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

Sidney P. Kuo, Hsin-Wei Lu, and Laurence O. Trussell

Neuroscience Graduate Program and Oregon Hearing Research Center and Vollum Institute, Oregon Health and Science University, Portland, OR 97239

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Corresponding Author:

Laurence O. Trussell
Oregon Health and Science University
3181 S.W. Sam Jackson Park Road
Mail code L335A
Portland, OR 97239
trussell@ohsu.edu

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Abstract

Multiple classes of inhibitory interneuron shape the activity of principal neurons of the dorsal cochlear nucleus (DCN), a primary target of auditory nerve fibers in the mammalian brainstem. Feed-forward inhibition mediated by glycinergic 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 glycinergic neurons express 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 spiking rates. Using paired recordings, 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.
Introduction

The dorsal cochlear nucleus (DCN) is a primary target of auditory nerve fibers in the mammalian auditory brainstem. Although the function of the DCN in auditory processing is not fully understood, the observation that DCN principal neurons are sensitive to sounds with complex spectral features has led to the hypothesis that the DCN contributes to the analysis of the spectral content of acoustic information. In particular, DCN principal neurons are proposed to detect spectral features of sounds that vary according to the position of sound sources with respect to the ears and therefore contribute to sound localization (Oertel and Young, 2004; Young and Davis, 2002).

Sensitivity to spectral information is thought to arise from the interaction of excitatory and inhibitory signals within the deep layer of the DCN, where auditory nerve fibers form glutamatergic synapses upon basal dendrites of principal cells as well as glycinergic interneurons called vertical cells (Rubio and Juiz, 2004). Evidence from in vivo electrophysiological recordings (Davis and Young, 2000; Nelken and Young, 1994; Rhode, 1999; Spirou et al., 1999; Voigt and Young, 1980; Voigt and Young, 1990) and anatomical work (Rhode, 1999) indicates vertical cells provide feed-forward inhibition that shapes the sound-evoked response properties of principal neurons. In fact, in some in vivo preparations, particularly decerebrate cats, principal neurons exhibit predominantly inhibitory responses to moderate and high intensity tones (“Type IV” units in the response map classification scheme (Young and Davis, 2002)), which has been attributed to vertical cell-mediated inhibition (Davis and Young, 2000; Spirou et al., 1999; Spirou and Young, 1991; Voigt and Young, 1990). Vertical cells are therefore hypothesized to contribute critically to the spectral analysis function of principal cells (Young and Davis,
However, the cellular and synaptic properties that determine how vertical cells influence DCN output are not well understood. We made use of transgenic mice in which glycinergic neurons express enhanced green fluorescent protein (EGFP) to target patch-clamp recordings of vertical cells in brainstem slices. We found previously unappreciated heterogeneity of intrinsic action potential firing properties in vertical cells. Additionally, paired recordings directly demonstrated functional synaptic connections between vertical cells and fusiform neurons, a primary type of principal neuron, as well as other vertical cells. However, postsynaptic targets received unexpectedly weak input from vertical cells and our experiments suggest activation of multiple vertical cells is necessary to influence the spiking output of fusiform cells.

Methods

Slice preparation

All animal care and handling procedures used in this study were approved by the OHSU 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 of P31-33 were used. To make slices, mice were deeply anesthetized using isofluorane, then killed by decapitation. After removing the skull to expose the
brain, the brainstem was isolated by making a coronal cut just rostral to the cerebellum. A sagittal cut was then made down the midline of the brainstem, and one half of the brainstem was removed from the skull and glued cut side-down to the stage of a vibratome (Leica VT1200S). The ventral side of the brainstem was turned to face the vibratome blade and was angled slightly downwards (~6° angle with horizontal plane).

During dissection and slicing, tissue was kept immersed in warm (~34°C) ACSF containing (in mM): 130 NaCl, 2.1 KCl, 1.7 CaCl₂, 1.0 MgSO₄, 1.2 KH₂PO₄, 20 NaHCO₃, 3 Na-HEPES, 11 glucose; saturated with 5% CO₂/ 95% O₂, ~300 mOsm.

Following slicing, slices were allowed to recover in 34°C ACSF for one hour, then either transferred to a recording chamber or maintained in ACSF at room temperature (~22°C) until use.

**Electrophysiology**

During recordings, slices were constantly perfused with ACSF (1-2 mL/min; chamber volume ~1.6 ml) maintained at 33 ± 1°C using an in-line heater (Warner Instruments). Cells were visualized using a 60X 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 using 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 based on location within the deep layer and somatic morphology (round or oval) and size (~10-15 μm).
diameter, see Figure 1B), even in wild-type tissue. Fusiform neurons were easily identified based upon location within the slice, lack of GFP expression in GlyT2-EGFP tissue, larger somatic size in comparison to 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 using 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 (WPI) and filled with a solution containing (in mM): 113 K+-Gluconate, 2.75 MgCl₂, 1.75 MgSO₄, 0.1 EGTA, 14 Tris₂-phosphocreatine, 4 Na₂-ATP, 0.3 Tris-GTP, 9 HEPES; osmolarity adjusted to ~290 mOsm with sucrose, pH adjusted to 7.25 with KOH. In some recordings, biocytin (0.3% weight/volume) was added to the internal solution. The red fluorescent dye AlexaFluor 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 gramicidin perforated-patch recordings established a reversal potential for glycine-elicited currents 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⁺-gluconate-based internal solution was supplemented with 20 mM glycine. Connections between cells recorded with the glycine-containing internal solution (3 total connections, one unidirectional, one reciprocal among three tested pairs
(six tested connections)) were included in the calculation of connection probability, but were not included in the reported measurements of unitary IPSC properties. In two of the vertical cell and fusiform cell pairs, the postsynaptic fusiform cell was recorded using an internal solution containing (in mM): 108 CsMeSO₃, 5 CsCl, 1 MgCl₂, 4 Mg-ATP, 0.4 Tris-GTP, 14 Tris₂-phosphocreatine, 5 EGTA, 10 HEPES, 3 QX-314, pH adjusted to 7.3 with CsOH, 290 mOsm. For recordings of unitary IPSCs (uIPSCs), postsynaptic cells were held at -60 mV, with the exception of the two recordings using the CsMeSO₃-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 (10-30V, 150-200 μsec) 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% (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 (Figure 8).

Electrophysiological data acquisition and analysis
Recordings were acquired using a Multiclamp 700B amplifier and pClamp 10 software (Molecular Devices). Signals were digitized at 50 kHz using 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 (τ_{membrane}) and input resistance (R_{input}) were measured in current-clamp 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 (V_{rest}) (no bias current injection). R_{input} was determined by measuring the voltage change from resting V_{rest} for the last 50 ms of current step and calculated using Ohm’s law. τ_{membrane} was measured by fitting a monoexponential function to the initial voltage response to the -20 pA current step. Action potential (AP) shape measurements were determined from responses to 1 ms suprathreshold current steps (typically 1 nA). AP threshold was defined as the first peak of the 3rd time-based derivative of the membrane voltage (V_{m}) before AP peak (Henze and Buzsaki, 2001). AP height was defined as the difference between action potential peak and threshold. AP half-width was defined as the width of the action potential at V_{m} halfway between peak and threshold. Mean spike frequency versus current injection relationships (Figure 3C) were fitted with Hill functions of the form: 

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

where I is the injected current, F_{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 versus 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 EPSCs and unitary IPSCs were determined by fitting biexponential or single exponential functions to the decay phase of currents, respectively. Spontaneous EPSCs were detected using 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 action potentials and current at which uIPSCs had achieved 20% of their peak amplitude. Unitary IPSC latencies and decay time constants 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. Conductance 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. Unitary IPSC 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 three 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 mean ± standard deviation (S.D.). Statistical significance (p< 0.05) was determined by ANOVA followed by Fischer’s PLSD post-hoc tests unless noted otherwise.

Biocytin labeling

Following 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 permeabilizing fixed tissue in 0.2% Triton-X100 solution (in PBS) for one hour at
room temperature, slices were incubated in a PBS solution containing Alex Fluor 568-conjugated steptravidin (1:2500 dilution; Invitrogen) overnight at 4°C, then rinsed and mounted on glass slides. Slices were dehydrated in an ascending series of alcohols, delipidized in xylene, then rehydrated and coverslipped using Fluoromount G (Southern Biotech). Fluorescence images were acquired using a confocal microscope (Olympus FV1000) by sequential scanning of GFP and Alexa Fluor 568 channels using a 40X 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 brainstem slices prepared from GlyT2-EGFP transgenic mice (Figure 1A-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 (Figure 1A) (Zeilhofer 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 solutions. 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 (Figure 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, action potentials elicited by depolarizing current steps were short in duration and could exhibit biphasic afterhyperpolarizations (Figure 1C), termed ‘double undershoots’ by Oertel and colleagues (Zhang and Oertel, 1993b), and spontaneous excitatory postsynaptic currents (EPSCs) recorded from GFP(+) neurons displayed rapid decay kinetics (Figure 1D; \( \tau_{\text{fast}} = 0.27 \pm 0.09 \text{ ms}, 77.81 \pm 10.74\% \text{ of decay}, \tau_{\text{slow}} = 1.65 \pm 0.41 \text{ ms}; n=5 \) (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 (Figure 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.

Heterogeneous spiking behavior in vertical cells

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 WT cells confirmed to be the same as cells from GlyT2-GFP mice by visualization of fluorescence) were also acquired in 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 action potential firing responses
to hyperpolarizing and depolarizing somatic current steps (200 ms duration) that we
classified into four general categories (Figure 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 following the offset of
depolarizing current steps (Figure 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; Figure 2B). In most cases, plateau
depolarizations could support action potential firing beyond the offset of current steps.
Another group of cells (9.0%; 11/122) exhibited plateau depolarizations but no rebound
spiking (“plateau” cells; Figure 2C). Finally, 12.3% of cells (15/122) did not exhibit
either plateau potentials or rebound spiking (“no plateau or rebound spiking” cells; Figure
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 to “rebound spiking” cells. No significant differences were
observed between groups for measurements (see Methods for details) of cell input
resistance or membrane time constant.

Action potential 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 action potential 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 3A and 3B 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 Figure 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 50-400 pA compared to “no plateau no rebound” cells or “rebound spiking”
cells, and for currents between 50-250 pA compared to “plateau” cells. All other
comparisons between groups were not significant with the exception of higher firing rates
in “rebound spiking” cells compared to “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 versus injected current relationships (Figure 3C) (see Methods)
(Silver 2010), were similar across the different subtypes, but with slightly higher gain in
“plateau and rebound spiking” cells (“rebound spiking” 0.88 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 compared instantaneous spike frequencies for
APs during identical, 300-pA current steps across different vertical cell categories (Figure
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 as compared to
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 to 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) (Figure 4E). In response to 300 pA steps, cells with
initial spike rates of \(<\sim 250\ \text{Hz}\) tended to exhibit spike frequency acceleration whereas those with initial rates \(>250\ \text{Hz}\) generally exhibited little change in spike frequency over the course of 200 ms current steps (Figure 4F).

**Short-term depression of excitatory inputs**

Excitatory inputs to vertical cells were evaluated by making voltage-clamp recordings 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 \(\mu\text{M}\) SR-95531 and 0.5 \(\mu\text{M}\) strychnine in the bath solution and NMDA receptors were blocked by including 50 \(\mu\text{M}\) 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 \(\mu\text{M}\); 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 (Figure 5A-B). However, in these cells, depression at the end of stimulus trains ranged between 80%-20% of initial EPSC amplitudes (see Figure 5C). In several cells (see Figure 5C), EPSCs at the end of a ten stimuli train were depressed to similar levels across a ten-fold range in stimulation frequencies (200 Hz EPSC10/EPSC1 vs. 20 Hz EPSC10/EPSC1 ratio >0.75 in 6/11 cells) so that the average depression of EPSCs was constant across stimulus frequencies in the eleven vertical cells with depressing excitatory synapses (Figure 5B-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.
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; Voigt and Young, 1990). We therefore directly tested for functional synaptic connections between vertical cells and fusiform neurons using dual whole-cell recordings.

Fusiform cells were identified based on their location within the fusiform cell layer, large, bipolar somata (see Figure 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 establishing a recording from a fusiform cell, 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 action potentials 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 ten 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”, 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 following 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 action potentials 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 pA. 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 cat suggests 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 action potentials, uIPSCs in fusiform cells usually exhibited moderate short-term facilitation (Figure 6B-C), suggesting a low initial release probability from vertical synapses. The mild facilitation of vertical cell-mediated synaptic currents indicates 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 out of 36 tested vertical cell pairs, with a bidirectional connection observed in one pair. We tested for connections in both directions in all but one 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”, one was a “plateau”, and one was a “no plateau or rebound spiking” cell. We characterized the spiking
behavior of the postsynaptic vertical cell in twelve 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.

Unitary IPSCs recorded in vertical cells were similar to those observed in fusiform cells in peak amplitude, decay kinetics, latency and failure rate (Tables 3, 4). However, uIPSCs elicited by presynaptic spiking were more clearly distinguishable from baseline currents because vertical cells received very little spontaneous IPSC input (see Figure 7B). The lack of spontaneous inhibitory input suggests 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 (Figure 7C).

Given previous in vivo work suggesting vertical cells provide robust inhibitory input to principal neurons, we were surprised to find that vertical cell-mediated uIPSCs were relatively weak, especially in comparison to 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 glycineergic transmission was potentially compromised in tissue from GlyT2-EGFP transgenic mice due to transgene expression. However, uIPSCs between vertical cells were not different in paired recordings from wild-type animals compared to those from
GlyT2-EGFP mice (peak conductance $2.2 \pm 3.0$ nS vs $1.6 \pm 1.7$ nS, respectively; $p=0.6$, unpaired t-test; wildtype $n=6$ pairs, transgenic $n=8$ pairs). Arguing against a reduction in neuronal glycine content over time during paired recordings, we did not notice systematic differences for connection probability or uIPSC amplitudes in relation to times when paired recordings were made after tissue preparation (up to ~7 hours post-slicing).

Unitary IPSCs were also stable for up to ~1 hour in recordings between connected neurons. Further, 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 ($1.5 \pm 0.78$ 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.

We also considered whether the slicing procedure led to artificially small uIPSCs, either due to severing of vertical cell axons or to a potential time-dependent decline in neuronal glycine content previously observed in acutely prepared DCN slices (Wickesberg et al., 1994). To address whether the slice procedure reduced uIPSC amplitudes by damaging vertical cell axons, we compared IPSCs evoked using a minimal stimulation procedure between vertical cells recorded in 210 $\mu$m slices (the thickness used in the paired recordings) and 310 $\mu$m 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 Figure 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 uIPSC amplitudes were not artificially small due to 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 experiments. However, minimal stimulation-evoked IPSCs recorded in vertical cells were not significantly different in tissue from P31-33 mice compared to those from P16-23 animals (p= 0.4, unpaired t-test) (Figure 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 ~4 out of 5 identical current injections (620-680 pA each, 20 Hz) while the presynaptic vertical cell was resting below spike threshold (Figure 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 (Figure 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 (Figure 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 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 Figure 9D-E, inset). In three cells, SR95531 (10 μM) was included in the bath
solution while another three recordings did not include SR95531. Stimulus position and
strength were adjusted to evoke detectable IPSPs in current-clamped fusiform cells
(Figure 9E, inset). When currents were recorded in voltage-clamp, the same stimuli
evoked IPSCs with ~100 pA peak amplitude (average value), which should correspond to
activity in ~3-5 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 100 Hz starting 10 ms prior
to somatic current injections in fusiform cells) significantly reduced fusiform cell spiking
in comparison to 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 subcategory, the duration of rebound or plateau activity varied from ~20 ms to several seconds beyond the end of current injection. Further, vertical cells could not generally be distinguished from each other based upon intrinsic membrane properties or action potential 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 to 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 non-specific 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 non-specific cation channel antagonist flufenamic acid (100 μM) reduced plateau potentials in vertical cells (not shown), spiking during steps was also severely
affected, likely due to non-specific actions of flufenamic acid (Ottolia 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, 1993). The maximum firing rates of vertical cells (with 650 pA injected current mean firing rate was 431 ± 55 Hz, range 315 to 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 action potentials 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 ≤~250 Hz, spike rates were higher at the end compared to 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 Figure 4A). 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 (Zhang and Oertel, 1993; Rubio and Juiz, 2004; Alibardi, 2006), 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 ten-fold range of stimulus frequencies (20 to 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 Figure 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 in comparison to uIPSCs mediated by molecular layer cartwheel interneurons. Further, 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 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 non-linear 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 unitary IPSC 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 mouse DCN compared to 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 best frequency tone-evoked inhibition compared to principal cells in cat DCN (mouse: Ma and Brenowitz, 2011; Portfors and Roberts, 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 (Wickesburg and Oertel, 1990). Whether vertical cell synapses in the VCN have functional properties similar to those we have shown for the feed-forward inhibitory function of vertical cells within the DCN remains to be examined.
Figure Legends

Figure 1. Vertical cells express 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 (left, top), one cartwheel cell (left, lower), and two vertical cells (lower 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 due to diffusion of the pipette solution into the cells). The fusiform cell did not express GFP. Most of the 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 -68 mV. D. Averaged spontaneous 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).
Figure 2. Heterogeneous spiking responses in vertical cells. A-D. Example responses to hyperpolarizing (-250 pA, gray trace) and depolarizing (+500 pA, black trace) current injections (200 ms duration, see bottom of (D)) in cells classified as (A) “rebound spiking” (66/122 recorded vertical cells), $V_{\text{rest}} = -67 \text{ mV}$; (B) “plateau and rebound spiking” (30/122), $V_{\text{rest}} = -71 \text{ mV}$; (C) “plateau” (11/122), $V_{\text{rest}} = -77 \text{ mV}$; (D) “no plateau or rebound spiking” (15/122), $V_{\text{rest}} = -73 \text{ mV}$.

Figure 3. Input-output relationships in vertical cells. A. Instantaneous spike frequencies between consecutive APs during 200 ms depolarizing current injections from +50 pA (bottom line) to +650 pA (topmost line) (50 pA increments). Current injection started at time zero 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 ± S.D. of mean spike frequencies for “rebound spiking” (circles, n= 21), “plateau and rebound spiking” (squares, n= 12), “plateau” (diamonds, n= 7) and “no plateau or rebound spiking” (triangles, n= 9) cells. Lines are Hill function fits to the mean values for each subtype. Maximal firing rates ($F_{\text{max}}$), half saturation values ($I_{1/2}$) and the exponent (n) from the fits (± S.D.) were $F_{\text{max}} = 582.48 \pm 49.8$, $I_{1/2} = 339.18 \pm 46.3$, n = 1.43 ± 0.098 for “rebound spiking”; $F_{\text{max}} = 572.56 \pm 129$, $I_{1/2} = 239.31 \pm 96.5$, n = 1.36 ± 0.45 for “plateau and rebound spiking”; $F_{\text{max}} = 520.9 \pm 151$, $I_{1/2} = 275.64 \pm 107$, n = 1.79 ± 0.098.
Figure 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 mean ± S.D. F. Plot of adaptation index vs. first instantaneous spike frequency for data in (A-D).

Figure 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 compared to the first stimulus-evoked EPSC 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 mean ± S.D. C. EPSC10/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 ± S.D. p= 0.08, paired t-test. All recordings for data in A-C were acquired with 50 μM D-APV, 0.5 μM strychnine, and 10 μM SR95531 in the bath solutions.
Figure 6. Paired recordings between vertical cells and fusiform neurons. A. Schematic of recording configuration. In 11 out of 91 tested pairs, spiking in presynaptic vertical cells elicited detectable unitary IPSCs in postsynaptic fusiform neurons. B. Example time-locked traces recorded simultaneously from a presynaptic vertical cell recorded in current-clamp mode (top, single sweep) and a postsynaptic fusiform cell held in voltage-clamp at -60 mV (bottom; gray traces are ten superimposed current sweeps, black trace is average of 20 sweeps). APs were elicited in the presynaptic vertical cell by 1 ms suprathreshold current injections applied ten times at 100 Hz (15 sec between trials). Presynaptic $V_{\text{rest}} = -75$ mV. C. Ratios of peak uIPSC amplitudes recorded in postsynaptic fusiform cells for each presynaptic vertical cell AP in a 100 Hz train compared to the first AP. Gray lines are data from individual cells. Black circles with error bars are mean ± S.D. (n= 7 pairs).

Figure 7. Paired recordings between vertical cells. A. Schematic of recording configuration. Unidirectional connections were found in 15 out of 36 tested pairs and one pair was connected in both directions (reciprocal connection). In all but one pair, synapses were tested in both directions, yielding a connection probability of 23.9% (17 out of 71 tested connections). B. Example time-locked traces recorded simultaneously from a presynaptic vertical cell recorded in current-clamp mode (top, single sweep) and a postsynaptic vertical cell held in voltage-clamp at -60 mV (bottom; gray traces are ten superimposed current sweeps, black trace is average of the ten individual sweeps). APs were elicited in the presynaptic vertical cell by 1 ms suprathreshold current injections.
applied ten times at 100 Hz (15 sec between trials). Presynaptic $V_{\text{rest}} = -69 \text{ mV}$. C. Ratios of peak uIPSC amplitudes recorded in postsynaptic cells for each presynaptic AP in a 100 Hz train compared to the first AP. Gray lines are data from individual cells. Black circles with error bars are mean ± S.D. (n= 10 pairs).

Figure 8. Comparison of minimal stimulation-evoked IPSCs between different slice thicknesses and tissue ages. A, Example traces of minimal stimulation protocol evoked IPSCs in a vertical cell in a 210 μm slice. Statistics for evoked IPSCs in this connection: 12 successes, 18 failures, 30 trials total. B, Average peak amplitudes of the successful events obtained in control (40.66±19.57 pA, n = 7), thick (46.84±12.59 pA, n = 7), and old (51.07±19.72 pA, n = 6) slices. Means are ± S.D. No significant differences were found when comparing control versus thick (p=0.50, unpaired t test) or old (p=0.36, unpaired t test) groups.

Figure 9. Effect of vertical cell-mediated inhibition on fusiform cell spike output. A- B. Example traces from a simultaneous recording acquired from a synaptically connected vertical cell and fusiform cell pair (see inset) in which spikes were evoked by just-suprathreshold current injection (“test injections”, arrows) in the postsynaptic fusiform cell either when the presynaptic vertical cell was at resting $V_m$ and did not fire spikes (control; example traces shown in (A)), or when a train of suprathreshold current steps (1 ms each) were applied to presynaptic vertical cells to elicit 50 APs at 100 Hz starting 50 ms prior to test current injections into the fusiform cell (vertical spiking; example traces in (B)). Sweeps with presynaptic vertical cell spiking were interleaved with control
sweeps in which vertical cells were silent. Identical test injections into the postsynaptic fusiform cell were used for both conditions and were constant throughout the experiment.

Presynaptic $V_{\text{rest}} = -79$ mV, postsynaptic $V_{\text{rest}} = -67$ mV. C. Summary of paired recording experiments as shown in (A-B). Spike probability values were determined for 25-30 trials in each condition and were calculated by dividing the number of observed spikes by the number of test injections. Gray circles connected by lines represent mean spike probabilities in individual pairs under the different experimental conditions. Black circles with error bars show mean ± S.D. for all tested pairs (n= 6 pairs). D-E. Example voltage traces in a fusiform cell ($V_{\text{rest}} = -65$ mV) in which just-suprathreshold current injections (arrows) were used to elicit spikes without (D) or with extracellular stimulation of inhibitory fibers starting 10 ms prior to test injections (E). Inset between (D-E) shows recording configuration. Gray inset in E is expanded region of voltage trace showing first four extracellular stimuli and first intracellular current injection. Note small (~2 mV) IPSPs following stimulus artifacts. Control and stimulus conditions were interleaved. F. Summary of experiments as in (D-E). For each cell, mean spike probabilities were determined from 30 sweeps in each condition (gray circles). Black circles with error bars are mean ± S.D for the 6 cells. For data shown in (F), three cells were recorded with and three cells were recorded without SR95531 (10 μM) in the bath solution. All recordings in (A-F) were acquired with D-APV (50 μM) and NBQX (10 μM) in the bath solutions.
REFERENCES


Table 1. Intrinsic membrane properties of vertical cells

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All comparisons between vertical cell subtypes not statistically significant except Vrest of "rebound spiking" and "no plateau or rebound spiking" cells ($p<0.01$)

Table 2. Action potential properties of vertical cells

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No statistically significant differences between vertical cell subtypes
### Table 3. Properties of vertical cell unitary IPSCs recorded from fusiform cells

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### Table 4. Properties of vertical cell unitary IPSCs recorded from vertical cells

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<tbody>
<tr>
<td>peak conductance (nS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>including failures</td>
<td>14</td>
<td>1.8</td>
<td>2.3</td>
<td>0.3 to 7.8</td>
</tr>
<tr>
<td>without failures</td>
<td>14</td>
<td>2.4</td>
<td>2.1</td>
<td>0.5 to 7.8</td>
</tr>
<tr>
<td>failure rate (%)</td>
<td>14</td>
<td>37.7</td>
<td>30.8</td>
<td>0 to 80</td>
</tr>
<tr>
<td>latency (ms)</td>
<td>14</td>
<td>0.58</td>
<td>0.15</td>
<td>0.33 to 0.84</td>
</tr>
<tr>
<td>τ decay (ms)</td>
<td>14</td>
<td>1.31</td>
<td>0.29</td>
<td>0.87 to 2.07</td>
</tr>
</tbody>
</table>

### Table 5. Properties of minimal stimulation IPSCs recorded from vertical cells, comparing slice thickness and animal age

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean</th>
<th>S.D.</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>peak conductance (nS)</td>
<td>210 μm young / 310 μm young / 210 μm old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>including failures</td>
<td>7 / 7 / 6</td>
<td>0.8 / 0.9 / 0.9</td>
<td>0.3 / 0.3 / 0.4</td>
<td>0.5 to 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 to 3.2 / 1.4-2.9 / 1.1-3.2</td>
</tr>
<tr>
<td>failure rate (%)</td>
<td>7 / 7 / 6</td>
<td>58.7 / 60.0 / 64.6</td>
<td>8.8 / 15.2 / 10.7</td>
<td>47 to 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*
Figure 1

A. Medial-dorsal region showing a high-magnification view.

B. Neuron image with scale bar of 10 μm.

C. Voltage traces showing 10 mV and 5 ms.

D. Current traces with time constants: τ_slow = 1.43 ms (27.3%) and τ_fast = 0.29 ms (72.7%).
Figure 2

A
"rebound spiking"

B
"plateau and rebound spiking"

C
"plateau"

D
"no plateau or rebound spiking"

+500 pA
-250 pA
Figure 3

A

B

C

inst. spike freq. (Hz)

inst. spike freq. (Hz)

mean spike freq. (Hz)

injected current (pA)
Figure 4

A) "rebound spiking"

B) "plateau and rebound spiking"

C) "plateau"

D) "no plateau or rebound spiking"

E) Adaptation index

F) 1st instantaneous spike frequency (Hz)
Figure 5

A

20 Hz

50 ms

10 ms

50 Hz

200 Hz

20 Hz

100 Hz

200 Hz

100 pA

B

EPSC(n)/EPSC1

20 Hz

50 Hz

100 Hz

200 Hz

1.0

0.8

0.6

0.4

0.2

0.0

EPSC number

C

n.s.

EPSC10/EPSC1

20 Hz

200 Hz
Figure 6

A
12.1% connection probability
pre-vertical
post-fusiform

B
pre
post

C
uIPSC(uIPSC)/uIPSC
0 1 2 3 4
1 2 3 4 5 6 7 8 9 10
uIPSC number
5 ms 10 pA

Figure 6
Figure 7

A

pre

23.9% connection probability

post

pre-vertical

post-vertical

B

pre

20 mV

post

20 pA

5 ms

C

uIPSC(n)/uIPSC1

uIPSC number
Figure 8
Figure 9

A

pre-vertical
post-fusiform

post

20 mV
50 ms

B

50 ms
12.5 ms

20 mV
5 mV

D

E

F

+/- stim

DF

pre-vertical post-fusiform

control
vertical spiking

control
stim

spike probability

***

*