Presynaptic GABAergic receptors modulate inhibitory synaptic feedback in the avian cochlear nucleus angularis

Running head: GABAergic modulation in the cochlear nucleus

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7 Figures

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.

44 **New and Noteworthy**

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

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54 **Introduction**

55 Inhibition plays a critical role in auditory processing, contributing in various ways to
56 sound level gain control (Peña et al., 1996), sculpting frequency tuning (Köppl and Carr, 2003;
57 Fukui et al., 2010), and enhancing temporal processing (Funabiki et al., 1998; Yamada et al.,
58 2013). In the avian system, nearly all cochlear nucleus neurons provide feedforward excitatory
59 projections to the ascending pathways, while inhibitory inputs principally arise from an
60 anatomically separate brain stem region, the superior olivary nucleus (SON) (Funabiki et al.,
61 1998; Yang et al., 1999; Lu and Trussell, 2000; Monsivais et al., 2000; Lu and Trussell, 2001).
62 The SON provides ipsilateral feedback inhibition to both divisions of the cochlear nucleus,
63 nucleus angularis (NA) and nucleus magnocellularis (NM), as well as to nucleus laminaris (NL)
64 (Burger et al., 2005a; Nishino et al., 2008). This inhibitory feedback loop to NM and NL has
65 been proposed to enact an intensity-dependent gain control that enhances coincidence detection
66 for interaural time difference. While NA provides a source of sound level information to the
67 SON (Lachica et al., 1994; Yang et al., 1999), how the reciprocal inhibitory feedback is
68 integrated into sound processing in NA is not yet clear.

69 The mechanisms underlying inhibitory control of the timing nuclei involve both
70 presynaptic and postsynaptic regulation. Inhibitory synaptic terminals in these nuclei release the
71 neurotransmitter γ–aminobutyric acid (GABA), along with glycine (Kuo et al., 2009).
72 Postsynaptic inhibitory responses are principally $GABA_A$ receptor mediated in NM (Tang et al.,
73 2009; Coleman et al., 2011; Tang and Lu, 2012a; Fischl and Burger, 2014), while responses in
74 NA are mediated by both $GABA_A$ and glycine receptors (Kuo et al. 2009). Presynaptic inhibition
75 is mediated via metabotropic GABA ($GABA_B$) receptors in NM: pharmacological activation of
76 $GABA_B$ receptors has a strong presynaptic suppressive effect on glutamatergic inputs to NM

77 neurons (Brenowitz et al., 1998; Brenowitz and Trussell, 2001a; 2001b). Activation of $GABA_B$
78 receptors also suppressed inhibitory inputs onto NM neurons, as well as onto NL neurons (Tang
79 et al., 2009), suggesting the inhibitory terminals were self-regulatory via homosynaptic feedback.
80 Less is known about the mechanisms and functional effects of presynaptic inhibition in
81 nucleus angularis. Immunohistochemical and electron microscopy studies showed $GABA_B$
82 receptors were also expressed in NA (Burger et al., 2005b). Like in NM, a previous study has
83 shown that $GABA_B$ agonists had a suppressive effect on excitatory synaptic inputs into NA via
84 presynaptic mechanisms (Shi and Lu, 2017). In this study, we examined the effects of activation
85 of $GABA_B$ mechanisms on the inhibitory inputs to NA neurons using in vitro whole cell patch
86 clamp electrophysiology in the brain stem slice preparation. The results provide evidence for
87 GABAergic presynaptic modulation of these putative SON inputs to NA, as well as similar
88 modulation of excitatory (presumptive nerve) input. These data suggest a common mechanism
89 for homosynaptic regulation was present at the inhibitory terminals at all three descending
90 targets of the SON.
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93 **Methods**

94 *Chick brain stem slice preparation*

95 Embryonic day 17-18 chicken embryos were anesthetized and rapidly decapitated in
96 accordance with the University of Maryland Institutional Animal Care and Use Committee
97 guidelines. The brain stem containing the cochlear nuclei was dissected out of the skull and
98 submerged in low sodium artificial cerebral spinal fluid (ACSF) (in mM: 97.5 NaCl, 3 KCl, 2.5
99 $MgCl_2$, 26 $NaHCO_3$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 10 dextrose, 3 HEPES, 57.5 sucrose) that was
100 being continuously oxygenated with 95% O_2- 5% CO_2. Transverse brain stem slices 250 µm
101 thick were cut using a vibrating tissue slicer (Leica Microsystems, Wetzlar, Germany), then
102 incubated in oxygenated normal ACSF (in mM: 130 NaCl, 3 KCl, 2 $MgCl_2$, 26 $NaHCO_3$, 2
103 $CaCl_2$, 1.25 NaH_2PO_4, 10 dextrose, 3 HEPES) heated to 34°C for 30 minutes, then held at room
104 temperature.

105 *Patch-clamp electrophysiology*

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

115 Oswego, OR). Two intracellular solutions were used: excitatory (AMPA-receptor mediated)
116 synaptic currents were measured with a cesium-based intracellular voltage-clamp solution (in
117 mM: 70 cesium sulfate, 5 QX-314 (lidocaine), 1 $MgCl_2$, 1 Na_2ATP, 0.3 Na_2GTP, 10
118 phosphocreatine, 4 NaCl, 10 HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5
119 BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), 32 sucrose and 0.2%
120 biocytin). Inhibitory (mixed GABA/glycine receptor mediated) synaptic currents were recorded
121 with a similar solution with cesium chloride to achieve a chloride reversal potential was close to
122 zero mV (in mM: 130 cesium chloride, 5 QX-314, 1 $MgCl_2$, 1 Na_2ATP, 0.3 Na_2GTP, 5
123 phosphocreatine, 10 HEPES, 5 BAPTA, and 0.2% biocytin). IPSCs that were recorded under
124 voltage clamp near resting potential were therefore measured as inward currents. To eliminate
125 GABA/glycine inhibitory currents for EPSC recordings, gabazine (20 μM) and strychnine, (3
126 μM) were included in the bath ACSF. Alternatively, to eliminate glutamatergic synaptic currents
127 for IPSC recordings DNQX (10 μM) and APV (25 μM) were included. $GABA_B$ receptor agonist
128 baclofen (20-50 μM, Sigma), antagonist CGP 52432 (10-20 μM, Tocris Cookson) and GABA
129 transporter inhibitor NNC-771 (20-80 μM, Tocris Cookson) were also bath applied. Spontaneous
130 events were recorded in the absence of tetrodotoxin as there is no evidence for spontaneous
131 firing activity in the cochlear nucleus in slice under our recording conditions and a previous
132 study showed no differences between spontaneous excitatory synaptic events and so-called
133 "miniature events" recorded in the presence of tetrodotoxin (MacLeod and Carr, 2005).
134 Spontaneous IPSC events measured using 20 μM (n=4) and 80 μM (n=4) NNC 771 were pooled
135 as experiments showed no differences in event properties or drug effect between concentrations.
136 Spontaneous sIPSC events measured with 10 μM (n=6) and 20 μM (n=2) CGP 52432 were also
137 pooled.

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

145 *Analysis and statistics*

146 Spontaneous [E/I]PSC kinetics were analyzed for recordings in which there were
147 sufficient numbers of events to test in both control and drug conditions (>30 events per
148 condition). Paired pulse ratio (PPR) was the second PSC amplitude relative to the initial
149 amplitude; the steady state ratio (SSR) was the average of PSC 8 through 10 of the train relative
150 to the initial PSC amplitude. Single or double exponential fits were performed using built in
151 curve fitting in Igor Pro v.8. Weighted time constants for double exponential fits were calculated
152 according to $\tau_{weighted} = \left(A_{fast} \cdot \tau_{fast} + A_{slow} \cdot \tau_{slow}\right) / \left(A_{fast} + A_{slow}\right)$ and fast time constant percentage
153 was calculated as $\%\,fast = A_{fast} / \left(A_{fast} + A_{slow}\right)$. Distributions were tested for differences by two-
154 sample Kolomogorov-Smirnov test with a signficance threshold of $p<0.05$. Summary statistics
155 were calculated using Microsoft Excel. Values reported in the text are mean±s.d. unless
156 otherwise indicated. Hypothesis testing used Student's paired *t* Test unless otherwise indicated.
157 Short-term plasticity during trains were analyzed with 2-way ANOVA with two within subject
158 factors of drug condition and stimulus number, and post hoc pairwise Student's *t* Test with

Bonferroni correction for multiple comparisons. Statistical analysis was performed using Microsoft Excel or the statistical software *R* (Version 0.98.1103 RStudio, Inc.) on a MacBook Pro.

164 **Results**

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

167 To investigate inhibitory modulation of synaptic transmission in the avian cochlear
168 nucleus angularis, the specific $GABA_B$ receptor agonist baclofen was applied during recordings
169 of inhibitory postsynaptic currents (IPSCs) in acute brain stem slices. Synaptic currents were
170 evoked by extracellular electrical stimulation and measured under whole-cell voltage clamp
171 mode. Baclofen (20 μM) reliably reduced the peak amplitudes of pharmacologically isolated
172 evoked IPSCs (Fig. 1A). On average, baclofen reduced IPSC amplitudes to 10.5% of control
173 levels (control: -0.38±0.18 nA, baclofen: -0.041±0.043 nA, n=12, $p=2.4x10^{-5}$, Student's paired *t*
174 Test; Fig. 1B). The baclofen effect on IPSC amplitudes was partially and inconsistently reversed
175 with washout of the drug (blue shaded region in Fig. 1A), recovering to 52% of control levels on
176 average (control: -0.37±0.19 nA, washout: -0.20±0.16 nA, n=10, p=0.0032).

177 Baclofen had little effect on the time course of the evoked currents (Fig. 1Ab). Most
178 evoked IPSCs could be best fit with a double exponential with a fast and slow time constant in
179 control which marginally decreased with baclofen (control: τ_{fast} =10.3±5.0 ms, τ_{slow} =66.2±37.7
180 ms; baclofen: τ_{fast} =8.3±6.3 ms, τ_{slow} =46.6±47.9 ms; n=10, no significant difference, p=0.12
181 and p=0.14, respectively; Fig. 1Ci). A small but significant decrease in the weighted time
182 constant of the current decay suggested a slight speeding up of the overall current (control: τ_{weight}
183 26.4±19.4 ms, baclofen: τ_{weight} =21.4±21.3 ms, n=10, p=0.012, Student's paired *t* Test). This
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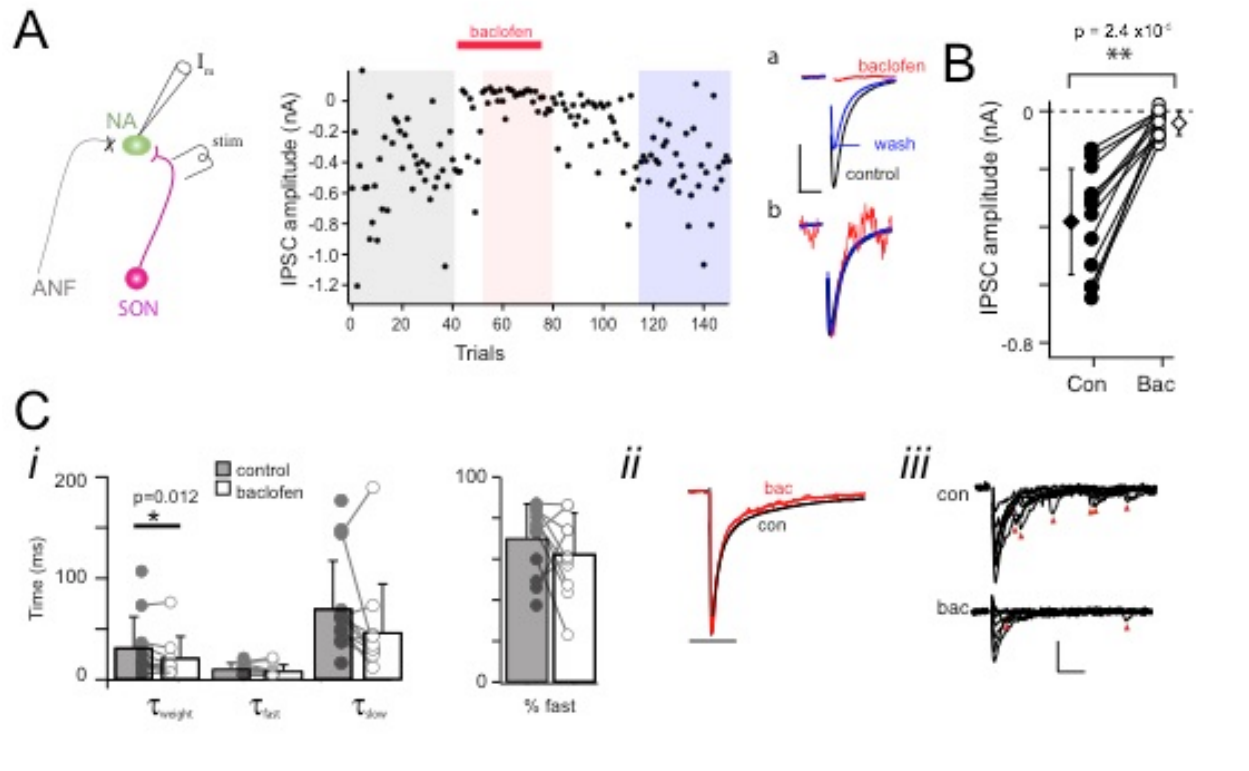

Figure 1.
Baclofen suppressed evoked inhibitory postsynaptic currents onto neurons in the cochlear nucleus angularis (NA). A) Schematic of recording arrangement (left). Time plot of amplitudes of evoked single IPSCs elicited by stimulating electrode at a low rate (0.1 Hz) before, during, and after bath application of baclofen (20 µM)(middle). Right: average IPSC traces in control (black trace), baclofen (red) and during partial washout (blue) overlaid (a) or normalized and overlaid (b). Traces correspond to averages of responses in gray, red and blue shaded regions, respectively, in time plot. ANF, auditory nerve fiber, SON, superior olivary nucleus. B) Summary data of IPSC absolute amplitude (n=12). Baclofen reduced the IPSCs by >90%. ** Student's paired *t* Test, $p=2.4x10^{-5}$. C) Kinetics analysis of the decay of the IPSCs fit with double exponential showed a slight decrease in time constants τ_{fast} and τ_{slow}, with significant effect on weighted time constant τ_{weight} (*i*). Grand average of all normalized IPSCs before (control) and after baclofen (*ii*). Individual traces from one recording showed numerous asynchronous events (red arrowheads) that likely contribute to the slower decay and were largely absent in baclofen (*iii*). Exponential fits of grand averages: τ_{fast}=8.4 ms, τ_{slow}=93 ms, $\%fast$ = 68% in control; τ_{fast}=6.8 ms, τ_{slow} =63.0 ms, $\%fast$ = 72% in baclofen. Horizontal scale bars: 10 ms (Aa), 50 ms (Cii), 20 ms (Ciii). Vertical scale bars: 200pA (Aa), 100 pA (C*iii*).

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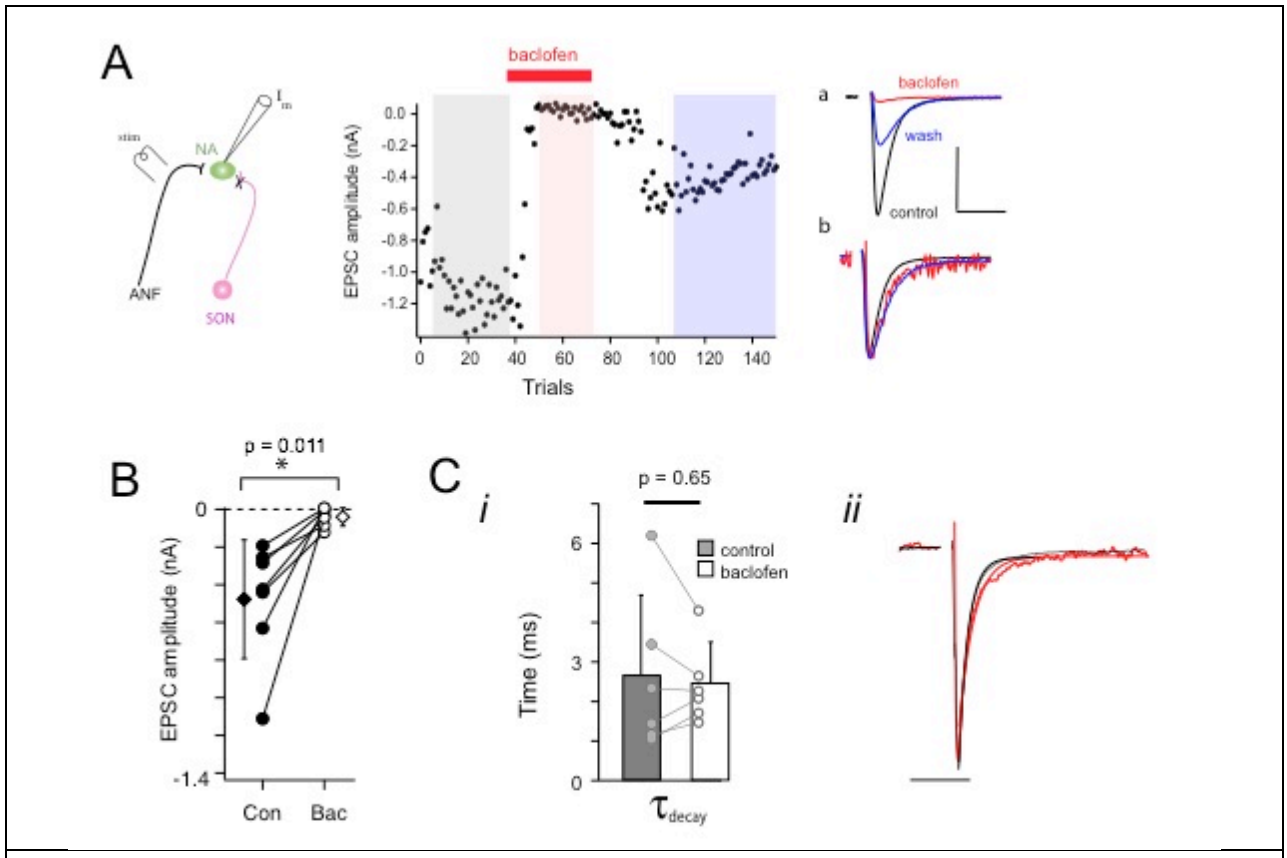

Figure 2.
Baclofen suppressed evoked excitatory postsynaptic currents onto neurons in the cochlear nucleus angularis. A) Schematic of recording arrangement (left). Time plot of amplitudes of evoked single EPSCs evoked at a low rate (0.1Hz) before, during, and after bath application of baclofen (20 µM)(middle). Right: average EPSC traces in control (black trace), baclofen (red) and during partial washout (blue) overlaid (a) or normalized and overlaid (b). Traces correspond to averages of responses in gray, red and blue shaded regions, respectively, in time plot. ANF, auditory nerve fiber, SON, superior olivary nucleus. B) Summary data of EPSC absolute amplitude (n=7). Baclofen also reduced the EPSCs by >90%. * Student's paired *t* Test, p=0.011. C) Kinetics analysis of the decay of the EPSCs fit with single exponential showed no significant effect (*i*). Grand average of all normalized EPSCs before (control) and after baclofen (*ii*). Exponential fits of grand averages: τ =1.6 ms in control; τ =2.5 ms in baclofen. Horizontal scale bars: 5 ms (Aa), 10 ms (Cii). Vertical scale bar: 600pA (Aa).

191 effect may be due to the reduction in asynchronous events that could be seen in some recordings
192 along with a decrease in the synchronous IPSC (Fig. 1Ciii).

193 We observed similar levels of $GABA_B$ receptor driven suppression on evoked EPSCs, the
194 putative auditory nerve inputs (Fig. 2A). Baclofen (20 μM) reliably suppressed the mean EPSC
195 peak amplitude to 8.4% of control levels (control: -0.48±0.32 nA; baclofen: 0.040±0.048 nA,
196 n=7, p=0.011; Fig. 2B). Washout of the baclofen effect on EPSCs was slow and inconsistent,
197 only measurable in a subset of neurons (control: -0.57±0.37, washout: -0.71±0.85, n=4, no
198 significant difference, p=0.60)(Fig. 2A). Baclofen had no significant effect on the time course of
199 evoked EPSCs fit with a single exponential curve (control: τ = 2.7±1.7 ms, baclofen: τ =
200 2.6±0.8 ms n=6, no significant difference, p=0.70; Fig. 2C).

201 For both excitatory and inhibitory synapses, the baclofen effect was blocked by co-
202 application of the specific $GABA_B$ receptor antagonist, CGP52432 (10 μM). Blockade was
203 complete for evoked IPSCs with no signficant difference between control IPSC amplitudes
204 IPSCs in presence of agonist and antagonist together (control: -0.53±0.23 nA; baclofen+CGP: -
205 0.53±0.11 nA, n=4, p=0.93). The same concentration of CGP52432 also substantialy blocked the
206 baclofen suppression of the evoked EPSCs, retaining 74.4% of control amplitudes (control: -
207 0.90±0.54 nA; baclofen+CGP: -0.64±0.40 nA, n=5, p=0.055).

208 *Presynaptic locus of suppression*

209 To determine whether baclofen was acting presynaptically, we examined the effect of the
210 drug on pharmacologically isolated spontaneous synaptic currents under voltage clamp. Baclofen
211 application significantly decreased the frequency of both inhibitory and excitatory spontaneous
212 PSCs (sIPSCs and sEPSCs, respectively)(Fig. 3Ai, Bi). Cumulative histograms of the interevent

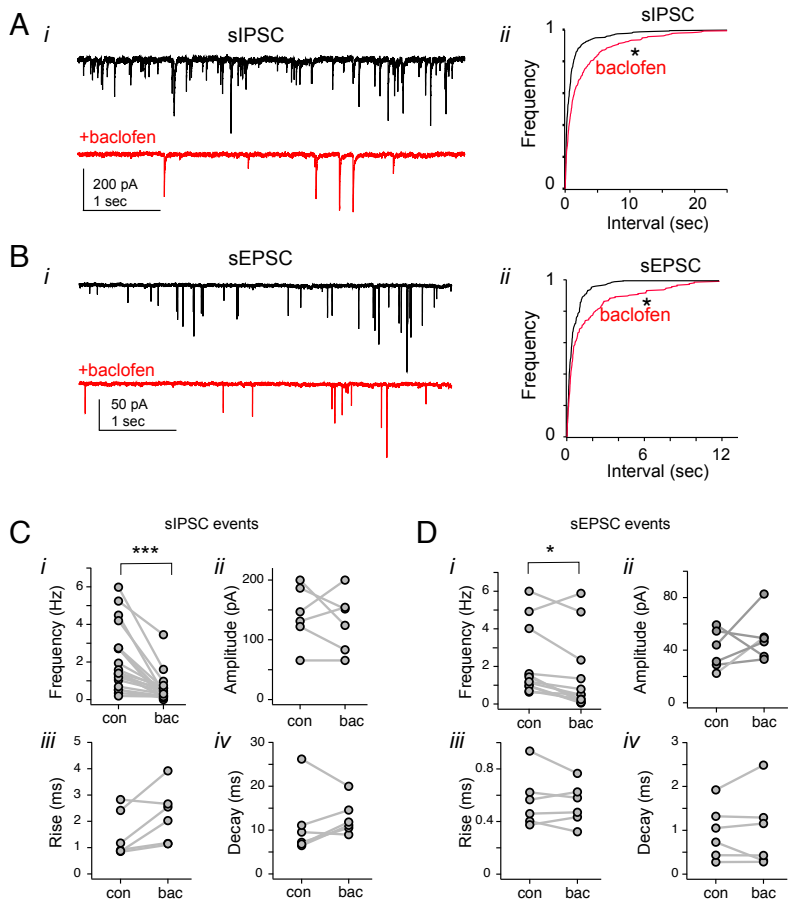

Figure 3.
Baclofen effects on spontaneous IPSC and EPSC events.Baclofen application (50 µM) reduced the frequency of spontaneous IPSC events (A) and spontaneous EPSC events (B). Current traces (i) and cumulative histograms of PSC intervent intervals (ii) before (black) and after application of baclofen (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). 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).

intervals for sIPSCs showed significant shifts to longer intervals (N=400 control events, N=397 baclofen events, $p=1.12x10^{-10}$, Kolmogorov-Smirnov test, Fig.3Aii). The average spontaneous event rate for sIPSCs was reduced by >70% when baclofen was bath applied (control: 2.1±1.7 Hz , baclofen: 0.60±0.76 Hz, 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 (N=250 control events, N=204 baclofen events, $p=4.54x10^{-4}$, 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 a mean 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 a mean amplitude of 0.040±0.015 nA in control and 49.4±0.018 nA in baclofen (p=0.44). 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=0.56), while sEPSCs had decay time constants of 0.96±0.61 ms in control and 0.99±0.85 ms in baclofen (p=0.92). 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 $GABA_BR$-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

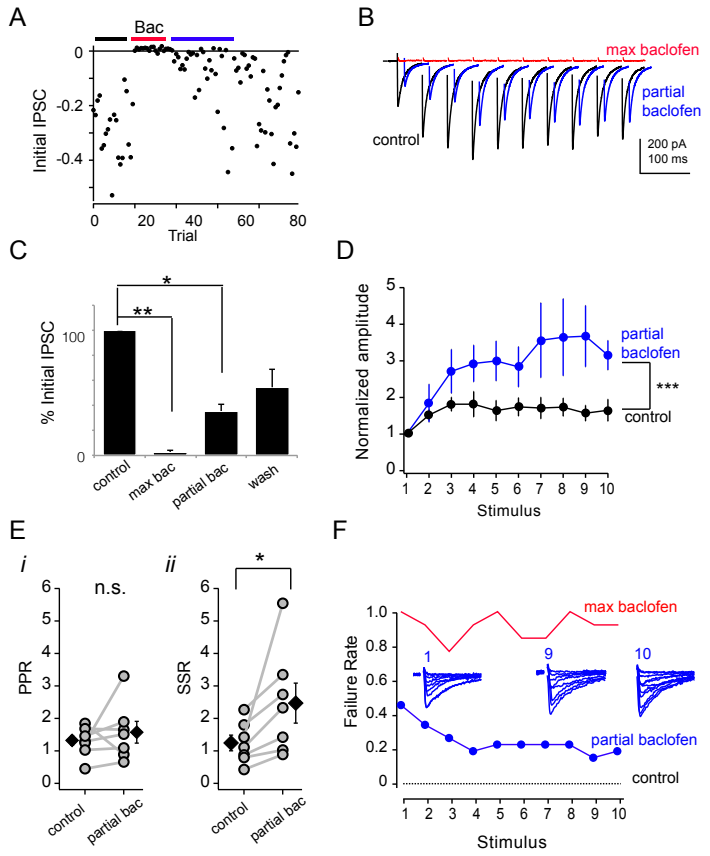

Figure 4.
Effect of baclofen on short-term synaptic plasticity during trains of electrically evoked IPSCs. Plasticity of IPSC train responses assessed during periods of partial GABA-B receptor activation with baclofen revealed enhanced facilitation. A) Amplitude of the initial evoked IPSC of the train 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) Normalized amplitude plots shows short-term plasticity of IPSCs over train. Sustained facilitation was typical of IPSC dynamics during moderate stimulation frequencies during control trials (black). Baclofen suppressed initial IPSC amplitude, but enhanced relative facilitation (blue). Mean±s.e.m. for 7 experiments; *, significant difference using Bonferroni adjusted pairwise Student's paired t Test. D) 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). E) Paired pulse ratio (PPR) during trains was unchanged during partial baclofen blockade (*i*), but sustained facilitation of later IPSCs in the train (SSR, steady state ratio) increased significantly (*, p<0.05)(*ii*). F) Failure rate at each stimulus in the train, uner 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 responses during partial blockade at the first (1), ninth (9), and tenth (10) stimulus of the train.

239 trace). Because the maximal baclofen application (20 μM) could sometimes reduce the initial
240 response amplitudes to negligible levels (e.g. 'max baclofen', Fig. 4A,B), changes in short-term
241 plasticity were evaluated using blocks of trials during which the initial IPSC was reduced to 38%
242 of the control amplitude (Fig.4A-D)(control: -0.29±0.16 nA; 'partial baclofen': -0.11±0.08 nA,
243 n=7, p=0.016). As baclofen reduced the initial IPSC amplitude, the relative amplitudes of IPSCs
244 later in the train were enhanced, showing greater facilitation compared to control (Fig. 4B,C;
245 $p=1.2x10^{-5}$, 2-way ANOVA). While the paired pulse ratio marginally increased from 1.28±0.46
246 to 1.58±0.88 (22% increase, p=0.43, no significant difference), the steady state ratio doubled
247 from 1.24±0.64 to 2.47±1.63 (99% increase, p=0.0495)(Fig. 4Ei,ii).

248 Partial blockade allowed investigation of how release probability changed over the course
249 of the train by measuring the failure rate. Under control conditions, the evoked IPSC failure rate
250 in response to the first stimulus was nearly zero (1.3±1.9%, n=7 experiments); as these were not
251 minimal stimulation experiments, responses likely comprised multiple inputs. In contrast, during
252 partial blockade the failure rate in response to the first stimulus was ~40% (one example shown
253 in Fig. 4F, 'partial baclofen', blue line). Over the course of the train, the failure rate then
254 declined to ~19% in response to the last stimulus. Over 7 experiments, during partial blockade
255 the failure rate was 38.8±31.3% for the initial IPSC and declined to 17.2±0.3% by the end of the
256 20 Hz train. During maximal blockade, we could also detect some decline in the failure rate over
257 the train (Fig. 4F, 'max baclofen', red line). For all experiments, under maximal blockade, the
258 failure rate in response to the first stimulus was on average 80% (79.6±21.6%, n=7), which then
259 decreased over the course of the train to 50% (49.9±37.9%). Together with the effects observed
260 on the spontaneous neurotransmitter release, these results suggest that activation of $GABA_B$

261 receptors acts by reducing the initial release probability, which then increased over the course of
262 the train via enhanced presynaptic facilitation.

263 Similar experiments were performed to determine how $GABA_B$ receptor activation
264 affected the excitatory synaptic dynamics. In these experiments, under control conditions the
265 EPSC amplitude depressed to approximately 60% of initial amplitude during a 20 Hz constant
266 frequency train (Fig. 5A, B; control: black trace, partial baclofen: blue trace). To measure
267 whether baclofen had comparable effects as on IPSCs, short-term plasticity of the EPSCs was
268 evaluated using blocks of trials during which the initial EPSC was reduced approximately to
269 ~26% of the control amplitude (control: 1.46±0.73 nA; 'partial baclofen': -0.39±0.18 nA, 74%
270 reduction, n=5 neurons). As baclofen reduced the initial EPSC amplitude, the relative amplitudes
271 of EPSCs later in the train were enhanced, showing less depression compared to control
272 (p=0.00412, 2-way ANOVA). Under partial baclofen suppression, the paired pulse ratio for
273 EPSCs showed no significant change (control: 0.80±0.17, baclofen: 0.92±0.12, n=5, p=0.24,
274 Student's paired t Test)(Fig. 5Di), while the steady state ratio increased significantly by 44%
275 (control: 0.52±0.10, baclfoen: 0.75±0.17, p=0.031). Thus under conditions of a moderate, but not
276 maximal, activation of $GABA_B$ receptors, there was a small but significant shift in short-term
277 plasticity towards less depression in their excitatory train responses.

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

279 To test whether $GABA_B$ receptors could endogenously modulate synaptic dynamics
280 during physiological activation in vitro, we elicited trains of evoked IPSCs before and after
281 applying an antagonist of the receptor, CGP52432 (10 μM), and measured the effects on short-
282 term plasticity. Unexpectedly, bath application of CGP52432 increased the initial IPSC

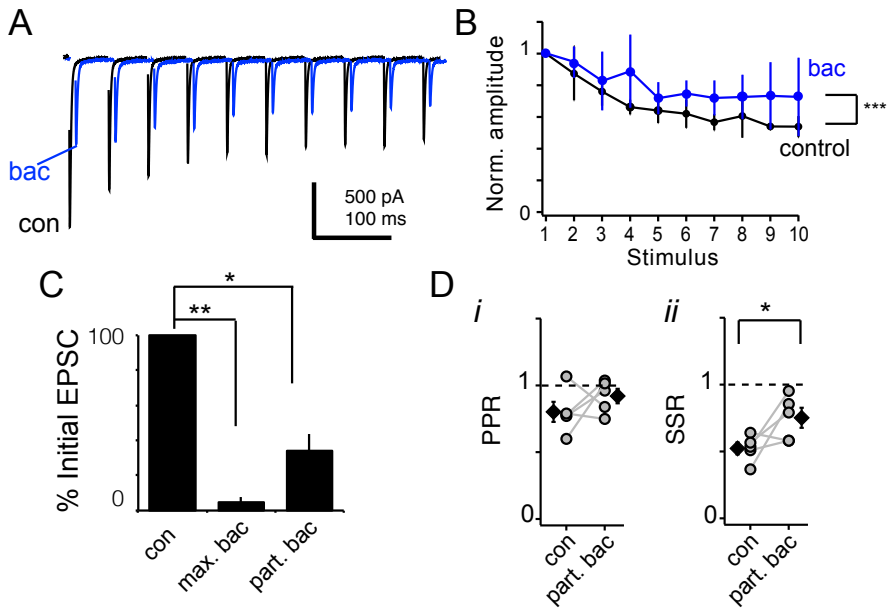

Figure 5.
Partial GABA-B receptor activation with baclofen altered the short-term synaptic plasticity of EPSCs during short trains. A) Comparison of mean traces of train of EPSCs (20 Hz) before (black trace) and after partial suppression with baclofen (blue trace) in one recording (traces are horizontally offset for clarity). B) Normalized amplitude plots shows short-term plastitiy of EPSCs over train. Baclofen suppressed initial EPSC amplitude, but enhanced relative amplitudes (blue). Mean±s.e.m. for 4 experiments; posthoc testing did not reveal significant differences. C) Initial EPSC suppression during control, maximal blockade (>90% blocked, **p<0.01), and partial blockade (>66% blocked, * p<0.05) showed comparable levels to IPSC experiments. D) Paired pulse ratio (PPR) of the EPSCs during trains was unchanged during partial baclofen blockade (*i*), but the short-term synaptic depression of later responses in the train decreased significantly (steady state ratio increased, SSR, *, p<0.05) (*ii*).

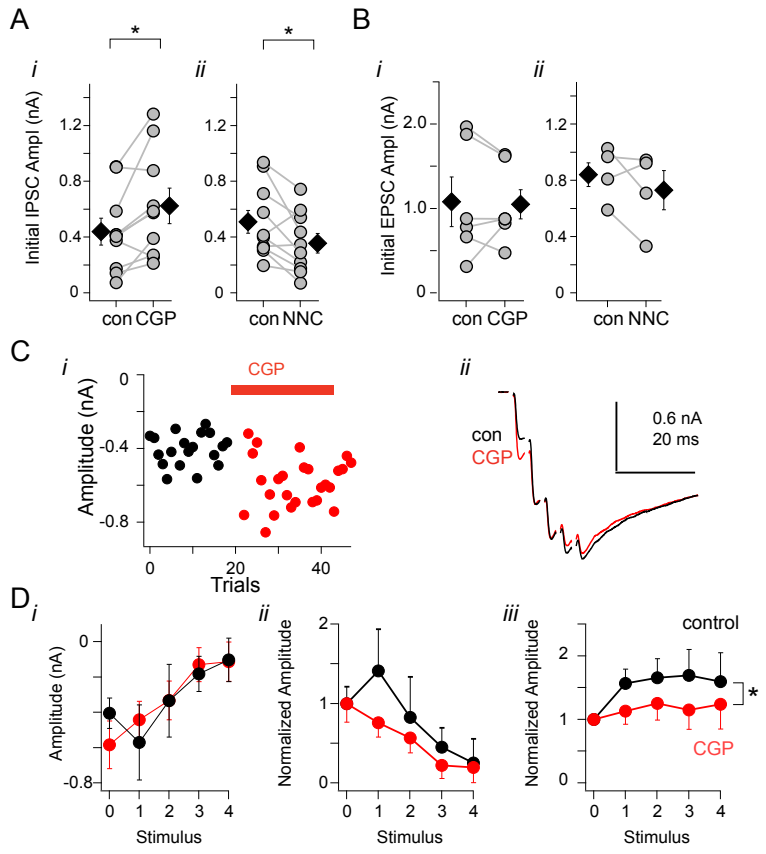

Figure 6.
Blocking GABA reuptake (with GABA trasnsporter inhibitor NNC 771) or $GABA_B$ receptor (with antagonist CGP 52432) has opposing effects on baseline inhibitory synaptic release and short-term synaptic plasticity. A) Evoked IPSC amplitudes were enhanced with application of $GABA_B$ receptor antagonist CGP 52432 (*i*) and suppressed with inhibition of GABA reuptake transporter NNC771 (*ii*). B) Evoked EPSCs were unaffected by either drug. Diamond markers in panels A and B show mean±s.e.m. C) Example experiment of CGP 52432 effects on short, high frequency (200 Hz) trains of IPSCs. CGP 52432 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 CGP 52432 (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). Error bars are ±s.e.m.

287 amplitude (Fig. 6A). On average, the initial IPSC increased by 41% (control: -0.44±0.29 nA,
288 CGP52432: -0.62±0.38 nA, n=10, p=0.018, paired Students *t*-Test)(Fig. 6Ai). Conversely,
289 inhibiting GABAergic reuptake with NNC771 (20 μM) decreased the evoked IPSC amplitude by
290 30% (control: -0.51±0.26 nA, NNC771: -0.36±0.22, n=10, p=0.022)(Fig. 6Aii). No significant
291 effects were observed by either drug on the amplitude of evoked EPSCs (initial EPSC NNC771
292 experiments: control -0.84±0.17 nA, NNC, -0.73±0.28 nA, n=4, p=0.31; initial EPSC CGP52432
293 experiments: control -1.08±0.72 nA, CGP52432 -1.05±0.42 nA, n=6, p=0.83)(Fig. 6B).

294 The changes observed in short-term plasticity during IPSC trains were consistent with the
295 change in initial amplitude: an increase the initial amplitude under CGP52432 was accompanied
296 by a decrease in facilitation (n=5, $p=2.14x10^{-4}$, 2-way ANOVA). Conversely, we asked whether
297 the reduction in the initial inhibitory synaptic amplitude in the presence of the GABA reuptake
298 inhibitor would be accompanied by an increase in facilitation. However, blocking the reuptake of
299 GABA during trains with reuptake inhibitor NNC 771 (20 μM) was found to have minor and
300 inconsistent effect on the short-term-plasticity with no significant effect on the train facilitation
301 overall (n=4, p=0.083, 2-way ANOVA).

302 The effects of CGP 52432 and NNC 771 on the basal release properties during evoked
303 trains implied ongoing, baseline activation of $GABA_BR$, suggesting that the GABA chronically
304 in the milieu was sufficient to affect these receptors. To determine whether spontaneous release
305 was also affected by the same pharmacological manipulations, we measured spontaneous IPSC
306 frequency before and after application of NNC 771 (20-80μM; Fig. 7A-C) and CGP52432 (10-
307 20 μM; Fig. 7D-F). When NNC 771 was applied, the distribution of the interevent intervals
308 between sIPSCs showed a significant shift toward longer intervals (Fig. 7B)($N_{control}$=378, N_{NNC}=
309 324, $p=3.2x10^{-6}$, Kolmogorov-Smirnov test), while the sIPSC frequency was reduced by 29%

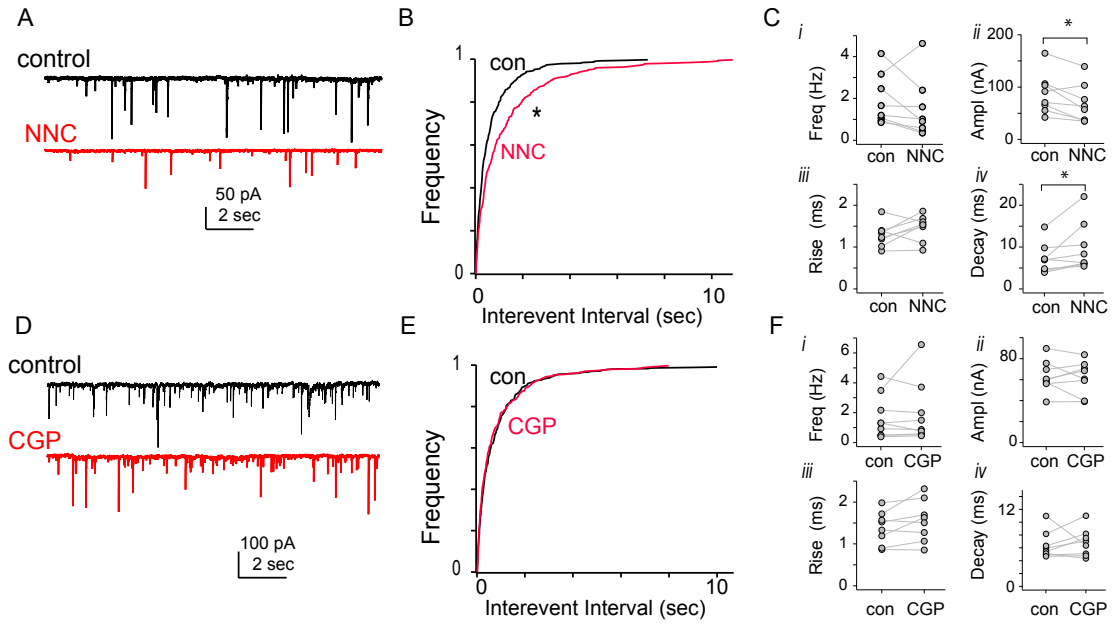

Figure 7.
Effects of GABA reuptake inhibitor NNC771 (A-C) and $GABA_B$ receptor blockade CGP 52432 (D-F) on spontaneous IPSC activity and kinetics. A) Example current traces showing spontaneous IPSC events before (top black trace) and after (bottom red trace) application of NNC 771. B) Cumulative histograms of sIPSC intervent intervals before and after application of NNC 771. The distribution of time intervals between events shifted toward longer intervals with drug application. C) Summary statistics on n=8 recordings showing mean sIPSC spontaneous rate frequency, peak amplitude, 10-90 rise time, and single exponential decay time constant before and after drug application. No signficant differences were found in average frequency or rise time; a signficant 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 CGP 52432. E) Cumulative histogram of sIPSC intervent intervals before and after application of CGP 52432 showed no changes. F) Summary statistics reveal no signficant changes with CGP 52432 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).

312 (1.9±1.4 Hz in control to 1.5±1.0 Hz in NNC771; n=8, p=0.107, Wilcoxon Rank Sum test)(Fig.
313 7Ci). In these neurons, NNC 771 application appeared to have small but detectable effects on the
314 synaptic current kinetics and amplitude. The sIPSC amplitude showed a small but significant
315 decrease (Fig. 7Cii; control, -86.5±37.9 pA; NNC, -67.0±36.4 pA, p=0.039, Wilcoxon Rank Sum
316 test) and the decay time course slowed slightly (Fig. 7Civ; control, 7.3±3.6 ms; NNC 771,
317 10.0±5.9 ms, p=0.039, Wilcoxon Rank Sum test), but no change in the rise time was observed
318 (Fig. 7Ciii; control, 1.3 ±0.2 ms; NNC, 1.5±0.3 ms, p=0.148, Wilcoxon Rank Sum test). These
319 mild postsynaptic effects on quantal event amplitude and kinetics with NNC771 indicate possible
320 desensitization of postsynaptic ionotropic GABA receptors (Tang and Lu, 2012b).

321 In contrast, $GABA_B$ receptor antagonist CGP 52432 had no effect on the spontaneous
322 IPSC frequency, amplitude or kinetics (Fig. 7D). The cumulative histograms of the interevent
323 intervals of sIPSCs were unchanged by application of CGP 52432 (Fig. 7E)($N_{control}$=363, N_{NNC}=
324 338, p=0.656, Kolmogorov-Smirnov test), while the frequency marginally increased from
325 1.8±1.3 Hz in control conditions to 2.1±0.9 Hz in CGP 52432 (no significant effect; Fig. 7Fi).
326 No changes in the kinetics or amplitude of the sIPSCs were observed with the antagonist (Fig.
327 7Fii-iv).

328 **Discussion**

329 These results suggest that both excitatory and inhibitory inputs may be modulated by the
330 release of GABA via metabotropic receptors. The functional role of presynaptic $GABA_B$
331 receptors in sensory processing at synapses in the NA is as yet undetermined, but in vitro
332 heterosynaptic and homosynaptic effects of GABA were similar in the three important ascending
333 auditory brain stem nuclei. Direct inhibitory modulation of NA neurons occurs via postsynaptic

334 synapses and ionotropic GABA and glycine receptors, but the release of GABA may also
335 indirectly influence information processing in these circuits via presynaptic mechanisms.
336 Heterosynaptic effects on the auditory afferent inputs and homosynaptic effects on the inhibitory
337 terminals that were equally suppressive in their actions, which could maintain the excitatory
338 versus inhibitory balance while providing common negative feedback.

339 *Presynaptic GABAergic inhibition at inhibitory and excitatory synapses in NA.*

340 Physiological evidence suggested that the strong suppression of inhibitory and excitatory
341 evoked currents occurred via a presynaptic mechanism. An immunohistochemical study
342 previously showed $GABA_B$ receptors were expressed in both avian cochlear nuclei and electron
343 microscopy data further verified the presynaptic localization, at least within NM (Burger et al.,
344 2005b). The clear decline in the frequency of spontaneous events that occurred with the $GABA_B$
345 agonist supports a presynaptic locus of action, in agreement with a previous study (Shi and Lu,
346 2017). At the same time, spontaneous synaptic events showed no significant changes in event
347 amplitude or time course with direct pharmacological activation of the $GABA_B$ receptor,
348 suggesting there was suggesting no postsynaptic effect on the channels that underlie the
349 ionotropic IPSCs or EPSCs. Although evoked IPSCs showed a slightly faster time course, this
350 could be explained by a reduction in asynchronous release that occurred in some recordings, a
351 common feature with inhibitory inputs onto neurons inNM (Tang and Lu, 2012b) and onto
352 spherical bushy cells in the AVCN (Nerlich et al., 2014). Although we did not further test
353 presynaptic mechanisms, presynaptically localized $GABA_B$ receptors have been shown in other
354 systems to suppress neurotransmitter release via associated G-protein pathways and their
355 downstream effectors. Downregulation of neurotransmitter release may occur by Gβγ subunit

356 suppression of presynaptic voltage gated calcium channels or direct binding of vesicle release
357 proteins inhibiting fusion (Gassmann and Bettler, 2012).

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

367 *Role of inhibitory feedback in the timing pathway.*

368 In the avian cochlear nucleus, the majority of known inhibitory feedback arises from an
369 anatomically separate brainstem nucleus, the superior olivary nucleus (SON) (Funabiki et al.,
370 1998; Yang et al., 1999; Lu and Trussell, 2000; Monsivais et al., 2000; Lu and Trussell, 2001).
371 The two divisions of the cochlear nuclei, NA and NM, receive a common inhibitory feedback
372 projection from the ipsilateral SON, as does the tertiary nucleus, NL (Burger et al., 2005a). NM
373 and NL together comprise the circuit known for binaural processing of timing information
374 important for localization sound in the azimuthal plane. How the inhibitory feedback affects the
375 ascending sound information processing and whether there is functional heterogeneity in those
376 effects across brain structures is yet to be fully understood. Because the timing circuit is highly
377 specialized, while the ascending pathways via NA deal with sound intensity and spectrotemporal

378 processing more broadly (Köppl and Carr, 2003; Steinberg and Peña, 2011; Fontaine et al.,
379 2014), inhibition could have quite different roles in each area.

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

394 Our findings suggest that the modulation of excitatory and inhibitory inputs of NA
395 neurons via $GABA_BR$ activation appears to mirror previous studies of GABAergic modulation of
396 synaptic transmission in NM (Brenowitz et al., 1998; Brenowitz and Trussell, 2001a; 2001b).
397 Agonists of $GABA_BR$ had an strong presynaptic suppressive effect on excitatory, presumptive
398 nerve, inputs to NM neurons. These axosomatic calyceal terminals are highly specialized endings
399 thought to reliably evoke large, suprathreshold glutamatergic potentials sufficient to elicit an
400 action potential without fail. These relatively high-release probability synapses also show strong

401 activity-dependent synaptic depression. Upon activation of presynaptic $GABA_B$ receptors, the
402 baseline release probability was reduced and the initial amplitude was reduced (but still
403 suprathreshold), but later responses in a train of activity were, paradoxically, less likely to fail to
404 produce action potentials due to reduction in synaptic depression and relief from receptor
405 desensitization.

406 In NA, presynaptic modulatory effects may similarly alter the synaptic dynamics:
407 $GABA_BR$ activation likewise suppressed baseline release and shifted the synaptic plasticity away
408 from depression. However, because the excitatory inputs to NA neurons tend to be subthreshold
409 (requiring summation to elicit an action potential) and exhibit less synaptic depression than NM
410 under control conditions, presynaptic $GABA_B$ modulation could have markedly different end
411 effects on this network. Excitatory inputs to NL, which originate from NM, do not appear to be
412 modulated by presynaptic $GABA_B$ receptors, suggesting differential regulation of excitation by
413 SON feedback relative to the cochlear nuclei (Lu et al., 2005; Tang et al., 2009).

414 *Heterosynaptic versus homosynaptic inhibition.*

415 In summary, we conclude that inhibitory terminals from the SON are subject to negative
416 feedback or self-regulation in all three nuclei to which it projects. Thus the SON provides 1)
417 direct inhibitory input to the projection neurons of NA via ionotropic GABA/glycine receptors
418 (Kuo et al., 2009), 2) indirect, heterosynaptic inhibitory control of excitatory drive via
419 metabotropic $GABA_B$ receptors on glutamatergic terminals (Lu et al., 2005; Lu, 2009), and 3) a
420 negative feedback regulatory signal via homosynaptic inhibitory metabotropic $GABA_B$ receptors
421 on the SON terminals (this study). The agonist effectiveness on the excitatory and inhibitory
422 release appeared to be comparable, causing >90% suppression. Under what physiological

423 conditions modulation by presynaptic $GABA_B$ receptor occurs is yet to be determined, but our
424 experiments suggest the inhibitory terminals may be more susceptible to modulation. During
425 mild to moderate stimulation conditions, GABA release was sufficient to suppress baseline
426 transmission at inhibitory synapses (see Fig. 6). In addition, blocking reuptake with an inhibitor
427 of the GABA transporter, presumably causing transmitter pooling in the synaptic cleft, was
428 sufficient to reduce spontaneous inhibitory event frequency, but not spontaneous excitatory event
429 frequency (see Fig. 7). On the other hand, blocking $GABA_B$ receptors, however, had no effect on
430 spontaneous inhibitory (nor excitatory) event frequency, suggesting ambient spontaneous
431 neurotransmitter release is not sufficient to elicit chronic, baseline suppression via this pathway.
432 These results suggest that under normal physiological activity, the SON input may itself be under
433 chronic, mildly suppressive negative feedback.

434 Our experiments failed to produce evidence for physiologically-evoked, endogenous
435 heterosynaptic suppression of excitatory inputs to NA neurons. Neither NNC771 nor CGP52432
436 had any effect on the initial or singly-evoked EPSCs (see Fig. 6) or on the short-term plasticity
437 during EPSC trains (data not shown). Because the EPSCs were pharmacologically isolated, it
438 could not be determined whether SON fibers were also releasing GABA under the same
439 stimulation paradigm that elicited the EPSCs. Nevertheless, our current results suggest that
440 previous studies on synaptic plasticity using trains of stimulation in conditions in which
441 metabotropic receptors were unblocked were probably not complicated by modulatory effects in
442 slice (MacLeod et al., 2007). Heterosynaptic effects that rely on neurotransmitter spillover may
443 be more likely in NM, where large calyceal axosomatic synapses interdigitate with small,
444 punctate axosomatic inhibitory terminals. Whether the relative position of excitatory and

445 inhibitory synaptic inputs to NA neurons facilitates such indirect modulatory effects in the intact
446 circuit remains to be investigated.

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