Chemical synaptic transmission onto superficial stellate cells of the mouse dorsal cochlear nucleus

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Abstract

The dorsal cochlear nucleus (DCN) is a cerebellum-like auditory brainstem region whose functions include sound localization and multisensory integration. While previous in vivo studies show that glycinergic and GABAergic inhibition regulate the response properties of several DCN cell types in response to sensory stimuli, data regarding the synaptic inputs onto DCN inhibitory interneurons remain limited. Using acute DCN slices from mice, we examined the properties of excitatory and inhibitory synapses onto the superficial stellate cell, a poorly understood cell type that provides inhibition to DCN output neurons (fusiform cells) as well as to local inhibitory interneurons (cartwheel cells). Excitatory synapses onto stellate cells activated both NMDA receptors and fast-gating, Ca\textsuperscript{2+}-permeable AMPA receptors. Inhibition onto superficial stellate cells was mediated by glycine and GABA\textsubscript{A} receptors with different temporal kinetics. Paired recordings revealed that superficial stellate cells make reciprocal synapses and autapses, with a connection probability of ~18-20%. Unexpectedly, superficial stellate cells co-released both glycine and GABA, suggesting that co-transmission may play a role in fine-tuning the duration of inhibitory transmission.

Introduction

The dorsal cochlear nucleus (DCN) is a mammalian auditory brainstem region composed of diverse neuronal cell types (Oertel and Young, 2004). The contribution of the DCN to auditory processing is not clear, but previous studies suggest that it may be involved in sound localization in the vertical plane and orientation of the head towards sounds of interest (May, 2000; Kanold and Young, 2001; Sutherland et al., 1998). Interestingly, the local circuitry of the DCN closely resembles that of the cerebellum and
cerebellum-like nuclei found in mammals and fish (Oertel and Young, 2004) and this homology suggests that the DCN may also function to generate "negative images" of predictable auditory input, thereby filtering out self-generated sounds (Bell et al., 2008; Requarth and Sawtell, 2011).

Regardless of the precise role of the DCN in auditory processing, previous studies have established that the glutamatergic afferent neurons (fusiform and giant cells) integrate auditory information from the ear with sensory input from non-auditory brain regions (Oertel and Young, 2004). Sound information is relayed via the auditory nerve and perhaps from T-stellate cells of the ventral cochlear nucleus (Oertel, et al., 2011) that synapse onto the basal dendrites of fusiform cells. Non-auditory information enters via an array of cerebellum-like, granule cell parallel fibers that synapse onto fusiform cell apical dendrites. DCN parallel fibers also contact two types of molecular layer interneuron: glycinergic, Purkinje-like cartwheel cells, and GABAergic superficial stellate cells (henceforth “stellate cells”) that closely resemble the stellate/basket cells of the cerebellum (Wouterlood et al., 1984; Mugnaini, 1985; Bell et al., 2008).

Previous studies show that parallel fibers recruit feedforward glycinergic inhibition onto fusiform cells, presumably mediated by cartwheel cells (Davis et al., 1996; Davis and Young, 1997; Kanold and Young, 2001; Roberts and Trussell, 2010; Kuo and Trussell, 2011; Doiron et al., 2011). Given that even a single cartwheel cell can control the firing rate of fusiform cells (Roberts and Trussell, 2010), these findings imply that feedforward inhibition from interneurons effectively determines whether the DCN relays signals to downstream targets. Interestingly, in vivo work suggests that GABAergic inhibition also controls the response properties of cartwheel and fusiform cells (Davis and Young, 2000), and a recent in vitro study suggested that GABAergic inhibition controls the spread of parallel fiber excitation in the DCN (Middleton et al., 2011). Since cartwheel cell transmission is predominantly glycinergic (Golding and Oertel, 1997;
results suggest that stellate cells are a likely source of GABAergic inhibition. Little is known about chemical synaptic transmission onto DCN stellate cells. This lack of information is surprising, as stellate cells are common components of the outer layers of the DCN (Wouterlood et al., 1984) and may share homology with the GABAergic stellate and basket cells known to be crucial for controlling the output of the cerebellar cortex (Callaway et al., 1995; Barmack and Yakhnitsa, 2008; Oldfield et al., 2010; Coddington et al., 2013). Therefore, a comparison of cerebellar and DCN stellate cell properties might provide clues to DCN function. Nevertheless, there are no confirmed in vivo recordings from DCN stellate cells, and in vitro recordings are few (Zhang and Oertel, 1993; Golding and Oertel, 1997; Apostolides and Trussell, 2013b). We performed whole-cell recordings from visually identified DCN stellate cells in acute mouse brain slices and examined the properties of excitatory and inhibitory synapses. We find that similar to cerebellar stellate/basket cells, parallel fibers activate fast, Ca\(^{2+}\)-permeable AMPA receptors on DCN stellates. Unlike cerebellar stellate cells (Clark and Cull-Candy, 2002), however, single spikes in parallel fibers led to activation of both AMPA and NMDA receptors in DCN stellate cells. We also find that DCN stellate cells inhibit one another via reciprocal synapses as well as autapses. While it has been previously suggested that DCN stellate cells were GABAergic, we found that they express the glycine transporter GlyT2 required for functional glycinergic transmission (Gomez et al., 2003; Rousseau et al., 2008), and most stellate cells tested co-released both glycine and GABA. Our findings shed light on the synaptic properties to DCN stellate cells, highlighting significant functional similarities and differences between the DCN and the cerebellum.
Methods

Slice Preparation

All protocols involving animals were approved by OHSU's Institutional Animal Care and Use Committee. Mice P15-P24 days of age of wild-type (C57/Bl6 or CBA), GlyT2-GFP, or GAD65-GFP background were used for the majority of these experiments. For optogenetic experiments in Figure 7, we used two mutant mouse lines expressing channelrhodopsin2 in DCN fusiform cells (Thy1-ChR2-YFP line 18 [Jax stock #007612] and VGluT2-ChR2-YFP [Jax stock #017978]; Apostolides and Trussell 2013b). Mice were anesthetized with isofluorane, decapitated, and slices (200-250 µm thick) containing the DCN were cut in an ice-cold sucrose solution containing (in mM): 87 NaCl, 25 NaHCO3, 25 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgCl2, and bubbled with 5% CO2/95% O2. After cutting, slices were allowed to recover for 30-45 min at 34°C in an artificial cerebrospinal fluid (ACSF) solution containing (in mM): 130 NaCl, 2.1 KCl, 1.7 CaCl2, 1 MgSO4, 1.2 KH2PO4, 20 NaHCO3, 3 Na-HEPES, 10-12 glucose, bubbled with 5% CO2/95% O2 (300-310 mOsm). This solution was used as the standard perfusate for all experiments. For experiments in 6 week old animals, mice were anesthetized with an i.p. injection of 2% Avertin, and the brain sliced in an ice-cold solution composed of (in mM) 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 10 MgSO4, 0.5 ascorbic acid, 2 thiourea, 3 Na-pyruvate, 25 Glucose, 0.5 CaCl2, 0.005 MK801, 12 n-acetyl-l-cysteine, 300-310 mOsm, pH adjusted to 7.3-7.4 with HCl. Slices subsequently recovered at 34°C in NMDG solution for 10-15 min followed by a 40-60 min recovery period at room temperature in standard ACSF. In some experiments 5 µM R-CPP or 50 µM D-APV were added to the cutting solution and/or recovery chamber. After recovery, slices were maintained at ~22°C until recording. Experiments were typically performed within 5 hours of slice preparation.
Electrophysiology

Slices were mounted in a recording chamber and continuously perfused at 3-5 ml/min with ACSF heated to 31-33° C (total chamber volume: 1-2 ml). Neurons were visualized using Dodt contrast optics with a 40x or 63x objective on a Zeiss Axioscop2 microscope. For paired recordings, the pipette internal solution contained 60 K-gluconate, 10 K-glutamate, 70 KCl, 4.8 MgCl₂, 4 ATP, 0.5 GTP, 10 Tris-phosphocreatine, 0.1-0.2 EGTA, 10 HEPES, pH adjusted between 7.2-7.3 with KOH (~290 mOsm). This high intracellular Cl⁻ solution increased the driving force for Cl⁻ and thus generated larger absolute GABAₐ and glycine currents. For recording miniature events and broadening action potentials in Figure 7, K-gluconate, K-glutamate and KCl were replaced by 120 mM CsCl. For evoked and spontaneous EPSC recordings, the internal contained (in mM) 118 CsMeSO₃, 5 TEA-Cl, 5 QX314-Cl, 5 Cs₄-BAPTA, 10 HEPES, 4 Mg-ATP, 0.5 GTP, 10 Tris-Phosphocreatine. In some experiments, 30 mM CsF was substituted for equimolar CsMeSO₃ to facilitate gigaseal formation.

Whole-cell recording pipettes (final resistance: 2.5-5 MOhm) were made from borosilicate glass (#8250, World Precision Instruments) using a Narishige P97 puller. Parallel fibers were stimulated by delivering voltage pulses (50-200 µs) through a theta glass pipette filled with ACSF positioning within 20-40 µm of the cell. Series resistance in voltage-clamp (<30 MOhm) was monitored frequently and compensated 60-80% 'correction', 90% 'prediction' (bandwidth=3 kHz) and data were not analyzed if series resistance varied >20-25% over the course of the recording. Optogenetic experiments were performed using light from a custom-made, TTL-gated blue LED passed through the epifluorescence port of the microscope.

Identification of stellate cells

Most experiments were performed using wild-type mice. Stellate cells were
identified by their location in the slice (at the outer edge of the molecular layer and below
the ependymal surface) and basic membrane properties. The main types of neurons
found in the DCN superficial molecular layer are stellate and cartwheel cells (Wouterlood
et al., 1984; Osen and Mugnaini, 1981). Stellate cells were easily distinguished from
cartwheel cells by the size of their soma and input resistance which typically differed by
an order of magnitude (Apostolides and Trussell, 2013b). In experiments with transgenic
mice, GFP fluorescence was observed using illumination from a 100W Hg bulb placed in
the epi-fluorescence port of the microscope and passed through a GFP filter set.

Data Acquisition and Analysis

Traces were recorded with a Multiclamp 700B amplifier and a Digidata 1322A
analog-digital converter board using pClamp 9 software. Signals were low-pass filtered
at 10-20 kHz and digitized at 20-50 kHz. Data were analyzed offline after filtering the
traces at 2-10 kHz. For voltage-clamp data, all measurements were made on the peak
amplitude of averaged traces unless otherwise stated. Spontaneous and miniature
events were detected using a template algorithm or amplitude threshold in Axograph X
or Clampfit, and each event was visually inspected for false positives or malformed
events. Events were not included for averaging if they overlapped significantly with the
decay or rising phase of other events. Decay kinetics were measured from averages of
aligned events fitted with a single, double, or triple exponential decay function. Weighted
decay time constants were calculated from double or triple exponential fits as

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\frac{(A_{\text{slow}} \cdot \tau_{\text{slow}} + A_{\text{medium}} \cdot \tau_{\text{medium}} + A_{\text{fast}} \cdot \tau_{\text{fast}})}{(A_{\text{slow}} + A_{\text{medium}} + A_{\text{fast}})}. \]

All values are reported as mean ± SEM. Statistical significance was determined using paired or unpaired Student's
t-tests where appropriate. For AMPA/NMDA ratios, the AMPA component was
calculated by measuring the amplitude of EPSCs recorded at +33 mV at a time point
corresponding to the peak amplitude of events at -67 mV. The NMDA component was
calculated by measuring the amplitude of EPSCs at +33 mV 5-7 ms after the time point used to calculate the AMPA component.

Histology
Mice were transcardially perfused with warm (38°C) 100 mM phosphate buffered saline (PBS) solution, pH 7.4, followed by ice-cold 4% paraformaldehyde in PBS. The brains were dissected from the skull and incubated overnight in 4% paraformaldehyde for complete tissue fixation. Next, the brains were rinsed in PBS and brainstem coronal sections were acquired at 30 μm on a vibratome (VT1000S, Leica). After sectioning, the tissue was washed in PBS solution for 30 minutes. Sections were then incubated for 1 hour in block solution (2% goat serum, 0.2% Triton X-100 (in PBS), 0.3% BSA) and subsequently incubated overnight at 4°C with anti-GFP, rabbit polyclonal antibody, Alexa Fluor 488 conjugate (1:200 in block solution, Invitrogen Molecular Probes). Next day, sections were washed in PBS for 1 hour and mounted on 0.3% gelatin coated slides. After drying, sections were dehydrated in ascending alcohols, and delipidized in xylenes. The tissue was then rehydrated and coverslipped using Fluoromount G medium (Southern Biotechnology Associates). Fluorescence images were acquired using a confocal microscope (Olympus FV1000) by sequential scanning of GFP signals using an oil-immersion objective (60×, numerical aperture 1.42) with Olympus Fluoview-1000 software. Image analysis was conducted using NIH ImageJ software.

Reagents
NBQX, APV, CPP, SR95531 were purchased from Ascent Scientific/Abcam. Strychnine was purchased from Sigma-Aldrich.
Results

Single parallel fibers activate AMPA and NMDA receptors

Previous studies in cerebellar stellate/basket cells showed that excitatory post-synaptic currents (EPSCs) evoked by parallel fiber stimulation lack a prominent NMDA receptor component (Glitsch and Marty, 1999). Consequently, NMDA receptors on cerebellar stellate cells are thought to be extra-synaptic and primarily activated by glutamate spillover during sustained activity of multiple parallel fibers (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Nahir & Jahr, 2013). We tested whether NMDA receptors were similarly excluded from postsynaptic sites in DCN stellate cells by evoking EPSCs while voltage-clamping the cell at negative or positive potentials. All recordings were performed in 10 µM SR95531 and 0.5-2 µM strychnine to block GABA\textsubscript{A} and glycine receptors, respectively. Single parallel fiber shocks from a bipolar stimulating electrode (0.5-6 V), delivered while the cell was held at -67 to -77 mV, evoked fast EPSCs typical of AMPA receptor-mediated transmission (Fig. 1A, lower trace). By contrast, EPSCs at +33 mV displayed a prominent slow component (Fig. 1A, upper trace) that was blocked by the selective NMDA receptor antagonist R-CPP (5 µM, n=6 cells; Fig. 1B). NBQX (10 µM) blocked the fast EPSC component (n=5 cells; Fig. 1C), confirming that it was mediated by AMPA receptors.

However, even relatively weak shocks employed in these experiments likely activated more than one presynaptic parallel fiber (Roberts and Trussell, 2010). It could be argued that pooling of glutamate from even a few closely spaced synapses, or the repetitive activation of single parallel fibers (Isope et al., 2004; Nahir and Jahr, 2013), might suffice to activate high-affinity, extrasynaptic NMDA receptors. We therefore tested whether NMDA receptors were activated by single parallel fibers by analyzing spontaneous EPSCs (sEPSCs) occurring due to random firing of presynaptic granule cells. At +33 mV, sEPSCs displayed a prominent slow component similar to evoked
EPSCs (Fig. 1D). Given that the decay phases of the average sEPSCs were best fit with
the sum of two or three exponentials (4/8 cells fit with 2 exponents), we quantified the
decay kinetics of average sEPSCs by calculating a weighted decay time constant before
and after bath application of a NMDA receptor antagonist. Under baseline conditions, the
average weighted decay time constant of sEPSCs was 36.2±6.3 ms (n=8 cells). This
value was not significantly different from the weighted decay time constant of evoked
EPSCs recorded in absence of NMDA receptor blockers (39.5±9.2 ms; p=0.76, unpaired
t-test). 5 µM R-CPP or 50 µM D-APV selectively blocked the slow decay component,
thereby reducing the weighted decay time constant to 1.8±0.2 ms (Fig. 1E,F; n=8,
p<0.001, paired t-test). However, NMDA receptor antagonists had minimal effect on the
peak amplitude of spontaneous events (Fig. 1F. baseline: 26.3±2.1 pA, NMDA blockers:
21.5 ±3.4 pA, n=8, p=0.20). Furthermore, the ability to resolve the sub-millisecond rise
kinetics of AMPA sEPSCs (10-90% rise time: 0.48 ± 0.05 ms, n=8 cells) suggested that
the slow decay of the NMDA component is unlikely to be affected by voltage-clamp error
in these experiments. We also tested whether the synaptic localization of NMDA
receptors in DCN stellate cells was developmentally stable by comparing the
AMPA/NMDA ratio of EPSCs in 2-3 week versus 6-week-old mice. The AMPA/NMDA
ratio was similar between the two age groups (AMPA/NMDA ratio in 2-3 week old:
1.6±0.3; 6-week-old mice: 2.3 ±0.4, p=0.2, unpaired t-test). Altogether these results
show that in contrast to cerebellar stellate/basket cells, glutamate released from single
parallel fibers can readily access both AMPA and NMDA receptors on DCN stellate cells.

DCN stellate cells contain fast, Ca^{2+}-permeable AMPA receptors.

Cerebellar stellate cells express Ca^{2+}-permeable, GluA2-lacking AMPA receptors
with sub-millisecond decay kinetics (Liu and Cull-Candy, 2000; Carter and Regehr,
2002; Jackson and Nicoll, 2011). Interestingly, previous studies in the cochlear nuclei
found that the biophysical properties of AMPA receptors correlate with the identity of the presynaptic terminal: AMPA receptors at auditory nerve synapses have rapid kinetics and are typically Ca\(^{2+}\)-permeable, whereas AMPA receptors at parallel fiber synapses gate slowly and are impermeable to Ca\(^{2+}\) (Gardner et al., 1999; 2001). Thus, we asked whether DCN stellate cells contained Ca\(^{2+}\)-permeable AMPA receptors with rapid kinetics similar to cerebellar stellate cells, or if their AMPA receptor properties followed the pattern proposed by Gardner et al. (2001).

We first quantified the kinetics of synaptic AMPA receptors by recording miniature EPSCs (mEPSCs) in DCN stellate cells. D-APV, SR95531, and strychnine were added to the bath solution to isolate AMPA mEPSCs, and 1 µM tetrodotoxin (TTX) was added to block spikes. Quantal events typically ranged in amplitude between 20 and 200 pA, with an average amplitude of 45.2±6.5 pA, a 10-90% rise time of 0.17±0.01 ms, and a weighted decay time constant of 0.71±0.07 ms (n=12 cells). This decay time is somewhat faster than that for evoked AMPA EPSCs described above, presumably because of dispersion of the EPSC by the release time course. Data from an example cell are shown in Figure 2A, with the corresponding amplitude histogram of its individual events shown in Figure 2B. mEPSCs in this cell could exceed 200 pA, which, given the high input resistance of stellate cells (Apostolides & Trussell, 2013b) indicate that even small synapses are relatively potent. These amplitude and kinetic values are similar to those reported in cerebellar stellate/basket cells (Carter and Regehr, 2002; Crowley et al., 2007), and suggest that AMPA receptors on DCN stellate cells might also lack the GluA2 subunit and be Ca\(^{2+}\)-permeable (Liu and Cull-Candy, 2000; Jackson and Nicoll, 2011).

We further tested for expression of Ca\(^{2+}\)-permeable AMPA receptors by evoking EPSCs with a stimulating electrode while voltage clamping stellate cells at different holding potentials. Ca\(^{2+}\)-permeable AMPA receptors exhibit an inward rectification due to
intracellular polyamine block at positive potentials, while GluA2-containing AMPA receptors are Ca\(^{2+}\)-impermeable and have a linear current-voltage (IV) relationship (Isaac et al., 2007). AMPA EPSCs were isolated with D-APV/SR95531/strychnine and exogenous polyamines (spermine, 100 µM) were added to the pipette internal solution. Parallel fibers were stimulated 3 times at 100 Hz (delivered once every 10-15 s) while holding the postsynaptic cell between -50 and +50 mV. AMPA EPSCs exhibited paired-pulse facilitation, typical of parallel fiber synapses in the DCN (Manis, 1989; Tzounopoulos et al., 2004; Kuo and Trussell, 2011) (Fig. 2C): The amplitude of the third EPSC at -50 mV was on average 6.28±0.56 times larger than the first EPSC (n=12 cells). As expected from Ca\(^{2+}\)-permeable AMPA receptors, the IV curve of EPSCs showed significant inward rectification (Fig. 2D, E). We calculated a "rectification index" (RI) for each cell, defined as the actual EPSC amplitude recorded at +50 mV divided by the value extrapolated from a linear fit of EPSCs recorded at negative potentials. 10 of 12 DCN stellate cells tested exhibited significant inward rectification (RI=0.58±0.05, p<0.0001, one sample t-test; Fig 2E,F), and rectification was absent when EPSCs were recorded without exogenous polyamines added to the pipette solution (RI=1.06±0.06, n=6, p=0.5; (Kamboj et al., 1995); Fig 2F). Thus, inward rectification was due neither to poor voltage-clamp at positive potentials, nor to the activation of glutamatergic synapses on electrically-coupled neurons (Apostolides and Trussell, 2013b). Altogether, these data suggest that approximately half of the AMPA EPSCs in stellate cells are generated by Ca\(^{2+}\)-permeable receptors. The degree of inward rectification we observed was comparable to that reported in rat cerebellar stellate cells (Liu and Cull-Candy, 2000; Soto et al., 2007). Given the low release probability of parallel fiber synapses, we measured the final EPSC in a train of 3 parallel fiber stimuli delivered at 100 Hz. This analysis may therefore underestimate the degree of inward rectification if the receptors experienced use-dependent unblock by spermine (Rozov and Burnashev, 1999).
However, paired-pulse facilitation was not significantly different between EPSCs recorded at negative and positive potentials (EPSC3/EPSC1 at -50 mV was 6.27±0.47 versus 6.79±0.93 at +50 mV; p=0.47, paired t-test), suggesting that any use-dependent unblock of GluA2-lacking receptors occurring during these short trains was negligible.

Stellate cells are GABA/glycine co-releasing interneurons.

DCN stellate cells receive inhibitory inputs both from cartwheel cells and other stellate cells (Manis et al., 1994; Wouterlood et al., 1984). While cartwheel cells are dual GABA-glycinergic neurons (Roberts et al., 2008; Apostolides and Trussell, 2013a), previous immunohistochemical and anatomical studies suggested that stellate cells are GABAergic interneurons (Wouterlood et al., 1984; Mugnaini, 1985). Indeed, in order to train ourselves to recognize these tiny neurons for patch clamping in thick brain slices, we initially utilized a mouse line in which GFP expression was driven by the promoter for GAD65 (Hughes et al., 2005), a synthetic enzyme for GABA. In the DCN molecular layer in this mouse line, small cells could be observed lying close to the ependymal layer, with numerous fine processes extending towards the cell body layer (Fig 3A), consistent with superficial stellate cells. Labeled small cells are also very common in the molecular layer of the cerebellum of this mouse (not shown). However, we were surprised that small cells can also be readily observed in a mouse line (Zeilhofer et al., 2005) in which GFP expression is driven by the promoter for GlyT2, a marker of glycinergic neurons (Fig 3B). These observations suggested that inhibition by stellate cells may be mediated by both GABA and glycine.

GABAergic and glycinergic transmission onto stellate cells

We therefore quantified the amplitude and kinetics of GABAergic and glycinergic miniature inhibitory postsynaptic currents (mIPSCs). GABA mIPSCs were isolated with
10 µM NBQX, 50 µM D-APV, 500 nM strychnine and 1 µM TTX. The decay phase of averaged GABA<sub>A</sub> mIPSCs were fit by the sum of two exponentials (Fig. 4A). The fast component decayed with a time constant 2.2±0.1 ms and represented 82±2% of the total amplitude, whereas the slow component decayed with a time constant of 9.8±1.3 ms. The weighted decay time constant for GABA<sub>A</sub> mIPSCs was 3.4±0.2 ms (n=12 cells). GABA<sub>A</sub> mIPSCs had an average amplitude of 44.4±7.1 pA and a 10-90% rise time of 0.24±0.01 ms (n=9).

Glycine mIPSCs (isolated with NBQX, D-APV and SR95531) were prevalent in all cells tested and had an average amplitude of 42.9±5.5 pA and a 10-90% rise time of 0.17±0.01 ms (n=14). The decay of glycine mIPSCs was fit with a double exponential function in 13/14 cells, whereas one cell was best fit by a single exponential. Similar to GABA<sub>A</sub> mIPSCs, glycinergic events were dominated (72±6% of total amplitude) by a fast decay time constant of 1.1±0.1 ms and a minor slow component of 3.6±0.2 ms (n=14). The average weighted decay time constant for glycine mIPSCs was 1.7±0.2 ms (Fig. 4C). Glycine mIPSCs rose significantly faster than GABA<sub>A</sub> events (p<0.001, unpaired t-test). Furthermore, both fast and slow decay components were significantly faster for glycinergic compared to GABA<sub>A</sub> mIPSCs (p<0.01, unpaired t-test), although the relative weighting of fast vs. slow did not differ significantly (p=0.19). The peak amplitude of the glycine and GABA<sub>A</sub> mIPSCs did not differ significantly (p=0.82). Figures 4B and 4D show amplitude histograms for GABA and glycine mIPSCs recorded in two different cells, illustrating that for both classes of event, amplitudes could exceed 300-400 pA. These results show that stellate cells receive similarly potent glycinergic and GABAergic inhibition, albeit with different temporal kinetics. These results do not however indicate whether these different events arose from cartwheel cell or stellate cell synaptic terminals.
Previous anatomical studies suggested the existence of reciprocal synaptic connections between DCN stellate cells (Mugnaini, 1985), in agreement with the connectivity profile of stellate/basket cells of the cerebellum (Palay and Chan-Palay, 1974). We searched for reciprocal connections by making paired recordings from neighboring (<80 µm intersomatic distance) DCN stellate cells. Similar to reports on cerebellar stellate cells (Kondo and Marty, 1998), we found that 11/60 (18.3%) of attempted stellate connections (each pair = 2 connections) were synaptically coupled. An example pair is shown in Figure 5A. The presynaptic cell was held in voltage clamp and action potentials were elicited at 10 Hz by voltage steps from -70 to +5 mV for 100 µs (top trace). Five consecutive sweeps from the postsynaptic cell are shown in black, and an average of these sweeps is shown in red. Interestingly, a similar proportion of cells also displayed a prominent autaptic connection (19/89 cells tested, 21.3%), observed as an evoked IPSC that appears after triggering an escaping action potential in the recorded neurons, thus highlighting another parallel between the DCN and cerebellar stellate cells (Pouzat and Marty, 1998). An example autaptic recording is shown in Figure 5B. The top trace is an average of twenty trials showing the Na⁺ action current evoked by a voltage step. The middle traces are an overlay of 20 consecutive sweeps (4 failures) evoked at 0.5 Hz in black, with an average of successes in red. The transmission probability in this recording was 83.9% (131/156 successes). Overall, transmission fidelity was highly variable between connections, with success probability ranging from 16.8% to 100% when IPSCs were evoked at 0.5-1 Hz. The mean rate of successes, ie, when an IPSC followed a presynaptic spike, was 56±9%, n=6 autapses and 4 paired recordings. The GABAₐ receptor antagonist SR95531 blocked the majority of transmission in 7 unitary connections (5 autapses and 2 paired recordings). The lower traces in Figure 5B are 20 consecutive sweeps (black) and average (red) after the
addition of 10 µM SR95531, showing that this current was predominantly due to GABA release. Figure 5C plots the amplitude of autaptic events from the recording in Figure 5B as a function of time as the GABA<sub>A</sub> receptor blocker was added.

In some recordings, we noticed a small residual IPSC in the presence of SR95531, which together with the GlyT2 expression described above, suggests that DCN stellate cells co-release glycine. Due to the high failure rate of these connections, however, it was difficult to determine whether these IPSCs originated specifically from the stellate cell of interest, or if these were spontaneous glycinegic IPSCs coincidently originating from another presynaptic neuron. We therefore repeated the pharmacological experiments in stellate cells loaded with a CsCl-rich internal solution. By blocking K<sup>+</sup> currents that would normally repolarize presynaptic action potentials, intracellular Cs<sup>+</sup> should enhance exocytosis, thereby increasing the absolute IPSC size and reducing the variability of unitary IPSCs (Vincent and Marty, 1996). Under these conditions, autaptic IPSCs showed almost no failures (Fig. 6A). Bath application of 10 µM SR95531 reduced the peak IPSC to 29±7% of baseline (n=13 cells). In 11/13 experiments, a significant SR95531-insensitive component remained and this component was subsequently blocked by 0.5-1 µM strychnine (Fig. 6B). Thus, glycine and GABA are functionally co-released from DCN stellate cells. Although transmission was mostly mediated by GABA and GABA<sub>A</sub> receptors in the majority of experiments, there was a significant variability in percentage of transmission mediated by glycine in the grouped data (Fig. 6B). This variability could reflect differential postsynaptic expression of glycine versus GABA<sub>A</sub> receptors, or different vesicular ratios of glycine and GABA. Furthermore, although we performed these pharmacology experiments soon after establishing a whole-cell recording, differential washout of intracellular GABA versus glycine (Apostolides and Trussell, 2013a) may also contribute to the observed variability.
Activating fusiform cells generates GABA and glycinergic IPSCs in stellate cells.

We sought another method for determining the transmitter phenotype of stellate cells that would avoid the dialysis of presynaptic neurons which may alter the effectiveness of inhibitory transmission (Apostolides and Trussell, 2013a). The approach is based upon the recent demonstration that stellate cells are electrically coupled to fusiform principal cells, and that optogenetic activation of fusiform cells leads to action potentials in stellate cells (Apostolides and Trussell 2013b). This provides a means for selectively activating presynaptic stellate cells without doing presynaptic recordings. We therefore recorded from stellate cells in transgenic mice expressing channelrhodopsin2 in glutamatergic fusiform cells (Apostolides and Trussell 2013b; Thy1-ChR2 line 18: Arenkiel et al., 2007 and VGlut2-ChR2: Hägglund et al., 2010). Stellate cells were recorded with a cesium-based internal solution and voltage-clamped between 0 and +40 mV to establish an outward Cl⁻ driving force. Pulses of blue light delivered to the slice often led to electrically conducted photocurrents and spikelets, as previously shown. However, we noticed that holding the stellate cell at positive potentials for extended periods (>5 min) of recording weakened the gap-junction mediated events on the recorded cell, leaving only chemical transmission (Figure 7A). This enabled us to record IPSCs in stellate cells generated by the neighboring still-coupled stellate cells. Blue light pulses led to vigorous bursts of IPSCs (Fig 7B). We determined the transmitter phenotype of stellate cells by averaging individual detected IPSCs (see Methods) evoked under baseline conditions and in the presence of GABAₐ receptor blockers (Figure 7C). GABA-glycine co-transmission was evident in 10/12 cells tested: SR95531 reduced the peak amplitude of average IPSCs to 47±10% of baseline (range: 20-80% remaining in SR95531. n=6 and n=4 in Thy1-ChR2 and VGlut2-ChR2 mice, respectively). In two cells, SR95531 completely abolished light evoked IPSCs (1 cell from each genotype). The fraction remaining in SR95531 in these experiments was not significantly different from the CsCl-filled autapses (p=0.45,
unpaired t-test). Together with the results of Figures 3 and 6, these data show that DCN stellate cells co-release GABA and glycine from autaptic and reciprocal synaptic connections.

**Discussion**

We have examined chemical synaptic transmission onto DCN stellate cells, contrasting the results with known properties of synapses onto cerebellar stellate cells. While general parallels exist between these two types of interneuron in terms of GABAergic phenotype, subtype of AMPA receptors and existence of gap junctions (see Apostolides and Trussell 2013b), several differences were apparent, notably in NMDA and glycine receptor activation. Given the kinetic properties of these receptors, our results suggest that the time course of synaptic excitation and inhibition may be quite different in stellate cells of the two regions.

**Glutamate receptors on DCN stellate cells are Ca\(^{2+}\)-permeable**

We observed that NMDA and AMPA receptors in DCN stellate cells could be activated by single stimuli to parallel fibers. This contrasts with cerebellar molecular layer interneurons, where the NMDA component of parallel fiber EPSCs is small (Glitsch and Marty, 1999; Clark and Cull-Candy, 2002) and is primarily recruited by glutamate spillover during intense presynaptic activity (Carter and Regehr, 2000; Clark and Cull-Candy, 2002; Nahir and Jahr, 2013). However, similar to cerebellar stellate cells, intracellular polyamines cause inward rectification of current-voltage relations for AMPA receptor EPSCs, indicating the presence of GluA2-lacking, Ca\(^{2+}\)-permeable AMPA receptors. Thus, single action potentials in granule cells may activate two types of glutamate-gated, Ca\(^{2+}\)-permeable conductance on DCN stellate cells, mediated by AMPA and NMDA receptors. DCN parallel fiber synapses onto cartwheel and fusiform
cells are differentially depressed or potentiated by similar patterns of Ca\(^{2+}\) influx through NMDA receptors during spike-timing dependent plasticity (Tzounopoulos et al., 2004; Tzounopoulos et al., 2007). The presence of Ca\(^{2+}\)-permeable AMPA receptors, which lack the outward rectification endowed by voltage-dependent Mg\(^{2+}\) block of NMDA receptors, suggests the possibility that parallel fiber plasticity rules in DCN stellate cells may be distinct from those previously described in cartwheel and fusiform cells (e.g. Lamsa et al., 2007). Moreover, the submillisecond decay kinetics of stellate cell AMPA receptors contrast sharply with the properties of AMPA receptors at parallel fiber synapses onto cartwheel cells and the apical dendrites of fusiform cells (Gardner et al., 1999; Gardner et al., 2001), suggesting that similar patterns of parallel fiber activity will result in cell-type specific spike output.

Inhibitory transmission is mediated by glycine and GABA

Glycinergic and GABAergic mIPSCs had similar amplitudes but different time courses, suggesting that the potency of both transmitter systems is approximately equal, but also that the temporal profiles of inhibition may differ in a transmitter-specific manner. Based on labeling of small superficial cells similar to the ones we routinely record from, DCN stellate cells probably express the plasma membrane glycine transporter GlyT2, which transports glycine from the extracellular space into the nerve terminal for subsequent vesicular packaging (Gomeza et al., 2003; Rousseau et al., 2008). Most DCN stellate cells tested co-released glycine and GABA, although transmission was predominantly mediated by GABA\(_A\) receptors. This suggests that stellate cells release more GABA than glycine, or alternatively, that postsynaptic GABA\(_A\) receptors are more abundant and/or have higher conductance than glycine receptors. Although our data do not distinguish between these possibilities, cartwheel cells of the DCN appear to maintain a largely glycinergic phenotype through a preponderance of
glycine receptors rather than a mismatch in GABA/glycine synthesis (Apostolides and Trussell, 2013a).

Stellate cells probably also receive inhibitory inputs from cartwheel cells (Manis et al., 1994), and we have occasionally noticed large IPSC bursts typical of the cartwheel cell's "complex spike" firing pattern (data not shown; Golding and Oertel, 1997; Roberts et al., 2008). Thus, some proportion of glycine and GABA_A mIPSCs in our data sets likely originate from cartwheel as well as stellate cell synapses.

Function of DCN stellate cells

A recent study from Middleton et al. (2011) demonstrated that brain slices made from acoustically traumatized mice exhibited enhanced spread of electrical activity through the DCN molecular layer, apparently due to reduced GABAergic control of excitation. Given that GABA is the primary transmitter of stellate cells, it may be that reduced activity of stellate cells or their synapses may underlie this cellular correlate of tinnitus, and this possibility highlights the need to clarify how stellate cells function within the context of molecular layer circuitry. However, it should be noted that cartwheel cells of the molecular layer can also release GABA (Roberts et al., 2008; Apostolides and Trussell, 2013a). Thus, the relative contribution of GABAergic inhibition from stellate or cartwheel cells in controlling the spread of activity in the DCN is still unclear.

The three targets of parallel fibers, stellate cells, cartwheel cells and fusiform cells, together form a remarkably sophisticated, interacting network. Cartwheel cells provide powerful inhibition to fusiform cells (Roberts and Trussell, 2010), with synapses targeting near the fusiform soma (Rubio and Juiz, 2004). Stellate cells, given their size and position, primarily reach the distal portions of fusiform dendrites (Apostolides and Trussell, 2013b), and probably distal regions of cartwheel dendrites as well. It is therefore possible that they serve to locally control excitation by parallel fibers in those
distal compartments. However, the recent observation that fusiform cells communicate to stellate cells via gap junctions suggests a model in which fusiform cell activity, triggered either by parallel fibers or auditory nerve, is carried back into the molecular layer by stellate cells (Apostolides and Trussell, 2013b). The inhibitory synapses stellate cells made onto one another and onto other cell types, as well as the gap junctions between stellate cells, may provide a mechanism for distributing these ‘retrograde’ signals from fusiform cells in complex ways throughout the molecular layer. Further understanding of how such signals are processed will require clarifying the organization of these cell types with respect to the tonotopic axis and the subtypes of modalities represented by parallel fibers activity.

Author Contributions:
PFA conducted all electrophysiology recordings. LOT performed confocal imaging of GFP mice. PFA and LOT designed the experiments, analyzed the data, and interpreted the results. PFA, and LOT wrote the manuscript.

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Figure Legends:

Figure 1: Parallel fibers activate AMPA and NMDA receptors.
A) Example average EPSCs recorded in a stellate cell at negative (lower trace) and positive (upper trace) holding potentials. Of note is the slow decay component at +33 mV, typical of NMDA receptor-mediated transmission.
B) In the same cell, bath application of the NMDA receptor antagonist R-CPP (5 µM) selectively blocks the slow component.
C) Subsequent application of the AMPA receptor blocker NBQX (10 µM) abolishes the fast EPSC.
D) Average of sEPSCs recorded at +33 mV, highlighting a slow component similar to evoked EPSCs. These traces are from a different cell from the one shown in panels A-C.
E) Average of sEPSCs recorded at +33 mV in the presence of R-CPP, showing that the slow decay is due to NMDA receptor activation.
F) Quantification of 6 experiments similar to panels D-E. Left panel shows the peak amplitudes of average sEPSCs before and after NMDA receptor blockade, indicating that NMDA receptors contribute minimally to the peak. The rightmost panel shows the effect of NMDA receptor blockers on the weighted decay time constant of sEPSCs. Black lines connect data from individual experiments, red dot is mean ± SEM. Asterisks denote statistical significance.

Figure 2: AMPA receptors are Ca\(^{2+}\)-permeable.
A) Example average mEPSC from a DCN stellate cell. The decay phase is fit with a double exponential decay (weighted decay τ = 0.42 ms).
B) Amplitude histogram of individual mEPSCs from the same cell as in (A).
C) Example average EPSCs evoked at different holding potentials. Arrows denote time of parallel fiber stimulation. The shock artifact is blanked for clarity.
D) IV curve for the third EPSC in the example traces shown in (A). The red line is a linear fit to the data recorded at negative holding potentials.

E) Summary IV curve for 12 cells, normalized to the amplitude at -50 mV. Error bars are generally smaller than the symbols.

F) Group data showing the rectification index (ratio of response recorded at +50 mV to that predicted from linear fit to points at negative potentials) of individual stellate cells. Note that all cells recorded with a spermine-free internal solution displayed a linear IV.

Figure 3: Stellate cells express molecular markers of GABA/glycine co-releasing interneurons.

A) Photomicrograph of the DCN in a GAD65-GFP mouse, showing that stellate cells express the GABA synthesizing enzyme GAD65. Arrows point to stellate cells located at the ependymal edge of the DCN.

B) Photomicrograph of the DCN in a section from a GlyT2-GFP mouse. Arrows point to small labeled neurons at the ependymal edge of the brainstem, suggesting that stellate cells also express the plasma membrane transporter required for functional glycine release. The larger neurons in the DCN molecular layer are presumably cartwheel cells, while the intense background reflects the high density of glycinergic fibers throughout the DCN. Scale bar in A applies also to B.

Figure 4: Inhibition is mediated by glycine and GABA

A) Left: Summary data showing the distribution of fast, slow, and weighted decay time constants for average GABAₐ mIPSCs. Right, example average GABAₐ mIPSC. Red curve is the double exponential fit to the decay phase. Rate constants for the fit were: τ-fast, 2.1 ms (73%), τ-slow 7.1 ms (27%), weighted τ, 3.41 ms.
B) Histogram showing the amplitude distribution of GABA$_A$ mIPSCs from a single cell. These data are from a different cell than that shown in panel A.

C) A) Left: Summary data showing the distribution of fast, slow, and weighted decay time constants for average glycine mIPSCs. Right, example average glycine mIPSC. Red curve is the double exponential fit to the decay phase. Rate constants for the fit were: $\tau$-fast, 0.9 ms (75%), $\tau$-slow 3.0 ms (25%), weighted $\tau$, 1.5 ms.

D) Histogram showing the amplitude distribution of glycine mIPSCs from a single cell. The data are from a different cell as panel C.

Figure 5: Reciprocal synaptic connections and autapses in DCN stellate cells.

A) Example recording of a synaptically coupled pair of stellate cells. The presynaptic cell (upper trace) is held in voltage clamp and escaping spikes were triggered by short voltage steps. Middle black traces are four consecutive sweeps showing time-locked IPSCs in a postsynaptic stellate cell. Lower red trace is an average of multiple trials from this cell. These experiments were performed in the presence of NBQX/R-CPP to block AMPA and NMDA receptors. IPSCs are inward due to a high Cl$^-\,$ internal solution.

B) Upper panel: A voltage step evokes a single escaping spike in a stellate cell. The arrow points to a small but significant synaptic current observed after the action current, typical of autaptic transmission. Middle panel: Example autaptic IPSCs from this stellate cell. Note the difference in scale bar from the upper panel. Black traces are individual trials, red trace is an average of successful transmission events. The lower panel is after bath application of SR95531. The escaping spike artifact was removed from these traces by digitally subtracting an average of transmission failures from these traces.

C) Time course of the experiment shown in panel (B)
Figure 6: Stellate cells co-release GABA and glycine

A) Example traces from a stellate cell filled with a CsCl internal solution. Black traces are individual sweeps, red trace is an average of sweeps in each individual condition. SR95531 blocks the majority of the autaptic IPSC in this cell. The remaining component is blocked by strychnine.

B) Summary data showing the fraction of IPSC (normalized to a baseline period) remaining after the addition of SR95531 and strychnine. Notice the spread of data points, with some cells displaying no glycine component, whereas some were predominantly glycinergic.

Figure 7: Reciprocal connections between neighboring stellate cells are also mixed GABA/glycinergic.

A) Single traces from an experiment in a Thy1-ChR2 mouse, recorded at different time points after clamping the cell at +33 mV. ΔT 0 min was recorded ~3 min after initiating the whole-cell recording. Blue traces denote optogenetic activation of fusiform cells. The arrow points to gap junction-mediated spikelet events that represent action potentials in prejunctional fusiform cells (Apostolides and Trussell, 2013b). Note the marked decrease in spikelet amplitude in the ΔT 3 min trace and the absence of spikelet events in the ΔT 9 min trace.

B) Example experiment from a Thy1-ChR2 mouse. The stellate cell is held at +33 mV and fusiform cells are activated by blue light stimuli (500 ms) denoted by the blue bar. Four example sweeps are shown superimposed, recorded in baseline (black), SR95531 (red), and SR95531 + strychnine (gray).

C) Average of IPSCs in baseline (black) and SR95531 (red) detected with a template algorithm. Data are from the same cell as in panel (A).
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Evoked EPSCs
Baseline

+33 mV

-67 mV

100 pA
10 ms

+NMDA blocker

Apostolides and Trussell, Figure 1
Apostolides and Trussell, Figure 2
Apostolides and Trussell, Figure 3
Apostolides & Trussell, Figure 4
Apostolides and Trussell, Fig 6
Baseline +10 μM SR95531 +10 μM strychnine

Average of detected events

Apostolides & Trussell, Figure 7