GABAergic and glycinergic inhibition modulate monaural auditory response properties in the avian superior olivary nucleus

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Keywords: GABA\textsubscript{A} receptor, Sound localization, Superior Olivary Nucleus, Glycine Receptor, Inhibition, Interaural time disparity

Running Head: Inhibition modulates response properties in avian SON
Abstract:

The superior olivary nucleus (SON) is the primary source of inhibition in the avian auditory brainstem. While much is known about the role of inhibition at the SON’s target nuclei, little is known about how the SON itself processes auditory information or how inhibition modulates these properties. Additionally, the synaptic physiology of inhibitory inputs within the SON has not been described. We investigated these questions using in vivo and in vitro electrophysiological techniques in combination with immunohistochemistry in the chicken, an organism for which the auditory brainstem has otherwise been well characterized. We provide a thorough characterization of monaural response properties in the SON and the influence of inhibitory input in shaping these features. We found that the SON contains a heterogeneous mixture of response patterns to acoustic stimulation, and that in most neurons these responses are modulated by both GABAergic and glycinergic inhibitory inputs. Interestingly, many SON neurons tuned to low frequencies have robust phase-locking capability and the precision of this phase-locking is enhanced by inhibitory inputs. On the synaptic level, we found that evoked and spontaneous inhibitory postsynaptic currents (IPSCs) within the SON are also modulated by both GABAergic and glycinergic inhibition in all neurons tested. Analysis of spontaneous IPSCs suggests that most SON cells receive a mixture of both purely GABAergic terminals, as well as terminals from which GABA and glycine are coreleased. Evidence for glycinergic signaling within the SON is a novel result that has important implications for understanding inhibitory function in the auditory brainstem.
Introduction:

Interaural time disparities (ITDs) are the main cue that animals use to localize low frequency sounds. Many features of neural circuitry that process this cue are similar between birds and mammals. For example, both systems involve specialized coincidence detecting neurons that detect the timing differences of spikes arriving from both ears. These neurons comprise the medial superior olive (MSO) in mammals (Goldberg and Brown 1969; Yin and Chan 1990) and nucleus laminaris (NL) in birds (Parks and Rubel 1975; Sullivan and Konishi 1986; Carr and Konishi 1990; Peña et al. 1996; Burger and Rubel 2008; Grothe et al. 2010).

Additionally, both systems include inhibitory feedback pathways to monaural and binaural processing centers originating from one or more nuclei located in the superior olive (Caspary et al. 1994; Lachica et al. 1994; Ebert and Ostwald 1995a,b; Westerberg and Schwarz 1995; Smith et al. 1998; Backoff et al. 1999; Yang et al. 1999; Kopp-Scheinpflug et al. 2002; Burger et al. 2005).

In birds, the superior olivary nucleus (SON) is the primary source of inhibition to both cochlear nuclei nucleus angularis (NA) and nucleus magnocellularis (NM), as well as the coincidence detecting neurons in NL (Carr et al. 1989; Lachica et al. 1994; Westerberg and Schwarz 1995; Yang et al. 1999, Burger et al. 2005). The SON receives excitatory inputs from the ipsilateral NA and NL, and a putatively inhibitory input from the contralateral SON (Yang et al. 1999; Monsivais et al. 2000; Burger et al. 2005; Fukui et al. 2010). In vitro studies on chicken brainstem slices have shown that GABAergic input from the SON evokes a depolarizing GABAergic conductance in both NL and NM that improves ITD selectivity and phase-locking to auditory stimuli (Yang et al. 1999; Monsivais et al. 2000). These studies suggested that GABAergic inhibition at NL and NM arising from the SON has relatively slow kinetic
Inhibition modulates response properties in avian SON properties, and is unlikely to contribute to temporal properties on a cycle-by-cycle basis. More recent work has shown that inhibitory signaling within the avian auditory brainstem is not solely GABAergic, but also includes a glycinergic component endowed with faster kinetics (Kuo et al. 2009). The source of this glycinergic input is unknown, but appears to derive from corelease of GABA and glycine from some inhibitory terminals (Kuo et al. 2009).

Recent studies have contributed much to our understanding of SON function in shaping ITD selectivity in birds. Peña et al. (1996) proposed that inhibition contributes to maintenance of ITD selectivity over a wide range of intensities (Peña et al. 1996, Viete et al. 1997), and more recent models based on new anatomical evidence expanded upon this idea (Monsivais et al. 2000; Burger et al. 2005; Dasika et al. 2005). We proposed that in addition to the inhibitory feedback mechanism at the ipsilateral NA, NM, and NL, the putative inhibitory connection between the two SONs would serve to balance input strength for coincidence detecting neurons when stimulus amplitude was bilaterally unequal (Burger et al. 2005; Dasika et al. 2005). Recent work has supported this hypothesis by demonstrating that the SON modulates both ITD encoding in NL and rate/intensity coding in NM bilaterally (Nishino et al. 2008; Fukui et al. 2010).

Despite its central role in these pathways, the nature of acoustic response properties or inhibitory signaling within the SON has not been thoroughly investigated. While there has been much gained from investigation of the influence of inhibition and SON function at its targets, very little is known about how the SON itself processes auditory information. Only a few studies to date report in vivo recordings from SON cells (Moiseff and Konishi 1983; Lachica et al. 1994; Tabor et al. 2011), but these recordings were incidental to the primary goals of these studies. Thus, a thorough characterization of chicken SON auditory response properties is lacking. Since the SON plays a central role in maintaining ITD selectivity as the primary source
of inhibition for many targets in the avian auditory system, we sought: 1) to characterize the 
monaural response properties of SON cells to acoustic stimulation, 2) to investigate how these 
properties are modulated by inhibition, and 3) to investigate the nature of inhibitory synaptic 
transmission at SON neurons. We pursued these questions using *in vivo* and *in vitro* 
electrophysiological recording techniques and immunohistochemical analysis. We found that the 
SON contains a heterogeneous mixture of cell types with diverse monaural response patterns to 
acoustic stimulation, and that these responses are modulated by both GABAergic and glycinergic 
inhibitory inputs in a majority of neurons. Surprisingly, many SON cells have strong phase-
locking capability, and the precision of this phase-locking is enhanced by inhibitory inputs. On 
the synaptic level, we found in all SON neurons that we sampled evoked and spontaneous IPSCs 
were modulated by both GABAergic and glycinergic inhibition. Immunohistochemical evidence 
and analysis of sIPSCs revealed that most SON cells receive a mixture of both purely 
GABAergic terminals, as well as terminals from which GABA and glycine are coreleased. 
Evidence for glycinergic signaling within the SON is a novel result that may have important 
implications for the role of inhibition in the auditory brainstem.
Materials and Methods:

Animals/Surgery:

All procedures were approved by the Lehigh University Institutional Animal Care and Use Committee. Eighty white Leghorn chickens aged postnatal day 5-30 were used for the in vivo experiments. Fertilized eggs were obtained from a commercial poultry supplier (Moyer’s Chicks, Quakertown, PA) and raised at Lehigh University's central animal facility. Chicks were initially anesthetized for surgery with a single dose of ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) at 80 mg/kg (im) and pentobarbital (Sigma-Aldrich, Saint Louis, MO) at 80 mg/kg (ip). For recording, anesthesia was switched to urethane (full dose: 2.5g/kg, given in quarter doses approximately every 30-45 minutes, im). Small supplemental doses were administered if a chick showed any signs of discomfort. After chicks were deeply anesthetized, feathers were removed from the head and the guard feathers were removed from the opening of the ear canal with scissors and depilatory cream. A layer of skin was reflected on the neck to expose the trachea. A small incision was made and the trachea was intubated using a blunt-ended dispensing needle secured with thread. Following tracheotomy, chicks breathed normally during the experiment. Throughout experiments, body temperature was maintained at 39.5° C via a TC-1000 Temperature Controller and cloacal thermistor probe (CWE, INC., Ardmore, PA). The head was secured in a stereotaxic apparatus (Stoelting Co., Wood Dale, IL) with ear bars and adjusted to approximately a 45° angle with respect to the horizontal plane. An incision was made in the scalp, and the tissue was reflected from the top of the skull. Any remaining fascia on the skull was cauterized and the skull was covered with a thin layer of cyanoacrylate adhesive and small glass beads. A brass rod was then secured to rostral skull at the midline with dental acrylic. After the dental acrylic hardened, the chick was transferred to a custom stereotaxic
Inhibition modulates response properties in avian SON apparatus (Schuller et al. 1986) inside a sound-attenuation booth (Industrial Acoustics, Bronx, NY). The head was then adjusted to a 45° pitch angle, 0° roll, and 0° yaw with respect to the horizontal axis. A hole was drilled in the skull overlying the cerebellum approximately 2.1 mm lateral to the midline. The dura was excised and reflected from the cerebellum. Speakers were coupled to the ears with tubes insulated with acoustic foam (Etymotic Research, Inc., Elk Grove Village, IL), and a ground wire was inserted into the exposed neck muscle at the base of the head.

In vivo recording configuration:

The SON was located using a single blunt-tipped glass electrode (filled with 3M KCl, 0.5-1 MΩ resistance) advanced through the brainstem using a remotely driven actuator (MC1000e -1 Controller; Siskiyou Design Instruments, Oregon, USA) while presenting 50 ms noise bursts in the ipsilateral ear. The SON was identifiable by characteristic responses to noise bursts that evoked strong sustained responses lasting over a penetration depth of approximately 500 μm. The SON was typically located 2.1 to 2.2 mm from the midline, rostral to NM, and approximately 8-9 mm from the surface of the cerebellum. After locating the SON, the blunt electrode was replaced by either a single sharp-tipped borosilicate recording electrode or by a “piggy-back” multibarrel electrode (Havey and Caspary 1980) used for simultaneous recording and local iontophoresis of drugs. Recording electrodes were filled with 3M KCl, and typically had a resistance range of 5-15 MΩ. Multibarrel electrodes were fabricated from 3-barrel borosilicate glass blanks pulled to a sharp tip and then broken manually under a microscope to a tip with 10-20 μm total diameter (A-M Systems, Carlsborg, WA). A sharp single recording electrode was bent to approximately a 20° angle and glued to the multibarrel tip so that it
protruded approximately 20 μm. The electrodes were then secured with dental acrylic and allowed to dry. Multibarrels were loaded with drug solutions in two barrels, and the third barrel was filled with 3M KCl for current balance. Drug solutions contained either: bicuculline methiodide (5mM in 0.9% NaCl, pH 3), SR95531 hydrobromide (3mM in 0.9% NaCl, pH 3), or strychnine HCl (10mM in 0.9% NaCl, pH 3.5). Bicuculline methiodide and SR95531 hydrobromide were obtained from Tocris bioscience (Bristol UK), and strychnine HCl was obtained from Sigma-Aldrich (St. Louis, MO). Drug/balance barrels were connected by silver-silver chloride wires to a headstage unit controlled by an MVCS-02 iontophoresis module (NPI electronic GmbH, Tamm, Germany). Drugs were held in the pipette with -15 to -18 nA retaining current. To expel drugs the current was switched to the positive range, typically 50-70 nA. Drugs were applied until their effects reached a stable saturated level, suggesting that as many receptors were blocked as were available. Recovery times ranged from 5-43 minutes for bicuculline and SR95531, and 10-87 minutes for strychnine treatment.

Acoustic stimuli and data acquisition:

Acoustic stimuli were created using freely available Spike software (Brandon Warren, University of Washington) and output to Eartone 3A Insert Earphones (Etymotic Research, Inc., Elk Grove Village, IL) through a HB7 Headphone Driver (Tucker-Davis Technologies, Alachua, FL). Speaker output was controlled by a PA5 Programmable Attenuator (Tucker-Davis Technologies, Alachua, FL). Speaker intensities were calibrated using a ¼” Free Field microphone (model 2520, Larson-Davis, Provo, UT) and microphone preamp (model 2221; Larson-Davis, Provo, UT). Stimuli consisted of 50 ms tones with 5 ms rise/fall times with either frequency or intensity presented in pseudo-random order.
Extracellular signals were band-pass filtered between 0.3 – 3 kHz (Model 3362 Filter, Krohn-Hite Corporation, Brockton, MA), and amplified using a Neuroprobe Amplifier Model 1600 (A-M Systems, Carlsborg, WA). Action potential event times were recorded using a voltage window discriminator (model 121; World Precision Instruments, Sarasota, FL) and processed by an RX6 Multifunction Processor (Tucker-Davis Technologies, Alachua, FL).

Labeling of recording sites:

In order to anatomically confirm recording sites, neurobiotin (Vector Labs, Burlingame, CA) was iontophoresed from the recording electrode (5% Neurobiotin in 2M K acetate, as described in Köppl and Carr 2008) with 0.5 \( \mu A \) of positive current for 60 seconds using an iontophoresis pump (BAB-501, Kation Scientific, Minneapolis, MN) in several experiments. This protocol ejected a minimal amount of neurbiotin, which was sufficient to label just a few (<10) local neurons (Fig 1D). After a minimum rest time of 30 minutes to allow cellular uptake, the chick was transcardially perfused first with PBS and then with 4% paraformaldehyde (PFA) in PBS. Following perfusion, the brain was removed from the skull and post-fixed in 4% PFA in PBS overnight at 4° C. Tissue was sliced on a vibratome and then processed using the Vectastain ABC kit (Vector Labs, Burlingame, CA).

In vivo data analysis and statistics:

Data were analyzed using Spike software. Spike counts were obtained from a time window equal in duration to the stimulus, and adjusted to the minimum first spike latency of the response. Response patterns of neurons were classified from PST histograms at a level 20 dB SPL above threshold. Neuron threshold was defined as either 1) the intensity at which the spike
rate during the stimulus time exceeded 2 standard deviations of the average spontaneous rate, or 2) the intensity at which the spike rate during the stimulus time exceeded a 50% increase in the average spontaneous rate. The latter method was necessary to accommodate the small proportion of cells with high spontaneous rates, as the standard deviation of the spontaneous rates in these cells was quite low compared to the actual spontaneous rate. Spontaneous rates were calculated from a time window preceding stimulation and averaged across all trials. Dynamic range was defined as the intensity range from threshold to the intensity at which firing rate is maximal. Rate-intensity slope values were calculated from linear fits of 10-90% of dynamic range. Input-output functions were classified as nonmonotonic if the spike rate decreased >10% of the peak rate. Characteristic frequency (CF) was defined as the frequency at which threshold was crossed at the lowest intensity. If multiple frequencies evoked threshold crossing at a single intensity, the frequency with the highest spike rate at that intensity was chosen. Frequency tuning curves were assessed by analyzing Q10 dB values, calculated as the CF divided by the response bandwidth 10 dB SPL above threshold. Vector strength (Goldberg and Brown 1969) was calculated as $\text{VS} = \sqrt{\sum \sin(\alpha_i) + \sum \cos(\alpha_i)} + N$, where $\alpha_i$ is the phase position of a single spike, and $N$ is the number of spikes, as described (Fukui et al. 2010). Vector strength values were obtained from a time window excluding any onset peak response to the end of the stimulus time. This time window varied between cells, but was always greater than 20 ms (range 22-50 ms). Vector strength values were tested for statistical significance using the Rayleigh test. P values were approximated using equation 27.4 from Zar (1999) $P = \exp\left[\sqrt{(1+4N+4(N^2-R_n^2)-(1+2N)}\right]$ where $R$ is vector strength, $N$ is the number of spikes, and $R_n$ is $R \cdot N$, as described in Berens (2009). Unless otherwise noted, data values reported in text are presented as mean ± standard deviation, and all data shown in figures are presented as...
mean ± standard error. Data were tested for statistical significance using Student’s paired t-test with two-tailed distribution.

In vitro brain-slice preparation:

For synaptic physiology, 12 white leghorn chickens aged E17-P5 were rapidly decapitated and the brainstem containing auditory nuclei was removed, blocked, and submerged in oxygenated artificial cerebrospinal fluid (ACSF) (containing in mM: 130 NaCl, 3 KCl, 10 glucose, 1.25 NaH2PO4, 26 NaHCO3, 3 CaCl2, 1 MgCl2) at 22°C. The brainstem was placed rostral surface down on the stage of a vibrating microtome (Microm 650V, Walldorf, Germany). Coronal sections (150-200 μm) containing the SON were collected, submerged in an incubation chamber of continuously oxygenated ACSF and incubated at 37°C for approximately one hour. Slices were then maintained at room temperature until used for recording.

Brainstem slices were placed in a custom recording chamber on a retractable chamber shuttle system (Siskiyou Design Instruments, Oregon, USA) and neurons were visualized with a Nikon FN-1 Physiostation microscope using infrared differential interference contrast optics. Video images were captured using a CCD camera (Hammamatsu C7500-50, Hamamatsu City, Japan) coupled to a video monitor. The recording chamber was continuously perfused with ACSF at a rate of 2-4 ml/min. An inline feedback temperature controller and heated stage were used to maintain chamber temperature at 35 ± 1°C (TC344B, Warner Instruments, Hamden, CT, USA).

IPSC Recordings:
For evoked IPSC recordings, a concentric bipolar electrode with tungsten core (WPI TM53CCINS, Sarasota, FL) was lowered to the tissue surface with a micromanipulator and placed in a position dorsomedial to the SON. Principal SON neurons were identified based on their characteristic round morphology. Patch pipettes were pulled from thick walled borosilicate glass capillary tubes (WPI 1B120F-4) to a resistance of 4-8 MΩ using a two-stage puller (Narishige PC-10, Tokyo, Japan) and back-filled with internal solution (containing in mM: 130 CsCl, 1 CaCl2, 1 MgCl2, 10 EGTA, 10 HEPES, 2 ATP, 0.3 GTP, 10 phosphocreatine, pH adjusted to 7.3 with CsOH). 5 mM QX314 was added to the internal solution to prevent antidromic action potentials. In many cases, 0.4% biocytin was added to the internal solution to label the neurons following the protocol of Scott et al. (2005). SON principal neurons had an average whole-cell capacitance of 36.7 ± 14.3pF and an average series resistance of 10.2 ± 4.1MΩ. In voltage clamp, series resistance was compensated at 60-80%. Evoked and spontaneous IPSCs were recorded during bath application of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (40μM) and D-2-amino-5-phosphonopentanoic acid (AP5) (50μM) in order to block glutamatergic input. Membrane voltage was clamped at -60 mV using a Multiclamp 700B amplifier. The signal was digitized with a Digidata 1440 data acquisition board and recorded using Clampex software (Molecular Devices, Sunnyvale, CA). IPSCs were evoked with 50 μsec stimulus pulses with a stimulus isolation unit (Isoflex, A.M.P.I. Inc., Israel) through a bipolar electrode. Stimulus magnitude (range 10-90 V) was gradually increased until IPSC amplitudes stabilized at their maximum. Spontaneous IPSC data was collected by recording 30-60 second epochs while clamping the membrane at -60mV. Miniature IPSCs were also collected in the presence of 1μM TTX to block the contribution of presynaptic action potentials to spontaneous events. In our recordings, no significant differences were observed between spontaneous and
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miniature events in any condition and therefore the data were pooled (Oleskevich and Walmsley 2002), and will be referred to as sIPSCs hereafter. After collection of control data, SR95531 (20μM) and strychnine (500nM) were sequentially applied to block GABA_A and glycine receptors (GlyRs) respectively. In several cells, SR95531 and strychnine were applied simultaneously during data collection. IPSC amplitudes and kinetics were analyzed using Clampfit software. Rise and decay time constants, expressed hereafter as tau (τ_{rise} and τ_{decay}) values, were calculated from standard exponential fits from 10-90% of the peak of IPSCs. τ_{decay} values were obtained using either single or double exponential fits. Goodness of fit was determined by comparing the sum of the squared errors. Double exponential fits were chosen if the sum of the squared errors was less than half that of the single exponential fit. A weighted τ_{decay} value was calculated for double exponential fits using the equation: weighted τ = τ_1 (A_1/A_1+A_2) + τ_2 (A_2/A_1+A_2), as in Kuo et al. (2009). Spontaneous IPSC frequency (Hz) was also calculated. Drug and recovery condition amplitudes and kinetic measures were normalized to control and each treatment group was assessed for statistical significance using paired Student's t-tests. Data in the results are expressed as percent of control ± standard deviation. Error bars in figures represent standard error of the mean. Raw data values for evoked, spontaneous, and miniature IPSCs are found in Table 1.

Immunohistochemistry, Receptor staining:

Four chickens aged embryonic day 19 to postnatal day 21 were deeply anesthetized with ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) at 160 mg/kg (im) and pentobarbital (Sigma-Aldrich, Saint Louis, MO) at 80mg/kg (ip). Chickens were transcardially perfused first with PBS and then 4% PFA in PBS, pH 7.4. The brain was removed from the skull
Inhibition modulates response properties in avian SON and post fixed in 4% PFA overnight at 4°C. Following PBS rinse, a section of brainstem containing the auditory nuclei was sliced into 50 μm coronal sections using a vibratome. An additional fixation step of methanol:acetic acid (95:5) for 10 min at -20°C (Dumoulin et al. 2001) was applied to the sections. Sections were blocked in 10% normal goat serum, then incubated overnight at 4°C in primary antibody solution (0.3% PBST, 5% normal goat, and mAb4a [1:1000; Synaptic Systems; Göttingen, Germany; lot #146011/13]). This antibody was raised against the glycine receptor from rat spinal cord (Pfeiffer et al. 1984), and recognizes amino acids 96-105 of the α1 subunit (Schröder et al. 1991). A BLAST search revealed that this amino acid sequence is identical in the chicken GlyR α1 subunit (Altschul et al. 1997). Additionally, this antibody has been previously used in the chicken brain (Tsen et al. 2000).

After PBS rinses, the sections were incubated for two hours with secondary antibody conjugated to a fluorophore (1:200 AlexaFluor 488 goat anti-mouse lot # 558866, Invitrogen, Eugene, Oregon). A fluorescent Nissl stain (NeuroTrace 640/660, Invitrogen, Eugene Oregon) was applied to visualize nuclei (1:100, for 25 minutes). Sections were mounted between coverslips in Glycergel (Dako, Carpenteria, CA) and images were obtained with a confocal microscope (Zeiss LSM 510 Meta, Thornwood, NY). Images were processed using Photoshop (Adobe Systems Inc., San Jose, CA) to match pixel intensity distributions between color channels. No staining was observed when primary antibodies were absent. Additionally, antibody preabsorption experiments were performed using a custom peptide (GenScript; Piscataway, NJ) matching the GlyR sequence 96-105 (WNDPRLAYNE) with amidation at the C-terminus, as described in Schröder et al. (1991). Peptide concentrations at a 400 fold ratio to primary concentration eliminated cell specific staining, confirming the specificity of the mAb4a antibody (data not shown).
In order to anatomically confirm GABA and glycine neurotransmitter at terminals, immunohistochemistry following the protocol of Kuo et al., (2009) was performed. Briefly, three mature chickens ranging in age from P17-P23 were deeply anesthetized as previously described and transcardially perfused with PBS then 2% PFA and 2% glutaraldehyde (Grade 1; Sigma) in PBS, pH 7.4. The brains were removed from the skulls, rinsed in PBS and the section of brainstem containing the auditory nuclei was sliced into 30 μm coronal sections using a vibratome. Sections were rinsed thoroughly in PBS and then incubated in freshly made 1% sodium borohydride (Sigma) in PBS at room temperature for 30 minutes to reduce glutaraldehyde auto-fluorescence. After thorough PBS rinses, the sections were blocked in 2% normal goat serum, 1% BSA and 0.1% saponin in PBS for one hour at room temperature. Primary antibodies were raised with neurotransmitter conjugated to BSA and glutaraldehyde. Anti-glycine (polyclonal rabbit anti-glycine; 1:500; AB 139, lot #NG1740903; Millipore) and anti-GABA (monoclonal mouse anti-GABA; 1:5000; mAB 3A12, lot ps2; Swant, Switzerland) primary antibodies were incubated concurrently overnight at 4°C in block solution. Both antibodies have been used in chick tissue previously (Matute and Streit 1986; Kalloniatis and Fletcher 1993; Kuo et al. 2009). After primary incubation, sections were rinsed in PBS and incubated in secondary antibodies (Alexa Fluor 488 goat anti-mouse; Lot #774904; Alexa Fluor 568 goat anti-rabbit; Lot #514959; 1:500; Invitrogen) for two hours at room temperature. Sections were mounted on gelatin coated slides, allowed to dry overnight, dehydrated in ascending alcohols and cleared in xylene. Sections were then rehydrated in descending alcohols and coverslipped with Vectashield mounting medium (Vector laboratories, Burlingame, CA). Confocal images were obtained and edited as previously described.
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Results

Monaural response properties of SON cells to acoustic stimulation were investigated using in vivo recording techniques. The modulation of these properties by inhibitory signaling was also evaluated. Additionally, the nature of inhibitory synaptic transmission at these neurons was investigated using whole cell voltage clamp recording techniques. In vivo data were collected from 109 SON cells, from 80 white-leghorn chicks age range P5-P30. The characteristic frequencies (CFs) of sampled cells ranged from 0.19 to 4.8 kHz. In vitro data were obtained from late stage embryos and early posthatch chicks aged E17-P5. A total of 27 SON neurons from 12 chicks were used for analysis of synaptic physiology.

SON neurons have diverse response patterns to acoustic stimulation.

SON neurons were stimulated at BF with 50 ms tone bursts. The responses of SON cells to acoustic stimulation segregated into two broad categories. The great majority (94.5%) of SON cells were driven by acoustic stimulation, while the remaining population was suppressed by acoustic stimulation (Fig 1). Of neurons with acoustically driven responses, 69.7% had a sustained response. These cells typically had a strong initial peak response followed by tonic firing throughout a stimulus tone (Fig 1A). 24.8% had an onset response during acoustic stimulation, in which only one or two spikes fired at the beginning of a stimulus tone (Fig 1B). The spike rates of most cells increased monotonically with intensity (64% of all cells), while the remainder of cells were nonmonotonic, exhibiting >10% depression from the peak rate at high intensities. The proportion of cells exhibiting nonmonotonic rate functions was greater for sustained cells (41% of sustained cells, 22% of onset cells). Acoustically driven neurons had an
average threshold of 26 dB SPL ± 21, average dynamic range of 40 dB SPL ± 17, and an average first spike latency of 9.3 ms ± 4.2.

We recorded from 6 neurons that were suppressed by acoustic stimuli. This population tended to have relatively high spontaneous firing rates (29 spikes/sec ± 13, n = 6) that were reduced or eliminated during acoustic stimulation (Fig 1C). It should be noted that because this cell type was encountered infrequently, a thorough characterization of this population was not possible. However, in several neurons we observed that this spike suppression was dependent on both stimulus intensity and frequency. A representative example of a suppressed neuron is shown in Figure 2. The acoustically driven suppression of spiking in this SON cell occurred over a 1.4 octave frequency range (Fig 2A). The suppressive effect occurred over a relatively broad frequency range for all neurons where tuning information was obtained (1.8 octaves ± 0.9, n = 3). In every neuron tested (n = 6), the suppressive effect increased with sound intensity (Fig 2B). Our observations are consistent with those reported for 2 neurons in Tabor et al. (2011).

We also investigated the frequency-tuning properties of 27 SON cells with sustained or onset responses. The population's CFs were distributed across the entire chicken audiogram, and the frequency range did not differ for sustained and onset cells (Fig 3A). Tuning curves for both types were generally broad, and representative examples are shown in Figure 3B (mean Q10: 2.0 ± 1.2). The distribution of Q10 values for all neurons for which tuning data were obtained demonstrates that most SON cells were broadly tuned, regardless of response type (Fig 3C).

We also investigated whether SON neurons were capable of encoding temporal aspects of auditory signals by analyzing phase-locking to tone stimuli. Previous studies have suggested that temporal information is not likely to be encoded by SON cells (Lachica et al. 1994; Yang et al. 1999; Monsivais et al. 2000). Surprisingly, we observed that many SON cells have robust phase-
Inhibition modulates response properties in avian SON locking capabilities. We calculated vector strength values from a time window that excluded the initial peak response (as judged from PST histograms) through the end of the stimulus for 74 cells with sustained responses. Raster plots of phase-locked discharges and their corresponding vector strengths are shown for two neurons with CFs of 0.41 kHz and 1.04 kHz in Figure 4A. Figure 4B shows a scatter plot of peak vector strength values as a function of CF for the entire population. Vector strength values were tested for significance using the Rayleigh test (see Methods): 40/74 cells had peak vector strengths with p values < 0.05 according to this test (filled squares in Figure 4B). Of these 40 cells, 6 had CFs greater than 1.5 kHz.

Response properties of SON neurons are modulated by both GABAergic and glycinergic inhibitory inputs.

It has not previously been investigated whether inhibition influences SON responses. The SON is known to receive a putatively inhibitory input from its contralateral counterpart and immunostaining against GABA_A receptors or glutamic acid decarboxylase (GAD) showed strong reactivity in the SON (Carr et al. 1989; Code and Churchill 1991). We next investigated how the monaural response properties of SON cells are modulated by inhibitory inputs. To test whether SON neurons are modulated by GABAergic inputs, we employed pharmacological block of GABA_A receptors by iontophoresis of either SR95531 hydrobromide or bicuculline methiodide using multibarrel electrodes.

Both SR95531 and bicuculline had similar effects on response properties, so data for GABA_A receptor block were pooled for population analysis. GABA_A antagonist application increased acoustically driven spike rates in every cell tested (25/25 cells). A representative example of the effects of GABA_A block on the response properties of an SON neuron is shown
in Figure 5. Figure 5A demonstrates that application of bicuculline increased the number of
spikes in this neuron at every intensity. In this case, the spike count increased by 130% at 60 dB
SPL and by 200% at 80 dB SPL. Following cessation of bicuculline application, the spike count
recovered to control levels within 5 minutes for this neuron. Rate-level functions during the
control, bicuculline, and recovery conditions for the neuron shown in A are shown in Fig 5Bi.
Two important features of GABA_A function are demonstrated in this example. First, bicuculline
application increased the spike rate, particularly at suprathreshold intensities. Across the
population of tested cells, GABA_A block significantly increased the spike rate at the stimulus
intensity that evoked the peak firing rate in the control condition (p < 0.05, Fig 5Bi). However,
GABA_A block did not significantly affect the threshold (22 dB SPL ± 20 for control; 21 dB SPL ± 22 for GABA_A block, p > 0.05, n = 23). The effect of GABA_A block on spontaneous firing
rate was variable, increasing in some cells but not changing in others. Across the population,
there was a mild trend for the spontaneous rate to increase during GABA_A block, but this effect
was not statistically significant (24 spikes/sec ± 27 for control; 29 spikes/sec ± 30 for GABA_A
block, p > 0.05, n = 23). GABA_A block did not affect the first spike latency (8.7 ms ± 2.9 for
control; 8.0 ms ± 2.0 for GABA_A block, p > 0.05, n = 22) of SON neurons.

The second major feature of GABA function is that it decreases the slope of the input-
output function. Across the population, GABA_A block significantly steepened the input-output
function. We quantified this change by calculating response slope over 10-90% of the dynamic
range of the response for each condition (see Methods). The intensity boundaries of the dynamic
range were unchanged by GABA_A antagonist application (45 dB SPL ± 16 for control; 47 dB
SPL ± 18 for GABA_A block, p > 0.05, n = 23). However, GABA_A block significantly increased
the slope of the response through the dynamic range (p < 0.01, Fig 5Bi).
Glycinergic signaling is prominent in SON.

A recent study showed that in addition to GABAergic signaling, prominent glycinergic signaling also exists in NA (Kuo et al. 2009), although the source of the glycinergic inhibition is not known. To test whether SON neurons also receive glycinergic inhibitory inputs, we locally iontophoresed strychnine (a GlyR antagonist) while recording acoustic responses. Surprisingly, we found that similar to GABA_A block, GlyR block also increased acoustically driven spikes in 16/19 cells. A representative SON neuron in which glycinergic transmission was manipulated is shown in Figure 6. Figure 6A shows that strychnine treatment increased the spike count similarly throughout most of the dynamic range of this cell (about 26%), but had a slightly larger effect at the loudest intensity tested (90 dB SPL, 38% increase). After iontophoresis of strychnine was turned off, the spike count recovered nearly to control levels at higher intensities within 40 minutes; this cell was lost before complete recovery could be obtained. Figure 6Bi shows rate-level functions during the control, strychnine, and recovery conditions for the same cell in panel A, illustrating two important features of glycinergic modulation. First, despite a general rise in spike rate, the slope of the dynamic range did not change for this neuron. Across the population, and in contrast to GABA_A block, strychnine did not significantly affect the slope of the input-output function. The second feature of interest is that nonmonotonic responses are frequently mediated by inhibitory input to SON neurons. For this cell, the control input-output function was nonmonotonic but became monotonic during strychnine treatment. This change from nonmonotonic to monotonic input-output functions was observed for 2/2 neurons treated with strychnine as well as 5/8 GABA_A block neurons.
Across the population of tested cells, GlyR block significantly increased the spike rate at the intensity evoking the peak rate in the control condition (p < 0.05, Fig 6Bii). Similar to GABA_A block, GlyR block did not affect the threshold intensity (21 dB SPL ± 21 for control; 21 dB SPL ± 20 for strychnine, p > 0.05, n = 15), the intensity limits of the dynamic range (40 dB SPL ± 18 for control; 43 dB SPL ± 19 for strychnine, p > 0.05, n = 15), or the first spike latency (8.4 ms ± 1.8 for control; 8.5 ms ± 2.1 for strychnine, p > 0.05, n = 15) of SON neurons. Unlike GABA_A block, GlyR block had no affect on the slope of the dynamic range (Fig 6Bii). The effect of GlyR block on spontaneous firing rate was variable between neurons, similar to GABA_A block. Across the population, there was a mild trend for the spontaneous rate to increase during strychnine treatment, but this effect was not statistically significant (11 spikes/sec ± 14 for control; 17 spikes/sec ± 21 for strychnine, p > 0.05, n = 15).

GABAergic and glycinergic inhibitory inputs enhance phase-locking in SON neurons.

In this report we show that some SON cells show strong phase-locking capabilities (Fig 4). To investigate how inhibitory inputs may influence the phase-locking of SON neurons, we measured changes in peak vector strength during pharmacological block of GABAergic and glycinergic inhibitory receptors (using similar methods as in the previous experiments). We found that both types of inhibitory inputs enhance the precision of phase-locking in SON cells. GABA_A block reduces the peak vector strength in every cell tested, and this affect is significant across the population (p < 0.0001, Fig 7Ai,ii). Similar to GABA_A block, GlyR block with strychnine also significantly reduces the peak vector strength of SON cells (p < 0.0001, Fig 7Bi,ii).
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Evoked inhibitory synaptic transmission in the SON is mediated by both GABAergic and
glycinergic components.

The in vivo experiments demonstrated modulation of SON response properties with
application of both glycine and GABA\textsubscript{A} receptor antagonists. Both antagonists increased
acoustically driven spike rates and diminished the precision of phase-locking. Additionally,
GABA\textsubscript{A} block increased the slope of the input-output function through the dynamic range. To
further characterize inhibitory transmission in the SON, we used in vitro whole cell voltage
clamp techniques to record evoked and spontaneous IPSCs while pharmacologically
manipulating GABAergic and/or glycinergic transmission.

IPSCs were evoked by placing a stimulating electrode on input fibers dorsomedial to the
border of SON while excitatory inputs were blocked with 40 \(\mu\)M DNQX and 50 \(\mu\)M AP5.
Evoked IPSCs (eIPSC) were observed in 20/21 neurons tested. eIPSC amplitude was variable
with an average peak amplitude of \(-975 \pm 957\) pA (Table 1). In order to isolate the GABAergic
component of eIPSCs, 500 nM strychnine was bath applied. Peak eIPSC amplitude was reduced
in 12/12 cells on average to 50.6 \(\pm\) 16.8\% of the control value (Fig 8A). Similarly, blocking
GABA\textsubscript{A} receptors with SR95531 (20\(\mu\)M) reduced eIPSC amplitude to 45.2 \(\pm\) 19.4\% of control (n
= 14). Simultaneous application of both SR95531 and strychnine completely abolished eIPSCs
in 9 of 13 cells. The residual current for the population was 5.3 \(\pm\) 6.9\% of control (Table 1). In
addition to amplitude modulation, changes in the eIPSC waveform kinetics were also evident
(Fig 8A). We compared the halfwidth and \(\tau_{\text{decay}}\) values of isolated GABAergic and glycinergic
eIPSC components. Blocking GlyRs broadened the eIPSC and increased both the halfwidth
(145.3 \(\pm\) 59.9\% of control, \(p < 0.05\), n = 12) and \(\tau_{\text{decay}}\) (158.1 \(\pm\) 72.1\% of control, \(p < 0.05\), n =
Inhibition modulates response properties in avian SON. Blocking GABA\(_A\) receptors with SR95531 had a complementary effect on kinetics, narrowing the eIPSC waveform by reducing halfwidth (65.3 ± 25.6% of control, p < 0.01, n = 14) and \(\tau_{\text{decay}}\) (66.5 ± 19.7% of control, p < 0.01, n = 14; Fig 8C, D). Area under the eIPSC waveform was also reduced in both drug conditions (strychnine: 60.7 ± 22.1% of control, p < 0.01, n = 12; SR95531: 31.1 ± 22.4%, p < 0.01, n = 14; Fig 8B). These data suggest that both GABAergic and glycinergic transmission occur in the SON, that each contribute equally to the peak eIPSC amplitude, but that the GABAergic transmission provides the majority of the total eIPSC current.

**Glycine and GABA are coreleased at some inhibitory terminals in SON.**

Kuo et al. (2009) showed evidence suggesting that the most likely source of glycine in NA was from terminals that corelease GABA and glycine. Given our findings that both GABAergic and glycinergic synaptic transmission act on SON neurons, several possible input arrangements exist for these two modes of inhibition: 1) SON neurons could receive independent, purely GABAergic and purely glycinergic inputs, 2) these inputs could be provided by GABA/glycine corelease terminals as reported in NA (Kuo et al. 2009), or 3) there could be a mixture of single transmitter and corelease terminals. To differentiate between these possible input arrangements, we evaluated the properties of spontaneous and miniature IPSCs. Statistical analysis of spontaneous and miniature events revealed no significant differences so the data sets were pooled and will be referred to as sIPSCs (see Methods).

We held SON cells at -60mV for 30 or 60 second epochs while blocking excitatory input with DNQX and AP5 in 24 neurons. sIPSCs were common with an average frequency of 8.42 ± 6.25Hz (Table 1). Blocking GABA\(_A\) receptors significantly reduced the sIPSC frequency (27.8
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± 23.5% of control, p < 0.01, n = 21), suggesting that many sIPSCs were purely GABAergic.

SR95531 application also reduced the average peak amplitude of the remaining sIPSCs (76.4 ±
19.1% of control, p < 0.001, n = 21) (Fig 9A,C,D). In contrast, strychnine application had no
significant effect on sIPSC frequency, but there was a slight trend toward fewer events (85.6 ±
25.6% of control, p > 0.05, n = 17; Fig 9C). However, peak sIPSC amplitude was significantly
reduced (66.2 ± 32.3% of control, p < 0.01, n = 17; Fig 9A,D). No sIPSCs were observed during
simultaneous application of strychnine and SR95531 (n = 13). One additional observation was
that during the control condition there was typically a few very large (>400pA) events as seen in
Figure 9A. These events were absent in the drug conditions, but returned with recovery. These
data taken together are consistent with an input arrangement where each SON neuron receives a
substantial portion of purely GABAergic synaptic terminals, but few, if any, purely glycinergic
terminals. Rather, the glycinergic component appears to be coreleased with GABA at some
terminals. We further analyzed the distributions of sIPSC kinetics in each condition to confirm
these findings.

The evoked IPSC data show that isolated GABAergic and glycinergic conductances have
very different decay kinetics. If sIPSCs arose from purely GABAergic or glycinergic terminals,
then we would expect a bimodal distribution of sIPSC kinetics. However, $\tau_{\text{decay}}$ values for
sIPSCs recorded in the control condition typically had a broad continuous distribution from slow
to fast events. Figure 9Bi shows example events from the control condition in panel A
illustrating this heterogeneity. The first event (arrow) has a slow decay, whereas the second
event (arrowhead) is notably faster. Application of strychnine shifted the sIPSC population
towards slower events (Fig 9Bii,E,F). Conversely, SR95531 application resulted in mostly faster
sIPSCs (Fig 9Biii,E,F). Analysis of the cumulative probability plot for all $\tau_{\text{decay}}$ values from 14
Inhibition modulates response properties in avian SON cells in which both drugs were applied shows a monophasic rise in the control condition consistent with the broad and continuous distribution of values (Fig 9E). $\tau_{\text{decay}}$ kinetics of sIPSCs in both strychnine and SR95531 were significantly different from the control condition and different from each other (Kolmogorov-Smirnov test, p < 0.01). Taken together the observations of antagonist effects on $\tau_{\text{decay}}$ distributions in conjunction with their influences on sIPSC frequency and amplitude modulation shown above suggests that the most parsimonious model of SON input arrangements includes both purely GABAergic terminals and some GABA/glycine corelease terminals. While we cannot rule out the possibility that SON neurons receive some purely glycinergic terminals, our data do not support this hypothesis.

**Immunohistochemical analysis of glycinergic signaling in SON.**

Glycinergic signaling in the avian brainstem auditory system has only been reported in two studies that we know of (Code and Rubel 1989; Kuo et al. 2009), but our physiological results suggest glycinergic transmission is an important transmitter system in the SON. Further, analysis of sIPSCs suggest that glycine is coreleased with GABA. We performed immunohistochemical analysis to confirm these two sets of physiological findings.

First, we performed immunohistochemistry using an antibody originally raised against glycine receptor (GlyR) from rat spinal cord (Pfeiffer et al. 1984, see Methods) in 4 chickens aged E19-P21. GlyR immunoreactivity was abundant throughout the SON and intensely stained individual neurons. Figure 10 (A-C) shows representative staining from a P7 brain. Figure 10A shows at high magnification individual neurons in the SON positively stained for GlyR (*green* label). Panel B shows a low power image of a 50 $\mu$m section through the SON (*dashed white line*) with widespread GlyR immunofluorescence. Figure 10C shows only nissl staining (*red nuclei*) and the absence of non-specific staining when primary antibody was excluded.
Second, we used antibodies targeting GABA and glycine following the method of Kuo et al., (2009) to examine the distribution of these neurotransmitters in terminals and somas of SON cells in 3 animals aged P17-23. Figure 10D shows NM with both doubly and singly labeled terminals at high magnification confirming the pattern observed in Kuo et al., (2009), while two images from the SON are shown in E and F. Singly labeled GABAergic terminals appear green and are labeled with arrows while terminals positive for both GABA and glycine (red label) appear yellow and are indicated with arrowheads in Fig 10D, E, and F. SON somas were previously reported to be immunopositive for GABA (von Bartheld et al. 1989), and green labeled somas shown in panels E and F confirm this earlier finding. This pattern is in marked contrast to NM, where somatic staining is absent in panel D. We also observed positive soma staining for both transmitters in SON as shown in panels E and F. The relative intensity of staining for GABA and/or glycine was highly variable between cells. These results suggest that a candidate source of GABA and glycine corelease to NM, NA, NL, and SON is the SON itself.
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**Discussion:**

In this report we characterized the monaural response properties of SON cells during acoustic stimulation and the modulation of these responses by GABAergic and glycinergic inhibition. We showed that GlyR immunoreactivity is prominent in the SON. We also investigated the synaptic physiology of inhibition in the SON. We found that SON neurons demonstrate a heterogeneous mixture of acoustic response patterns. These responses are modulated by both GABAergic and glycinergic inhibitory inputs in the majority of SON neurons. Many SON neurons have robust phase-locking capability, and the precision of this phase-locking is enhanced by inhibitory inputs. On the synaptic level, we found that evoked and spontaneous IPSCs within the SON are also mediated by GABAergic and glycinergic inhibition. Analysis of sIPSC data suggests that the most likely input arrangement for most SON cells is a mixture of purely GABAergic terminals, and terminals from which GABA and glycine are coreleased. These results were supported with immunohistochemistry demonstrating that many synaptic terminals in the SON are positive for both GABA and glycine.

**In vivo SON physiology.**

To date, only a few studies have investigated SON response characteristics *in vivo.* Moiseff and Konishi (1983) reported a general characterization of SON cells in the barn owl, in which most cells were only excited monaurally. Lachica et al. (1994) reported PST histograms of SON cells from chicken that demonstrated a primary-like response pattern, and cells with a strong phasic onset response followed by a steadily decreasing tonic response. Additionally, this study reported a few sample tuning-curves that were relatively broad. The present study has expanded upon the previous data by showing that in addition to neurons with a sustained
response that compose the majority of the tested population, other response patterns to acoustic stimulation also exist within the SON. Specifically, we found that many cells only respond with a phasic onset pattern to tones (onset cells). Interestingly, we also observed a third class comprising a small proportion of SON neurons that were suppressed in response to acoustic stimulation.

We investigated the frequency tuning properties of 27 sustained and onset cells. The majority of these cells were very broadly tuned, with average Q10 values of approximately 2. This result agrees well with recent anatomical and physiological studies that investigated inhibitory feedback to SON’s ipsilateral targets. Fukui et al. (2010) recorded from individual NM neurons and demonstrated tuning of inhibitory responses was broader than that of excitatory responses. Tabor et al. (2011) recently showed that individual SON neurons innervate NL broadly across the tonotopic domain. These data taken together with the data presented here suggest that SON derived inhibitory feedback in the chicken auditory system functions broadly in the frequency domain.

Possible functions of the different SON cell types.

It has previously been shown that the SON forms a negative feedback circuit in the avian auditory brainstem. Excitatory inputs from NA and NL drive the ipsilateral SON (Conlee and Parks 1986; Takahashi and Konishi 1988; Westerberg and Schwarz 1995), which in turn sends inhibitory projections back to the ipsilateral NA, NM, and NL (Yang et al. 1999; Monsivais et al. 2000; Burger et al. 2005). Additionally, a second output pathway from the SON forms reciprocal connections between the two SONs (Yang et al. 1999; Monsivais et al. 2000). Our previous study demonstrated that these two output pathways from the SON arise from distinct
Inhibition modulates response properties in avian SON populations of SON neurons (Burger et al. 2005). This and other studies proposed that in addition to the inhibitory feedback mechanism at the ipsilateral NA, NM, and NL, the putative inhibitory connection between the two SONs would serve to balance input strength for coincidence detecting neurons in NL by coupling inhibitory feedback to their inputs from NM (Burger et al. 2005; Dasika et al. 2005). More recent studies have supported this hypothesis by demonstrating that the SON modulates ITD encoding in NL (Nishino et al. 2008). Additionally, a given SON will suppress responses in the ipsilateral NM, but disinhibit the contralateral NM (Fukui et al. 2010). While the targets of the various cell types could not be identified in the current study, the response properties of the majority of neurons reported in this study are consistent with the current understanding of SON function. The acoustically driven SON cells reported in this study had an average dynamic range of 40 dB SPL ± 16, with approximately 60% of cells responding monotonically with increasing stimulation intensity. Fukui et al. (2010) reported that the effects of GABAergic inhibitory inputs were strongest at higher intensities (higher than 60 dB) in NM. Most SON cells responded robustly at high intensities and thus are well suited to provide an intensity dependent inhibitory signal to their targets.

We found a small population of SON cells that were suppressed by acoustic stimulation, consistent with observations of two neurons reported in Tabor et al. (2011). These neurons had high spontaneous rates and discharges were reduced or eliminated by tone stimulation. The suppression that we observed was dependent on stimulus frequency and intensity, and typically the suppression appeared to last for the duration of the stimulus. These neurons were encountered relatively rarely and we did not have the opportunity to fully characterize this population. For example, it was not determined in this study if the suppression in these cells is mediated by inhibitory inputs to the suppressed neurons themselves. It is possible that the
suppressed cells inherit their response properties from the NA (a primary source of excitatory input to the SON), as a similar response type (type IV) has been reported in the NA of barn owl (Köppl and Carr, 2003). The aforementioned study suggested that these cells may be involved in encoding spectral information. The functional role of the SON cells with the suppressed response type is not clear and it is unknown if these cells are also inhibitory neurons as are the majority of SON neurons.

Temporal properties of SON cells.

Previous physiological investigations suggest that the inhibitory input from the SON to the NM and NL are GABAergic in nature, depolarizing, and have remarkably slow kinetics. These inputs readily summate, and are likely to have a relatively long-lasting effect on postsynaptic neurons (Yang et al. 1999; Lu and Trussell 2000; Monsivais et al. 2000; Howard et al. 2007; Howard and Rubel 2010). A limited sample of whole cell physiological recordings suggested that SON neurons do not respond to electrical input with temporal precision (Yang et al. 1999). These results suggested that SON cells were unlikely to convey a temporally structured inhibition on a cycle-by-cycle basis with respect to sound stimuli. Surprisingly, we found that many low CF SON neurons respond to acoustic stimulation with phase-locked discharges, and this phase-locking was enhanced by inhibitory inputs. These results suggest that some SON neurons may be providing temporally patterned inhibition to their targets. Interestingly, 6 cells with CF higher than 1.5 kHz were found to be significant by the Rayleigh test. While the peak vector strengths for these neurons were relatively low (0.19-0.23), significance by the Rayleigh test suggests that the occurrence of sustained spikes in these cells is not random with respect to the stimulus waveform. Recently, Kuo et al. (2009) demonstrated
that rapid glycinergic signaling within the NA is quite prominent. This study did not identify the
source of the glycine but showed that many terminals immunoreactive to antisera against GABA
also appeared to contain glycine. One intriguing possibility is that the glycinergic input arises
from the SON.

Our immunostaining for GABA and glycine revealed SON somas positive for both
transmitters, in contrast to the glutamatergic NM, where somatic staining is absent. The GABA
positive somatic staining was reported in von Bartheld et al., (1989) but not shown. Our GABA
staining confirms this finding, and is complemented with glycine immunoreactivity in some
SON neurons. The somatic staining pattern in SON, as well as coimmunopositive terminal
staining among the target nuclei of SON projections shown in both Kuo et al., (2009) and the
current study, support the hypothesis that the SON is a source of GABA/glycine corelease
terminals in the chicken auditory brainstem.

If indeed the SON is a major source of glycinergic inhibition to its target nuclei, the SON
may provide phase-locked release of glycine, a neurotransmitter that has rapid kinetics, and
therefore provide a source of temporally patterned inhibition to targets in NA or elsewhere.
Rapid glycinergic transmission is a hallmark of many lower auditory nuclei including those
involved in temporal processing in mammals (Grothe and Sanes 1993; Brand et al. 2002; Smith
et al. 2000; Awatramani et al. 2004; Magnusson et al. 2005). Similar function for glycinergic
transmission has not been demonstrated in vivo in birds. Future studies will be necessary to
investigate whether the glycine in the avian auditory brainstem arises from the SON, and what
the functional consequences of this output are for its targets.

Inhibitory inputs to the SON.

The in vivo data reported in this study demonstrate that most SON cells receive both
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Our *in vitro* data and immunohistochemistry supported these findings. During tone stimulation, both modes of inhibition influenced acoustically driven spike rate but not the spontaneous rate. Further, both modes of inhibition contribute to the precision of phase-locking. Finally, GABAergic signaling influenced the slope of the dynamic range, however glycinergic signaling had no effect on this property. While the source of the inhibitory inputs to SON neurons observed in this study remain unknown, one possibility is that the inhibition arises from the contralateral SON. Although we presented tones monaurally, the contralateral SON could have been driven in one of two ways. First, it has long been known that the middle ears of the chicken are acoustically coupled by the interaural canal, through which low frequency sound waves can readily pass and thereby influence the opposite ear drum (for review, Hyson 2005). Second, ipsilateral NM cells project bilaterally to each NL (Parks and Rubel 1975; Rubel and Parks 1975), and the contralateral NL which projects in part to the contralateral SON (Conlee and Parks 1986) can be monaurally driven (Peña et al. 1996). Alternatively, intrinsic SON inhibitory connections cannot presently be ruled out. Further experiments will be necessary to investigate these different possibilities.

Our *in vitro* data provide additional insights into the nature of GABA and glycinergic input to SON neurons. In all of the cells tested, we observed a GABAergic and glycinergic component during evoked IPSCs. Isolated GABAergic currents had a longer duration while the glycinergic component was shorter when compared to control. In the SON, the GABAergic component contributed about 65% of the total inhibitory current. Evoked IPSCs in the control condition were similar in kinetics but larger in magnitude than evoked IPSCs seen in the NA (Kuo et al. 2009) where GABA and glycine appear to be coreleased. Our analysis of sIPSCs suggested a similar input arrangement of inhibitory transmission in the SON. We found that
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blocking GABAergic transmission resulted in a significant decrease in the frequency of spontaneous events, indicating that the SON receives many inhibitory inputs that are purely GABAergic. In contrast blocking GlyRs did not reduce sIPSC frequency, but did significantly reduce sIPSC amplitudes suggesting that the glycinergic component of sIPSCs was not independent of GABA release. Kinetic analysis of sIPSCs were consistent with a corelease model of GABA and glycine. First, if GABA and glycine were released in separate vesicles, one would predict a bimodal distribution of sIPSC decay kinetics reflecting each of the two putative sources of input. Instead we observed that sIPSCs in the control condition had a continuous distribution of $\tau_{\text{decay}}$ values. Second, sIPSC amplitude decreased significantly in both drug conditions consistent with GABA and glycine corelease. Finally, the control condition was the only condition where we saw very large (>400pA) events (Fig 9A). We hypothesize that these larger events, which tended to have fast kinetics (data not shown), are the result of summation of coreleased transmitters.

Role of Corelease in the Avian Brainstem

Corelease of GABA and glycine has been demonstrated at many synapses in the brain (Jonas et al. 1998; Tanaka and Ezure 2004; Dugué et al. 2005; Wojcik et al. 2006; Lu et al. 2008). Kuo et al., (2009) reported coimmunolocalization of GABA and glycine in NA, NM and NL, however, physiological recording of glycinergic transmission was only observed in the NA. Burger et al. (2005) showed that many inhibitory terminals across NA, NM, and NL arise from collateral branches of single SON neurons. In the present study, we observed GlyR staining in the SON as well as mixed GABA/glycinergic synaptic transmission in vitro. In the mammalian auditory brainstem circuit one source of inhibitory input, the medial nucleus of the trapezoid
body, is known to corelease GABA, glycine and glutamate from terminals innervating the lateral superior olive early in development (Gillespie et al. 2005). Release from MNTB terminals shifts to primarily glycinergic output following hearing onset (Kandler and Friauf 1995; Kotak et al. 1998; Kim and Kandler 2003; Nabekura et al. 2004; Gillespie et al. 2005). Our data suggest that corelease in the SON is not likely due to developmental processes since we observed the physiological and anatomical hallmarks of corelease in animals up to P23. Hearing onset is around E11 in chickens, and the auditory system is considered mature before hatching (by E18 for review see: Rubel and Fritzsch 2002). However, a comprehensive developmental study will be required to completely rule out further developmental changes that may occur throughout maturation.

While the source of the GABA/Gly corelease terminals remains unknown, an appealing hypothesis is that SON neurons are providing both GABA and glycine to their targets. Coimmunostaining for GABA and glycine was observed in NM, NA, and NL Kuo et al., (2009) and the SON is known to provide the dominant inhibition to these targets. However, at present, it appears that the mode of inhibition in those targets is determined by the complement of receptors expressed by the postsynaptic cells. A similar multi-target corelease arrangement has been described in a mammalian hindbrain circuit (Dugué et al. 2005). This arrangement seems plausible in the avian auditory brainstem where the primary source of inhibition (SON) provides input to both nuclei that process timing information and areas involved in intensity processing (Takahashi and Konishi 1988). The kinetically slow GABAergic input to NM and NL has been shown to improve temporal selectivity and coincidence detection (Funabiki et al. 1998; Yang et al. 1999; Monsivais et al. 2000; Fukui et al. 2010). The functional significance of GABA/glycine corelease within the SON is at present unknown. The \textit{in vivo} experiments
performed in this study demonstrated that GABAergic and glycinergic inhibitory inputs modulated the response properties of SON neurons similarly. The sIPSC analysis strongly suggests that SON neurons receive mixed inhibitory inputs composed of both corelease and purely GABAergic terminals. The possibility for diverse functional roles for this complement of inhibitory circuitry in the avian auditory brainstem should be investigated in future studies.

**Summary:**

We have reported several major findings in this study. First, we characterized the monaural response properties of chicken SON cells *in vivo*, which until now has been a notable gap in our understanding of auditory processing in the avian brainstem. SON cells have diverse response properties to acoustic stimulation, and some have strong phase-locking capabilities. Second, we have shown that these response properties are modulated by both GABAergic and glycinergic inhibitory inputs. On the synaptic level, we have shown that evoked and spontaneous IPSCs are modulated by GABAergic and glycinergic inputs. Third, analysis of sIPSCs was consistent with a model of inhibitory input to SON neurons composed of both purely GABAergic terminals and terminals from which GABA and glycine are coreleased. Glycinergic signaling within the SON is a novel result with important implications for our understanding of acoustic processing in birds. The source(s) of inhibitory input to the SON and further investigation of the role of glycinergic signaling in avian brainstem auditory circuitry will be a focus of future study.
Acknowledgements:

The authors would like to thank Dr. Lynne Cassimeris for her help with confocal microscopy, Dr. Martin Richter for his comments on statistical analysis and Gina Notaro for her help in processing tissue following the in vivo experiments. We would like to thank Stefan Oline for editorial comments. We also thank the two anonymous reviewers whose expert comments and suggestions greatly improved our manuscript. This work was supported by grants from the Deafness Research Foundation and the National Institutes of Health /NIDCD DC008989.
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**Figure Legends:**

**Figure 1:** SON cells have diverse responses to tone stimulation. A. PST histogram of a cell with a sustained response, showing the typical peak initial response followed by tonic firing throughout the duration of the stimulus. B. PST histogram of a cell with an onset response, which typically fires one or two spikes only at the beginning of a stimulus tone. C. PST histogram of a suppressed cell, which have high spontaneous rates that are reduced or eliminated during a tone stimulus. D. Recording sites were confirmed by iontophoresis of neurobiotin from the recording electrode following several experiments. In this case, neurobiotin labeled just a few cells within the SON. Scale bar: 200 μm.

**Figure 2:** The spike rate reduction of suppressed SON cells is frequency and intensity dependent. A. Raster plot demonstrating the stimulus frequency dependence of acoustically driven reduction in spike rate in a suppressed SON cell. Cell was stimulated using 50 ms tones (black bar) at 20 dB SPL above suppression threshold at 0.7 kHz, our estimate of CF. B. Rate-level function for the same cell in A at 0.7 kHz. The degree of spike suppression increases with intensity. Inset: PST histograms showing the number of spikes before the threshold for suppression has been crossed (upper left, 101 spikes), and when suppression of spike rate is maximal (lower right, 39 spikes). Spike counts were obtained from the entire histogram shown (40 trials).

**Figure 3:** SON neurons are broadly tuned. A. Characteristic frequencies (CFs) of sampled SON cells are distributed across the entire chicken audiogram and include both sustained filled bars and onset neurons open bars. B. Example tuning curves from cells with sustained and onset
Inhibition modulates response properties in avian SON responses. Tuning curves are similar and generally broad for both cell types. C. Distribution of Q10 values re: CF from sustained and onset cells.

Figure 4: Some SON neurons respond to acoustic stimulation with phase-locked discharges. A. Raster plots of responses to CF tones for two SON cells that demonstrated high vector strength values. Values were computed for responses at all suprathreshold intensities for neurons with robust sustained responses. * represents p < 0.05 by Rayleigh test (see Methods).

B. Distribution of peak vector strength re: CF. Many of the sustained cells with CF <1.5 kHz have peak vector strength values 0.3 or higher. Filled squares: p < 0.05 by Rayleigh test (see Methods). Arrows denote the peak vector strengths of the neurons shown in A.

Figure 5: Response properties of SON neurons to acoustic stimulation are modulated by GABAergic inhibitory inputs. A. Example of SON responses during local iontophoresis of bicuculline during acoustic stimulation. PST histograms are shown for the control, bicuculline, and recovery conditions. The number of spikes increases in the presence of bicuculline, particularly during suprathreshold stimulation and then recovers when iontophoresis of the drug is turned off. Black bar represents the 50 ms stimulus tone. Spike counts are the total for entire PST histogram shown. Bi. Rate-level functions for control, bicuculline, and recovery conditions from the same cell as in A. Bii. Summary population data for GABA_A receptor block. GABA_A block significantly increases the peak spike rate (re: the peak control rate; p < 0.05). GABA_A block also significantly increases the slope of the dynamic range (calculated over the intensity range derived from 10-90% of threshold to maximum rate in control; p < 0.01).
Inhibition modulates response properties in avian SON

**Figure 6:** Response properties of SON neurons to acoustic stimulation are modulated by glycinergic inhibitory inputs. A. Example of SON acoustic responses during local iontophoresis of strychnine. PST histograms are shown for the control, strychnine, and recovery conditions. Spike count increases at all intensities in the presence of strychnine, and then recovers when iontophoresis of the drug is turned off. For this cell, the input-output function that was nonmonotonic in the control condition became monotonic during strychnine treatment. Black bar indicates 50 ms stimulus tone. Spike counts are the total for entire PST histogram shown. Bi. Rate-level functions for control, strychnine, and recovery conditions from the same cell as in A. Bii. Population data for GlyR block. GlyR block significantly increases the peak spike rate (re: the peak control rate; p < 0.05). GlyR block did not significantly affect the slope of the dynamic range.

**Figure 7:** The phase-locking of sustained SON neurons is enhanced by both GABAergic and glycinergic inhibitory inputs. Ai. GABA_A block decreases the peak vector strength of sustained SON cells (pooled bicuculline and SR95531 data). Filled circles: p < 0.05 by Rayleigh test (see Methods). Aii. Summary population data of the effect of GABA_A block on peak vector strength. GABA_A block significantly decreases the peak vector strength (p < 0.0001). Bi. GlyR block also decreases the peak vector strength of sustained SON cells. Filled triangles: p < 0.05 by Rayleigh test (see Methods). Bii. Summary population data of the effect of GlyR block on peak vector strength. GlyR block significantly decreases the peak vector strength (p < 0.0001).
Figure 8: Evoked IPSCs in the SON contain GABAergic and glycinergic components. A. Evoked IPSC (eIPSC) traces from a representative SON neuron during pharmacological treatments. B. Population data showing the effect of treatments on eIPSC area. C. Normalized eIPSC traces showing kinetic modulations during drug treatments (average of 10 traces in each condition). Ci. Bath application of SR95531, a GABA<sub>A</sub> receptor antagonist results in faster eIPSC kinetics. Cii. Blockade of GlyRs with strychnine yielded slower eIPSC kinetics. D. Population data for τ<sub>decay</sub> measured in each condition. *Significantly different from control, p<0.05, **p<0.01. ***Significantly different from all other conditions, p<0.01.

Figure 9: Spontaneous postsynaptic currents are modulated by blockade of both GABA and glycine receptors. A. Representative 5 second epochs of sIPSC records in each experimental condition. B. Expanded 100 ms segments from the traces in A showing condition dependent decay kinetics. Bi. Control condition showing heterogeneous kinetic profiles including slow arrow and fast arrowhead events. Bii. Isolated GABAergic sIPSCs tended to have slower kinetics (quantified in panel F). Biii. Isolated glycinergic sIPSCs had faster kinetics. C. Population data for normalized frequency of sIPSCs. Frequency was significantly reduced during application of SR95531, but not strychnine. D. Population data for normalized amplitude of sIPSCs. Both SR95531 and strychnine significantly reduced sIPSC amplitude when bath applied. E. Cumulative probability analysis of τ<sub>decay</sub> values revealed a monophasic rise in control conditions that was significantly shifted to shorter or longer values in SR95531 and strychnine, respectively (Kolmogorov-Smirnov test, p<0.01). F. Quantification of kinetic measures show a decrease in halfwidth and τ<sub>decay</sub> values in SR95531 and an increase
Inhibition modulates response properties in avian SON. Strychnine did not significantly change halfwidth. *Significantly different from control, p<0.05, **p<0.01. Pooled data includes both sIPSCs and mIPSCs.

Figure 10: Immunohistochemistry of glycinergic signaling in the SON. A-C, images of coronal sections of a P7 chicken SON: images are maximum intensity projections from confocal z-stacks through 50 µm slices. A. GlyR immunoreactivity (green) is abundant among SON somas and processes. Nissl stain is shown in red. B. Lower magnification image from the same SON as in A, GlyR is broadly distributed throughout the SON. Dashed line denotes the borders of the SON. Orientation bars indicate dorsal D and medial M. C. Immunofluorescence for GlyR is absent in tissue processed without primary antibody (Nissl staining shown in red). D-F show staining for neurotransmitters glycine red and GABA green, images are single confocal sections from a P23 chicken brainstem sectioned at 30 µm. D. GABA/glycine immunostaining in NM show singly labeled GABA terminals arrow and robust double labeled terminals arrowheads. Somatic staining was completely absent in NM. E-F, SON neurons also have both doubly labeled GABA/Gly immunopositive terminals as well as singly labeled GABAergic terminals. In contrast to NM, SON somas are differentially immunopositive for GABA or glycine or both. Scale bars: A&C: 50 µm, B: 200 µm, DEF: 20 µm.
Fig 1

A  Sustained, 69.7%

B  Onset, 24.8%

C  Suppressed, 5.5%

D

Bar scale: [Insert scale bar here]
Fig 2

A  Tuning function

B  Rate function
Fig 5

A

<table>
<thead>
<tr>
<th>SPL</th>
<th>Control</th>
<th>Bicuculline</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>90 dB</td>
<td>spike count</td>
<td>179</td>
<td>437</td>
</tr>
<tr>
<td>80 dB</td>
<td>139</td>
<td>415</td>
<td>167</td>
</tr>
<tr>
<td>70 dB</td>
<td>81</td>
<td>253</td>
<td>100</td>
</tr>
<tr>
<td>60 dB</td>
<td>50</td>
<td>115</td>
<td>62</td>
</tr>
<tr>
<td>50 dB</td>
<td>49</td>
<td>114</td>
<td>67</td>
</tr>
<tr>
<td>40 dB</td>
<td>58</td>
<td>134</td>
<td>66</td>
</tr>
</tbody>
</table>

Bi

Spike rate (spikes/sec)

Bii

Spike rate (spikes/sec)

Δ Spike rate/Δ Intensity

Con GABA recovery block

n = 25

n = 11

p < 0.05

p < 0.01
Fig 6

A

<table>
<thead>
<tr>
<th>Intensity (dB SPL)</th>
<th>Control</th>
<th>Strychnine</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>90 dB SPL</td>
<td><img src="Image" alt="Histogram" /></td>
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<td>70 dB SPL</td>
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<tr>
<td>60 dB SPL</td>
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<tr>
<td>50 dB SPL</td>
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<tr>
<td>40 dB SPL</td>
<td><img src="Image" alt="Histogram" /></td>
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<tr>
<td>30 dB SPL</td>
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<tr>
<td>20 dB SPL</td>
<td><img src="Image" alt="Histogram" /></td>
<td><img src="Image" alt="Histogram" /></td>
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<tr>
<td>10 dB SPL</td>
<td><img src="Image" alt="Histogram" /></td>
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</tr>
</tbody>
</table>

Bi

- Control
- Strychnine
- Recovery

Bii

- Spike rate (spikes/sec)
- Intensity (dB SPL)

- Spike rate (spikes/sec) vs Intensity (dB SPL)
- Spike rate (spikes/sec) vs Intensity (dB SPL)
Fig 8

A

Control  Strychnine  Strychnine + SR95531  Recovery

B

Normalized Area

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>SR</th>
<th>SN</th>
<th>SR+SN</th>
<th>Rec</th>
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</thead>
<tbody>
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<td><strong>K</strong></td>
<td>1.0</td>
<td>0.6</td>
<td>0.4</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>*</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
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<tr>
<td>**</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
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</table>

Ci

SR

Control  Recovery

Cii

SN

D

\( \tau_{\text{decay}} \) (ms)

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>SR</th>
<th>SN</th>
<th>Rec</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K</strong></td>
<td>4.0</td>
<td>2.0</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>*</td>
<td>2.0</td>
<td>1.0</td>
<td>0.8</td>
<td>2.0</td>
</tr>
<tr>
<td>**</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Fig 9

A

Control

Strychnine

SR95531

SN + SR

Recovery

B

Bi

20 ms

100 pA

Bii

Biii

C

D

Normalized Freq.

Con

SR

SN

Rec

Normalized Amp.

E

Con
gSR
gSN

gCumulative Prob.

0

5

10

15

20

25

\( \tau \) Decay (ms)

F

Normalized Value

SR

SN

**

Halfwidth

\( \tau \) Decay

**
Table 1. Raw data values for evoked, spontaneous and miniature IPSCs during in vitro whole cell voltage clamp experiments

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak Amp. (pA)</th>
<th>Area (pA)</th>
<th>Halfwidth (ms)</th>
<th>τ Rise (ms)</th>
<th>τ Decay (ms)</th>
<th>Frequency (Hz)</th>
</tr>
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<tbody>
<tr>
<td><strong>Evoked IPSCs</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Control (n = 20)</td>
<td>-947 ± 957</td>
<td>-4339 ± 3557</td>
<td>3.92 ± 2.29</td>
<td>1.56 ± 0.83</td>
<td>4.41 ± 2.93</td>
<td>--</td>
</tr>
<tr>
<td>Strychnine (n = 12)</td>
<td>-510 ± 611</td>
<td>-2807 ± 2513</td>
<td>5.45 ± 3.26</td>
<td>1.70 ± 0.85</td>
<td>7.03 ± 3.55</td>
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</tr>
<tr>
<td>SR95531 (n = 14)</td>
<td>-310 ± 216</td>
<td>-741 ± 353</td>
<td>2.35 ± 1.03</td>
<td>1.23 ± 0.77</td>
<td>2.34 ± 0.88</td>
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</tr>
<tr>
<td>SN + SR (n = 13)</td>
<td>-35.6 ± 48.0</td>
<td>-135 ± 108</td>
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</tr>
<tr>
<td>Recovery (n = 15)</td>
<td>-676 ± 672</td>
<td>-3227 ± 2584</td>
<td>3.83 ± 2.25</td>
<td>1.53 ± 0.58</td>
<td>5.35 ± 3.82</td>
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<tr>
<td><strong>Spontaneous IPSCs</strong></td>
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<tr>
<td>Control (n = 18)</td>
<td>-114 ± 48.2</td>
<td>-428 ± 192</td>
<td>1.93 ± 0.61</td>
<td>0.91 ± 0.22</td>
<td>4.35 ± 1.61</td>
<td>7.45 ± 6.51</td>
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<tr>
<td>Strychnine (n = 12)</td>
<td>-73.3 ± 35.6</td>
<td>-329 ± 147</td>
<td>2.32 ± 1.10</td>
<td>0.99 ± 0.31</td>
<td>6.48 ± 4.41</td>
<td>8.49 ± 4.58</td>
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<tr>
<td>SR95531 (n = 16)</td>
<td>-95.3 ± 53.6</td>
<td>-278 ± 169</td>
<td>1.39 ± 0.43</td>
<td>0.84 ± 0.37</td>
<td>2.91 ± 1.69</td>
<td>1.87 ± 1.38</td>
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<tr>
<td>Recovery (n = 14)</td>
<td>-103 ± 61.5</td>
<td>-398 ± 209</td>
<td>2.09 ± 1.00</td>
<td>1.06 ± 0.55</td>
<td>5.59 ± 4.10</td>
<td>7.76 ± 6.75</td>
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<tr>
<td><strong>Miniature IPSCs</strong></td>
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<tr>
<td>Control (n = 6)</td>
<td>-141 ± 48.7</td>
<td>-496 ± 112</td>
<td>2.26 ± 0.48</td>
<td>0.90 ± 0.33</td>
<td>4.71 ± 1.26</td>
<td>11.3 ± 7.02</td>
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<tr>
<td>Strychnine (n = 5)</td>
<td>-63.1 ± 17.2</td>
<td>-244 ± 60.4</td>
<td>2.11 ± 0.44</td>
<td>1.06 ± 0.32</td>
<td>7.46 ± 1.12</td>
<td>9.11 ± 6.61</td>
</tr>
<tr>
<td>SR95531 (n = 5)</td>
<td>-92.2 ± 41.2</td>
<td>-308 ± 113</td>
<td>1.81 ± 0.26</td>
<td>0.87 ± 0.24</td>
<td>4.34 ± 1.01</td>
<td>1.81 ± 0.98</td>
</tr>
<tr>
<td>Recovery (n = 5)</td>
<td>-122 ± 84.6</td>
<td>-436 ± 255</td>
<td>2.20 ± 0.39</td>
<td>0.97 ± 0.20</td>
<td>5.09 ± 1.08</td>
<td>10.8 ± 6.51</td>
</tr>
</tbody>
</table>

Values indicate mean ± SD. SN Strychnine, SR SR95531