Slowly emerging glycinergetic transmission enhances inhibition in the sound localization pathway of the avian auditory system

Glycinergic transmission in the avian brainstem

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Abstract

Localization of low frequency acoustic stimuli is processed in dedicated neural pathways where coincidence detecting neurons compare the arrival time of sound stimuli at the two ears, or interaural time disparity (ITD). ITDs occur in the submillisecond range and vertebrates have evolved specialized excitatory and inhibitory circuitry to compute these differences. Glycinergic inhibition is a computationally significant and prominent component of the mammalian ITD pathway. However, evidence for glycinergic transmission is limited in birds where GABAergic inhibition has been thought to be the dominant or exclusive inhibitory transmitter. Indeed, previous work showed that GABA antagonists completely eliminate inhibition in avian nuclei specialized for processing temporal features of sound, nucleus magnocellularis (NM) and nucleus laminaris (NL). However, more recent work shows that glycine is co-expressed with GABA in synaptic terminals apposed to neurons in both nuclei (Kuo et al. 2009; Coleman et al. 2011). Here we show complementary evidence of functional glycine receptor (GlyR) expression in NM and NL. Additionally, we show that glycinergic input can be evoked under particular stimulus conditions. Stimulation at high but physiologically relevant rates evokes a slowly emerging glycinergic response in NM and NL that builds over the course of the stimulus. Glycinergic response magnitude was stimulus rate dependent, representing 18% and 7% of the total inhibitory current in NM and NL, respectively, at the end of the 50-pulse, 200Hz stimulus. Finally, we show that the glycinergic component is functionally relevant as its elimination reduced inhibition of discharges evoked by current injection into NM neurons.
Introduction

Animals use differences in the arrival time of sound at each ear, or interaural time disparities (ITDs), to compute the location of low frequency sound sources (Rayleigh 1907). In vertebrates, ITDs are computed by binaural coincidence detecting neurons in the brainstem. Coincidence detecting neurons reside in nucleus laminaris (NL) in birds (Parks and Rubel 1975; Sullivan and Konishi 1986; Carr and Konishi 1990; Pena et al. 1996; Burger et al. 2011) and in the medial superior olive (MSO) in mammals (Goldberg and Brown 1969; Yin and Chan 1990). In order to perform sound localization computations, these neurons integrate bilateral excitatory and prominent inhibitory inputs while modulating their firing rate based on submillisecond differences in ITDs.

Inhibitory synaptic transmission is a key feature of sound localization circuitry, contributing to temporal precision over a broad range of sound intensities. In mammals, precisely timed feed-forward glycinergic input from the medial nucleus of the trapezoid body (MNTB) modulates the ITD selectivity of MSO neurons (Brand et al. 2002; Pecka et al. 2008). Until recently, investigation of avian systems has focused on slow, depolarizing, GABAergic feedback inhibition from the superior olivary nucleus (SON) to its targets in the brainstem (Hyson et al. 1995; Yang et al. 1999; Monsivais et al. 2000; Monsivais and Rubel 2001; Yamada et al. 2013), while no physiological observations of glycinergic transmission in these nuclei have been documented. Also, previous studies have shown that glycine immunoreactivity is sparse in NM and NL (Code and Rubel 1989), especially when compared to GABA immunoreactivity (Carr et al. 1989, Code and Churchill 1991). Further, whole-cell recordings in these nuclei have indicated that both spontaneous and evoked inhibitory synaptic currents were completely blocked by
application of GABA<sub>A</sub> receptor antagonists (Funabiki et al. 1998; Yang et al. 1999; Monsivais et al. 2000; Lu and Trussell 2000). Recent work has revealed glycinergic transmission in nucleus angularis (NA) and SON, components of this circuitry that are not considered to be specialized for temporal processing. Inhibitory input to NA (Kuo et al. 2009) and SON (Coleman et al. 2011) is marked by co-release of GABA and glycine at some synapses. These studies further showed that GABA and glycine co-localize in the terminals that synapse onto NA and SON neurons (Kuo et al. 2009; Coleman et al. 2011). Paradoxically this staining pattern is also present surrounding neurons in NM and NL where glycinergic transmission has never been detected electrophysiologically (Kuo et al. 2009). Release of multiple transmitters is known to occur in developing synapses (Gillespie et al. 2005; Awatramani et al. 2005) but has also been demonstrated in mature neurons in the avian (Kuo et al. 2009; Coleman et al. 2011) and mammalian (Lu et al. 2008) auditory brainstem. Recent work in the mammalian cochlear nucleus, where glycine is the dominant inhibitory transmitter, has demonstrated GABA release emerges when synapses are stimulated at high frequencies (Nerlich et al. 2013). Given these recent findings regarding the presence of multiple inhibitory transmitters in the auditory system, we searched for hallmarks of glycinergic transmission in the NM and NL; two nuclei involved in temporal processing of acoustic signals in the avian brainstem.

Here we report expression of GlyRs in all principal nuclei of the mature avian ITD computing circuit, including NM and NL. Physiological recordings in NM and NL show that the expressed GlyRs are functional and generate current in response to exogenous glycine application. Finally, we demonstrate evoked, frequency dependent synaptic release of glycine in response to long duration, high frequency stimulation in
NM and NL. Functional experiments suggest that this glycinergic component is sufficiently potent to influence the overall efficacy of inhibition in NM during high frequency stimulation.
Methods

All procedures were approved by the Lehigh University Animal Care and Use Committee. In this study, 57 white leghorn chickens *Gallus domesticus* (Moyers, Quakertown, PA) aged E17-P5 and of both sexes were used.

Immunohistochemistry

Immunohistochemical staining for GlyR followed protocols described in Coleman et al., (2011). Briefly, four P5 chickens were deeply anesthetized and transcardially perfused with PBS followed by 4% PFA in PBS, pH 7.4. Brains were removed and postfixed in 4% PFA overnight at 4°C. Brains were rinsed and blocked, then sectioned at 30μm (HM650V, Microm). Sections were transferred to a solution for antigen retrieval (10mM sodium citrate; 0.05% Tween 20; pH 6.0) and maintained at 80°C in a water bath for 30 min. Sections were cooled to 27°C, rinsed and then blocked in 10% normal goat serum for 1 hour. Sections were incubated overnight at 4°C in solution containing: 5% normal goat serum, anti-neurofilament (cat.#: AB1987; 1:200; Millipore) and anti-GlyR (clone mAb4a, cat.#: 146011; 1:1,000; Synaptic Systems). Sections were rinsed then incubated for 2h with secondary antibody conjugated to AlexaFluor 488 goat anti-mouse and AlexaFluor 633 goat anti-rabbit to label GlyR and Neurofilament, respectively (Invitrogen). Sections were mounted in Vectashield (Vector Laboratories) and confocal images were captured (LSM 510 Meta, Zeiss). Images were processed using Photoshop (Adobe Systems) to match pixel intensity distributions between color channels. No staining was observed when primary antibodies were absent (Fig. 2E) or when GlyR antibody was preabsorbed with antigen peptide (Coleman et al. 2011).
Western blot analysis

Chicken lung, chicken brainstem, and gerbil brainstem tissue were homogenized in lysis buffer (10mM Tris-HCl pH 7.4, 0.32M sucrose, 5mM EDTA pH 8) supplemented with 0.1mM PMSF and cOmplete-mini EDTA-free protease inhibitor cocktail (Roche), mixed with an equal volume of 4% SDS, and sonicated. Membrane fractions were collected by centrifugation at 13,000 x g (4°C) for 20min with three washes in supplemented lysis buffer. Membranes were resuspended in modified RIPA buffer (25mM Tris pH 7.6, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS) supplemented with 1mM PMSF for 10min at room temperature and measured using the DC Protein Assay (Bio-Rad). 20µg of protein was separated by 10% reducing SDS-PAGE and then electrotransferred onto a 0.2µm PVDF membrane for Western analysis. Antibodies used: mouse anti-GlyR (1:500; Synaptic Systems, clone mAb4a, cat#:146011); HRP-conjugated goat anti-mouse IgG (1:50,000; Promega). ECL-Plus (GE Healthcare) was used for chemiluminescent detection with Kodak BioMax film.

In vitro brain slice preparation:

For in vitro physiology, 53 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 (HM650V, Microm). Coronal sections (150-200 µm) containing the auditory brainstem
nuclei 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) 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 (Hamamatsu C7500-50)_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).

In vitro whole-cell recordings:

Borosilicate capillary glass pipettes (1B120F-4, WPI) were pulled to a resistance
of 4-8 MΩ using a two-stage puller (PC-10, Narishige) and back-filled with internal
solution (for voltage clamp containing in mM: 105 CsMeSO3, 35 KCl, 5 EGTA, 10
HEPES, 1 MgCl2, 4 ATP-Mg, and 0.3 GTP-Na, pH 7.2 adjusted with KOH). In current
clamp experiments, CsMeSO3 was exchanged for K-gluconate and CsCl for KCl. A
liquid junction potential of 10mV was corrected for in both internal solutions. 5mM
QX314 was added to the internal solution to prevent antidromic spiking. When clamping
the membrane at -70mV, these internal solutions yielded depolarizing inhibitory inputs
normally observed in the avian brainstem. In voltage clamp, series resistance was
compensated at 60-80% (Multiclamp 700B, Molecular Devices). The signal was digitized
with a Digidata 1440 data acquisition board and recorded using Clampex software (Molecular Devices).

Inhibitory inputs were pharmacologically isolated in ACSF containing 6,7-dinitroquinoxaline-2,3-dione (DNQX) (40μM) and D-2-amino-5-phosphonopentanoic acid (AP5) (50μM) to block AMPA and NMDA receptors. GlyRs were blocked using strychnine (0.5-1μM). GABA_A receptors (GABA_ARs) were blocked using SR95531 (20μM). GABA_BRs were blocked with CGP55845 (2μM) during high frequency stimulation protocols. Pharmacological agents were supplied from Tocris except where indicated.

GlyR activation via pressure application of glycine

Pipettes for pressure application of glycine were pulled to a resistance of ~1MΩ and placed within 50μm of the neuron soma. 100ms glycine (Sigma) (0.5-1mM in ACSF containing DNQX and AP5) puffs were applied using ~2.5psi pressure injection (PLI 100A, Warner Instruments).

Synaptic activity in NM and NL

Inhibitory postsynaptic potentials or currents were evoked with 50μsec stimulus pulses with a stimulus isolation unit (Isoflex, A.M.P.I. Inc.) through a concentric bipolar electrode (TM53CCINS, WPI) placed to the ventrolateral perimeter of NM or the dorsolateral perimeter of the NL. A schematized view of the recording setup is shown in Figure 1. Stimulus magnitude (range 10-90V) was gradually increased until amplitudes stabilized at their maximum. Stimulation protocols ranged from single events to trains of
50 pulses at frequencies from 20 to 200Hz. The average of 5-10 traces was used for comparison between each condition (depicted in Fig 4C,D). For 50-pulse trains, the glycineric component was analyzed on a pulse-by-pulse basis where the average amplitude at each pulse was compared between the control and GABA<sub>A</sub>R block conditions. The average residual component remaining during block of both GABA<sub>A</sub>Rs and GlyRs was subtracted from each condition before comparisons were made. Differences in the magnitude of the glycineric component were compared between frequencies by averaging the last five pulses (pulses 46-50) and using Student’s t-test (Fig. 5B &D). Decay time constants, τ<sub>decay</sub> values, were calculated from standard exponential fits from 10–90% of the peak of IPSCs. The τ<sub>decay</sub> 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<sub>decay</sub> value was calculated for double exponential fits using the equation: weighted τ<sub>decay</sub> = τ<sub>1</sub>[A1/(A1+A2)] + τ<sub>2</sub>[A2/(A1+A2)], as in Kuo et al. (2009).

Functional testing of glycineric component

The functional test for glycineric efficacy consisted of suprathreshold current injection trains (duration 0.5-0.6ms ranging from 850-1650pA) at 50 Hz for 50 pulses. The minimum current which evoked 90-100% spiking in the absence of inhibitory input fiber stimulation was used. During the current injection train, synaptic inhibitory input was evoked at 200 Hz for 40 pulses (200ms) using a bipolar tungsten electrode. Spike probability was calculated and compared between control and strychnine conditions.
during the 250ms period initiated at the first pulse of inhibitory stimulation.
Results

GlyR immunohistochemistry in the auditory brainstem

Recent work has confirmed Code and Rubel's (1989) finding of weakly immunopositive glycinergic synaptic terminals apposed to neurons in several avian auditory nuclei, including NM, NL and SON (Kuo et al. 2009; Coleman et al. 2011). These results are paradoxical considering that GABAergic input accounted for nearly all of the inhibitory current to NM and NL in previous studies using a variety of stimulus configurations (Funabiki et al. 1998; Yang et al. 1999; Monsivais et al. 2000; Lu and Trussell 2000) and glycinergic transmission has never been physiologically documented in NM and NL. Studies in other systems have shown that postsynaptic responses from multi-transmitter releasing terminals are determined solely by the complement of receptors expressed by the postsynaptic neuron (Dugue et al. 2005), suggesting that glycine may be released but not received postsynaptically in NM and NL. To resolve whether glycine is a component of synaptic signaling in temporal processing nuclei, we investigated expression of GlyR in NM and NL (Fig. 2). Anti-GlyR immunoreactivity was indeed robust in NM and NL (Fig. 2A-C) where subcellular staining appeared punctate and was absent from the nucleus. Anti-GlyR immunopositive neurons in NA and SON supports physiological and immunohistochemical evidence of glycinergic synaptic transmission in these nuclei (Kuo et al. 2009; Coleman et al. 2011) (Fig. 2D). Antibody specificity was confirmed using antigen preabsorption prior to tissue staining (Coleman et al. 2011) and Western blot analysis (Fig. 2F) where the glycine receptor α1 subunit was detected at the predicted molecular weight (~48kD) and at a similar
molecular weight observed in gerbil brainstem tissue (Fig. 2Fii), a known GlyR positive brain region in rodents (Friauf et al. 1997, Korada and Schwartz, 1999).

Exogenous glycine application evokes strychnine sensitive currents in NM and NL

Our immunohistochemical evidence suggests that GlyRs are expressed in NM and NL. Since glycinergic transmission has never been recorded physiologically in these nuclei, we tested whether glycinergic responses could be observed during exogenous glycine application. 100ms glycine puffs were applied to neurons in NM, NL and SON in the presence of glutamate and GABA receptor antagonists. Application of glycine evoked inward current that was almost entirely abolished during bath application of strychnine (0.5-1μM), a GlyR antagonist. Figure 3A shows the glycine response in control, strychnine, and washout conditions for an E19 NM neuron. Figure 2B depicts the population for NM responses (gray bars) in strychnine and recovery conditions normalized to the control response (strychnine: 0.077 ± 0.065, n=9; recovery: 0.76 ± 0.49, n=5; mean ± SD). Similar responses were also observed for all neurons tested in SON (strychnine: 0.039 ± 0.029, n=10; recovery: 0.67 ± 0.23, n=8; black bars) and NL (strychnine: 0.016 ± 0.019, n=8; recovery: 0.68 ± 0.34, n=6; light gray bars) (Fig. 3B). Responses were consistent across the age range (E18-P5). Kuo et al. (2009) demonstrated similar results for NA. Taken together with the immunohistochemical results, these data indicate that functional GlyRs exist in all four brainstem nuclei at ages considered mature for the chick auditory system (Rubel and Fritzsch 2002).

High frequency stimulation evokes glycine release in NM and NL
A number of studies have indicated that inhibitory transmission in the NM and NL is completely blocked by GABAAR antagonists (Funabiki et al. 1998; Yang et al. 1999; Monsivais et al. 2000; Lu and Trussell 2000). Indeed, we too observed no evidence of glycinergic activity in spontaneous events (Fig. 4A) or responses evoked by single-pulse stimuli (Fig. 4B) in voltage (n=4) or current clamp (n=3). However, few of these studies evoked inhibitory synaptic transmission at high stimulation rates approaching those driven in SON by high intensity acoustic signals. Previous work from our group suggests that SON neurons can reach spike rates exceeding 200Hz during intense acoustic stimulation (Coleman et al. 2011). Thus, we tested whether prolonged high frequency stimulation could evoke glycine release in the NM and NL. Our protocol consisted of 50-pulse stimulus trains at 200Hz during whole cell recordings in voltage or current clamp. Figure 4C shows averaged IPSC traces from a representative NM neuron stimulated at 200Hz in each pharmacological condition in voltage clamp. Control IPSCs were evoked in the presence of DNQX and AP5 to block glutamate receptors and CGP55845 to block GABA\textsubscript{B}Rs. These control IPSCs tended to sum over the first several pulses and then plateau or depress for the remainder of the stimulus train (black trace). The \( \tau_{\text{decay}} \) of the current in this condition was 103 ± 59 ms for the population (n=12). The GABA\textsubscript{A}R antagonist SR95531 reduced but did not eliminate the evoked IPSC (red trace). The remaining IPSC appeared to accumulate over the course of the stimulus (red arrow in Fig 4C) and reached approximately 18% of the control IPSC by pulse 50 (population data for 200Hz: 17.5 ± 2.1%, n=13, Fig 5A,B). This component had a \( \tau_{\text{decay}} \) of 168 ± 51 ms (n=12, \( p<0.001 \) vs. control, paired t-test) and was eliminated with the
addition of 0.5μM strychnine (*blue trace*) and recovered after washout (*green trace*) (Fig. 4C). IPSC amplitude recovered near control levels after SR95531 washout (*gray trace*).

Neurons in NL responded similarly to those in NM under the same conditions. Figure 4D shows the averaged traces of a representative NL neuron at 200Hz stimulus rate in each of the pharmacological conditions tested. Again, GABA<sub>A</sub>R block reduced, but did not eliminate the inhibitory currents. For the population of NL neurons the glycine component reached approximately 7% at 200Hz (6.9 ± 0.8%, n=11; measured at pulse 50, Fig 5C,D). The $\tau_{\text{decay}}$ of the control and SR95531 condition were 193 ± 84 ms and 168 ± 66 ms, respectively (n=12, $p<0.05$, paired t-test).

Evoked IPSPs followed a similar response pattern in current clamp in the NM. Again, application of SR95531 resulted in an incomplete suppression of the IPSP and the residual component was nearly abolished after the addition of strychnine. The glycine voltage was greatest at pulse 50 and represented 30% of the control potential at 200 Hz stimulus frequency (30.7 ± 7.9%, n=4, not depicted). These results suggest that glycine contributes to inhibition in both NM and NL under high but physiologically relevant firing rates.

**Magnitude of glycine component is frequency dependent**

Our previous results indicated that glycine transmission was a component of inhibition during high frequency stimulation in NM and NL. Next, we varied the stimulus rate to determine the frequency dependence of the glycine component. In voltage clamp, we tested the 50-pulse protocol at three additional frequencies; 20, 50 and 100Hz. These input frequencies are representative of physiologically relevant firing rates.
that range from spontaneous activity (20Hz) to maximum rates observed during intense auditory stimulation (200Hz in SON, Coleman et al. 2011). Figure 5A & C shows the glycinergic contribution at each pulse at the four frequencies tested for the population of cells. In the NM we found that the 20Hz frequency evoked a modest amount of glycinergic signaling in 9 of 11 cells. This glycinergic component represented 5.5 ± 4.2% of the IPSC amplitude when compared to control (mean ± SD of the last 5 pulses, n=11, Fig. 5B). In the NL, 6 of 11 cells showed a small glycinergic component at 20Hz (2.5 ± 3.9%, n=11, Fig. 5D). As the stimulation frequency increased, the glycinergic component also increased. In the NM there was a large increase between 20 and 50Hz and then smaller gains at 100 and 200Hz (50Hz, 14.2 ± 4.7%, n=8; 100Hz, 15.7 ± 4.5%, n=9; 200Hz, 17.5 ± 6.8%, n=13; Fig 5A,B). In the NL, the glycinergic component increased more incrementally and appeared to plateau in the 200Hz condition (50Hz, 4.5 ± 3.3%, n=11; 100Hz, 6.1 ± 2.9%, n=12; 200Hz, 6.9 ± 2.7%, n=11; Fig 5C,D). These results indicate that the recruitment of glycine is frequency dependent and that the threshold for glycinergic transmission is close to 20Hz for the majority of neurons tested.

GlyR block reduces the efficacy of inhibition in NM

To test the functional efficacy of glycinergic input to NM, we used a protocol similar to Monsivais et al., (2000) (see Methods) where 50 Hz suprathreshold current pulse trains were injected into NM neurons to evoke spiking while inhibitory fibers were simultaneously stimulated during a 200ms window (40 pulses at 200Hz) (Fig 6A,B). Representative traces are shown in Figure 6A for an NM neuron in each condition. Activation of inhibitory inputs in the control condition generally reduced spiking by at least 40% during the 250ms following the onset of evoked inhibition (see Methods).
Blocking the glycinergic component of the inhibition with strychnine led to a significant increase in spike probability during inhibitory input activation (control: 0.19 ± 0.12; strychnine 0.50 ± 0.22, mean ± SD; n=7, p<0.005, paired t-test; Fig. 6B). After strychnine washout, spike probability returned to near control values (0.27 ± 0.15, n=3). These results suggest that glycine release evoked under physiologically relevant stimulus conditions contributes to the overall efficacy of the inhibition, and modulates the excitability of NM neurons.
Discussion

Physiological evidence of glyciner gic transmission in the sound localization circuitry of birds has only recently been documented (Kuo et al. 2009; Coleman et al. 2011). These studies demonstrated the existence of glyciner gic signaling in neurons generally associated with the encoding of sound intensity information, the NA and SON (Takahashi et al. 1984). In the present report, we show for the first time robust expression of GlyRs and evidence of synaptically evoked glycine release in nuclei specialized for temporal processing in the avian auditory system, the NM and NL. We confirm the functionality of the GlyRs and demonstrate that glyciner gic transmission is frequency dependent. We also show that the glyciner gic transmission is functionally relevant, adding to the efficacy of inhibition to reduce spiking.

GlyR immunohistochemistry and receptor function in the sound localization pathway

Our immunohistochemical results show robust staining for the GlyR in all four of the main nuclei in the avian auditory brainstem. These data are complementary to previous work, which indicated that glycine could be labeled in presynaptic terminals around these nuclei (Code and Rubel 1989; Kuo et al. 2009; Coleman et al. 2011). These studies along with our present physiological evidence of functional GlyRs in NM and NL show that the anatomical pre- and postsynaptic hallmarks of glyciner gic transmission observed in these nuclei are indeed indicative of glyciner gic signaling in all principal nuclei of this circuitry.

Previous studies suggested that glyciner gic transmission accounts for approximately 50% of the amplitude of single-pulse evoked IPSCs in the NA and SON.
(Kuo et al. 2009; Coleman et al. 2011). In NM we confirmed previous results that glycine transmission is not evident from recordings of spontaneous release or release evoked by transient stimuli such as single-pulses (Fig. 4A,B). Rather, our results indicate that glycine transmission was only induced during high frequency stimulation. One possibility is that the glycine receptors may be in an extrasynaptic location that requires spillover for activation. The threshold stimulus for detection of the glycine release was typically between 20 and 50 Hz pulse stimulation. Additionally, the percentage of total inhibitory current attributable to glycine increased over the course of the response. Our protocols simulated high but physiologically relevant input rates, within the range of firing rates observed for SON neurons in response to acoustic stimuli in vivo (Coleman et al. 2011). Given that the spontaneous rates of neurons in this system are similar to the threshold rates for glycine recruitment, (>20Hz, Warchol and Dallos 1990; Manley et al. 1991; Lippe 1994; Nishino et al. 2008; Coleman et al. 2011) it is possible that a low, basal level of glycine is released even in silence in vivo.

Roles for glycine in sound location computation

Glycinergic transmission in NA and SON results from the co-release of GABA and glycine from inhibitory terminals. One function of glycine was described in vivo for SON neurons where glycine sharpens phase-locking to acoustic stimuli (Coleman et al. 2011). The role of glycine in NM and NL has not yet been investigated in vivo. Given the results of the current study, we propose that glycinergic transmission would be recruited during intense stimulation when GABA release may be subject to synaptic depression (Lu et al. 2005 and Fig. 4C). This hypothesis is plausible for several reasons. First, glycine and GABA are trafficked by the same vesicular transport protein, vesicular amino
acid transporter (VIAAT \textit{also known as VGAT}) (Wojcik et al. 2006). VIAAT has been localized at both GABA- and glycineric terminals (Chaudhry et al. 1998; Dumoulin et al. 1999). Since terminals surrounding NM and NL neurons are immunopositive for both GABA and glycine, co-release is a possibility as similar terminal profiles have been demonstrated to co-release these transmitters in NA and SON (Kuo et al. 2009; Coleman et al. 2011). The finding that glycine is only released during high frequency stimulation may suggest that glycine is only recruited into vesicles by VIAAT when GABA is depleted in the terminal. Previous studies have demonstrated that transmitter transport into vesicles is concentration dependent and that the relative abundance of GABA or glycine may suppress transport of the complementary transmitter into vesicles (Burger 1991). Additionally, it was recently shown that glycineric transmission can be suppressed, and GABA transmission potentiated via GLYT2 block or increase in GABA synthesis (respectively) in cartwheel cells that co-release GABA and glycine (Apostolides and Trussell 2013). Thus, depletion of GABA during intense and prolonged inhibitory stimulation in NM may lead to the recruitment of glycine into vesicles to maintain the inhibitory tone. Our functional test demonstrated that GlyR block does reduce the efficacy of inhibition in the NM. Evidence for a similar frequency dependent recruitment has recently been documented in the mammalian cochlear nucleus where GABAergic transmission is observed during high frequency stimulation of primarily glycineric inputs to bushy cells (Nerlich et al. 2013). These results indicate a similar scenario for inhibitory neurotransmission in mammals and birds where one mode of transmission dominates the activity in each system, but interestingly, the complementary mode plays a role in maintaining the circuit.
Kinetic features of inhibitory transmission

Co-release of inhibitory transmitters has been shown to affect the decay kinetics of IPSCs. For example, in the mammalian MNTB, co-release of GABA with glycine acts to speed up the kinetics of IPSCs (Lu and Trussell 2008). The kinetics of inhibition in this circuit are diverse and dependent on the specific nucleus. For IPSCs evoked using single stimuli, decay kinetics in the NM were observed to be approximately 3-fold slower than those recorded in NA, NL and SON (Kuo et al. 2009; Coleman et al. 2011). In the NA and SON, where glycine was a prominent component of these IPSCs, the isolated glycinergic component had faster kinetics. While kinetic analysis was not a focus of this study, we did analyze IPSC decay kinetics. For high frequency stimuli (50-pulses at 200Hz), we observed that the decay in NM was about 2-fold faster than for NL in the control condition (NM: 103 ± 59 ms, NL: 193 ± 84 ms, p<0.005, t-test). This is the opposite of what was observed in the single pulse stimulus responses where decay was faster in the NL. Additionally, we found that blocking GABA\(_A\)Rs with SR95531 had different effects on the kinetics in each nucleus. When compared to the control condition, decay kinetics in the NM were slower in SR95531, whereas in the NL they were faster. The kinetics of the glycinergic component remaining after SR95531 application were remarkably similar for both nuclei (NM: 168 ± 51 ms, NL: 168 ± 66 ms). There are a number of possible mechanisms that could contribute to these rather complex differences in kinetics including variations in extrasynaptic receptors, rates of spillover or cross-reactivity of receptors (reviewed in Muller et al. 2008). There was also a considerable amount of asynchronous release in both nuclei that prolonged the decay times (>100ms) as was well described previously in NM (Lu and Trussell 2000).
Source(s) of glycine

The source of glycinergic terminals in NM and NL is unknown, but one possibility is the SON. The somas of some SON neurons stain positively for glycine and GABA suggesting that high levels of both GABA and glycine are present in populations of these cells (Coleman et al. 2011). These neurons would seem to be the most parsimonious source of glycine, but it is puzzling that spontaneous glycine release occurs in NA and SON, but not NM and NL, as these nuclei share SON collateral inputs (Burger et al. 2005). However, it is possible that different subsets of SON fibers project to subsets of target nuclei or that collateral terminals have different release characteristics.

Whether the GABA/glycine positive SON neurons are the population projecting to NM and NL is unresolved and requires further investigation. Alternatively, several other studies have described a small population of neurons located between the NM and NL that are immunopositive for markers of GABA and glycine (Müller 1987; Carr et al. 1989; von Bartheld et al. 1989; Kuo et al. 2009). Yamada et al. (2013) recently showed that these neurons receive excitatory input from NM and provide a stimulus-locked inhibitory input to low frequency NL neurons. It is unknown if these neurons also have collateral postsynaptic targets in the NM, and thus could explain the NM data presented in this report.

Summary

This report shows that functional GlyRs are expressed in four principal nuclei of the avian auditory brainstem and that endogenous glycine is released in NM and NL by high frequency stimulation. Importantly, glycine transmission was only recruited during long repetitive stimulus trains albeit within the range of in vivo SON firing frequencies.
This glycinergic transmission contributes to the efficacy of the overall inhibition by suppressing spikes evoked by current injections into NM neurons. These findings indicate that glycinergic inhibition is more ubiquitous in the avian brainstem than previously understood, and that models of ITD processing circuitry in avians must incorporate glycinergic components of inhibition for intense or long duration stimuli.
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References


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Figure Legends

**Figure 1** Schematic of a chicken auditory brainstem slice and recording setup. *Left:* The avian auditory brainstem is composed of four main nuclei; two cochlear nuclei, the nucleus magnocellularis (NM) and nucleus angularis (NA); the site of binaural coincidence detection, the nucleus laminaris (NL); and the superior olivary nucleus (SON) which is the source of inhibitory feedback in the circuit. *Right:* Recording setup for whole cell recordings in the NM with the approximate positioning of the stimulating electrode. Stimulator placement was similar during NL recordings.

**Figure 2** Glycine receptors are expressed in four principal auditory brainstem nuclei. A. Low magnification confocal image of temporal processing nuclei (NM and NL). B. GlyR staining (*green*) is robust in NM soma co-labeled for neurofilament (*red*). C. NL neurons D. Low magnification image of NA confirms GlyR expression. E. Image of NM neurons shows no GlyR staining when the primary antibody was omitted. F. Western blot analysis using anti-GlyR antibody detects the glycine receptor α1-subunit (Pfeiffer et al. 1984) in chick brainstem tissue (*Fiii*) at a molecular weight (~48kD) comparable to that in gerbil brainstem (positive control, *Fii*). Immunoreactivity of anti-GlyR is absent in chicken lung tissue (negative control, *Fi*). Scale bars: A,D = 200μm; B,C,E = 50μm.

**Figure 3** Exogenous glycine application evokes strychnine sensitive currents in SON, NM, and NL. A. Representative traces from an E19 NM neuron shows current responses to a 100ms puff of glycine (0.5mM) in control, strychnine and recovery conditions. B.
Population data from the 100ms glycine puff reveals glycinergic currents in all nuclei that are suppressed by GlyR Block and recovered after washout. Numbers above (strych) and inside bars represent the \( n \) for each condition.

Figure 4  High frequency stimulation evokes glycinergic synaptic transmission in NM and NL. A. Traces from an NM neuron show spontaneous IPSCs that are completely abolished during SR95531 application suggesting purely GABAergic events (\( n=4 \), population data not shown). B. Overlay of averaged IPSPs evoked during single pulse stimulation (gray triangle) confirm purely GABAergic events in NM (\( n=3 \)). C. Averaged IPSC traces evoked using a 200Hz, 50-pulse stimulus in each pharmacological condition in an NM neuron. GABA\(_A\)R block reduces the summed IPSC amplitude leaving a gradually increasing component that is eliminated by strychnine application. D. Averaged IPSC traces from 200Hz stimulus train in the NL shows similar trend during pharmacological manipulations. Color code (inset in C) for pharmacological condition is the same for A-D.

Figure 5  Recruitment of glycinergic transmission is dependent on stimulation frequency. A. Population data for NM neurons at each frequency for mean amplitude of the glycinergic IPSC component analyzed pulse-by-pulse during the high frequency train stimuli. The glycinergic component was calculated by dividing the IPSC amplitude at pulse \( n \) in SR95531 by the IPSC amplitude at pulse \( n \) in control. B. Population data for the magnitude of the glycinergic component at each frequency tested. Value is the
average of the last 5 pulses of the stimulus ± SEM. C. Pulse-by-pulse analysis of glycinergic component in NL at each frequency. D. Population data for the average magnitude of the last 5 pulses at each frequency in NL. a = significantly different from 20Hz (p<0.001), b = significantly different from 50Hz (p<0.05).

Figure 6  Blocking GlyRs decreases the ability of evoked IPSPs to suppress action potentials in NM. A. Representative current clamp traces from an E19 NM neuron in each pharmacological condition. Application of strychnine resulted in an increase in the firing rate during the 250ms time window beginning at inhibition onset (asterisks in strychnine condition represent action potentials suppressed in the control). B. Population data shows a significant increase in spike probability during strychnine application compared to control (n=7, p<0.005). Grey lines connect the spike probability at each condition for each neuron tested.
inhibitory stimulation

whole-cell recording