Intrinsic and synaptic properties of vertical cells of the mouse dorsal cochlear nucleus

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The dorsal cochlear nucleus (DCN) is a primary target of auditory nerve fibers in the mammalian brain stem. Feedforward inhibition mediated by glycnergic vertical cells (also termed tuberculoventral or corn cells) is thought to contribute importantly to the sound-evoked response properties of principal neurons, but the cellular and synaptic properties that determine how vertical cells function are unclear. We used transgenic mice in which glycnergic neurons express green fluorescent protein (GFP) to target vertical cells for whole cell patch-clamp recordings in acute slices of DCN. We found that vertical cells express diverse intrinsic spiking properties and could fire action potentials at high, sustained firing rates. Paired recordings, which we directly examined synapses made by vertical cells onto fusiform cells, a primary DCN principal cell type. Vertical cell synapses produced unexpectedly small-amplitude unitary currents in fusiform cells, and additional experiments indicated that multiple vertical cells must be simultaneously active to inhibit fusiform cell spike output. Paired recordings also revealed that a major source of inhibition to vertical cells comes from other vertical cells.

METHODS

Slice preparation. All animal care and handling procedures used in this study were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee. Parasagittal slices (210 μm), which should be approximately parallel to the strial axis of the DCN (Blackstad et al. 1984), were prepared from 16- to 23-day-old heterozygous GlyT2-EGFP transgenic mice (Zeilhofer et al. 2005) or their wild-type littermates. GlyT2-EGFP mice were backcrossed into the C57BL/6J genetic background (Jackson Labs) and were genotyped and maintained as previously described (Roberts et al. 2008). In one set of experiments GlyT2-EGFP mice at P30–33 were used. To make slices, mice were deeply anesthetized with isoflurane and then killed by decapitation. After the skull was removed to expose the brain, the brain stem was isolated by making a coronal cut just rostral to the cerebellum. A sagittal cut was then made down the midline of the brain stem, and one half of the brain stem was removed from the skull and glued cut side down to the stage of a vibratome (Leica VT1200S). The ventral side of the brain stem was turned to face the vibratome blade and was angled slightly downward (~6° angle with horizontal plane). During dissection and slicing, tissue was kept immersed in warm (~34°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 130 NaCl, 2.1 KCl, 1.7 CaCl2, 1.0 MgSO4, 1.2 KH2PO4, 20 NaHCO3, 3 Na-HEPES, and 11 glucose, saturated with 5% CO2–95% O2, ~300 mosM. After slicing, slices were allowed to recover in 34°C ACSF for 1 h and then either transferred to a recording chamber or maintained in ACSF at room temperature (~22°C) until use.
**Physiology of DCN Vertical Cells**

**Electrophysiology.** During recordings, slices were constantly perfused with ACSF (1–2 ml/min; chamber volume ~1.6 ml) maintained at 33 ± 1°C with an in-line heater (Warner Instruments). Cells were visualized with a ×60 magnification objective on the stage of an upright microscope (Olympus BX51W) equipped with infrared gradient contrast and fluorescence optics. GFP-expressing neurons were identified by briefly illuminating tissue via a mercury lamp light source and viewing fluorescence with a GFP filter set (Chroma). Vertical cells were initially identified based on location within the deep layer of DCN slices and GFP fluorescence in tissue from GlyT2-EGFP mice. With some experience, we could routinely target vertical cells for recordings on the basis of their location within the deep layer and somatic morphology (round or oval) and size (~10–15 µm diameter; see Fig. 1B), even in wild-type tissue. Fusiform neurons were easily identified on the basis of location within the slice, lack of GFP expression in GlyT2-EGFP tissue, larger somatic size compared with vertical cells, and bipolar somatic morphology. Fusiform neurons had spiking properties consistent with previous reports (see, for example, Tzounopoulos et al. 2004; Zhang and Oertel 1994), and fusiform cells visualized with fluorescent dyes or biocytin labeling had spiny apical dendrites extending through the molecular layer and long, smooth basal dendrites that projected into the deep layer (Blackstad et al. 1984; Tzounopoulos et al. 2004; Zhang and Oertel 1994). Whole cell recording electrodes (2–4 MΩ) were pulled from borosilicate glass (VP) and filled with a solution containing (in mM) 113 K+-glucuronate, 2.75 MgCl₂, 1.75 MgSO₄, 0.1 EGTA, 14 Tris-phosphocreatine, 4 Na₂ATP, 0.3 Tris-GTP, 9 HEPES, HEPES adjusted to ~290 mosM with sucrose, pH adjusted to 7.25 with KOH. In some recordings, biocytin (0.3% wt/vol) was added to the internal solution. The red fluorescent dye Alexa Fluor 555 (20 µM) was also routinely included in recording solutions. The composition of the internal solution was designed to give a calculated reversal potential of ~84 mV for Cl⁻ conductances because previous work using gamicidin-perforated patch recordings established a reversal potential of ~83.9 ± 0.7 mV in fusiform neurons (Kim and Trussell 2009). All reported membrane potential values are corrected for a junction potential of ~10 mV that was measured between the internal and bath solutions. In a subset of paired recordings between vertical cells, the K⁺-glucuronate-based internal solution was supplemented with 20 mM glycine. Connections between cells recorded with the glycine-containing internal solution ([3 total connections, 1 unidirectional, 1 reciprocal among 3 tested pairs (6 tested connections)]) were included in the calculation of connection probability but were not included in the reported measurements of unitary inhibitory postsynaptic current (uIPSC) properties. In two of the vertical cell and fusiform cell pairs, the postsynaptic fusiform cell was recorded with an internal solution containing (in mM) 108 CsMeSO₄, 5 CsCl, 1 MgCl₂, 4 Mg-ATP, 0.4 Tris-GTP, 14 Tris-phosphocreatine, 5 EGTA, 10 HEPES, 3 QX-314, pH adjusted to 7.3 with CsOH, 290 mosM. For recordings of uIPSCs, postsynaptic cells were held at ~60 mV, with the exception of the two recordings using the CsMeSO₄-based internal solution, in which postsynaptic fusiform cells were held at ~30 mV. Extracellular stimulation of auditory fibers or vertical cell axons was achieved by applying voltage pulses (10–30 V, 150–200 µs) via ACSF-filled double-barreled glass electrodes (theta pipettes, lengthwise tip diameter ~5 µm; Sutter Instruments) placed in the deep layer within ~50 µm of the somas of recorded cells. For minimal stimulation, a bipolar theta glass electrode was used. Stimulus strength was adjusted to generate transmission failures in at least 40% of the trials; in most cases the failure rate exceeded 50% (see Table 5). Responses were accepted as candidate single-axon connections if IPSCs were clearly distinguishable from failures and increases of stimulus strength of 10–20% did not noticeably change the amplitude of successful stimuli (see Fig. 8).

**Electrophysiological data acquisition and analysis.** Recordings were acquired with a Multiclamp 700B amplifier and pCLAMP 10 software (Molecular Devices). Signals were digitized at 50 kHz with a Digidata 1322A (Molecular Devices) and low-pass filtered at 10 kHz. For voltage-clamp experiments, pipette series resistance (<20 MΩ) was compensated by 80%. In current-clamp recordings, bridge balance was used to compensate series resistance. Membrane time constant (τmembrane) and input resistance (Rinput) were measured in current-clamp mode from averaged voltage responses (10–20 sweeps) to 200- to 400-ms-long ~20-pA current steps delivered while vertical cells were at resting membrane potential (Vrest) (no bias current injection). Rinput was determined by measuring the voltage change from Vrest for the last 50 ms of current step and calculated with Ohm’s law. τmembrane was measured by fitting a monoexponential function to the initial voltage response to the ~20-pA current step. AP shape measurements were determined from responses to 1-ms supra-threshold current steps (typically 1 nA). AP threshold was defined as the first peak of the third time-based derivative of the membrane voltage (Vm) before AP peak (Henze and Buzsaki 2001). AP height was defined as the difference between AP peak and threshold. AP half-width was defined as the width of the AP at Vm halfway between peak and threshold. Mean spike frequency vs. current injection relationships (see Fig. 3C) were fitted with Hill functions of the form

\[ F(I) = \frac{F_{\text{max}}}{1 + \left(\frac{I}{I_{1/2}}\right)^n} \]

where \(F\) is the injected current, \(F_{\text{max}}\) is the maximum firing rate, \(I_{1/2}\) is the current injection level at which the firing rate is half-maximal, and \(n\) is an exponent factor. Gain of the spike frequency vs. current injection relationships was defined as the mean initial slope of the fitted Hill functions and was calculated by differentiating over the portion of the fit between 0 and 300 pA injected current. Decay kinetics of spontaneous excitatory postsynaptic currents (EPSCs) and uIPSCs were determined by fitting biexponential or single-exponential functions to the decay phase of currents, respectively. Spontaneous EPSCs were detected with the template function event detection feature of Axograph X (Clements and Bekkers 1997). In paired recordings, latencies of uIPSCs were defined as the time difference between the peaks of presynaptic APs and current at which uIPSCs had achieved 20% of their peak amplitude. uIPSC latencies and decay times were usually measured from the first uIPSC in a train but were sometimes measured from the second or third uIPSC when the first uIPSC was obscured by spontaneous IPSCs or was very small. Conduction values for unitary events were calculated from the driving force for Cl⁻ currents (usually 23.8 mV) and peak current amplitudes relative to baseline current for the first uIPSC in a train. uIPSC latency, decay time constant, and peak amplitude were measured from averaged currents from 10–33 sweeps, with the exception of peak conductance values that excluded failures, in which averaged currents were from 3 or more sweeps. Failures were defined as trials in which a rapid outward current deflection rising above baseline noise levels was not observed within 1 ms after a presynaptic AP. Correct identification of failures was confirmed by averaging together trials in which failures were counted. All data are presented as means ± SD. Statistical significance (P < 0.05) was determined by ANOVA followed by Fisher’s protected least significant difference (PLSD) post hoc tests unless noted otherwise.

**Biocytin labeling.** After some recordings in which biocytin was included in the internal solution, slices were fixed overnight in a cold (4°C) phosphate-buffered saline (PBS) solution (0.1 M, pH 7.4) containing 4% formaldehyde. After fixation, slices were rinsed in PBS and stored for up to a week at 4°C in PBS until processing for biocytin labeling. After fixed tissue was permeabilized in 0.2% Triton X-100 solution (in PBS) for 1 h at room temperature, slices were incubated in a PBS solution containing Alexa Fluor 568-conjugated streptavidin (1:2,500 dilution; Invitrogen) overnight at 4°C and then rinsed and mounted on glass slides. Slices were dehydrated in an ascending series of alcohols, delipidized in xylenes, and then rehydrated and cover-
slipped with Fluoromount-G (Southern Biotech). Fluorescence images were acquired with a confocal microscope (Olympus FV1000) by sequential scanning of GFP and Alexa Fluor 568 channels with a ×40 oil-immersion objective.

**RESULTS**

To study vertical cells, we made whole cell patch-clamp recordings from GFP-expressing (GFP⁺) neurons located in the deep layer of the DCN in brain stem slices prepared from GlyT2-EGFP transgenic mice (Fig. 1, A and B). The neuronal glycine transporter GlyT2 is a reliable marker of glycinergic neurons in mammals (Friauf et al. 1999; Zafra et al. 1995), and GFP is selectively expressed in almost all glycinergic neurons in GlyT2-EGFP mice (Zeilhofer et al. 2005). Many neurons in the DCN express GFP in GlyT2-EGFP mice (Fig. 1A) (Zielhofer et al. 2005), consistent with the large number of glycinergic neurons in the DCN. The morphology of recorded cells was routinely assessed by examining fluorescent signals from Alexa Fluor 555, which was usually included in pipette solution. In a few recordings, deep GFP⁺ cell morphology was also examined in cells filled with biocytin. Most GFP⁺ neurons in the DCN deep layer had a few (~3–5) relatively unbranched, smooth (aspy) dendrites extending from the cell body (Fig. 1B), similar to previous anatomical descriptions of vertical cells (Lorente de No 1981; Rhode 1999; Zhang and Oertel 1993b). Additionally, almost all deep-layer GFP⁺ neurons had intrinsic and synaptic properties consistent with those previously described for anatomically identified vertical cells. Specifically, APs elicited by depolarizing current steps were short in duration and could exhibit biphasic afterhyperpolarizations (Fig. 1C), termed “double undershoots” by Oertel and colleagues (Zhang and Oertel 1993b), and spontaneous EPSCs recorded from GFP⁺ neurons displayed rapid decay kinetics (Fig. 1D; τfast = 0.27 ± 0.09 ms, 77.81 ± 10.74% of decay, τslow = 1.65 ± 0.41 ms; n = 5) (Gardner et al. 1999). Vertical cells were easily distinguishable from cartwheel cells, which are usually found in the molecular layer and fusiform cell layer and make up the other major population of glycinergic neurons in the DCN (Lorente de No 1981). Cartwheel cells exhibit EPSCs with slower kinetics (Gardner et al. 1999), have spiny dendrites that branch extensively in the molecular layer (Fig. 1A) (Mugnaini 1985; Zhang and Oertel 1993a), and are unique among DCN neurons in their ability to fire high-frequency bursts of APs (“complex spikes”) (Manis et al. 1994; Zhang and Oertel 1993a). Thus targeting deep-layer GFP⁺ neurons provided a reliable approach for acquiring recordings from vertical cells.

**Vertical cells express green fluorescent protein (GFP) in GlyT2-EGFP transgenic mice.** We initially performed current-clamp recordings from deep GFP⁺ cells to characterize the intrinsic spiking properties of vertical cells. Some recordings (33/122 total; morphology of all wild-type cells confirmed to be the same as cells from GlyT2-EGFP mice by visualization of fluorescence) were also acquired in

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**Fig. 1.** Vertical cells express green fluorescent protein (GFP) in GlyT2-EGFP transgenic mice. A: confocal z-projection (maximum-intensity projection of 50.4 μm in z-axis, images acquired at 0.4-μm intervals) of fixed coronal slice from a GlyT2-EGFP mouse in which cells were filled with biocytin. GFP fluorescence is in cyan. Biocytin labeling is in magenta. One fusiform cell (top left), 1 cartwheel cell (bottom left), and 2 vertical cells (bottom right) were filled with biocytin. The basal dendrites of the labeled fusiform cell and axon of the cartwheel cell were truncated in this slice. Prior to recordings, the labeled cartwheel cell and vertical cells exhibited strong GFP fluorescence (intensity of the GFP signal decreased during the vertical cell recordings, perhaps because of diffusion of the pipette solution into the cells). The fusiform cell did not express GFP. Most of the GFP-expressing (GFP⁺) cells in the deep layer are likely vertical cells. Most medium-sized cells within the superficial molecular layer and fusiform cell layer are likely cartwheel cells, with Golgi cells also probably labeled. Small cells in the outermost region of the molecular layer are mostly stellate cells. B: enlarged negative image of red fluorescence channel from boxed region in A. Note the round somatic morphology and smooth, relatively unbranched dendrites, which were typical of vertical cells. C: example voltage trace from a GFP⁺ deep-layer neuron (different cell than shown in B) from the beginning of the response to a 50-pA, 200-ms-long current injection. Note the biphasic afterhyperpolarizations following the spikes. Resting membrane potential (Vrest) ~68 mV. D: averaged spontaneous excitatory postsynaptic current (EPSC) recorded at ~70.2 mV from a GFP⁺ deep-layer neuron (average from 133 events occurring during 1 min of recording). Different cell than in B or C.
tissue from wild-type littermates of GlyT2-EGFP transgenic mice to assess whether transgene expression altered vertical cell characteristics. No differences between wild-type and GlyT2-EGFP cells were observed, so results were pooled.

In contrast to cartwheel cells of the molecular layer, ~70–75% of which spike spontaneously under our slice conditions (Kim and Trussell 2007; Kuo and Trussell 2011), vertical cells very rarely exhibited spontaneous spiking. This is consistent with the in vivo behavior of type II units (Shofner and Young 1985; Spirou et al. 1999), which are presumed vertical cells (Rhode 1999).

In our recordings, we observed several different AP firing responses to hyperpolarizing and depolarizing somatic current steps (200-ms duration) that we classified into four general categories (Fig. 2). In over half of recordings (54.1%; 66/122 cells), cells fired rebound spikes after the offset of hyperpolarizing current steps and membrane potential quickly returned to resting levels after the offset of depolarizing current steps (Fig. 2A). We termed these cells “rebound spiking” cells. In another subset of recordings (24.6%; 30/122 cells), cells also exhibited rebound spiking after hyperpolarizing current injection, but depolarizing steps evoked plateau depolarizations in which membrane potential remained depolarized beyond the offset of current injection (“plateau and rebound spiking” cells; Fig. 2B). In most cases, plateau depolarizations could support AP firing beyond the offset of current steps. Another group of cells (9.0%; 11/122) exhibited plateau depolarizations but no rebound spiking (“plateau” cells; Fig. 2C). Finally, 12.3% of cells (15/122) did not exhibit either plateau potentials or rebound spiking (“no plateau or rebound spiking” cells; Fig. 2D). In some cells with rebound or plateau spiking, the spiking behavior following current injection offset could last up to several seconds, but more typically rebound and plateau depolarizations lasted 100–300 ms.

Despite differences in spiking behavior, cells among the different categories could not be clearly differentiated from each other based on intrinsic membrane properties (Table 1). Resting membrane potentials ($V_{\text{rest}}$) were not significantly different between spiking phenotypes except for slightly hyperpolarized $V_{\text{rest}}$ values in “no plateau or rebound spiking” compared with “rebound spiking” cells. No significant differences were observed between groups for measurements (see METHODS for details) of cell input resistance or membrane time constant.

AP characteristics, which were measured in a subset of cells in which 1-ms suprathreshold current steps were applied to trigger spikes, were also similar among the different spiking phenotypes. No significant differences were observed between spiking phenotypes for AP threshold, spike height, or spike half-width (Table 2) (see METHODS for definitions).

All cells, regardless of spiking phenotype, exhibited sustained, repetitive firing during long depolarizing current steps. Figure 3, A and B, show plots of instantaneous spike frequencies during 200-ms current injections from 50 to 650 pA (50-pA increments) for different example cells that were near opposite ends of the range for spike frequencies observed in our recordings. As illustrated by these plots as well as the summary of mean spike frequencies versus current injection in Fig. 3C, vertical cells responded to increasing levels of current injection with steadily higher firing rates. Mean firing rates were significantly higher in “plateau and rebound spiking” cells at current injection levels between 50 and 400 pA compared with “no plateau no rebound” cells or “rebound spiking” cells and for currents between 50 and 250 pA compared with “plateau” cells. All other comparisons between groups were not significant, with the exception of higher firing rates in “rebound spiking” cells compared with “no rebound no plateau” cells at 50 pA. The gains of the input-output relationships, which were determined by fitting Hill equations to the mean spike frequency vs. injected current relationships (Fig. 3C) (see METHODS) (Silver 2010), were similar across the different subtypes, but with slightly higher gain in “plateau and rebound spiking” cells (9.0 Hz/pA; “plateau and rebound spiking” 1.09 Hz/pA; “plateau” 0.93 Hz/pA; “no rebound no plateau” 0.86 Hz/pA).

Vertical cell spiking remained consistent over the course of 200-ms current injections. In a subset of recordings, we com-
pared instantaneous spike frequencies for APs during identical 300-pA current steps across different vertical cell categories (Fig. 4). Although a range of initial spike frequencies was observed at this current level, in general spike frequency changed very little by the end of 200-ms steps compared with initial spike rates. This was quantified by calculating the ratio of mean instantaneous spike frequency for the last 20 ms versus the first 20 ms of the 200-ms current steps (adaptation index). Within each subtype of vertical cell, some cells showed slight spike frequency adaptation (adaptation index < 1) and others exhibited some spike frequency acceleration (adaptation index > 1), but on average spike rate was similar at the end of current steps compared with the start and were not significantly different between vertical cell subtypes (adaptation indexes: “rebound spiking” 1.0 ± 0.18, n = 21; “plateau and rebound spiking” 1.1 ± 0.17, n = 13; “plateau” 1.2 ± 0.27, n = 7; “no plateau or rebound spiking” 1.3 ± 0.38, n = 9; P = 0.06) (Fig. 4E). In response to 300-pA steps, cells with initial spike rates of less than ~250 Hz tended to exhibit spike frequency acceleration, whereas those with initial rates greater than 250 Hz generally exhibited little change in spike frequency over the course of 200-ms current steps (Fig. 4F).

Short-term depression of excitatory inputs. Excitatory inputs to vertical cells were evaluated by making voltage-clamp record-ings of currents in response to stimuli applied through an extracellular electrode positioned in the deep layer of DCN. In these experiments, inhibitory transmission was blocked by including 10 μM SR-95531 and 0.5 μM strychnine in the bath solution and NMDA receptors were blocked by including 50 μM D-APV. EPSCs were likely mediated by AMPA receptors because we did not observe inward currents (evoked or spontaneous) in the presence of NBQX (10 μM; not shown) and vertical cells were previously shown to express GluA2 subunit-lacking AMPA receptors (Gardner et al. 1999, 2001). Consistent with auditory inputs to fusiform cells (Irie and Ohmori 2008), EPSC amplitudes depressed in response to repetitive stimulation in 11 of 12 vertical cells (Fig. 5, A and B). However, in these cells, depression at the end of stimulus trains ranged between 80% and 20% of initial EPSC amplitudes (see Fig. 5C). In several cells (see Fig. 5C), EPSCs at the end of a 10-stimulus train were depressed to similar levels across a 10-fold range in stimulation frequencies (200 Hz EPSC1/ EPSC10 ratio > 0.75 in 6/11 cells) so that the average depression of EPSCs was constant across stimulus frequencies in the 11 vertical cells with depressing excitatory synapses (Fig. 5, B and C). However, the lack of difference was also likely due in part to variability in the extent of depression across the sample of recorded cells.

Table 2. Action potential properties of vertical cells

<table>
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<tr>
<th>Vertical Cell Subtype</th>
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<th>SD</th>
<th>Range</th>
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<td>-56.5</td>
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<td></td>
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<td>232</td>
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<td>194 to 274</td>
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No statistically significant differences between vertical cell subtypes.
Weak synaptic connections between vertical cells and postsynaptic targets. Vertical cell-mediated inhibition is hypothesized to strongly shape DCN principal neuron activity (Young and Davis 2002). However, existing evidence that vertical cells provide inhibition to principal neurons is indirect and comes primarily from anatomical studies (Rhode 1999) and cross-correlation analysis of paired extracellular recordings between principal neurons and vertical cells (Voigt and Young 1980, 1990). We therefore directly tested for functional synaptic connections between vertical cells and fusiform neurons, using dual whole cell recordings.

Fusiform cells were identified on the basis of their location within the fusiform cell layer, large bipolar somata (see Fig. 1A), large amplitude spikes (peak ~20 mV more depolarized than vertical cells), and lack of GFP expression in tissue from GlyT2-EGFP mouse tissue. We also usually visualized the dendritic morphology of fusiform cells by including Alexa Fluor 555 in pipette solutions. In almost all recordings in which dendritic morphology was examined, dendrites appeared to be intact in our sagittal slice preparations. Fusiform cells had extensive spiny dendrites extending throughout the molecular layer and long, aspiny, and less branched dendrites extending over large areas of the deep layer. After a recording from a fusiform cell was established, whole cell recordings were acquired from vertical cells located both nearby the fusiform cell soma as well as along the fusiform cell basal dendrites up to ~150 μm distal to the soma to test for synaptic connections. Often, we sequentially recorded from several vertical cells while simultaneously recording from the same fusiform cell (1–5 vertical cells were tested for a given fusiform cell).

We found that APs in vertical cells elicited detectable postsynaptic currents in fusiform cells in 11/91 of tested pairs (12.1% connection probability). We examined spiking responses in presynaptic vertical cells in 10 of the connected pairs to examine which vertical cell subtypes synapse upon fusiform cells. Five presynaptic cells were “rebound spiking” cells, four were “plateau and rebound spiking” cells, and one was a “plateau” cell. Thus at least three of the four subtypes of vertical cell can form synapses upon fusiform cells.

Measurements of peak conductance, latency, decay kinetics, and failure rate for unitary postsynaptic currents (uIPSCs) elicited by presynaptic vertical cell spiking are summarized in Table 3. Consistent with monosynaptic connections between vertical cells and fusiform cells, uIPSCs were evoked with short latencies after presynaptic vertical cell APs (Table 3).

Unitary currents had fast decay kinetics (Table 3), similar to spontaneously occurring IPSCs, which likely arose primarily from cartwheel cells (Golding and Oertel 1997; Roberts and Trussell 2010), the majority (~75%) of which spontaneously fire APs under our slice conditions (Kim and Trussell 2007; Kuo and Trussell 2011). Vertical cell-mediated uIPSCs recorded in fusiform neurons were usually small in amplitude and were often obscured by spontaneous IPSCs, which could exhibit peak amplitudes of several hundred picoamperes. The weak vertical cell inputs were in sharp contrast to the more than 10-fold larger amplitudes of cartwheel cell-mediated uIPSCs recorded in fusiform cells recorded from cartwheel cell and fusiform cell pairs under identical conditions (peak conductance 24.0 ± 18.0 nS, range 6.4–63.0 nS, n = 11 pairs; data taken from recordings reported in Kuo and Trussell 2011). Connections between presynaptic vertical cells and postsynaptic fusiform neurons were likely often mediated by only a few synapses, because presynaptic spikes frequently failed to evoke uIPSCs (Table 3). This was unexpected because evidence from in vivo recordings in cats suggests that vertical cells can powerfully inhibit principal cell output (Voigt and Young 1980; Young and Davis 2002).

The short-term dynamics of vertical cell synapses onto fusiform cells were examined in a subset of paired recordings in which spontaneous IPSCs were not too large and frequent to complicate measurement of uIPSC amplitudes in response to trains of presynaptic APs. In response to 100-Hz trains of presynaptic APs, uIPSCs in fusiform cells usually exhibited moderate short-term facilitation (Fig. 6, B and C), suggesting a

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**Fig. 3.** Input-output relationships in vertical cells. A: instantaneous spike frequencies between consecutive action potentials (APs) during 200-ms depolarizing current injections from +50 pA (bottom line) to +650 pA (top line) (50-pA increments). Current injection started at time 0 on the x-axis. This cell was a “rebound spiking” neuron and was near the lower end of the observed range for mean spike frequencies elicited by depolarizing current injections. B: same as in A but for a cell that was near the upper end of the range for mean spike frequencies in response to current injection. This cell was a “plateau and rebound spiking” neuron. C: summary of mean frequencies for spikes occurring within the 200-ms duration of different levels of current injections. Symbols and error bars show mean ± SD of mean spike frequencies for “rebound spiking” (□, n = 21), “plateau and rebound spiking” (□, n = 12), “plateau” (○, n = 7) and “no plateau or rebound spiking” (●, n = 9) cells. Lines are Hill function fits to the mean values for each subtype. Maximal firing rates (F_max), half-saturation values (I_1/2), and the exponent (n) from the fits (±SD) were F_max = 582.48 ± 49.8, I_1/2 = 339.18 ± 46.3, n = 1.43 ± 0.098 for “rebound spiking”; F_max = 572.56 ± 129, I_1/2 = 239.31 ± 96.5, n = 1.36 ± 0.45 for “plateau and rebound spiking”; F_max = 520.9 ± 151, I_1/2 = 275.64 ± 107, n = 1.79 ± 0.63 for “plateau”; and F_max = 547.67 ± 89.2, I_1/2 = 315.68 ± 66.7, n = 1.97 ± 0.42 for “no plateau or rebound spiking” cells.
low initial release probability from vertical synapses. The mild facilitation of vertical cell-mediated synaptic currents indicates that vertical cells can provide sustained inhibitory input to fusiform cells.

Although vertical cells are hypothesized to receive inhibitory input from D-stellate cells of the ventral cochlear nucleus (Nelken and Young 1994), sources of inhibition to vertical cells are not well known. We also used dual recordings to explore whether vertical cells synapse upon other vertical cells. Functional connections were detected in 16 of 36 tested vertical cell pairs, with a bidirectional connection observed in 1 pair. We tested for connections in both directions in all but one

![Fig. 4. Sustained spike rates in vertical cells. A–D: plots of instantaneous spike frequencies over the duration of 200-ms current steps at 300 pA. Connected lines represent spike frequency measurements for individual cells. A: data from 21 “rebound spiking” cells. B: “plateau and rebound spiking” cells (n = 13). C: “plateau” cells (n = 7). D: “no plateau or rebound spiking” cells (n = 9). E: summary of adaptation index measurements (mean instantaneous spike frequency of last 20 ms divided by that of first 20 ms of 200-ms step) for different vertical cell subtypes (symbols as in A–D) from data shown in A–D. Filled gray symbols show means ± SD.](http://jn.physiology.org/)

![Fig. 5. Short-term depression of excitatory inputs to vertical cells. A: example EPSCs recorded from a vertical cell in response to stimulus trains applied to auditory fibers at different frequencies. B: summary of ratio of EPSC peak amplitudes [EPSC(n)] compared with the first stimulus-evoked EPSC (EPSC1) over the course of stimulus trains for 11 cells that exhibited depression of EPSC amplitudes (out of 12 recorded vertical cells). Symbols and error bars show means ± SD. C: EPSC10-to-EPSC1 ratios for 20-Hz and 200-Hz stimulus trains. Gray circles connected by lines show data from individual cells acquired at each stimulus frequency. Filled black circles and error bars show mean ± SD. P = 0.08, paired t-test. n.s., Not significant. All recordings for data in A–C were acquired with 50 μM D-APV, 0.5 μM strychnine, and 10 μM SR-95531 in the bath solutions.](http://jn.physiology.org/)
vertical cell pair. Thus a total of 71 possible connections were examined, which yields a connection probability of 23.9% (17 connected/71 tested connections). We assessed the spiking phenotype of the presynaptic vertical cell in seven pairs. Four presynaptic neurons were “rebound spiking” cells, one was a “plateau and rebound spiking” cell, one was a “plateau” cell, and one was a “no plateau or rebound spiking” cell. We characterized the spiking behavior of the postsynaptic vertical cell in 12 of the connected pairs. Eight of these postsynaptic neurons were “rebound spiking” cells, two were “plateau and rebound spiking” cells, and two were “plateau” cells. Thus all vertical cell subtypes were observed to synapse upon other vertical cells, and all spiking phenotypes besides “no plateau or rebound spiking” neurons were found to receive synaptic input from other vertical cells.

uIPSCs recorded in vertical cells were similar to those observed in fusiform cells in peak amplitude, decay kinetics, latency, and failure rate (Tables 3 and 4). However, uIPSCs elicited by presynaptic spiking were more clearly distinguishable from baseline currents because vertical cells received very little spontaneous IPSC input (see Fig. 7B). The lack of spontaneous inhibitory input suggests that vertical cells receive little or no input from the spontaneously active cartwheel cell population. On average, vertical cell uIPSCs elicited by 100-Hz trains of presynaptic spikes exhibited moderate short-term facilitation, although the behavior of individual connections ranged from depression to strong facilitation (Fig. 7C).

Given previous in vivo work suggesting that vertical cells provide robust inhibitory input to principal neurons, we were surprised to find that vertical cell-mediated uIPSCs were relatively weak, especially compared with cartwheel cell inputs. We therefore considered several potential reasons we might have inaccurately measured the size of unitary connections between vertical cells and their targets. One possibility was that glycnergic transmission was potentially compromised in tissue from GlyT2-EGFP mice (peak conductance 2.2 ± 0.78 nS; P = 0.6, unpaired t-test; wild type n = 6 pairs, transgenic n = 8 pairs).

We also considered whether the slicing procedure led to artificially small uIPSCs, because of either severing of vertical cell axons or a potential time-dependent decline in neuronal glycine content previously observed in acutely prepared DCN slices (Wickesberg et al. 1994). Arguing against a reduction in neuronal glycine content over time during paired recordings, we did not note systematic differences for connection probability or uIPSC amplitudes in relation to times when paired recordings were made after tissue preparation (up to ~7 h after slicing). uIPSCs were also stable for up to ~1 h in recordings between connected neurons. Furthermore, we did not observe significantly different uIPSC amplitudes in additional paired recordings between vertical cells in which pipette solutions were supplemented with 20 mM glycine (1.5 ± 0.78 nS peak conductance without failures, range 0.95 to 2.4 nS, n = 3 connections; P = 0.5 unpaired t-test). The large amplitudes of cartwheel cell-mediated uIPSCs also argue against slicing-induced rundown of neuronal glycine content.
might account for the small-amplitude uIPSCs observed in our experiments. However, minimal stimulation-evoked IPSCs recorded in vertical cells were not significantly different in tissue from P30–33 mice compared with those from P16–23 animals (P = 0.4, unpaired t-test) (Fig. 8, Table 5).

Control of fusiform cell spiking requires multiple vertical cell inputs. We next investigated the functional impact of vertical cell-mediated inhibition on principal neuron spike output. In a first set of experiments, we made simultaneous current-clamp recordings between synaptically connected vertical cells and fusiform neurons. Brief (3 ms) current steps were applied to postsynaptic fusiform cells via somatic patch pipettes in order to elicit spiking on approximately four of five identical current injections (620–680 pA each, 20 Hz) while the presynaptic vertical cell was resting below spike threshold (Fig. 9A). Trials without vertical cell activity were then alternated with those in which a train of 50 spikes at 100 Hz was evoked in the presynaptic vertical cell by suprathreshold current steps starting 50 ms prior to the first current injection into the postsynaptic fusiform cell (Fig. 9B). Comparison of fusiform cell spiking probability in response to identical current steps in either condition demonstrated a slight, but significant, reduction in spike probability when presynaptic vertical cells fired trains of spikes (Fig. 9C; spike probability 0.797 ± 0.078 vs. 0.710 ± 0.115, respectively; P < 0.05, paired t-test). This experiment indicated that even with robust presynaptic activity, a single vertical cell does not strongly influence fusiform cell spiking. To examine whether activity in multiple vertical cells could have greater impact on postsynaptic spiking, we performed a similar experiment but replaced the presynaptic recording pipette with an extracellular stimulating electrode.

whether the slice procedure reduced uIPSC amplitudes by damaging vertical cell axons, we compared IPSCs evoked by a minimal stimulation procedure between vertical cells recorded in 210-μm slices (the thickness used in the paired recordings) and 310-μm slices (see METHODS). By this means we were able to record from a vertical cell deep in the slice and assess the strength of its lowest threshold axonal inputs. Amplitudes of IPSCs evoked by minimal stimulation were not significantly different from those measured in the paired recordings (P > 0.05, unpaired t-test). As shown in Fig. 8 and Table 5, no significant difference in minimal-stimulation IPSC amplitude was found between the different slice thicknesses (P = 0.5, unpaired t-test), suggesting that uIPSC amplitudes were not artificially small because of the use of 210-μm slices. Taken together, these results indicate that artifacts from tissue slice preparation or transgene expression do not account for the small amplitude of vertical cell synaptic connections we observed.

It is possible that we measured uIPSC amplitudes prior to complete maturation of vertical cell-mediated inhibition, which might account for the small-amplitude uIPSCs observed in our
Table 5. Properties of minimal-stimulation IPSCs recorded from vertical cells, comparing slice thickness and animal age

<table>
<thead>
<tr>
<th>n</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>Range</td>
</tr>
<tr>
<td>Peacteria conductance (210 μm young/310 μm young/210 μm old), nS</td>
<td>7/7/6</td>
<td>0.80/0.9/0.9</td>
<td>0.3/0.3/0.4</td>
<td>0.5–1.3/0.5–1.2/0.3–1.5</td>
</tr>
<tr>
<td>Without failures</td>
<td>7/7/6</td>
<td>1.7/2.0/2.1</td>
<td>0.8/0.5/0.8</td>
<td>0.9–2.1/1.4–2.9/1.1–3.2</td>
</tr>
<tr>
<td>Failure rate, %</td>
<td>7/7/6</td>
<td>57.6/60.0/64.6</td>
<td>8.8/15.2/10.7</td>
<td>47–72/41–82/50–78</td>
</tr>
</tbody>
</table>

Peak conductances from each group were not significantly different (P > 0.05, unpaired t-test) from those in the paired recordings.

positioned in the deep layer near to the recorded fusiform cells and blocked excitatory transmission with 10 μM NBQX and 50 μM D-APV (see Fig. 9, D and E, inset). In three cells SR-95531 (10 μM) was included in the bath solution, while another three recordings did not include SR-95531. Stimulus position and strength were adjusted to evoke detectable inhibitory postsynaptic potentials (IPSPs) in current-clamped fusiform cells (Fig. 9E, inset). When currents were recorded in voltage-clamp mode, the same stimuli evoked IPSCs with ~100-pA peak amplitude (average value), which should correspond to activity in approximately three to five vertical cells. Presumptive vertical cell-mediated IPSCs, which exhibited moderate facilitation in response to trains of stimuli, were easily distinguished from those likely arising from cartwheel cells because cartwheel inputs to fusiform cells exhibit short-term depression and are typically large in amplitude (Mancilla and Manis 2009; Kuo and Trussell 2011). Extracellular stimuli (50 stimuli at 10 Hz starting 10 ms prior to somatic current injections in fusiform cells) significantly reduced fusiform cell spiking compared with control conditions without extracellular stimuli (spike probability in control: 0.808 ± 0.062, with stim: 0.286 ± 0.096; P < 0.0001, paired t-test). Thus coordinated activity in several vertical cells can reduce fusiform cell output.

DISCUSSION

Intrinsic properties of vertical cells. Vertical cells exhibited diverse firing responses to step current injections. We used the presence or absence of persistent activity lasting beyond the duration of hyperpolarizing or depolarizing steps (rebound...
spiking or plateau depolarizations) to group vertical cells into different subtypes. However, within each subtype the duration of rebound or plateau activity varied from ~20 ms to several seconds beyond the end of current injection. Furthermore, vertical cells could not generally be distinguished from each other on the basis of intrinsic membrane properties or AP shape. Also, repetitive firing of vertical cells during depolarizing current steps was not distinctive between subcategories, with the exception of higher firing rates in cells exhibiting both plateau depolarizations and rebound spiking compared with other subtypes. The different spiking patterns we observed therefore appear to reflect a continuous distribution of response properties across the vertical cell population.

An unexpected feature of many vertical cells was the ability to generate plateau potentials that outlasted the duration of depolarizing current steps. In other cell types, the generation of similar plateau depolarizations has generally been attributed to calcium-dependent activation of a nonspecific cation conductance (Chang and Kim 2004; Egorov et al. 2002; Lee and Tepper 2007; Morisset and Nagy 1999). Whether a similar mechanism accounts for vertical cell plateau depolarizations is currently unclear. Although the nonspecific cation channel antagonist flufenamic acid (100 μM) reduced plateau potentials in vertical cells (not shown), spiking during steps was also severely affected, likely because of nonspecific actions of flufenamic acid (Ottoilia and Toro 1994; Poronnik et al. 1992; Yau et al. 2010).

Vertical cells could spike at high firing rates, consistent with previous work (Zhang and Oertel 1993b). The maximum firing rate of vertical cells (with 650-pA injected current mean firing rate was 431 ± 55 Hz, range 315–560 Hz; n = 47 cells across subtypes) was at the high end of reported maximum firing rates in several classes of cells in brain slices that exhibit rapid spiking including hippocampal and cortical fast-spiking interneurons (McCormick et al. 1985), vestibular nucleus neurons (Bagnall et al. 2007), and chicken cochlear nucleus neurons (Fukui and Ohmori 2003). In these other cell types, fast-spiking phenotypes have been shown to rely upon expression of Kv3 family K⁺ channels (Erisir et al. 1999; Fukui and Ohmori 2003; Gittis et al. 2010; Lien and Jonas 2003). Similar to other fast-spiking neurons, vertical cell APs were narrow (half-width ~230 μs) and repolarized rapidly, consistent with an important role for K⁺ conductances in shaping spike output.

A particularly striking feature of most vertical cells was the ability to sustain very high firing rates (up to 400–500 Hz) throughout the duration of depolarizing current steps. In fact, in several cells, particularly those in which initial spike rates were less than ~250 Hz, spike rates were higher at the end compared with the beginning of a 300-pA current step. Even cells with high initial spike rates often showed some acceleration of instantaneous spike frequencies after an initial drop in frequency at the start of current steps (see especially individual traces for “plateau and rebound spiking” cells in Fig. 4B). The mechanisms contributing to the maintenance (or acceleration) of high firing rates in vertical cells were not explored in this study, but it is possible that the same conductances underlying the generation of plateau potentials in some vertical cells might also contribute to sustained high-frequency firing by providing a depolarizing drive during excitatory input.

**Synaptic properties of vertical cells.** Excitatory synaptic inputs to vertical cells, thought to arise from auditory nerve fibers and possibly axons of T-stellate cells of the ventral cochlear nucleus (Albardi 2006; Rubio and Juiz 2004; Zhang and Oertel 1993b), exhibited short-term depression in response to repetitive stimulation. This is consistent with the dynamics of auditory inputs to fusiform neurons (Irie and Ohmori 2008) and contrasts with strong short-term facilitation of parallel fiber synapses in the DCN molecular layer (Roberts and Trussell 2010; Kuo and Trussell 2011). Interestingly, on average, the depression of EPSC amplitudes in vertical cells was similar across a 10-fold range of stimulus frequencies (20–200 Hz). Although this in part reflects cell-to-cell variability in the extent of depression of excitatory inputs at the different stimulus frequencies, the lack of difference in synaptic depression at low or high input frequencies was observed for approximately half of the cells examined (see Fig. 5C). Frequency-independent synaptic transmission has recently been described at vestibular nerve synapses in the vestibular nuclei (Bagnall et al. 2008) and at vestibular mossy fiber synapses onto granule cells in the cerebellum (Arenz et al. 2008), where frequency-independent transmission is proposed to support linear vestibular behaviors. Because changes in sound intensity are encoded as increases or decreases in the spike rate of auditory afferents, the apparent frequency independence of transmission of at least some auditory fibers onto vertical cells may preserve information regarding the intensity of auditory stimuli.

To our knowledge, this study is the first to directly examine vertical cell synaptic input to fusiform neurons. Paired whole cell recordings demonstrated that vertical cells made synapses onto fusiform cells with a moderate connection probability, but we were surprised to find that vertical cell-mediated unitary currents recorded from fusiform cells were usually small in amplitude, particularly compared with uIPSCs mediated by molecular layer cartwheel interneurons. Furthermore, current-clamp recordings indicated that activity in a single presynaptic vertical cell is not usually sufficient to affect fusiform cell spike output. Instead, our experiments suggest that activity in multiple vertical cells must be coordinated to inhibit fusiform cell spiking.

Using dual recordings, we also discovered that vertical cells synapse upon other nearby vertical cells (within ~100 μm) with high connection probability. Together with the low frequency of spontaneous inhibitory currents observed in vertical cells, which indicates that vertical cells do not receive significant input from inhibitory neurons that are spontaneously active in DCN slices, most prominently cartwheel cells (Kim and Trussell 2007; Kuo and Trussell 2011), this high connection probability suggests that a major source of inhibition to vertical cells comes from other vertical cells. Thus vertical cell-mediated inhibition may underlie the inhibitory responses to non-best-frequency tones (inhibitory sidebands) recorded from vertical cells in vivo and could therefore potentially contribute to the narrow frequency tuning of vertical cells (Rhode 1999; Spirou et al. 1999). However, whether connected vertical cells are usually tuned to different characteristic frequencies remains to be examined. It seems unlikely that vertical cell-mediated inhibition could underlie the lack of vertical cell responses to broadband noise, which has been suggested to arise from inhibition arising from “onset chopper” units that respond robustly to broadband sounds (Nelken and Young 1994; Young and Davis 2002), and which are thought to be
D-stellate neurons of the ventral cochlear nucleus (Smith and Rhode 1989).

Implications for vertical cell function. Although heterogeneous, the intrinsic properties of vertical cells do not appear to account for the various tone-evoked response patterns observed in vivo, which include spiking responses classified according to the shapes of tone-evoked peristimulus time histograms as chopper (regular discharge pattern sustained throughout tone presentation), onset (spiking at tone onset), and “unusual” responses with elements of both onset and chopper responses (Shofner and Young 1985). Rather, the regular, sustained firing responses in response to depolarizing current steps observed in all vertical cells are most consistent with the chopper response type. An interesting possibility is that inhibition provided by other vertical cells contributes to the onset and “unusual” spiking phenotypes observed in some vertical cells.

Together, vertical cell intrinsic spiking properties and excitatory synaptic inputs to vertical cells appear suited to support enhanced vertical cell activity in response to increasing rates of auditory input. Indeed, vertical cells recorded in vivo usually exhibit increasing firing rates in response to best-frequency tones presented at increasing sound intensities for moderate sound levels (Joris 1998; Rhode 1999; Shofner and Young 1985; Spirou et al. 1999). This sensitivity to sound level has been hypothesized to contribute to the nonlinear in vivo response properties of principal neurons, which exhibit predominantly inhibitory responses to moderate- and high-intensity sounds (Young and Davis 2002). However, our data indicate that vertical cell-mediated inhibition in mouse DCN is not as robust as previously hypothesized from in vivo cat studies. In slice preparations, truncation of axonal arbors might contribute to a reduction in synaptic strength. While this may still be a possibility in our recordings, we found that a significant increase in slice thickness had no effect on uIPSC conductance. One possibility is that the strongly inhibitory responses seen in some principal neurons are due to tone-evoked activity in a population of vertical cells converging on those principal neurons. Another possibility is that vertical cell-mediated inhibition plays a more subtle or different role in shaping principal cell output in DCN in mouse compared with cat. In support of differing roles for inhibition across species, in vivo studies using awake or decerebrate rodent preparations have demonstrated that gerbil and mouse principal neurons receive less frequent or evoked inhibition compared with principal cells in cat DCN (mouse: Ma and Brenowitz 2011; Roberts and Portfors 2008; gerbil: Davis et al. 1996; Navawongse and Voigt 2009). In addition to targeting cells locally within the DCN, vertical cells also project axon collaterals into the ipsilateral VCN (Lorente de No 1981; Wickesberg et al. 1991; Zhang and Oertel 1993b) and likely provide inhibitory input to T-stellate and bushy cells (Wickesberg and Oertel 1990). Whether vertical cell synapses in the VCN have functional properties similar to those we have shown for the feedforward inhibitory function of vertical cells within the DCN remains to be examined.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


