996; Tang et al., 2013). Our working hypothesis is that activation of mGluRs or GABA_BRs modulates glutamate transmission and cellular excitability in NA neurons, and the modulation may be cell type dependent given the heterogeneity of NA neurons. Using *in*

vitro whole-cell patch recordings from acute brain slices, we showed that activation of either mGluRs or GABA_BRs by exogenous agonists suppressed glutamate transmission and regulate cellular excitability of NA neurons. Contrary to our expectation, neuromodulation in NA neurons was not cell type dependent except for the action of group I mGluRs on synaptic excitation, suggesting a relatively universal modulatory role of these receptors in the sound intensity-coding pathway in birds. These modulatory actions may dynamically regulate the input-output functions of NA neurons, providing proper intensity information of sounds to the LLD for ILD encoding.

2. Methods

2.1. Slice preparation and *in vitro* whole-cell recordings

Brainstem slices (250–300 μ m in thickness) were prepared from chick embryos (E17–19), as described previously (Tang et al., 2009), with modification of the components of the artificial cerebrospinal fluid (ACSF) used for dissecting and cutting the brain tissue. The ACSF used for dissecting and slicing the brain tissue contained (in mM): 250 glycerol, 3 KCl, 1.2 KH₂PO₄, 20 NaHCO₃, 3 HEPES, 1.2 CaCl₂, 5 MgCl₂, and 10 dextrose, pH 7.4 when gassed with 95% O₂ and 5% CO₂. The procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) at Northeast Ohio Medical University, and are in accordance to NIH policies on animal use. Slices were incubated at 34–36°C for >1 h in normal ACSF containing (in mM): 130 NaCl, 26 NaHCO₃, 3 KCl, 3 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄ and 10 glucose (pH 7.4). For recording, slices were transferred to a 0.5 mL chamber mounted on a Zeiss Axioskop 2 FS Plus microscope (Zeiss, Germany) with a 40 $\times$ water-immersion objective and infrared, differential interference contrast 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). Recordings were performed at 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 μ m tip diameter using borosilicate glass micropipettes (inner diameter of 0.86 mm, outer diameter of 1.60 mm) (VWR Scientific, Seattle, WA). The electrodes had resistances between 3 and 7 M Ω 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 osmolality between 280 and 290 mOsm/L. The Cl[−] concentration (37 mM) in the internal solution approximated the physiological Cl[−] concentration in NA neurons. The use of a K⁺-based internal solution allows for the identification of cell type based on intrinsic firing properties recorded in current clamp, followed by studies of synaptic responses recorded in voltage clamp. Placement of recording 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. Voltage and current clamp experiments were performed with an AxoPatch 200B (Molecular Devices, Union City, CA). Voltage clamp recordings were obtained at a holding potential of −60 mV, unless indicated otherwise. Data were low-pass filtered at 3–10 kHz, and digitized with a Data Acquisition

Interface ITC-18 (Instrutech, Great Neck, NY) at 20 kHz. Recording protocols were written and run using the acquisition and analysis software AxoGraph X (AxoGraph Scientific, Sydney, Australia).

2.2. Synaptic stimulation and recordings of synaptic responses

Extracellular stimulation was performed using concentric bipolar electrodes with a tip core diameter of 127 μm (World Precision Instruments, Sarasota, FL). The stimulating electrodes were placed using a Micromanipulator NMN-25 (Narishige, Japan). Square electric pulses of 200 μs duration were delivered through a Stimulator A320RC (World Precision Instruments, Sarasota, FL). Evoked excitatory postsynaptic currents (EPSCs) were elicited with electrical shocks delivered to the afferent fibers ventral and lateral to NA. EPSCs were recorded in the presence of GABA_A receptor blocker SR95531 (Gabazine, 10 μM), and glycine receptor blocker strychnine (1 μM). Optimal stimulation parameters were selected for each cell to give rise to postsynaptic currents close to maximal amplitude.

All chemicals and drugs were obtained from Sigma (St. Louis, MO) except for 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), 6-Imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid (SR95531), and baclofen, which were obtained from Tocris (Ballwin, MO). Drugs were bath-applied using a gravity-driven perfusion system.

2.3. Data analysis

The resting membrane potential was read from the amplifiers immediately after whole-cell configuration was established. Detection of spontaneous EPSCs (sEPSCs) was performed using protocols published previously (Lu, 2007). Briefly, a template with defined rise time and decay time constant was used to detect sEPSCs. The values of the parameters for the template were determined based on the average of real sEPSC events. The detection threshold was 3 times the noise standard deviation, which allowed the detection of most of the events with the least number of false-positives.

Statistical analyses were performed using Excel (Microsoft, Redmond, WA) and Statview (Abacus Concepts, Berkeley, CA), and graphs were made in Igor (Wavemetrics, Lake Oswego, OR). Means and standard errors of the mean (SEM) are reported (n in parenthesis indicates number of cells). Statistical differences were determined by ANOVA post hoc Fisher's test.

2.4. mGluR immunostaining

Chick hatchlings were deeply anesthetized with Fatal-Plus (Vortech Pharmaceuticals Ltd., Dearborn, MI) and transcardially perfused with 4% paraformaldehyde (PFA, pH 7.4) in phosphate buffer. Brains of chicken embryos were dissected out after decapitation without transcardial perfusion. The brains were post-fixed in PFA for 2 h at room temperature and then overnight at 4 °C, rinsed thoroughly in phosphate-buffered saline (PBS), and vibratome-sliced (50 μm in thickness). Free-floating sections were processed for immunohistochemistry with standard avidin-biotin-peroxidase methods (Vectastain Elite

ABC; Vector Laboratories, Burlingame, CA). Sections were incubated in 10% normal goat serum in Tris-buffered saline (TBS) containing 0.3% Triton X-100 and avidin (200 $\mu\text{L}/\text{mL}$) for 3–4 h. After a brief rinse in TBS, the sections were incubated in the mGluR II and III-specific antibodies (rabbit, Abcam Inc; 1:200 for both group II and III mGluRs) in TBS containing 2% normal goat serum and biotin (200 $\mu\text{L}/\text{mL}$) overnight at room temperature. After rinsing in TBS, the sections were incubated in biotinylated goat anti-rabbit IgG (1:1,000) for 1 h, rinsed in TBS, and incubated in ABC Elite solution (5 $\mu\text{L}/\text{mL}$) for 1 h. After rinsing in PBS, the sections were processed with 0.06% 3,3-diaminobenzidine HCl and 0.006% H_2O_2 diluted in PBS for 15 minutes. The immunolabeling was enhanced by incubation in 0.003% osmium tetroxide in PBS for 30 seconds. After thorough rinsing, the sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Negative control experiments were performed with omission of the primary antibody. Images were taken with a high-resolution CCD camera system (Spot camera, Diagnostic instruments Inc, Sterling Heights, MI) mounted on an Olympus Provis AX70 microscope (Japan).

3. Results

3.1. Basic intrinsic neuronal properties of NA neurons

Unlike NM neurons, NA neurons are heterogeneous in their morphology and physiology, and have no immediate homologous counterpart in the mammalian system (review in MacLeod and Carr, 2007). Based on their intrinsic spiking properties in response to prolonged somatic current injections and their morphology, NA neurons are classified into three different cell types (one-spike or onset, damped, and tonic) in late chicken embryos (Soares et al., 2002), at which age the neuronal properties in the auditory brainstem are relatively mature (Gao and Lu, 2008). The one-spike and tonic firing patterns, but not the damped pattern, persist into hatchlings (Fukui and Ohmori, 2003). We first confirmed the basic intrinsic properties of NA neurons, using protocols similar to previous studies (Soares et al., 2002; MacLeod and Carr, 2005). One-spike (Onset) cells fired a single spike at the onset of the depolarizing suprathreshold current injections, followed by a subthreshold voltage plateau (Fig. 1A). This firing pattern is a characteristic hallmark of time coding neurons in the auditory system (Oertel, 1999). Damped cells produced a few spikes at the onset of suprathreshold current injections, followed by spikes with usually reduced amplitude and subthreshold membrane oscillations (Fig. 1B). This type of firing pattern was observed in late embryos (Soares et al., 2002; and this study), but not in hatchlings (Fukui and Ohmori, 2003). Tonic cells responded with trains of action potentials to suprathreshold current injections, without obvious reduction in spike amplitude (Fig. 1C). When the number of action potentials was plotted against the amplitude of the injection currents relative to the current threshold (the minimal current required to elicit spikes), one-spike cells ($n=46$) had a flat input-output function. Damped cells ($n=22$) showed adaptation in spiking activity, with an increase in the number of spikes in response to increasing currents and a slightly reduced spiking to more depolarizing current steps. Tonic cells ($n=30$) showed an increase in the number of action potentials with increasing current amplitude.

To further distinguish these cell types, we compared the basic membrane properties as well as parameters of their sEPSCs (Table 1). There were no significant differences in their

resting membrane potential (RMP) among different cell types. However, tonic cells had significantly higher input resistance and lower current threshold (I_{th}) than one-spike cells, consistent with observations in the mammalian cochlear nucleus, where bushy cells (one-spike firing) had higher input resistance than stellate cells (tonic firing) at various developing stages (Lu et al., 2007). For sEPSCs, onset cells had higher frequency and amplitude but faster decay kinetics than the other two cell types (Fig. 1; Table 1).

3.2. Activation of groups I mGluRs increased excitability and suppressed EPSCs of NA neurons in a cell type dependent manner

Eight mGluRs have been identified, which are further classified into 3 groups (group I: mGluR 1 and 5; II: mGluR2 and 3; and III: mGluR 4, 6, 7, and 8) based on their pharmacology, homology, and signal transduction pathways (review in Kew and Kemp, 2005; Nicoletti et al., 2011). Selective agonists for each group of mGluRs have been developed. For example, the agonist DHPG can activate group I mGluRs at an EC_{50} of 6–60 μ M. DCG-IV selectively activates group II mGluRs at an EC_{50} of 0.1–0.3 μ M. Meanwhile, L-AP4 activates mGluR 4, 6, and 8 at an EC_{50} less than 1 μ M, but a much higher concentration (>100 μ M) is required to activate mGluR7 (review in Cartmell and Schoepp, 2000). These three agonists were chosen to be used in this study, at a concentration at least three times higher than their EC_{50} .

Intrinsic neuronal properties including voltage-gated Na^+ and K^+ (Na_v and K_v) channels are subject to modulation by second messenger systems via G-protein-coupled receptors (review in Cantrell and Catterall, 2001; Steinert et al., 2008). To examine whether cellular excitability of NA neurons was modulated by mGluRs, we activated mGluRs using bath application of agonists selective for each group while recording membrane voltage responses of NA neurons to somatic current injections.

Activation of group I mGluRs increased cellular excitability of non one-spike firing neurons, without changing their firing patterns. DHPG (100 μ M) increased the spike counts in response to prolonged suprathreshold current injections, and the responses were not recovered after washout of DHPG (Fig. 2A, B). In contrast, one-spike firing cells fired only one spike in response to prolonged suprathreshold current injections under all conditions tested here (data not shown), suggesting little or no change in firing patterns. Because each cell had different current threshold and fired different number of spikes in response to the same current injection, we normalized the number of spikes of the non one-spike firing cells to the maximal spike count under the control condition, and plotted the normalized spike number against the amplitude of the injected currents relative to the threshold. Results showed increased excitability of NA neurons when group I mGluRs was activated, and the effect began when the injected current exceeded 200 pA above threshold (Fig. 2B, $n=7$).

Synaptic transmission can be modulated by each of the three groups of mGluRs, which can act as either autoreceptors or heteroreceptors (review in Cartmell and Schoepp, 2000). To determine whether the glutamatergic transmission in NA neurons was modulated by mGluRs, we activated mGluRs using bath application of agonists selective for each group while recording EPSCs from NA neurons. Synaptic responses of NA neurons were elicited by delivering electrical stimulation to the afferent fibers innervating the NA. We used

pharmacological agents to isolate EPSCs. It is known that glutamate mediates synaptic excitation, whereas GABA and/or glycine mediate synaptic inhibition in NA neurons (Kuo et al., 2009). Therefore, we applied GABA_AR antagonist SR95531 (10 μ M) and glycine receptor antagonist strychnine (1 μ M) to block synaptic inhibition in order to isolate EPSCs. DHPG (100 μ M) suppressed EPSCs in a cell type dependent manner. DHPG (100 μ M) significantly reduced the amplitude of EPSCs in nononset cells, without affecting the amplitude of EPSCs in onset cells (Fig. 2C–E, $P < 0.01$; $n=28$: 7 one-spike cells, 10 damped cells, and 11 tonic cells).

3.3. Activation of groups II and III mGluRs suppressed excitability and EPSCs of NA neurons

Activation of group II mGluRs by bath application of agonist DCG-IV suppressed cellular excitability of non one-spike firing neurons, without changing their firing patterns (Fig. 3A, B). DCG-IV (2 μ M) reduced the spike counts in response to prolonged suprathreshold current injections, and the responses largely recovered after washout of DCG-IV (Fig. 3A). In contrast, one-spike firing cells fired only one spike in response to prolonged suprathreshold current injections under all conditions tested here (data not shown), suggesting little or no change in excitability. The effect was especially prominent at higher current injections when the current exceeded 600 pA above threshold (Fig. 3B, $n=6$).

DCG-IV (2 μ M) suppressed EPSCs in a cell type independent manner. DCG-IV (2 μ M) significantly reduced the amplitude of EPSCs, with normalized EPSC amplitude during DCG-IV being 0.53 ± 0.08 compared to control in the non-onset cells whereas 0.68 ± 0.23 in onset cells (Fig. 3D–E, $P < 0.01$; $n=29$: 14 one-spike cells, 5 damped cells, and 10 tonic cells). LY341495 (200 nM), antagonist of group II mGluRs, slightly increased the overall spike count at high current injections (> 700 pA) ($n=5$). The effect seemed to be long lasting, without recovery after washout of LY341495 (Fig. 3C). LY341495 significantly increased the amplitude of EPSCs on the non-onset cells, indicating endogenous activity of group II mGluRs on the modulation of EPSCs (Fig. 3F, $n=16$: 8 onset, 8 non-onset cells). To support our physiological observations, the presence of group II mGluRs in the NA was studied using immunostaining techniques. Our data showed that antibodies against group II mGluRs detected relatively consistent expression of the receptors in NA neurons at three different ages (Fig. 3G–I). The same antibody also detected expression of group II mGluRs in the cerebellum, which was used as a positive control (data not shown).

Activation of group III mGluRs by L-AP4 (10 μ M) suppressed cellular excitability of NA neurons, and reduced spike counts of the cells in response to varying suprathreshold current injections, with relatively large variation among different neurons (Fig. 4A, B; $n=6$). L-AP4 (10 μ M) also significantly reduced the amplitude of EPSCs, with normalized EPSC amplitude during L-AP4 application being 0.44 ± 0.09 compared to the control in a cell type independent manner (Fig. 4D–E, $P < 0.01$; $n=25$: 8 one-spike cells, 8 damped cells, and 9 tonic cells). CPPG (10 μ M), antagonist of group III mGluRs, increased the overall spike count at higher current injections (> 550 pA) while decreased spike count at lower current injections (Fig. 4C, $n=7$), suggesting intensity-dependent modulation on excitability. CPPG significantly increased the amplitude of EPSCs of onset cells, but the effect was significant

after CPPG application, reflecting a delayed action (Fig. 4F, $n=23$: 10 onset, 13 non-onset cells). Immunostaining results showed expression of group III mGluRs in the NA, and the immunoreactivity appeared to become weaker in hatchlings (Fig. 4G–I), suggesting mGluR subtype-dependent developmental regulation in NA.

3.4. Activation of GABA_BRs suppressed excitability and EPSCs of NA neurons

Neurons in the NA receive inhibitory inputs primarily from the superior olive and also from locally distributed inhibitory neurons (Takhshi and Konishi, 1988; Carr et al., 1989; Yang et al., 1999; Monsivais et al., 2000). GABA is believed to be the major neurotransmitter mediating the inhibition in avian auditory brainstem, although glycinergic input is also present in NA neurons (Kuo et al., 2009). GABA can activate both ionotropic GABA_ARs as well as metabotropic GABA_BRs. GABA_BRs are highly expressed in the chicken auditory brainstem neurons including NA neurons (Burger et al., 2005). To test whether GABA_BRs modulate neuronal properties of NA neurons, we examined the effects of a specific GABA_BR agonist baclofen. Activation of GABA_BRs by baclofen at a saturating concentration (100 μ M) dramatically reduced the spike counts of NA neurons in response to suprathreshold current injections (Fig. 5A, B, $n=7$). The inhibitory effect appeared to be irreversible because we observed no recovery of cellular excitability after washout of baclofen. Baclofen (100 μ M) also significantly reduced EPSCs to $31\pm 8\%$ of the control independent of cell types (Fig. 5C–E, $P < 0.01$; $n=13$: 6 one-spike cells, 4 damped cells, and 3 tonic cells), and the effect sustained throughout the recording period.

3.5. Loci for mGluR- and GABA_BR-mediated modulation of glutamate transmission

The loci for G-protein-coupled receptor (GPCR) action on synaptic transmission can be presynaptic, postsynaptic, or both. Direct evidence supporting presynaptic modulation loci of GPCRs needs to derive from presynaptic physiological recordings. However, because the size of most presynaptic terminals is very small, such recordings have been successfully achieved in only few large synapses like the glutamatergic terminals at the medial nucleus of trapezoid body (Forsythe, 1994). Therefore, the conclusion about loci of GPCR modulation relies on indirect evidence from studies that examine the effects of GPCR activation on miniature transmitter release, pair-pulse ratios (PPR), and variation coefficients of synaptic responses. To determine the loci of mGluR- and GABA_BR-mediated modulation of glutamate transmission in the NA, we examined the effects of different mGluR and GABA_BR agonists and antagonist on spontaneous EPSCs (sEPSCs) and PPR of evoked EPSCs. In the NA, sEPSCs recorded in slice preparations represent miniature EPSCs (mEPSCs), because blocking action potential-dependent release does not affect sEPSCs in NA neurons (MacLeod and Carr, 2005). Group II mGluR agonist DCG-IV (2 μ M) significantly reduced the frequency of sEPSCs and PPR (Fig. 6A, $n=8$: 5 one-spike and 3 tonic cells, $P < 0.01$), whereas the amplitude of sEPSCs remained unchanged (Fig. 6A3, $n=8$, $P > 0.05$). Group III mGluR agonist L-AP4 (10 μ M) produced similar effects (Fig. 6B, $n=7$: 1 one-spike cell, 2 damped cells, and 4 tonic cells), and so did group I agonist DHPG (Fig. 6C, $n=19$: 8 one-spike, 6 damped, and 5 tonic cells). LY341495 (200 nM) did not change the frequency or the amplitude of sEPSCs (Fig. 6D, $n=15$: 8 one-spike, 4 damped, and 3 tonic cells). In contrast, CPPG (10 μ M) significantly reduced the frequency and increased the PPR, without affecting the amplitude of sEPSCs ($n=12$: 4 one-spike, 4

damped, and 4 tonic cells), suggesting that endogenous mGluR modulation of spontaneous release of glutamate in NA neurons was primarily mediated by group III mGluRs. GABA_BR agonist baclofen (100 μ M) reduced sEPSC frequency without affecting the amplitude (Fig. 6F, n=8: 3 one-spike and 5 tonic cells), consistent with effects on synaptic transmission commonly found in the CNS. These results suggest presynaptic modulatory actions of these GPCRs on synaptic excitation in NA.

4. Discussion

4.1. Neuromodulation of glutamate release and excitability of NA neurons

GPCR-mediated modulation of synaptic transmission has been found in numerous central neuronal systems, and the types of GPCRs and the mechanisms underlying the modulation dramatically differ among different systems. In the NA, agonists for various groups of mGluRs significantly reduced EPSCs independent of cell types, except that group I mGluRs did not affect EPSCs of onset NA neurons. The lack of effect of group I mGluRs in these neurons is unlikely due to an inadequate activation of the receptors. We chose the concentrations of 200, 2, and 10 μ M for 3,5-DHPG, DCG-IV, and L-AP4, respectively. These concentrations are at least three times higher than their EC₅₀ (review in Cartmell and Schoepp, 2000; Schoepp et al., 1999), so any possible activation of their corresponding receptors is expected to be nearly saturating. In addition, the specificity of these agonists has been tested in previous studies, which showed selectivity of these agonists 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). Interestingly, EPSCs of NA neurons were modulated by both group II and III mGluRs regardless of cell type. Modulation of synaptic transmission by multiple mGluRs at the same synapses is not uncommon (e.g. Drew and Vaughan, 2004; review in Knöpfel and Uusisaari, 2008; Zheng and Johnson, 2003). The clustering of different groups of mGluRs may account for such seemingly redundant modulation at different membrane loci, and they may exert modulation of glutamate release via different signaling pathways at different locations. Furthermore, it is possible that low density of each group of mGluRs exists on the presynaptic membrane, and simultaneous activation of multiple members from different groups can generate synergistic regulation of glutamate release. In some cases, interactions between receptors activated by different transmitters, for example, between mGluRs and GABA_BRs, also exist (Hirono et al., 2001).

The modulation loci for mGluRs and GABA_BRs on the excitatory transmission in NA neurons seem to be presynaptic. The effects of drugs on sEPSCs can indirectly indicate whether the modulation of glutamate transmission in NA arises from a presynaptic, postsynaptic, or dual mechanism. Modulation of the frequency but not the amplitude of sEPSCs would imply a presynaptic mechanism, modulation of the amplitude but not of the frequency of sEPSCs would imply a postsynaptic mechanism, and modulation of both the frequency and amplitude of sEPSCs 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). All the GPCR agonists used here that reduced EPSC amplitude of NA neurons produced a significant reduction in the frequency but not amplitude of sEPSCs. These effects

were consistent with previous studies (review in Cartmell and Schoepp, 2000; Schoepp, 2001; Ulrich and Bettler, 2007), particularly with the studies in NM and NL where presynaptic modulation of transmission by these receptors has been reported (Brenowitz et al., 1998; Brenowitz and Trussell, 2001; Lu et al., 2005; Lu, 2007; Otis and Trussell, 1996; Tang et al., 2009). Since evoked transmitter release is dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) in presynaptic terminals, and VGCCs are subject to modulation by mGluRs (reviewed in Catterall, 2000; Stefani et al., 1996) and GABA_BRs (Ulrich and Bettler, 2007), modulation of VGCCs by mGluRs and GABA_BRs is highly likely the mechanism underlying the presynaptic regulation of glutamate release at NA. On the other hand, reduced excitability by mGluRs and GABA_BRs supports the notion that these postsynaptic GPCRs except for group I mGluR reduce the overall excitation of NA neurons. The enhanced excitability of NA neurons by group I mGluRs is consistent with the inward current mediated by these receptors in many brain areas (review in Niswender and Conn, 2010). It remains interesting to determine whether and how the presynaptic and postsynaptic GPCRs interact to regulate the output of NA neurons.

4.2. Comparison of modulation of synaptic excitation between the two cochlear nucleus components, NM and NA

In the NM, regulation of synaptic strength of the glutamatergic inputs is critical for NM neuronal function. GABA modulates glutamate release at a presynaptic locus via metabotropic GABA_BRs, reducing synaptic depression (Brenowitz et al., 1998; Brenowitz and Trussell, 2001; Otis and Trussell, 1996). This modulation helps increase the synaptic efficacy and ensure high safety factor of glutamate transmission at the endbulb synapses, rendering NM neurons capability of following high frequency inputs. In the NA, the glutamatergic transmission is subject to a dual modulation by both mGluRs and GABA_BRs. The mGluR-mediated modulation of glutamate release in NA is a striking division from the timing-coding NM neurons where such autoreceptor-mediated modulation of glutamatergic transmission is absent (Otis and Trussell, 1996; Tang et al., 2013). One possible interpretation for the lack of mGluR modulation is that mGluRs are not expressed on the excitatory terminals of NM neurons. It is worth noting that mGluRs are found to be physiologically expressed in presynaptic inhibitory terminals of NM neurons, and modulate GABA release via presynaptic mechanisms (Lu, 2007; Tang et al., 2013). An alternative but less likely interpretation is that mGluRs on presynaptic glutamatergic terminals of NM neurons might be saturated or desensitized, and therefore no further activation can be observed when exogenous agonists are applied. The difference in mGluR-mediated regulation of glutamate release between NA and NM neurons may bear physiological implication based on their sensitivity to sound intensity. NA neurons have much steeper rate-intensity functions and larger dynamic ranges than NM neurons (Warchol and Dallos, 1990). Therefore, when sound intensity increases, NA neurons are expected to respond with increasing amount of glutamate release, leading to a high propensity of activation of mGluRs and subsequent modulation of glutamate release. In contrast, NM neurons are relatively less sensitive to changes in sound intensity, leading to a possible flat amount of glutamate release and hence a low propensity of activation of mGluRs regardless of sound intensity.

Interestingly, glutamatergic transmission in both NM and NA was subject to heteroreceptor GABA_BR-mediated modulation, via presynaptic mechanisms. In order to engage this modulatory mechanism under physiological conditions, synaptically released GABA needs to diffuse relatively long distance (spillover) to activate GABA_BRs on the excitatory terminals. Because synaptically released transmitter is actively uptaken into glial cells and neurons through transmitter transporters, it is normally difficult for transmitter molecules to reach their receptors at distance in order to initiate crosstalk between different types of synapses (Barbour and Häusser, 1997). However, spillover of transmitter molecules could be facilitated under certain circumstances. NM neurons are well known to have large glutamatergic synapses covering more than half of the cell body surface (Ryugo and Parks, 2003). GABAergic terminals innervating NM neurons are presumably located nearby these large glutamatergic terminals (Lachica et al., 1994). Such morphological features may facilitate interactions between the glutamatergic and GABAergic pathways and allow presynaptic GABA_BRs on the glutamatergic terminals to be activated by GABA spillover (Brenowitz et al., 1998; Brenowitz and Trussell, 2001; Lu et al., 2005; Otis and Trussell, 1996; Otis et al., 1996). In contrast, at NA, because both glutamate and GABA terminals are likely to be small bouton-like structures, activation of GABA_BRs on glutamate terminals may require relatively short distance of traveling of GABA molecules. In other words, we speculate that at least some of the glutamate and GABA synapses on NA neurons are spatially close to each other in order for spilled GABA to reach their receptors on the glutamatergic terminals and implement neuromodulatory actions.

The modulation of glutamate release in NA by both mGluRs and GABA_BRs may bear functional significance. Because group II mGluRs have high affinity of binding glutamate (review in Cartmell and Schoepp, 2000), a tonic activation of these receptors by spontaneous spike-evoked release of glutamate may exist limiting the basal level of glutamate release at NA. This may not be detectable in a slice preparation (Fig. 6D), because NA neurons do not spontaneously fire action potentials *in vitro*. However, NA neurons are considerably active *in vivo*, with a spontaneous firing rate of about 25 Hz (Warchol and Dallos, 1990). An even higher rate is expected when sound stimuli are present because during acoustic stimulation, the firing rate of cochlear nuclear synapses can be up to several times higher (Liberman, 1978; Manley et al., 1985, 1997; Sachs and Abbas, 1974; Sanes et al., 1998). At high-rate spiking activity, clearance of glutamate may be well delayed (Scanziani et al., 1997), rendering possible the presence of glutamate in the synaptic cleft and surrounding areas at a level that is sufficient in activating mGluRs and exerting a tonic regulation on its own release. While mGluR autoreceptor-mediated regulation of glutamate release provides a feedback control mechanism, GABA_BR-mediated modulation may be recruited as the GABAergic pathway is intensively activated under high sound intensity conditions. Both modulations can regulate the input-output functions of NA neurons, and determine the proper dynamic range and thus their intensity coding capability. In addition, some NA neurons (about 22%) display non-monotonic input-output functions (Warchol and Dallos, 1990), which is likely formed by inhibitory input. If this is the case, modulation of the inhibitory input, which is likely but yet determined, will inevitably affect the dynamic range of these neurons. Therefore, neuromodulation may participate in shaping the rate-intensity functions by regulating both the synaptic excitation and inhibition in NA neurons.

Acknowledgments

We thank Dr. Zheng-Quan Tang and other laboratory members for helpful discussion and comments. This work was supported by National Institute on Deafness and other Communication Disorders Grant R01DC008984 (Y.L.), and an Institutional Bridge Funding by Northeast Ohio Medical University.

ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AP	action potential
EPSCs	excitatory postsynaptic currents
GABA_BR	GABA _B receptor
GPCR	G protein-coupled receptor
ILD	interaural level difference
ITD	interaural time difference
LLD	dorsal nucleus of lateral lemniscus
mGluR	metabotropic glutamate receptor
NA	nucleus angularis
NM	nucleus magnocellularis
NL	nucleus laminaris
PPR	pair-pulse ratio
RMP	resting membrane potential
SON	superior olivary nucleus
VGCC	voltage-gated calcium channel

References

- Barbour B, Häusser M. Intersynaptic diffusion of neurotransmitter. *Trends Neurosci.* 1997; 20:377–384. [PubMed: 9292962]
- Brenowitz S, David J, Trussell LO. Enhancement of synaptic efficacy by presynaptic GABA_B receptors. *Neuron.* 1998; 20:135–141. [PubMed: 9459449]
- Brenowitz S, Trussell LO. Minimizing synaptic depression by control of release probability. *J Neurosci.* 2001; 21:1857–1867. [PubMed: 11245670]
- Burger RM, Pfeiffer JD, Westrum LE, Bernard A, Rubel EW. Expression of GABA_B receptor in the avian auditory brainstem, ontogeny, afferent deprivation, and ultrastructure. *J Comp Neurol.* 2005; 489:11–22. [PubMed: 15977167]
- Caramelo OL, Santos PF, Carvalho AP, Duarte CB. Metabotropic glutamate receptors modulate [(3)H] acetylcholine release from cultured amacrine-like neurons. *J Neurosci Res.* 1999; 58:505–514. [PubMed: 10533043]
- Carr CE, Fujita I, Konishi M. Distribution of GABAergic neurons and terminals in auditory system of the barn owl. *J Comp Neurol.* 1989; 286:190–207. [PubMed: 2794115]

- Cantrell AR, Catterall WA. Neuromodulation of Na⁺ channels: an unexpected from of cellular plasticity. *Nat Rev Neurosci.* 2001; 2:397–407. [PubMed: 11389473]
- Cartmell J, Schoepp DD. Regulation of neurotransmitter release by metabotropic glutamate receptors. *J Neurochem.* 2000; 75:889–907. [PubMed: 10936169]
- Catterall WA. Structure and regulation of voltage-gated Ca²⁺ channels. *Annu Rev Cell Dev Biol.* 2000; 16:521–555. [PubMed: 11031246]
- Chu Z, Moenter SM. Endogenous activation of metabotropic glutamate receptors modulates GABAergic transmission to gonadotropin-releasing hormone neurons and alters their firing rate: a possible local feedback circuit. *J Neurosci.* 2005; 25:5740–5749. [PubMed: 15958740]
- Drew GM, Vaughan CW. Multiple metabotropic glutamate receptor subtypes modulate GABAergic neurotransmission in rat periaqueductal grey neurons in vitro. *Neuropharmacology.* 2004; 7:927–934.
- Dutar P, Vu HM, Perkel DJ. Pharmacological characterization of an unusual mGluR-evoked neuronal hyperpolarization mediated by activation of GIRK channels. *Neuropharmacology.* 1999; 38:467–475.
- Forsythe ID. Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, in vitro. *J Physiol.* 1994; 479:381–387. [PubMed: 7837096]
- Fukui I, Ohmori H. Developmental changes in membrane excitability and morphology of neurons in the nucleus angularis of the chicken. *J Physiol.* 2003; 548:219–232. [PubMed: 12576492]
- Gao H, Lu Y. Early development of intrinsic and synaptic properties of chicken nucleus laminaris neurons. *Neuroscience.* 2008; 153:131–143. [PubMed: 18355968]
- Gomes AR, Cunha P, Nuriya M, Faro CJ, Haganir RL, Pires EV, Carvalho AL, Duarte CB. Metabotropic glutamate and dopamine receptors co-regulate AMPA receptor activity through PKA in cultured chick retinal neurones: effect on GluR4 phosphorylation and surface expression. *J Neurochem.* 2004; 90:673–682. [PubMed: 15255946]
- Hirono M, Yoshioka T, Konishi S. GABA(B) receptor activation enhances mGluR-mediated responses at cerebellar excitatory synapses. *Nat Neurosci.* 2001; 4:1207–1216.
- Kew JN, Kemp JA. Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology.* 2005; 179:4–29. [PubMed: 15731895]
- Kreimboog KM, Lester ML, Medler KE, Gleason EL. Group I metabotropic glutamate receptors are expressed in the chicken retina and by cultured retinal amacrine cells. *J Neurochem.* 2001; 77:452–465. [PubMed: 11299308]
- Knöpfel, Uusisaari M. Modulation of excitation by metabotropic glutamate receptors. *Results Probl Cell Differ.* 2008; 44:163–175. [PubMed: 17579817]
- Köpl C, Carr CE. Computational diversity in the cochlear nucleus angularis of the barn owl. *J Neurophysiol.* 2003; 89:2313–2329. [PubMed: 12612008]
- Kuo SP, Bradley LA, Trussell LO. Heterogeneous Kinetics and Pharmacology of Synaptic Inhibition in the Chick Auditory Brainstem. *J Neurosci.* 2009; 29:9625–9634. [PubMed: 19641125]
- Lachica EA, Rbsame R, Rubel EW. GABAergic terminals in nucleus magnocellularis and laminaris originate from the superior olivary nucleus. *J Comp Neurol.* 1994; 348:403–418. [PubMed: 7844255]
- Liberman MC. Auditory-nerve response from cats raised in a low-noise chamber. *J Acoust Soc Am.* 1978; 6:442–455.
- Lu Y, Rubel EW. Activation of metabotropic glutamate receptors inhibits high-voltage-gated calcium channel currents of chicken nucleus magnocellularis neurons. *J Neurophysiol.* 2005; 93:1418–1428. [PubMed: 15371493]
- Lu Y, Burge RM, Rubel EW. GABA_B receptor activation modulates GABA_A receptor mediated inhibition in chicken nucleus magnocellularis neurons. *J Neurophysiol.* 2005; 93:1429–1438. [PubMed: 15483063]
- Lu Y. Endogenous mGluR activity suppresses GABAergic transmission in avian cochlear nucleus magnocellularis neurons. *J Neurophysiol.* 2007; 97:1018–1029.
- Lu Y, Harris JA, Rubel EW. Development of spontaneous miniature EPSCs in mouse AVCN neurons over a critical period of afferent-dependent neuron survival. *J Neurophysiol.* 2007; 97:635–646. [PubMed: 17079338]

- Lu Y. Metabotropic glutamate receptors in auditory processing. *Neuroscience*. 2014; 274:429–445. [PubMed: 24909898]
- MacLeod KM, Carr CE. Synaptic physiology in the cochlear nucleus angularis of the chick. *J Neurophysiol*. 2005; 93:2520–2529. [PubMed: 15615833]
- MacLeod KM, Carr CE. Beyond timing in the auditory brainstem: intensity coding in the avian cochlear nucleus angularis. *Prog Brain Res*. 2007; 165:123–133. [PubMed: 17925243]
- Manley GA, Gleich O, Leppelsack HJ, Oeckinghaus H. Activity patterns of cochlear ganglion neurones in the starling. *J Comp Physiol A*. 1985; 157:161–181. [PubMed: 3837088]
- Manley GA, Köppl C, Yates GK. Activity of primary auditory neurons in the cochlear ganglion of the emu *Dromaius novaehollandiae*: spontaneous discharge, frequency tuning, and phase locking. *J Acoust Soc Am*. 1997; 101:1560–1573. [PubMed: 9069626]
- Monsivais P, Yang L, Rubel EW. GABAergic inhibition in nucleus magnocellularis: implications for phase locking in the avian auditory brainstem. *J Neurosci*. 2000; 20:2954–2963. [PubMed: 10751448]
- Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP. Metabotropic glutamate receptors: from the workbench to the bedside. *Neuropharmacology*. 2011; 60:1017–1041. [PubMed: 21036182]
- Niswender CM, Conn PJ. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol*. 2010; 50:295–322. [PubMed: 20055706]
- Oertel D. The role of timing in the brain stem auditory nuclei of vertebrates. *Annu Rev Physiol*. 1999; 61:497–519. [PubMed: 10099699]
- Otis TS, Trussell LO. Inhibition of transmitter release shortens the duration of the excitatory synaptic current at a calyceal synapse. *J Neurophysiol*. 1996; 76:3584–3588. [PubMed: 8930299]
- Otis TS, Wu YC, Trussell LO. Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J Neurosci*. 1996; 16:1634–1644. [PubMed: 8774432]
- Piet R, Bonhomme R, Theodosis DT, Poulain DA, Oliet SH. Modulation of GABAergic transmission by endogenous glutamate in the rat supraoptic nucleus. *Eur J Neurosci*. 2003; 17:1777–1785. [PubMed: 12752776]
- Ryugo DK, Parks TN. Primary innervation of the avian and mammalian cochlear nucleus. *Brain Res Bull*. 2003; 60:435–456. [PubMed: 12787866]
- Sachs MB, Abbas PJ. Rate versus level functions for auditory-nerve fibers in cats: tone-burst stimuli. *J Acoust Soc Am*. 1974; 56:1835–1847. [PubMed: 4443483]
- Sampaio LF, Paes-de-Carvalho R. Developmental regulation of group III metabotropic glutamate receptors modulating adenylate cyclase activity in the avian retina. *Neurochem Int*. 1998; 33:367–374. [PubMed: 9840228]
- Sanes DH, McGee J, Waish EJ. Metabotropic glutamate receptor activation modulates sound level processing in the cochlear nucleus. *J Neurophysiol*. 1998; 80:209–217. [PubMed: 9658042]
- Sato T, Fukui I, Ohmori H. Interaural phase difference modulates the neural activity in the nucleus angularis and improves the processing of level difference cue in the lateral lemniscal nucleus in the chicken. *Neurosci Res*. 2010; 66:198–212. [PubMed: 19914308]
- Scanzizni M, Salin PA, Vogt KE, Malenka RC, Nicoll RA. Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. *Nature*. 1997; 385:630–634. [PubMed: 9024660]
- Schoepp DD, Jane DE, Monn JA. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology*. 1999; 38:1431–1476. [PubMed: 10530808]
- Schoepp DD. Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. *J Pharmacol Exp Ther*. 2001; 299:12–20. [PubMed: 11561058]
- Soares D, Chitwood RA, Hyson RL, Carr CE. Intrinsic neuronal properties of the Chick Nucleus Angularis. *J Neurophysiol*. 2002; 88:152–162. [PubMed: 12091541]
- Sullivan WE, Konishi. Segregation of stimulus phase and intensity coding in the cochlear nucleus of the barn owl. *J Neurosci*. 1984; 4:1787–1799. [PubMed: 6737041]
- Stefani A, Pisani A, Mercuri NB, Calabresi P. The modulation of calcium currents by the activation of mGluRs. Functional implications. *Mol Neurobiol*. 1996; 13:81–95. [PubMed: 8892337]

- Steinert JR, Kopp-Scheinpflug C, Baker C, Challiss RA, Mistry R, Haustein MD, Griffin SJ, Tong H, Graham BP, Forsythe ID. Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. *Neuron*. 2008; 60:642–656. [PubMed: 19038221]
- Takahashi T, Konishi M. The projections of nucleus angularis and nucleus laminaris to the lateral lemniscal nuclear complex of the barn owl. *J Comp Neurol*. 1988; 274:212–238. [PubMed: 2463287]
- Tang ZQ, Gao H, Lu Y. Control of a depolarizing GABAergic input in an auditory coincidence detection circuit. *J Neurophysiol*. 2009; 102:1672–1683. [PubMed: 19571192]
- Tang ZQ, Liu YW, Shi W, Dinh EH, Hamlet WR, Curry RJ, Lu Y. Activation of synaptic group II mGluRs induces long-term depression at GABAergic synapses in CNS neurons. *J Neurosci*. 2013; 33:15964–15977. [PubMed: 24089501]
- Tasca CI, Cardoso LF, Souza DO. Effects of guanine nucleotides on adenosine and glutamate modulation of cAMP levels in optic tectum slices from chicks. *Neurochem Int*. 1999; 34:213–220. [PubMed: 10355488]
- Ulrich D, Bettler B. GABAB receptors: synaptic functions and mechanisms of diversity. *Curr Opin Neurobiol*. 2007; 17:298–303. [PubMed: 17433877]
- Valenti O, Marino MJ, Wittmann M, Lis E, DiLella AG, Kinney GG, Conn PJ. Group III metabotropic glutamate receptor-mediated modulation of the striatopallidal synapse. *J Neurosci*. 2003; 23:7218–7226. [PubMed: 12904482]
- Warchol ME, Dallos P. Neural coding in the chick cochlear nucleus. *J Comp Physiol (A)*. 1990; 166:721–734. [PubMed: 2341992]
- Yang L, Monsivais P, Rubel EW. The superior olivary nucleus and its influence on nucleus laminaris: a source of inhibitory feedback for coincidence detection in the avian auditory brainstem. *J Neurosci*. 1999; 19:2313–2325. [PubMed: 10066281]
- Zheng F, Johnson SW. Dual modulation of glutamate transmission by metabotropic glutamate receptors in rat ventral tegmental area. *Neuroscience*. 2003; 119:453–460. [PubMed: 12770559]

Research Highlights

- Neuronal properties of chicken cochlear nucleus angularis (NA) neurons are subject to neuromodulation.
- Group I mGluRs suppress EPSCs while increasing excitability of NA neurons.
- Group II and III mGluRs, and GABA_BRs suppress EPSCs of NA neurons.
- Group II and III mGluRs, and GABA_BRs suppress excitability of NA neurons.
- The modulation of glutamatergic transmission is via presynaptic mechanisms.

Fig. 1. Intrinsic neuronal properties of NA neurons

Based on the firing patterns in response to suprathreshold current injections into their cell bodies, NA neurons were classified into three types, consistent with a previous study (Soares et al. 2002). **A.** One-spike (onset) cells fired a single spike at the onset of the prolonged (200 ms) current injections. **B.** Damped cells generated a few spikes at the onset of the current injections, followed by spikes with reduced amplitude and subthreshold membrane oscillations. **C.** Tonic cells responded with trains of action potentials throughout the period of the current injections. **D.** Number of action potentials was plotted against the amplitude of the injected currents relative to the threshold current (the minimal current required to elicit

spikes). Onset cells (n=15) had a flat input-output function. Damped cells (n=14) showed adaptation, with an increase in the number of spikes in response to increasing currents and reduced spiking to more depolarizing current steps. Tonic cells (n=17) showed an increase in number of action potentials with increasing current amplitude. The scale bars in panel A also apply to panels B and C. Cells were held in current clamp at their resting membrane potential (RMP), which was -60, -59, and -53 mV for the neurons in A, B, and C, respectively. The chart recordings in panels A, B, and C are sEPSCs recorded under voltage clamp at -60 mV. The one-spike neuron appeared to have higher frequency sEPSCs than the other two types of neurons (see Table 1 for analysis). In this and subsequent figures, Means $\pm$ SEM are shown.

Fig. 2. Activation of group I mGluR enhanced excitability and suppressed EPSCs of nononset cells

A. Sample membrane voltage recordings of one tonic firing NA neuron (RMP: -66 mV) in response to a negative current injection (-0.4 nA) and two suprathreshold (0.25 and 0.6 nA) current injections. Bath-application of group I mGluR agonist DHPG (100 μ M) increased the number of spikes. **B.** DHPG increased spike firing of non-onset cells (n=7), and the effect was long lasting, without recovery after washout of DHPG. **C.** The EPSC amplitude of one NA neuron was plotted against time. The insets show the averaged EPSCs obtained under conditions of control (a), DHPG (b), and washout of the agonist (c). **D and E.** DHPG

significantly reduced the amplitude of EPSCs of non-onset cells (n=28: 7 one-spike, 10 damped, and 11 tonic cell).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. Activation of group II mGluRs suppressed excitability and EPSCs in a cell-type specific manner

A. Sample membrane voltage recordings of one tonic firing NA neuron (RMP: -53 mV) in response to a negative current injection (-0.4 nA) and two suprathreshold (0.2 and 0.75 nA) current injections. Bath-application of mGluR II agonist DCG-IV (2 μ M) reduced the number of spikes. **B.** The number of spikes of non one-spike cells was normalized to the maximal spike count under the control condition, and plotted against the amplitude of the injected currents relative to the threshold currents. DCG-IV reduced the overall spike count, and strongly suppressed cellular excitability at higher current injections (> 600 pA) ($n=6$).

C. mGluR II antagonist LY341495 (200 nM) increased the overall spike count at higher current injections (> 700 pA) ($n=5$), and the effect was long lasting, without recovery after washout of DCG-IV. **D.** EPSC amplitude of one NA neuron was plotted against time. The insets show the averaged EPSCs obtained under conditions of control (a), DCG-IV (b), and washout of the agonist (c). **E.** DCG-IV significantly reduced the amplitude of EPSCs on the non-onset cells and slightly reduced the amplitude of EPSCs on the onset cells ($n=29$: 14 one-spike, 5 damped, and 10 tonic cells). A partial recovery was observed after washout of DCG-IV. **F.** LY341495 significantly increased the amplitude of EPSCs on the non-onset cells, indicating endogenous activity of group II mGluRs on the modulation of EPSCs ($n=16$: 8 onset, 8 non onset). **G–I.** Expression of group II mGluRs in the NA (delineated by the red dashed line) was detected at different ages (E18, $n=2$ animals; P1–P2, $n=3$ animals; P7–P9, $n=2$ animals). In this and subsequent figures, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively (ANOVA post hoc Fisher's test unless indicated otherwise). Cells were held at the RMP and -60 mV for current and voltage clamp recordings, respectively.

Fig. 4. Activation of group III mGluRs suppressed excitability and EPSCs

A. Sample membrane voltage recordings of one tonic firing NA neuron (RMP: -54 mV) in response to a negative current injection (-0.4 nA) and two suprathreshold (0.2 and 0.75 nA) current injections. Bath-application of mGluR III agonist L-AP4 (10 μ M) reduced the number of spikes. **B.** L-AP4 reduced spike firing of non one-spike cells ($n=6$), and a partial recovery was observed after washout of L-AP4. **C.** CPPG (10 μ M), antagonist of group III mGluRs, increased the overall spike count at higher current injections (> 550 pA) ($n=7$). **D.** The EPSC amplitude of one NA neuron was plotted against time. The insets show the averaged EPSCs obtained under conditions of control (a), L-AP4 (b), and washout of the

agonist (c). **E.** L-AP4 significantly reduced the amplitude of EPSCs (n=25: 8 one-spike, 8 damped, and 9 tonic cells). **F.** After washout but not during application, CPPG significantly increased the amplitude of EPSCs on the onset cells, indicating delayed endogenous activity of group III mGluRs on the modulation of EPSCs (n=23: 10 onset, 13 non-onset). **G–I.** Expression of group III mGluR in the NA (delineated by the red dashed line) was detected at E13–E18 (n=2 animals), and the immunoreactivity diminished in hatchlings (P1–P2, n=2 animals; P7–P9, n=2 animals).

Fig. 5. Activation of GABA_BRs suppressed excitability and EPSCs

A. Sample membrane voltage recordings of one tonic firing NA neuron (RMP: -66 mV) in response to a negative current injection (-0.4 nA) and two suprathreshold (0.25 and 0.6 nA) current injections. Bath-application of GABA_BR agonist baclofen (100 μM) reduced the number of spikes. **B.** Baclofen reduced spike firing of non one-spike cells (n=7), and the inhibitory effect was long lasting, without recovery after washout of baclofen. **C.** The EPSC amplitude of one NA neuron was plotted against time. The insets show the averaged EPSCs obtained under conditions of control (a), baclofen (b), and washout of the agonist (c). **D and E.** Baclofen significantly reduced the amplitude of EPSCs (n=13: 6 one-spike, 4 damped,

and 3 tonic cell), and a sustained suppression of EPSCs was observed after washout of baclofen. Expression of GABA_BRs in the chicken auditory brainstem nuclei including NA was reported previously (Burger et al. 2005).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6. Presynaptic modulation of glutamate release by mGluRs and GABA_BRs

A1. Representative sEPSCs recorded under the conditions of control, DCG-IV (2 μ M), and washout. **A2 & A3.** DCG-IV (2 μ M) significantly reduced the frequency but not the amplitude of sEPSCs ($n=8$: 5 one-spike, and 3 tonic cells). **A4.** The suppression of EPSCs is associated with a reduction in paired-pulse ratio (PPR), indicating presynaptic modulation. **B.** L-AP4 (10 μ M) significantly reduced the frequency and PPR, without affecting the amplitude of sEPSCs ($n=15$: 7 one-spike, 4 damped, and 4 tonic cells). **C.** DHPG (100 μ M) significantly reduced the frequency and PPR, without affecting the amplitude of sEPSCs ($n=19$: 8 one-spike, 6 damped, and 5 tonic cells). **D.** LY341495 (200 nM) did not affect the frequency or the amplitude of sEPSCs ($n=15$: 8 one-spike, 4 damped, and 3 tonic cells). **E.** CPPG (10 μ M) significantly reduced the frequency and increased the PPR, without affecting

the amplitude of sEPSCs (n=12: 4 one-spike, 4 damped, and 4 tonic cells). **F.** Baclofen (100 μ M) produced similar effects (n=8: 3 one-spike, and 5 tonic cells). NS: not significant.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Comparisons of membrane properties and sEPSC in different cell types in NA.

	One-spike	Damped	Tonic	ANOVA p
N	46	22	30	
RMP (mV)	-58.3 ± 2.5	-57.7 ± 1.9	-56.0 ± 1.5	0.412
R_{in} (M Ω)	223 ± 22	289 ± 21	316 ± 13	0.008 ^{**}
I threshold (pA)	454 ± 38	237 ± 37	201 ± 46	0.010 [*]
sEPSC				
Frequency (Hz)	1.82 ± 0.21	1.02 ± 0.17	1.14 ± 0.24	0.004 ^{**}
Amplitude (pA)	22.26 ± 2.48	15.01 ± 1.80	19.29 ± 2.24	0.015 [*]
Rise time (ms)	1.59 ± 0.179	1.51 ± 0.16	1.24 ± 0.13	0.457
Decay tau (ms)	2.16 ± 0.24	4.23 ± 0.88	3.05 ± 0.54	0.002 ^{**}

Mean $\pm$ SEM are shown. N: number of cells.

^{*}
p < 0.05,

^{**}
p < 0.01, ANOVA. Post hoc Fisher's test detected significant difference in: 1) R_{in} and I threshold between one-spike and tonic neurons; 2) sEPSC frequency between one-spike and other two types of neurons; 3) sEPSC amplitude between onset and damped neurons, and between damped and tonic neurons; and 4) sEPSC decay tau between one-spike and other two types of neurons.