Presynaptic modulation of inhibitory synaptic feedback via $GABA_B$ receptors in the avian cochlear nucleus angularis

Running head: GABAergic modulation in the cochlear nucleus

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Abstract

Inhibition plays multiple critical roles in the neural processing of sound. In the avian auditory brain stem, the cochlear nuclei receive their principal inhibitory feedback from the superior olivary nucleus (SON) in lieu of local inhibitory circuitry. In the timing pathway, GABAergic inhibitory feedback underlies gain control to enhance sound localization. In the cochlear nucleus angularis (NA), which processes intensity information, how the inhibitory feedback is integrated is not well understood. Using whole cell patch clamp recordings in chick brain stem slices, we investigated the effects of GABA release on the inhibitory (presumed SON) and excitatory (8th nerve) synaptic inputs onto NA neurons. Pharmacological activation of the metabotropic $GABA_B$ receptors with baclofen profoundly suppressed both evoked excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs). Baclofen similarly reduced the frequency of spontaneous IPSCs and EPSCs, but had no significant effect on the current kinetics or amplitudes, indicating a presynaptic locus of modulation. Trains of IPSCs showed substantial transient and sustained short-term synaptic facilitation. Baclofen application reduced the initial IPSC amplitude, but enhanced the relative facilitation over the train via changes in release probability. Comparable levels of $GABA_B$ receptor mediated blockade also shifted short-term synaptic plasticity of EPSCs toward less depression. Evoked (but not spontaneous) release of GABA was sufficient to suppress basal release at inhibitory synapses in slices. Overall, the modulation of excitatory and inhibitory inputs of NA neurons via $GABA_B$ receptor activation appears to parallel that in the timing pathway.

New and Noteworthy

Avian cochlear nucleus angularis (NA) neurons are responsible for encoding sound intensity and provide level information for gain control feedback via the superior olivary nucleus. This GABAergic inhibitory feedback was itself modulated in NA via presynaptic, metabotropic $GABA_B$ receptor mediated suppression. Excitatory transmission was modulated by the same receptors, suggesting parallel homosynaptic and heterosynaptic mechanisms in both cochlear nuclei.

Introduction

Inhibition plays a critical role in auditory processing, contributing in various ways to sound level gain control (Peña et al., 1996), sculpting frequency tuning (Köppl and Carr, 2003; Fukui et al., 2010), and enhancing temporal processing (Funabiki et al., 1998; Yamada et al., 2013). In the avian system, nearly all cochlear nucleus neurons provide feedforward excitatory projections to the ascending pathways, while inhibitory inputs principally arise from an anatomically separate brain stem region, the superior olivary nucleus (SON) (Funabiki et al., 1998; Yang et al., 1999; Lu and Trussell, 2000; Monsivais et al., 2000; Lu and Trussell, 2001). The SON provides ipsilateral feedback inhibition to both divisions of the cochlear nucleus, nucleus angularis (NA) and nucleus magnocellularis (NM), as well as to nucleus laminaris (NL) (Burger et al., 2005a; Nishino et al., 2008). This inhibitory feedback loop to NM and NL has been proposed to enact an intensity-dependent gain control that enhances coincidence detection for interaural time difference. While NA provides a source of sound level information to the SON (Lachica et al., 1994; Yang et al., 1999), how the reciprocal inhibitory feedback is integrated into sound processing in NA is not yet clear.

The mechanisms underlying inhibitory control of the timing nuclei involve both presynaptic and postsynaptic regulation. Inhibitory synaptic terminals in these nuclei release the neurotransmitter γ–aminobutyric acid (GABA), along with glycine. Postsynaptic inhibitory responses are principally $GABA_A$ receptor mediated in NM (Kuo et al., 2009; Tang et al., 2009; Coleman et al., 2011; Tang and Lu, 2012a; Fischl and Burger, 2014). Presynaptic inhibition is known to occur via metabotropic GABA ($GABA_B$) receptors in NM. Pharmacological activation of $GABA_B$ receptors had a strong presynaptic suppressive effects on glutamatergic inputs to NM neurons (Brenowitz et al., 1998; Brenowitz and Trussell, 2001a; 2001b). Activation of $GABA_B$

receptors also suppressed inhibitory inputs onto NM neurons, as well as onto NL (Tang et al., 2009), suggesting the inhibitory terminals were self-regulatory via homosynaptic feedback.

Less is known about the mechanisms and functional effects of inhibition in nucleus angularis. Immunohistochemical and electron microscopy studies showed $GABA_B$ receptors were also expressed in NA (Burger et al., 2005b). Like in NM, a previous study has shown that $GABA_B$ agonists had a suppressive effect on excitatory synaptic inputs into NA via presynaptic mechanisms (Shi and Lu, 2017). In this study, we examined the effects of activation of $GABA_B$ mechanisms on the inhibitory inputs to NA neurons using in vitro whole cell patch clamp electrophysiology in the brain stem slice preparation. The results provide evidence for GABAergic presynaptic modulation of these putative SON inputs to NA, as well as similar modulation of excitatory (presumptive nerve) input. These data suggest a common mechanism for homosynaptic regulation was present at the inhibitory terminals at all three descending targets of the SON.

Results

Activation of metabotropic GABA receptors suppresses inhibitory and excitatory neurotransmitter release.

To investigate inhibitory modulation of synaptic transmission in the avian cochlear nucleus angularis, the specific $GABA_B$ receptor agonist baclofen was applied during recordings of inhibitory postsynaptic currents (IPSCs) in acute brain stem slices. Synaptic currents were evoked by extracellular electrical stimulation and measured under whole-cell voltage clamp mode. Baclofen (20 μM) reliably reduced the peak amplitudes of pharmacologically isolated evoked IPSCs to 10.5% of control levels (Figure 1A, B; IPSC in control: -0.38±0.18 nA; in baclofen: -0.041±0.043 nA; $p=2.4x10^{-5}$, n=12, paired Student's T test). The baclofen effect on IPSC amplitudes was partially and inconsistently reversed with washout of the drug (Fig. 1Ci), recovering to 52% of control levels on average (IPSC peak amplitude with washout: -0.20±0.16 nA, n=10, p=0.0032). Baclofen did not affect the time course of the currents (Fig. 1Cii).

We observed similar levels of $GABA_B$ receptor driven suppression on pharmacologically isolated evoked excitatory postsynaptic currents (EPSCs), the putative auditory nerve inputs. Baclofen reliably suppressed the mean EPSC peak amplitude to 8.4% of control levels (EPSC peak amplitude in control: -0.478±0.315 nA; baclofen: 0.040±0.048 nA, n=7, p=0.011). Washout of the baclofen effect on EPSCs was slow and inconsistent, only measurable in a subset of neurons (washout EPSC amplitudes: -0.71±0.85, compare to matched control EPSC amplitudes: -0.57±0.37, n=4; no significant difference, p=0.60)(Fig. 2Ci). Baclofen also did not affect the time course of the evoked EPSCs (Fig. 2Cii).

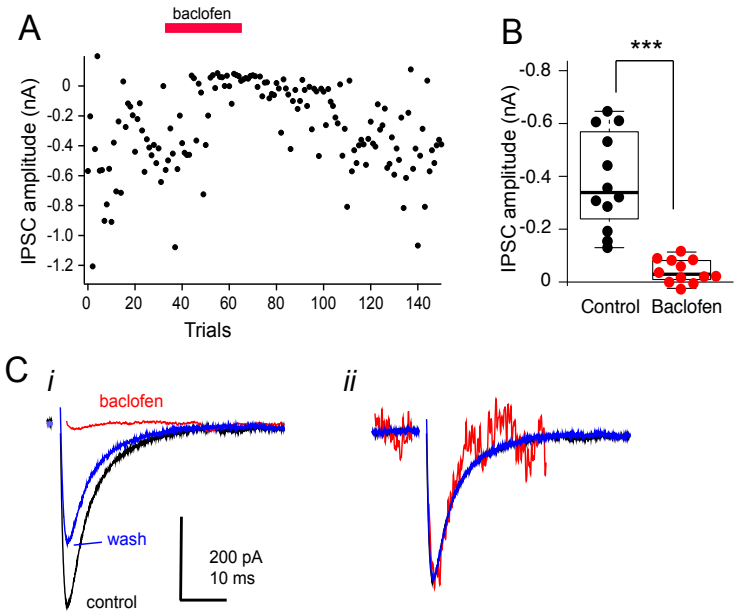

Figure 1 Baclofen suppressed the evoked inhibitory postsynaptic currents (IPSCs) onto neurons in the cochlear nucleus angularis. A) Single IPSCs recorded under patch clamp in one NA neuron evoked at a low rate (0.1 Hz) before, during, and after bath application of baclofen (20 µM). B) Summary data of IPSC peak amplitude (n=12, *** $p<2.4x10^{-5}$, paired Student's T test). C) Comparison of the average IPSC traces for each condition, overlaid (i) or scaled and overlaid (ii), showed the decline in amplitude but no change in kinetics occurred in baclofen (red trace) versus control (black trace). The IPSC partially recovered during washout (blue trace). Same recording as A.

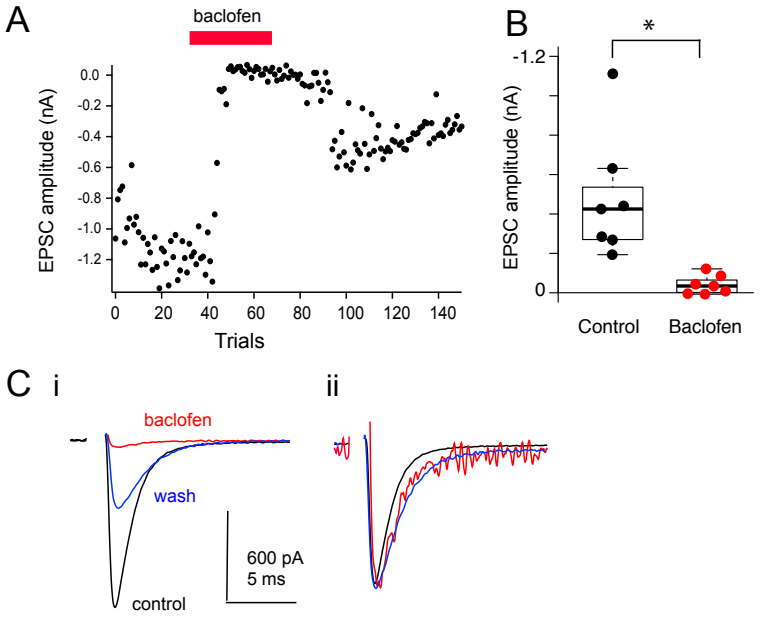

Figure 2 Baclofen also suppressed the evoked excitatory postsynaptic currents onto neurons in the cochlear nucleus angularis. A) Single EPSCs in one NA neuron evoked at a low rate (0.1 Hz) before, during, and after bath application of baclofen (20 µM). B) Summary data of IPSC absolute amplitude (n=7, p=0.011, paired Student's T test). C) Comparison of the average EPSC traces for each condition, overlaid (i) or scaled and overlaid (ii), showed the decline in amplitude but no change in kinetics occurred in baclofen (red trace) versus control (black trace). The EPSC partially recovered during washout (blue trace). Same recording as A.

For both excitatory and inhibitory synapses, the baclofen effect was blocked by co-application of the specific $GABA_B$ receptor antagonist, CGP52432 (10 μM). Blockade was complete for evoked IPSCs (n=4; control IPSC, -0.53±0.23 nA; baclofen+CGP, -0.53±0.11 nA, no significant difference, p=0.93). The same concentration of CGP52432 blocked more than half the baclofen suppression of the evoked EPSCs (n=5, control EPSC, -0.90±0.54 nA; baclofen+CGP, -0.64±0.40 nA, 74.4% of control but not significantly different, p=0.055).

Presynaptic locus of suppression

To determine whether baclofen was acting presynaptically, in separate experiments we examined the effect of the drug on pharmacologically isolated spontaneous synaptic currents under voltage clamp. Current traces of these events showed that baclofen application significantly decreased the frequency of both inhibitory and excitatory spontaneous PSCs (sIPSCs and sEPSCs, respectively)(Fig. 3Ai, Bi). Cumulative histograms of the interevent intervals for sIPSCs showed significant shifts to longer intervals ($p=1.12x10^{-10}$, N=400 control events, N=397 baclofen events, Kolmogorov-Smirnov test, Fig. 3Aii). The average spontaneous event rate for sIPSCs was reduced from 2.1±1.7 Hz in control conditions to 0.60±0.76 Hz when 50 μM baclofen was bath applied (n=20, $p=1.9x10^{-6}$, Wilcoxon rank sum test)(Fig. 3Ci).

Likewise, cumulative histograms of the interevent intervals of sEPSCs showed significant shifts to longer intervals ($p=4.54x10^{-4}$, N=250 control events, N=204 baclofen events, Kolmogorov-Smirnov test, Fig. 3Bii). The average spontaneous event rate sEPSCs was reduced from 1.5±1.3 Hz in control conditions to 1.0±1.9 Hz in baclofen (n=9, p=0.012, Wilcoxon rank sum test, Fig. 3Di). In contrast, baclofen application did not significantly affect the amplitudes and kinetics of either the sIPSCs (Fig. 3Cii-iv) or sEPSCs (Dii-iv). Spontaneous IPSCs had mean

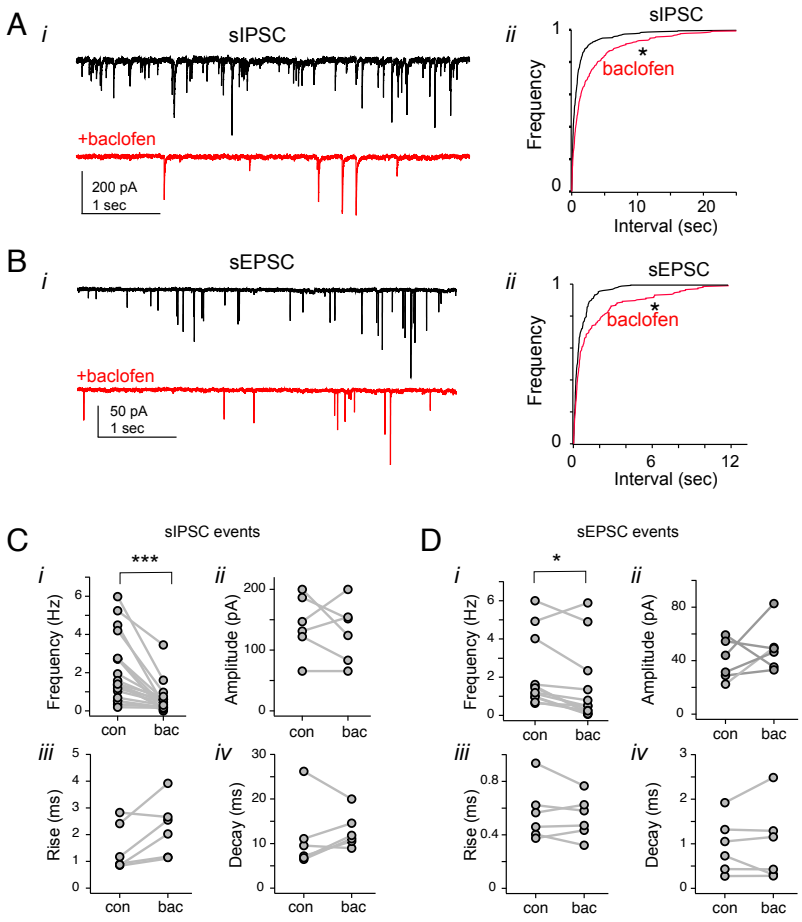

Figure 3 Baclofen effects on spontaneous IPSC and EPSC events.
(A-B) Baclofen application reduced the frequency of spontaneous IPSC events (A) and spontaneous EPSC events (B). Current traces (i) and cumulative histograms of PSC interevent intervals (ii) before (black) and after application of baclofen (red). Baclofen shifted the distribution toward longer intervals (IPSCs : N=400 control events, N=397 baclofen events, $p=1.12x10^{-10}$, Kolmogorov-Smirnov test; EPSCs: N=250 control events, N=204 baclofen events, $p=4.54x10^{-4}$, Kolmogorov-Smirnov test). (C-D) Summary of sIPSC (C) and sEPSC (D) statistics : (i) mean frequency, (ii) peak amplitude, (iii)10-90 rise time, and (iv) single exponential decay time constant before (Con) and after baclofen application (Bac). A significant decline in mean event frequency was observed for sIPSCs (n=20 experiments, *** $p=1.9x10^{-6}$, Wilcoxon rank sum test) and sEPSCs (n=9 experiments, * p=0.00122). No significant differences were found in sIPSC amplitude, rise time or decay kinetics following baclofen application (sIPSCs, n=6; sEPSCs, n=6).

amplitude of 0.11±0.56 nA in control and 0.091±0.059 pA in baclofen (n= 6, p=0.59), while sEPSCs had an amplitude of 0.040±0.015 nA in control and 49.4±0.018 nA in baclofen (n=6, p=0.438). Spontaneous IPSCs had single-exponential decay time constants of 12.6±7.5 ms in control and 19.1±15.9 ms in baclofen (p=0.563), while sEPSCs had decay time constants of 0.96±0.61 ms in control and 0.99±0.85 ms in baclofen (p=0.9163). These data suggest that baclofen may act via presynaptic $GABA_B$ receptors to alter release, but had little effect on postsynaptic determinants of the neurotransmitter response.

Effects of $GABA_B$R-mediated suppression on plasticity.

To determine how $GABA_B$ receptor activation might affect synaptic dynamics, we measured the IPSC amplitude profile during partial baclofen suppression on 20 Hz trains (Figure 4A). In the majority of recordings, the responses to trains of stimuli at this frequency revealed transient or sustained facilitation of the IPSC amplitude under control conditions (Fig. 4B, black trace). Because the maximal baclofen application could sometimes reduce the initial response amplitudes to negligible levels (e.g. 'max baclofen', Fig. 4A,B), changes in short-term plasticity were evaluated using blocks of trials during which the initial IPSC was reduced approximately to 30% of the control amplitude (Fig.4A-D)(control: -0.29±0.16 nA; 'partial baclofen': -0.11±0.08 nA, 63% reduction, p=0.016, n=7 neurons). As baclofen reduced the initial IPSC amplitude, the relative amplitudes of IPSCs later in the train were enhanced, showing greater facilitation compared to control (Fig. 4B,C; $p=1.2x10^{-5}$, 2-way ANOVA). While the paired pulse ratio marginally increased from 1.28±0.46 to 1.58±0.88 (22% increase, p=0.43, no significant difference), the steady state ratio doubled from 1.24±0.64 to 2.47±1.63 (99% increase, p=0.0495)(Fig. 4Ei,ii).

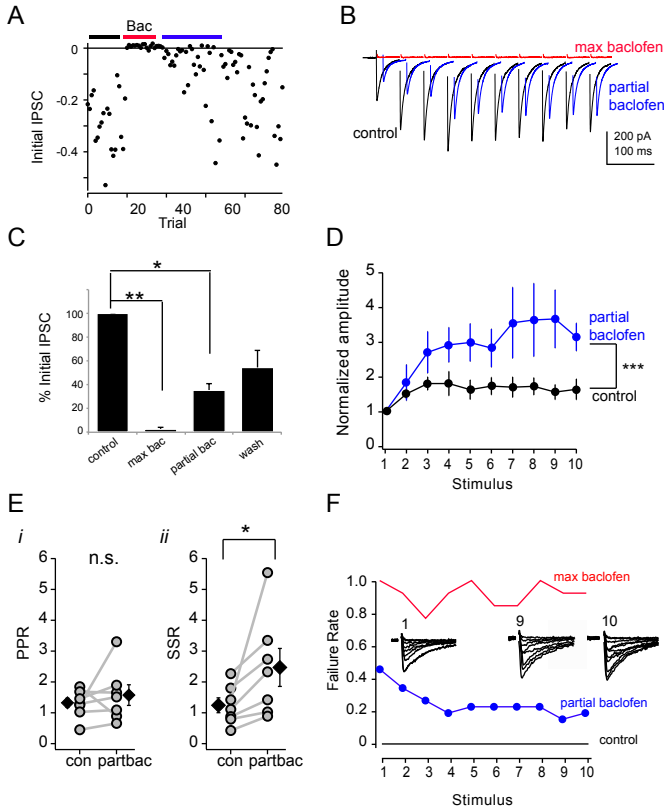

Figure 4 Baclofen enhanced the short-term synaptic facilitation during trains of electrically evoked IPSCs. A) Amplitude of the initial evoked IPSC of the train was profoundly suppressed during drug application (red bar), then recovered gradually. Blue bar shows time frame of trials analyzed for partial recovery. B) Comparison of mean current traces of train (20 Hz) before (black) and after maximal suppression (red), and during partial recovery (blue traces are horizontally offset for clarity). C) Initial PSC suppression during control, maximal blockade (>90% blocked, **p<0.01) partial blockade (>64% blocked, * p<0.05) and late washout (not significantly different from control). D) Normalized amplitude summary plot shows short-term plasticity of IPSCs over trains (mean±s.e.m., n=7 experiments). Sustained facilitation was typical of IPSC dynamics during moderate stimulation frequencies during control trials (black), which was enhanced during partial blockade (blue). E) Paired pulse ratio (PPR) was unchanged during partial baclofen blockade (*i*), but sustained facilitation (*ii*, SSR, steady state ratio, see Methods) increased significantly (*, p<0.05). Gray circles are individual experiments, filled diamonds are mean±s.e.m. F) Failure rate at each stimulus in the train in one experiment, under control conditions (black line~0), partial blockade (blue markers and line), and maximal blockade (red line). Partial baclofen suppression revealed decreasing failure rate over the course of the train. Inset traces show representative IPSCs during partial blockade at the first (1), ninth (9), and tenth (10) stimulus of the train.

Partial blockade allowed investigation of how release probability changed over the course of the train by measuring the failure rate. Under control conditions, the evoked IPSC failure rate in response to the first stimulus was nearly zero (1.3±1.9%, mean ± s.d., n=7 experiments); as these were not minimal stimulation experiments, responses likely comprised multiple inputs. In contrast, during partial blockade the failure rate in response to the first stimulus was ~40% (one example shown in Fig. 4F, 'partial baclofen', blue line). Over the course of the train, the failure rate then declined to ~19% in response to the last stimulus in this example. The average failure rate during partial blockade was a 38.8±31.3% (mean ± s.d., n=7 experiments) for the initial IPSC and declined to 17.2±0.3% by the end of the 20 Hz train. During maximal blockade, we could also detect some decline in the failure rate over the train (Fig. 4F, 'max baclofen', red line). For all experiments, under maximal blockade, the failure rate in response to the first stimulus was on average 79.6±21.6%, which then decreased over the course of the train to 49.9±37.9%. Together with the effects observed on the spontaneous neurotransmitter release, these results suggest that activation of $GABA_B$ receptors acts by reducing the initial release probability, which then increased over the course of the train via enhanced presynaptic facilitation.

Similar experiments were performed to determine how $GABA_B$ receptor activation affected the excitatory synaptic dynamics. In these experiments, under control conditions the EPSC amplitude depressed to approximately 60% of initial amplitude during a 20 Hz constant frequency train (Fig. 5A, B; control: black trace, partial baclofen: blue trace). To measure whether baclofen had comparable effects as on IPSCs, short-term plasticity of the EPSCs was evaluated using blocks of trials during which the initial EPSC was reduced approximately to ~26% of the control amplitude (control: 1.46±0.73 nA; 'partial baclofen': -0.39±0.18 nA, 74%

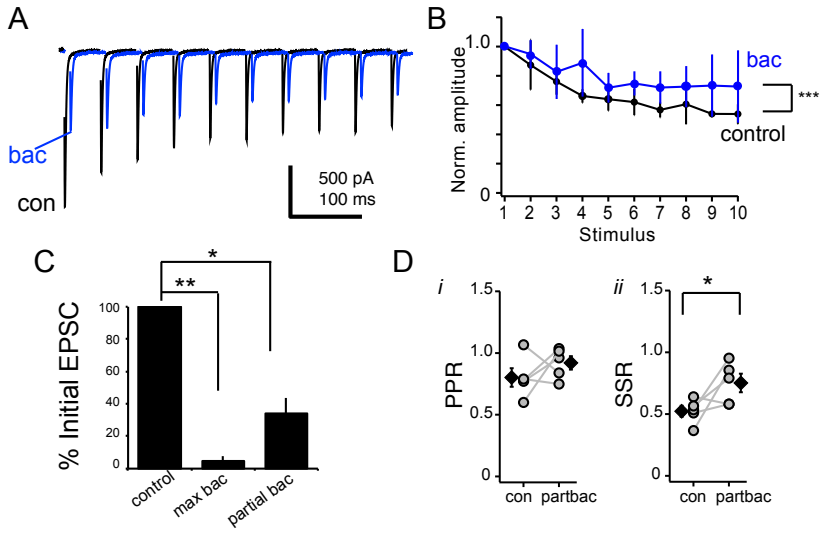

Figure 5 Baclofen reduced the short-term synaptic depression of EPSCs during short trains. A) Comparison of mean traces of train of EPSCs (20 Hz) after maximal suppression and during partial recovery (traces are horizontally offset for clarity). B) Normalized summaryamplitude plots shows short-term plasticity of EPSCs over train (mean±s.e.m., n=4). C) Initial EPSC suppression during control, maximal blockade (>90% blocked, **p<0.01), and partial blockade (>66% blocked, * p<0.05). D) Paired pulse ratio (PPR) was unchanged during partial baclofen blockade (i), but the short-term synaptic depression significantly decreased (steady state ratio increased, SSR, *, p<0.05) (ii). Gray circles are individual experiments, filled diamonds are mean±s.e.m.

reduction, n=5 neurons). As baclofen reduced the initial EPSC amplitude, the relative amplitudes of EPSCs later in the train were enhanced, showing less depression compared to control (p=0.00412, 2-way ANOVA). Under partial baclofen suppression, the paired pulse ratio for EPSCs showed no significant change from 0.80±0.17 to 0.92±0.12 (15% increase, p=0.24, Student's T test)(Fig. 5Di), while the steady state ratio increased from 0.52±0.10 to 0.75±0.17 (44% increase in the ratio, p=0.031). Thus under conditions of a moderate, but not maximal, activation of $GABA_B$ receptors, there was a small but significant shift in short-term plasticity towards less depression in their excitatory train responses.

Evidence for basal $GABA_B$ receptor activity on synaptic weight.

To test whether $GABA_B$ receptors could endogenously modulate synaptic dynamics during physiological activation in vitro, we elicited trains of evoked IPSCs and applied an antagonist of the receptor, CGP52432, and measured the effects on short-term plasticity. Unexpectedly, bath application of CGP52432 increased the initial IPSC amplitude (Fig. 6A). On average, the initial IPSC increased from -0.44±0.29 nA in control conditions to -0.62±0.38 nA with application of CGP52432 (41% increase, n=10, p=0.018, paired Students t-Test)(Fig. 6Ai). Conversely, inhibiting GABAergic reuptake with NNC771 decreased the evoked IPSC amplitude, from -0.51±0.26 nA to -0.36±0.22 (30% reduction, n=10, p=0.022)(Fig. 6Aii). No significant effects were observed by either drug on the amplitude of evoked EPSCs. The initial EPSC showed control amplitude of -0.84±0.17 nA followed by an EPSC amplitude in NNC of -0.73±0.28 nA (n=4, p=0.31). Similarly, the initial EPSC amplitude in control was -1.08±0.72 nA followed by an EPSC amplitude of -1.05±0.42 nA in CGP52432 (n=6, p=0.83)(Fig. 6B).

The changes observed in short-term plasticity during IPSC trains were consistent with the change in initial amplitude: an increase the initial amplitude under CGP52432 was accompanied by a decrease in facilitation (n=5, $p=2.14 \times 10^{-4}$, 2 way ANOVA). Conversely, we asked whether the reduction in the initial inhibitory synaptic amplitude in the presence of the GABA reuptake inhibitor would be accompanied by an increase in facilitation. However, blocking the reuptake of GABA during trains with reuptake inhibitor NNC771 was found to have minor and inconsistent

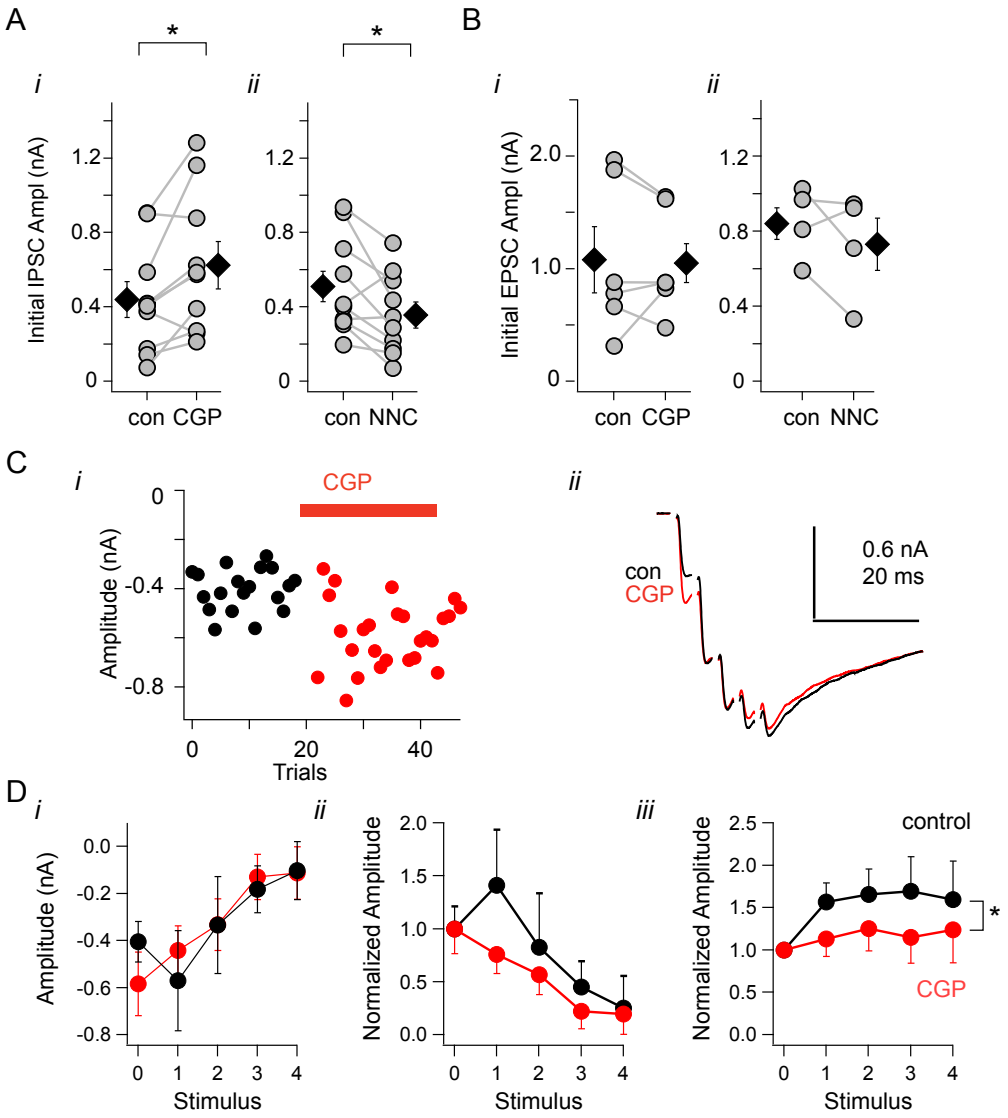

Figure 6 Blocking GABA reuptake (with GABA transporter inhibitor NNC771) or GABAB receptor (with antagonist CGP52432) has opposing effects on baseline inhibitory synaptic release and short-term synaptic plasticity. A) Evoked IPSC amplitudes were enhanced with application of GABA-B receptor antagonist CGP52432 (i) and suppressed with inhibition of GABA reuptake transporter NNC771 (ii). B) Evoked EPSCs were unaffected by either drug. C) Example experiment of CGP52432 effects on short, high frequency (200 Hz) trains of IPSCs. CGP52432 increased the amplitude of initial IPSC in the train (i), but not later IPSCs (ii). D) Measured net amplitude (summation subtracted) of each IPSC in the train before (black, "con") and after application of CGP52432 (red, 'CGP'). Increased amplitude of the initial response eliminated facilitation and resulted in further depression. Examples of single neurons responses showing raw (i) and normalized (ii) amplitudes and summary normalized short-term plasticity (iii)(n=5 experiments, 2-way ANOVA).

effects on the short-term-plasticity with no significant effect on the train facilitation overall (n=4, p=0.083, 2 way ANOVA).

The effects of CGP52432 and NNC771 on the basal release properties during evoked trains implied ongoing, baseline activation of $GABA_BR$, suggesting that the GABA chronically in the milieu was sufficient to affect these receptors. To determine whether spontaneous release was also affected by the same pharmacological manipulations, we measured spontaneous IPSC frequency before and after application of NNC771 (Fig. 7A-C) and CGP52432 (Fig. 7D-F). When NNC771 was applied, the distribution of the interevent intervals between sIPSCs showed a significant shift toward longer intervals (Fig. 7B; $N_{control}$=378, N_{NNC}= 324, p=3.2x10^{-6}, Kolmogorov-Smirnov test), while the sIPSC frequency was reduced by 29% (control: 1.9±1.4 Hz; NNC771: 1.5±1.0 Hz; Fig. 7Ci, n=8, p=0.107, Wilcoxon Rank Sum test). In these neurons, NNC771 application appeared to have small but detectable effects on the synaptic current kinetics and amplitude. The sIPSC amplitude showed a small but significant decrease (Fig. 7Cii; control: -86.5±37.9 pA; NNC: -67.0±36.4 pA, p=0.039) and the decay time course slowed slightly (Fig. 7Civ; control: 7.3±3.6 ms; NNC: 10.0±5.9 ms, p=0.039, Wilcoxon Rank Sum test), but no change in the rise time was observed (Fig. 7Ciii; control: 1.3 ±0.2 ms; NNC: 1.5±0.3 ms, p=0.148). These mild postsynaptic effects on quantal event amplitude and kinetics with NNC771 indicate possible desensitization of postsynaptic ionotropic GABA receptors (Tang and Lu, 2012b).

In contrast, $GABA_B$ receptor antagonist CGP52432 had no effect on the spontaneous IPSC frequency, amplitude or kinetics (Fig. 7D). The cumulative histograms of the interevent intervals of sIPSCs were unchanged by application of CGP52432 (Fig. 7E; $N_{control}$=363, N_{NNC}= 338, p=0.656, Kolmogorov-Smirnov test), while the frequency marginally increased from

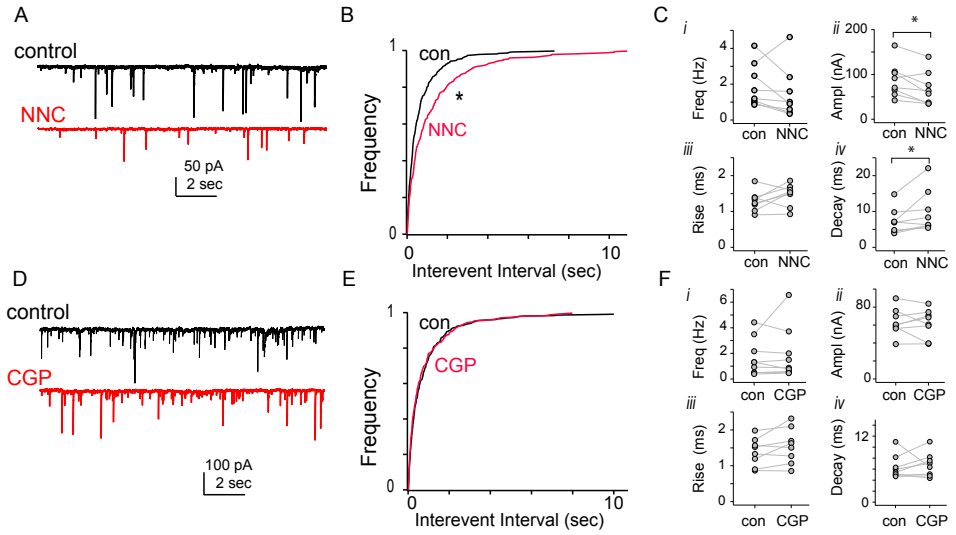

Figure 7

Figure 7 Effects of GABA reuptake inhibitor NNC771 (A-C) and $GABA_B$ receptor blockade CGP52432 (D-F) on spontaneous IPSC activity and kinetics. A) Example current traces showing spontaneous IPSC events before (top black trace) and after (bottom red trace) application of NNC771. B) Cumulative histograms of pooled sIPSC interevent intervals before and after application of NNC771. The distribution of time intervals between events shifted toward longer intervals with drug application. C) Summary statistics of mean sIPSC frequency, peak amplitude, 10-90 rise time, and single exponential decay time constant before and after drug application (n=8 experiments). No significant differences were found in average frequency or rise time; a significant decrease in mean amplitude and increase in decay were observed (p=0.039 and p=0.039, Wilcoxon Rank Sum test). D) Example current traces showing spontaneous IPSC events before (top black trace) and after (bottom red trace) application of CGP52432. E) Cumulative histogram of pooled sIPSC interevent intervals before and after application of CGP52432 showed no changes. F) Summary statistics reveal no significant changes with CGP52432 application showing mean sIPSC spontaneous rate frequency, peak amplitude, 10-90 rise time, and single exponential decay time constant before and after drug application (n=8 experiments).

1.8±1.3 Hz in control conditions to 2.1±0.9 Hz in CGP52432 (no significant effect, Fig. 7Fi). No changes in the kinetics or amplitude of the sIPSCs were observed with the antagonist (Fig. 7Fii-iv).

Discussion

These results suggest that both excitatory and inhibitory inputs may be modulated by the release of GABA via metabotropic receptors. The functional role of presynaptic $GABA_B$ receptors in sensory processing at synapses in the NA is as yet undetermined, but the presence of heterosynaptic and homosynaptic effects of GABA appears to function similarly in three important ascending auditory brain stem nuclei. Direct inhibitory modulation of NA neurons occurs via postsynaptic synapses and ionotropic GABA and glycine receptors, but the release of GABA may also indirectly influence information processing in these circuits via presynaptic mechanisms. Heterosynaptic effects on the auditory afferent inputs and homosynaptic effects on the inhibitory terminals were equally suppressive in their actions, which could maintain the excitatory versus inhibitory balance while providing common negative feedback.

Presynaptic GABAergic inhibition at inhibitory and excitatory synapses in NA.

Physiological evidence suggested that the strong suppression of inhibitory and excitatory evoked currents occurred via a presynaptic mechanism. An immunohistochemical study previously showed $GABA_B$ receptors were expressed in both avian cochlear nuclei and electron microscopy data further verified the presynaptic localization, at least within NM (Burger et al., 2005b). The clear decline in the frequency of spontaneous events that occurred with the $GABA_B$ agonist supports a presynaptic locus of action, in agreement with a previous study (Shi and Lu, 2017). At the same time, synaptic events showed no significant changes to event amplitude or

time course with direct pharmacological activation of the $GABA_B$ receptor, suggesting there was suggesting no postsynaptic effect on the channels that underlie the ionotropic IPSCs or EPSCs. Although we did not further test presynaptic mechanisms, presynaptically localized $GABA_B$ receptors have been shown in other systems to suppress neurotransmitter release via associated G-protein pathways and their downstream effectors. Downregulation of neurotransmitter release may occur by Gβγ subunit suppression of presynaptic voltage gated calcium channels or direct binding of vesicle release proteins inhibiting fusion (Gassmann and Bettler, 2012).

Previous research suggested that $GABA_B$ receptor agonists could modify the excitability of NA neurons directly via postsynaptically located receptors (Shi and Lu, 2017). It is unlikely that the baclofen effects observed here are due to postsynaptic changes in membrane properties or voltage gated channel activation, however, as synaptic currents in this study were measured under voltage clamp conditions using a cesium intracellular solution expected to block potassium currents, one likely endpoint of postsynaptic $GABA_B$ receptors. Even under current clamp conditions, baclofen did not induce changes in near-resting potential input resistance (Shi and Lu, 2017). Together these results suggest baclofen was acting solely via a presynaptic metabotropic pathway to modulate release in our study.

Role of inhibitory feedback in the timing pathway.

In the avian cochlear nucleus, the majority of known inhibitory feedback arises from an anatomically separate brainstem nucleus, the superior olivary nucleus (SON) (Funabiki et al., 1998; Yang et al., 1999; Lu and Trussell, 2000; Monsivais et al., 2000; Lu and Trussell, 2001). The two divisions of the cochlear nuclei, NA and NM, receive a common inhibitory feedback projection from the ipsilateral SON, as does the tertiary nucleus, NL (Burger et al., 2005a). NM

and NL together comprise the circuit known for binaural processing of timing information important for localization sound in the azimuthal plane. How the inhibitory feedback affects the ascending sound information processing and whether there is functional heterogeneity in those effects across brain structures is yet to be fully understood. Because the timing circuit is highly specialized and the ascending pathways via NA deal with sound intensity and spectrotemporal processing more broadly (Köppl and Carr, 2003; Steinberg and Peña, 2011; Fontaine et al., 2014), inhibition could have quite different roles in each area.

As sister nuclei that receive similar ascending nerve input and a common feedback, it is not surprising NA and NM have a number of similarities in their inhibitory synaptic properties. Among these are: co-release of GABA and glycine from presynaptic inhibitory terminals, likely arising from the same SON axons branching and projecting to each nucleus; chloride reversal potentials that are well above resting potential, resulting in depolarizing, inward currents; expression of postsynaptic fast ionotropic $GABA_A$ receptors ($GABA_AR$), postsynaptic glycine receptors (GlyR), and presynaptic and postsynaptic $GABA_B$ receptors (Brenowitz et al., 1998; Burger et al., 2005b; Kuo et al., 2009; Fischl and Burger, 2014; Shi and Lu, 2017). However, there are also a number of differences whose functional importance has yet to be explained. For example, in NA, the ionotropic postsynaptic inhibitory currents are mixed $GABA_AR$ /GlyR currents, while in NM (and NL) the inhibitory currents are largely carried by $GABA_AR$ (but see (Fischl et al., 2013; Fischl and Burger, 2014) for a possible role by GlyR during high activity levels). Furthermore, the inhibitory currents measured in NA are faster than in NM and NL, likely due to the faster kinetics of the GlyR component (Kuo et al., 2009).

Our findings suggest that the modulation of excitatory and inhibitory inputs of NA neurons via $GABA_BR$ activation appears to mirror previous studies of GABAergic modulation of

synaptic transmission in NM (Brenowitz et al., 1998; Brenowitz and Trussell, 2001a; 2001b). Agonists of $GABA_BR$ had an strong presynaptic suppressive effect on excitatory, presumptive nerve, inputs to NM neurons. These axosomatic calyceal terminals are highly specialized endings thought to reliably evoke large, suprathreshold glutamatergic potentials sufficient to elicit an action potential without fail. These relatively high-release probability synapses also show strong activity-dependent synaptic depression. Upon activation of presynaptic $GABA_B$ receptors, the baseline release probability was reduced and the initial amplitude was reduced (but still suprathreshold), but later responses in a train of activity were, paradoxically, less likely to fail to produce action potentials due to reduction in synaptic depression and relief from receptor desensitization.

In NA, presynaptic modulatory effects may similarly alter the synaptic dynamics: $GABA_BR$ activation likewise suppressed baseline release and shifted the synaptic plasticity away from depression. However, because the excitatory inputs to NA neurons tend to be subthreshold (requiring summation to elicit an action potential) and exhibit less synaptic depression than NM under control conditions, presynaptic GABAB modulation could have markedly different end effects on this network. Excitatory inputs to NL, which originate from NM, do not appear to be modulated by presynaptic GABAB receptors, suggesting differential regulation of excitation by SON feedback relative to the cochlear nuclei (Lu et al., 2005; Tang et al., 2009).

Heterosynaptic versus homosynaptic inhibition.

In summary, we conclude that inhibitory terminals from the SON are subject to negative feedback or self-regulation in all three nuclei to which it projects. Thus the SON provides 1) direct inhibitory input to the projection neurons of NA via ionotropic GABA/glycine receptors

(Kuo et al., 2009), 2) indirect, heterosynaptic inhibitory control of excitatory drive via metabotropic $GABA_B$ receptors on glutamatergic terminals (Lu et al., 2005; Lu, 2009), and 3) negative feedback regulatory signal via homosynaptic inhibitory metabotropic $GABA_B$ receptors on the SON terminals (this study). The agonist effectiveness on the excitatory and inhibitory release appeared to be comparable, causing >90% suppression. Under what physiological conditions modulation by presynaptic $GABA_B$ receptor occurs is yet to be determined, but our experiments suggest the inhibitory terminals may be more susceptible to modulation.

During mild to moderate stimulation conditions, GABA release was sufficient to suppress baseline transmission at inhibitory synapses (see Fig. 6). In addition, blocking reuptake with an inhibitor of the GABA transporter, presumably causing transmitter pooling, was sufficient to reduce spontaneous inhibitory event frequency, but not spontaneous excitatory event frequency (see Fig. 7). On the other hand, blocking $GABA_B$ receptors, however, had no effect on spontaneous inhibitory (nor excitatory) event frequency, suggesting ambient spontaneous neurotransmitter release is not sufficient to elicit chronic, baseline suppression via this pathway. These results suggest that under normal physiological activity, the SON input may itself be under chronic, mild suppressive negative feedback.

Our experiments failed to produce evidence for physiologically-evoked, endogenous heterosynaptic suppression of excitatory inputs to NA neurons. Neither NNC771 nor CGP52432 had any effect on the initial or singly-evoked EPSCs (see Fig. 6) or on the short-term plasticity during EPSC trains (data not shown). Because the EPSCs were pharmacologically isolated, it could not be determined whether SON fibers were also releasing GABA under the same stimulation paradigm that elicited the EPSCs. Nevertheless, our current results suggest that previous studies on synaptic plasticity using trains of stimulation in conditions in which

metabotropic receptor were unblocked were probably not complicated by modulatory effects in slice (MacLeod et al., 2007). Heterosynaptic effects that rely on neurotransmitter spillover may be more likely in NM, where large calyceal axosomatic synapses interdigitate with small, punctate axosomatic inhibitory terminals. Whether the relative position of excitatory and inhibitory synaptic inputs to NA neurons facilitates such indirect modulatory effects in the intact circuit remains to be investigated.

Acknowledgements

Support for this research was provided by NIH research grant R01DC10000 (K.M.M.). The authors thank P. Timi for assistance with analysis.

Methods

Chick brain stem slice preparation

Embryonic day 17-18 chicken embryos were anesthetized and rapidly decapitated in accordance with the University of Maryland Institutional Animal Care and Use Committee guidelines. The brain stem containing the cochlear nuclei was dissected out of the skull and submerged in low sodium artificial cerebral spinal fluid (ACSF) (in mM: 97.5 NaCl, 3 KCl, 2.5 $MgCl_2$, 26 $NaHCO_3$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 10 dextrose, 3 HEPES, 57.5 sucrose) that was being continuously oxygenated with 95% O_2- 5% CO_2. Transverse brain stem slices 250 μm thick were cut using a vibrating tissue slicer (Leica Microsystems, Wetzlar, Germany), then incubated in oxygenated normal ACSF (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 26 $NaHCO_3$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 10 dextrose, 3 HEPES) heated to 34°C for 30 minutes, then held at room temperature.

Patch-clamp electrophysiology

For recordings, slices were transferred to a submersion recording chamber continuously perfused with oxygenated ACSF (1–2 ml min^{-1}) and heated with a Warner TC 324B inline heating device (Warner Instr., Hamden, CT) to 30 ± 0.2°C. Whole cell patch-clamp recordings were made from visually-identified nucleus angularis neurons using IR/ DIC (infrared/differential interference contrast) video microscopy. Electrophysiological recordings were made with a Multiclamp 700B patch clamp amplifier (Molecular Devices, Sunnyvale, CA) in voltage-clamp mode. Initial micropipette resistances were 4.5–7 MΩ. The series resistance was corrected by 60–90% and the junction potentials were left uncorrected. Stimulation and recordings were controlled by a PC running custom software in IGOR Pro (Wavemetrics, Lake

Oswego, OR). Two intracellular solutions were used: excitatory (AMPA-receptor mediated) synaptic currents were measured with a cesium-based intracellular voltage-clamp solution (in mM: 70 cesium sulfate, 5 QX-314 (lidocaine), 1 $MgCl_2$, 1 Na_2ATP, 0.3 Na_2GTP, 10 phosphocreatine, 4 NaCl, 10 HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5 BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), 32 sucrose and 0.2% biocytin). Inhibitory (mixed GABA/glycine receptor mediated) synaptic currents were recorded with a similar solution with cesium chloride to achieve a chloride reversal potential was close to zero (in mM: 130 cesium chloride, 5 QX-314, 1 $MgCl_2$, 1 Na_2ATP, 0.3 Na_2GTP, 5 phosphocreatine, 10 HEPES, 5 BAPTA , and 0.2% biocytin). IPSCs that were recorded under voltage clamp near resting potential were therefore measured as inward currents. To eliminate GABA/glycine inhibitory currents for EPSC recordings, gabazine (20 μM) and strychnine, (3 μM) were included in the bath ACSF. Alternatively, to eliminate glutamatergic synaptic currents for IPSC recordings DNQX (10 μM) and APV (25 μM) were included. $GABA_B$ receptor agonist baclofen (Sigma), antagonist CGP-52432 (Tocris Cookson) and GABA transporter inhibitor NNC-771 (Tocris Cookson) were also bath applied.

Excitatory synaptic currents were evoked with a tungsten monopolar electrode placed amongst the 8th (auditory) nerve fibers tracts as they enter the nucleus angularis at the medial margin. Inhibitory synaptic currents were more difficult to isolate, but generally were stimulated from within the nucleus boundary, most often 100-200 μm medially or ventrally distant relative to the recording site. Biphasic stimulus waveforms passed through an analog, constant-current stimulus isolation unit (World Precision Instruments, Sarasota, FL). Stimulus artifacts were digitally removed for clarity in figure presentations.

Analysis and statistics

Spontaneous PSC kinetics were analyzed for recordings in which there were sufficient numbers of events to test in both control and drug conditions (>30 events per condition). Paired pulse ratio (PPR) was the second PSC amplitude relative to the initial amplitude; the steady state ratio (SSR) was the average of PSC 8 through 10 of the train relative to the initial PSC amplitude. Distributions were tested for differences by two-sample Kolmogorov-Smirnov test with a signficance threshold of $p<0.05$. Summary statistics were calculated using Microsoft Excel. Values reported in the text are mean±s.d. unless otherwise indicated. Box plots show median, interquartile range, and extremes. Hypothesis testing (Student's paired t-Test, Wilcoxon Rank Sum Test for paired data, 2-way ANOVA) was performed using Microsoft Excel or the statistical software R (Version 0.98.1103 RStudio, Inc.) on a MacBook Pro. Non-parametric tests were performed when the sample size was small or the data did not conform to a normal distribution.

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