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Detailed Terms
Response Features of Parvalbumin-Expressing Interneurons Suggest Precise Roles for Subtypes of Inhibition in Visual Cortex

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SUMMARY
Inhibitory interneurons in the cerebral cortex include a vast array of subtypes, varying in their molecular signatures, electrophysiological properties, and connectivity patterns. This diversity suggests that individual inhibitory classes have unique roles in cortical circuits; however, their characterization to date has been limited to broad classifications including many subtypes. We used the Cre/LoxP system, specifically labeling parvalbumin (PV)-expressing interneurons in visual cortex of PV-Cre mice with red fluorescent protein (RFP), followed by targeted loose-patch recordings and two-photon imaging of calcium responses in vivo to characterize the visual receptive field properties of these cells. Despite their relative molecular and morphological homogeneity, we find that PV+ neurons have a diversity of feature-specific visual responses that include sharp orientation and direction-selectivity, small receptive fields, and band-pass spatial frequency tuning. These results suggest that subsets of parvalbumin interneurons are components of specific cortical networks and that perisomatic inhibition contributes to the generation of precise response properties.

INTRODUCTION
The balance between excitation and inhibition is critical for normal brain development and function. Indeed, disruptions in this balance are associated with a variety of brain disorders, including autism and schizophrenia (Hensch, 2005; Lewis et al., 2005; Rubenstein and Merzenich, 2003). Intracortical inhibition is thought to be important not only for maintaining an appropriate dynamic range of cortical excitation, but also for shaping the response properties of cells and circuits in sensory cortices (Ferster and Miller, 2000; Marín et al., 2005; Via et al., 2003; Poo and Isaacson, 2009; Wehr and Zador, 2003; Zhang et al., 2003). The precise ways in which this is achieved remain unclear, however.

The elucidation of the roles for inhibition in cortical function is complicated by the vast diversity of inhibitory cell types. These cells can be distinguished based on their electrophysiological profiles, their morphologies, and their molecular signatures, suggesting that individual inhibitory cell classes may provide specific forms of inhibition and thus subserve unique functions (Burkhalter, 2008; Markram et al., 2004). For instance, the axons of some inhibitory neuron subtypes, such as calretinin- and somatostatin-positive cells, preferentially target neuron dendrites, while axons of others, such as parvalbumin-positive (PV+) basket cells and chandelier cells target the soma and axon initial segment, respectively (DeFelipe, 1997; DeFelipe et al., 1999; Kisvárday and Eysel, 1993; Markram et al., 2004). PV+ cells thus represent a distinct morphological subclass of inhibitory neurons, which are in an ideal position to efficiently suppress the output of their synaptic partners, while dendrite-targeting cells may have more subtle effects on neuronal responses and computations. Based on their wide dendritic geometry and extensive lateral axonal arbors (Kisvárday and Eysel, 1993; Stepanyants et al., 2009; Wang et al., 2002) and role in driving cortical synchrony (Cardin et al., 2009), a reasonable hypothesis is that PV+ cells have large integration fields resulting in relatively unselective responses that act generally to balance excitation. In contrast, based on their radial geometry and intracolumnar connectivity, dendrite-targeting interneurons may have small integration fields with feature-selective responses.

An ideal system for dissecting cell-specific roles in cortical information processing is the primary visual cortex (V1), where clear signatures of circuitry such as orientation and spatial frequency tuning arise and can be used to probe the function of specific cell types in vivo. A number of studies have addressed the general role of inhibition in orientation tuning by either measuring the net inhibition impinging on excitatory cells (Andersen et al., 2000; Ferster, 1986; Ferster et al., 1996; Marín et al., 2005; Monier et al., 2003) or by manipulating inhibition pharmacologically (Nelson et al., 1994; Sillito, 1975) or electrically (Chung and Ferster, 1998; Ferster et al., 1996). However, to
unravel the precise contributions of inhibitory interneurons and understand the specific roles played by different inhibitory cell types, direct measurement of the tuning properties of each cell type is necessary.

Physiologically identified fast-spiking cells or genetically identified inhibitory interneurons have been characterized in the visual cortex of cats, rabbits, and rodents, but direct measurements of the receptive field selectivity of presumed or confirmed inhibitory neurons have yielded somewhat conflicting results (Cardin et al., 2007; Hirsch et al., 2003; Liu et al., 2009; Niell and Stryker, 2008; Nowak et al., 2008; Sohya et al., 2007; Swadlow, 2003). Importantly, although fast-spiking behavior has been closely associated with PV+ inhibitory interneurons, the relationship is not one-to-one. Fast-spiking cells can also be somatostatin-positive and PV+ and can include a diverse array of morphologies (Burkhalter, 2008; Markram et al., 2004). Likewise, not all PV+ neurons are fast-spiking (Blatow et al., 2003). Furthermore, excitatory fast-spiking cells have been reported in sensory cortex (Dykes et al., 1988; Gray and McCormick, 1996), so that the precise nature of blindly recorded cell types often remains unclear. Thus, more definitive experimental approaches are needed to characterize the physiological properties of specific cell classes. Two recent studies, both using knockin mice expressing the GAD67-GFP (Δneo) transgene, have characterized the orientation tuning of inhibitory neurons (Sohya et al., 2007) and inhibitory neurons subclassified electrophysiologically into fast-spiking and regular-spiking inhibitory neurons (Liu et al., 2009). These studies found that inhibitory cells of all subtypes are broadly tuned for orientation, contradicting the findings of many studies in higher mammals, which have shown the existence of inhibitory neurons that are as sharply tuned as excitatory neurons (Cardin et al., 2007; Hirsch et al., 2003; Nowak et al., 2008). Importantly, these knockin mice develop with significant deficits in GABA production (Tamamaki et al., 2003), which is known to affect inhibitory circuitry (Chattoo-padhyaya et al., 2007). These unexpected findings thus need to be verified in mice with wild-type inhibitory circuitry, where the properties of subclasses of inhibitory neurons are more faithfully expressed, before it is concluded that the properties of inhibitory neurons are different between mice and higher mammals.

We have used recently developed genetic labeling techniques combined with in vivo two-photon guided loose-patch recording and calcium imaging to reveal the visual response properties of the PV+ soma/axon-targeting inhibitory neurons in layers 2/3 of visual cortex. Our measurements demonstrate that PV+ interneurons have a range of response features and include a significant proportion of cells with precisely tuned responses, small receptive fields, and band-pass spatial frequency tuning characteristics. We suggest that these cells are components of, and contributors to, highly specific networks that shape the selectivity of neuronal responses.

RESULTS

In order to better understand the role of inhibition provided by PV+ cells in visual cortical circuits, we characterized their visual response properties, including orientation and direction tuning, spatial frequency tuning, and receptive field size. We accomplished this by specifically expressing red fluorescent protein (RFP) in PV+ cells and then performing in vivo two-photon-guided loose-patch recordings, and in parallel experiments, two-photon imaging of calcium responses.

Neurons Labeled with RFP are PV+ GABAAergic Interneurons

We used the Cre/loxP system to selectively label PV+ cells with RFP (Kuhlman and Huang, 2008) by injecting an adenoassociated virus (serotype 2/9) containing a loxP-STOP-locxP-RFP construct (Figure 1A) into the primary visual cortex (V1) of PV-Cre knockin mice (Hippenmeyer et al., 2005). To examine the specificity of RFP expression to the PV+ inhibitory population, we perfused the mice and harvested their brains, staining alternate sections for PV or GABA after in vivo functional imaging. Immunostains for PV and GABA (Figures 1B and 1C) demonstrated qualitatively that RFP+ cells are PV+ and GABA+. Overall, up to 10% of GABA+ cells at the center of the viral injection site in PV-Cre mice were RFP+, a proportion consistent with estimates of PV+ interneurons in the cerebral cortex (Markram et al., 2004). In addition, we injected the virus into wild-type mice and did not observe RFP+ cells 2 weeks later, either in vivo or histologically (data not shown). Thus, RFP+ neurons targeted for in vivo recordings are PV+ GABAAergic interneurons.

Orientation and Direction Selectivity of RFP+ and RFP– Neurons Is Similar

A large proportion of PV+ neurons is comprised of large basket cells, whose axonal arbors can stretch across many cortical layers and across multiple cortical columns and preferentially innervate the somata of their targets (Gupta et al., 2000; Kisvárday and Eysel, 1993; Wang et al., 2002). Because of this wide-reaching geometry and soma-targeting output, we expected them to have broadly tuned responses and large receptive fields. However, we were surprised to find a diversity of orientation tuning characteristics in these cells, ranging from cells that responded to a broad range of orientations to others with highly selective responses that responded only to one orientation (Figures 2 and 3).

Loose-Patch Recordings

Under two-photon guidance, we targeted a dye-filled patch pipette to RFP+ and RFP– neurons. After characterizing the receptive field of each neuron, we ensured the identity of the recorded neuron by filling the cell (i.e., its soma and proximal dendrites) at the end of recording. Only RFP+ cells that were successfully and unambiguously filled (Figure 2A) were included in this study.

We recorded from 11 RFP+ and 17 RFP– visually responsive neurons. The spike waveforms of RFP+ and RFP– cells were distinguished by several parameters (Figures 2B and 2C). The ratio of the peak amplitude to the valley amplitude was
significantly lower in the RFP+ population (RFP+: 1.92 ± 0.9 SD, RFP−: 2.9 ± 1.1; p < 0.05, Wilcoxon rank sum test, here and for all comparisons below). The repolarization rate, defined as the maximum slope of the repolarization phase of the action potential, was significantly sharper in RFP+ cells (RFP+: 0.47 mV/ms ± 0.04; RFP−: 0.43 mV/ms ± 0.03, p < 0.05).

The mean spike-width, measured as the half-width at half-height of all recorded spikes of each cell, did not differ between the two populations (RFP+: 0.33 ms ± 0.10; RFP−: 0.34 ms ± 0.08; p = 0.48). These waveform characteristics (Figure S1A) suggest that PV+ cells are a diverse electrophysiological population and possibly include multiple functional subtypes. The measures that best distinguished RFP+ and RFP− cells, the repolarization rate and the peak:valley amplitude, probably reflect the strong colocalization of the Kv3.1 and PV proteins (Chow et al., 1999). The overlap between spike shape measures may additionally reflect the known diversity in expression of potassium channel subtypes in PV+ cells (Chow et al., 1999).

The spontaneous firing rates of the RFP+ and RFP− neurons were not significantly different (RFP+: 0.47 Hz ± 0.84; RFP−: 0.21 Hz ± 0.59; p = 0.29), nor were the visually evoked firing rates (RFP+: 1.53 Hz ± 2.16; RFP−: 1.16 Hz ± 3.45; p = 0.22).

We used two separate measures, the orientation selectivity index (OSI) and the orientation tuning width, to characterize the orientation preference of each neuron. We recorded from low-firing but highly selective cells in both populations that responded to only one orientation in one direction (Figures 2D and 2E). The OSIs of RFP+ and RFP− neurons were not statistically different (RFP+: 0.52 ± 0.36; RFP−: 0.76 ± 0.30; p = 0.18), and both populations included highly selective neurons with OSIs equal to 1 (Figure 2E). Furthermore, the direction selectivity index (DSI), computed by dividing the difference in the responses to the preferred orientation in two directions by the sum of the responses (Figure 2E), was not statistically different between the RFP+ and RFP− populations (RFP+: 0.47 ± 0.25; RFP−: 0.66 ± 0.26; p = 0.14).

Given the diversity of tuning characteristics in RFP+ cells, we considered whether waveform characteristics, which are known to vary in different types of basket cells and other PV+ inhibitory interneurons (Blatow et al., 2003; Wang et al., 2002), might correlate with orientation selectivity (Figure S1B). Although high OSIs were found in cells with all waveform characteristics, there was a weak relationship between certain spike shape parameters and OSI. In particular, a cluster of RFP+ cells with narrow spike widths and low peak:valley ratios, and also broad orientation tuning, can be qualitatively distinguished. These could potentially encompass a subclass of PV+ cells (see Discussion). In separate experiments, we used two-photon guided loose-patch recording to assess the orientation tuning of GFP+ (GABAergic) neurons and GFP− (non-GABAergic) neurons in...
adult mice heterozygous for the GAP67-GFP (Δneo) allele (Tammamaki et al., 2003). Replicating earlier findings in these mice (Liu et al., 2009; cf. Sohya et al., 2007), we found that GFP+ neurons were significantly more broadly tuned than GFP− cells (Figure S2), as assessed with OSI (GFP+: 0.33 ± 0.13, n = 12 cells; GFP−: 0.51 ± 0.21, n = 13 cells; p < 0.05) and with tuning width (GFP+: 55.19 ± 25.94; GFP−: 33.19 ± 31.51; p < 0.05). Furthermore, the OSI range of GFP+ neurons (0.1–0.5) was not only different from the OSI range of GFP− neurons (0.1–1) in the same mice but also from that of PV+ neurons in PV-Cre mice (0.1–1; Figure 2E) and of fast-spiking neurons in wild-type mice (0.1–1.0; Niell and Stryker, 2008). That is, inhibitory neurons with the highest orientation selectivity are absent in GAP67-GFP mice, suggesting that the tuning properties of these neurons develop abnormally.

**Calcium Imaging**

To compare responses in a larger sample of cells, we assessed the orientation tuning properties of GFP+ and GFP− neurons with two-photon calcium imaging (typically 20–30 GFP+ and 2–4 GFP− neurons were recorded simultaneously; Figures 3A and 3B).

We imaged 26 GFP+ and 173 GFP− visually responsive cells. The mean OSI in the two populations did not differ significantly (GFP+: 0.25 ± 0.12; GFP−: 0.25 ± 0.11; p = 0.72) (Figure 3C). In addition, the mean orientation tuning width was not significantly different in the two populations (GFP+: 36.02 ± 31.89; GFP−: 39.23 ± 31.44; p = 0.84). Thus, PV+ interneurons displayed a range of orientation selectivity preferences, which was comparable to the rest of the visually responsive population of cells.

RFP+ and RFP− cells with comparably sharp tuning were distributed among all animals and at all imaging depths. The mean OSI of the RFP+ cells (0.26 ± 0.11) also did not differ from that of the RFP− cells (0.27 ± 0.13; p = 0.72), although the highest OSIs of the RFP− cells were higher than those of the RFP+ cells.

**Spatial Frequency Tuning and Receptive Field Sizes Are Comparable in the RFP+ and RFP− Populations**

To further characterize the receptive fields of PV+ interneurons, we measured their spatial frequency tuning and receptive field sizes. The spatial frequency tuning characteristics of cells in the visual pathway, starting with retinal ganglion cells, reflect the spatial extent and magnitude of receptive field “centers” and “surrounds” (Enroth-Cugel and Robson, 1966). Responses to different spatial frequencies were recorded either by calcium imaging or loose-patch electrophysiology (Figure 4) and then fit to a difference-of-Gaussians (DOG) model (Enroth-Cugel and Robson, 1966; 1984; Shapley and Lennie, 1985).

The preferred spatial frequency of cells measured with calcium imaging (Figure 4A) did not differ significantly between the RFP+ and RFP− populations (RFP+: 0.031 cpd ± 0.019; RFP−: 0.033 cpd ± 0.024; p = 0.81). The tuning bandwidth was defined as the ratio of spatial frequencies with half-maximal responses. The mean bandwidths of the two populations were also similar (RFP+: 5.70 octaves ± 0.67; RFP−: 5.55 octaves ± 0.92; p = 0.97). The presence of a low-frequency roll-off in the spatial frequency tuning curves was consistent with a suppressive
Figure 3. In Vivo Two-Photon Calcium Imaging of RFP+ and RFP- Neurons Reveals Extensive Overlap in the Orientation Tuning Properties of PV+ Interneurons and the Unlabeled Population

(A) Two weeks after viral infection, a craniotomy was made, and the calcium indicator OGB was injected into the infected site. The RFP alone, OGB alone, and merged images are shown. Arrowheads point to the same cells in each image. Scale bar, 10 μm.

(B) (Left) Calcium indicator responses of representative RFP+ cells (red traces) and RFP- cells (black traces) to episodically presented oriented gratings at 20° intervals; each grating was drifted in a direction orthogonal to the grating orientation (gray shading, ON periods of stimulus presentation; white, OFF). Raw single-trial traces (thin lines) as well as the mean response trace (thick lines) are shown. (Right) Gaussian tuning curves were fitted to the calculated ΔF/F responses for each stimulus, as described in Experimental Procedures. The peak response is set to 180° for ease of comparison. Top RFP+ cell: OSI = 0.23, tuning width = 32°, DSI = 0.3. Bottom RFP+ cell: OSI = 0.53, tuning width = 19°, DSI = 0.5. Top RFP- cell: OSI = 0.13, tuning width = 81°, DSI = 0.5. Bottom RFP- cell: OSI = 0.48, tuning width = 12°, DSI = 0.5. Error bars on tuning curves denote SEM.

(C) Population histograms of the orientation and direction tuning properties of RFP+ (red) and RFP- (black) populations show the extensive overlap between the two cell populations in OSI, tuning width, and DSI. Arrowheads on histograms mark the mean of each population.
receptive field surround mechanism. The demonstration of this roll-off in some PV+ inhibitory neurons indicates that at least some inhibitory cells have suppressive receptive field components (Supplemental Text and Figure S3).

In a subset of visually responsive RFP+ neurons (n = 7) and RFP− neurons (n = 13), we measured the spatial frequency tuning with loose-patch electrophysiological recordings (Figure 4B). The mean preferred spatial frequency of the RFP+ cells (0.03 cpd ± 0.01) and of the RFP− cells (0.06 cpd ± 0.01) did not differ significantly (p = 0.16). Similarly, the mean tuning bandwidth of the two populations also did not differ (RFP+: 3.88 octaves ± 2.03; RFP−: 3.13 octaves ± 1.90; p = 0.58).

Measurement of the “classical center” of a receptive field provides an estimate of the spatial spread of inputs to a cell and of the influence of the cell’s activity within the visual cortex. Because recent work has shown that inhibitory cells in general may have larger receptive fields than excitatory cells (Liu et al., 2009; Niell and Stryker, 2008), we expected PV+ cells to follow this trend and even have some of the largest receptive fields of all cells.

We estimated receptive field “center” size in both the azimuth and elevation axes by measuring the calcium response to a bar (containing a contrast-reversing checkerboard) that was moved progressively across each axis and fitting a Gaussian function to the calcium response (Figure 5). The half-width at half-height (HWHH) of this function was used as an estimate of receptive field size. The RFP+ and RFP− cell populations had similar distributions of receptive field size: for RFP− cells, the mean HWHH was 19.84° (±7.37°) in the elevation axis and 21.80° (±10.02°) in the azimuth axis, whereas for RFP+ cells, the mean HWHH was 21.38° (±8.00°) in the elevation axis and 23.43° (±7.54°) in the azimuth axis. Although the RFP− cells tended to have larger receptive field sizes in both dimensions, the trend was not significant (azimuth, p = 0.36; elevation, p = 0.54). These receptive field features suggest that PV+ cells are likely involved in spatially local processing, despite their potentially large area of cortical influence (Kisvárday and Eysel, 1993; Somogyi et al., 1983; Stepanyants et al., 2009).

High Response Specificity Is Associated with Low Firing Rates

The loose-patch recordings of RFP+ and RFP− cells revealed an inverse relationship between orientation selectivity and peak response rate. The cells with the highest OSIs had low response rates (Figure S4A); this is also evident in the individual representative cells with high OSIs (Figure 2D). Since the calcium imaging was performed at a frame rate of 1 Hz, we suspected that some of the most selective cells would be missed, because at this frame rate calcium signals generated by the few spikes emitted by low-firing cells would lie near the threshold for detection. Indeed, while the calcium imaging data confirmed an inverse relationship between OSI and peak response (Figure S4B), cells with the highest OSIs were not included (see also Sohya et al., 2007, for a similar range of OSIs recorded with traditional calcium imaging). Thus, the OSIs of the RFP+ cells measured with calcium imaging were lower than those measured with loose-patch recording (p < 0.05), as were the OSIs of RFP− cells (p < 0.05), though the orientation tuning widths of RFP+ or RFP− populations were not different when measured with the two methods (p > 0.1). However, new fast-scan methods of rapidly assessing calcium responses along arbitrary scan paths that sample selected cells reveal as high OSIs of RFP+ and RFP− cells with calcium imaging as with electrophysiological recording, importantly including cells with low firing rates (Figures S4C and S4D).

DISCUSSION

We have shown that the receptive field properties of PV+ cells are diverse and indeed match the range of properties expressed in the general neuronal population in the primary visual cortex. Some PV+ cells respond broadly to all orientations, while others have sharp orientation selectivity. Their receptive field sizes are also similar to those of the general population, and they prefer a similar range of spatial frequencies. A difference-of-Gaussians model characterizing their spatial frequency responses shows...
et al., 2002) and are not neurochemically uniform (Chow et al., 2008; Markram et al., 2004; Wang et al., 2002). Since cortical PV+ cells include several subtypes of basket cells and chandelier cells (Burkhalter, 2008; Markram et al., 2004; Wang et al., 2002) and are not neurochemically uniform (Chow et al., 2009), it is perhaps not surprising that we have found diverse response properties and narrow as well as broad tuning in this cell population. In future studies, it would be informative to determine whether the individual gene expression patterns of PV+ cells correlate with unique response features (Wang et al., 2002).

The high response specificity of many PV+ cells indicates that they acquire this specificity in the same manner as do other, excitatory, cells. It has been argued that feature-selective responses in visual cortex, including orientation selectivity, may be explained by feedforward excitatory drive from thalamic neurons combined with membrane nonlinearities such as the spike threshold (Priebe and Ferster, 2008). In this view, excitatory and inhibitory neurons in cortex generate their orientation (and other) selectivity in similar ways, with precise feedforward connections from aligned geniculocortical afferents as the principal source of specificity (Chung and Ferster, 1998; Ferster et al., 1996).

Even in feedforward models, intracortical inhibition has been proposed to have two roles (Ferster and Miller, 2000; Lauritzen and Miller, 2003; Palmer and Miller, 2007). Feedforward push-pull models postulate the existence of inhibitory “pull” of opposing polarity to complement the excitatory “push” generated by thalamocortical projections in simple excitatory cells, which would further sharpen their stimulus selectivity. Sharp tuning of a subset of PV+ cells, as demonstrated here, is consistent with this proposal. In addition, broadly tuned inhibitory complex cells are proposed to be important for contrast-invariant orientation tuning (but see Finn et al., 2007). Another subset of the PV+ population is broadly tuned and well suited to provide such inhibition.

Models of cortical circuits that combine feedforward thalamocortical excitation with intracortical excitation and inhibition (Douglas et al., 1995; Somers et al., 1995; Sompolinsky and Shapley, 1997) also predict a range of orientation selectivities for inhibitory interneurons. Importantly, inhibitory conductances need to precisely balance excitatory conductances in order to generate sharp orientation tuning, particularly within orientation representations with diverse local neighborhoods (Marín et al., 2005). It is parsimonious to suggest that PV+ cells generate their response specificity via precise thalamocortical and intracortical connections and themselves contribute to the sharpening of selectivity of other neurons in the local network.

The present study is not the first attempt to measure the visual receptive field properties of a subclass of inhibitory interneurons, but important contradictions have been found across species. In the superficial layers of V1 in cats, studies using intracellular recording to classify electrophysiological profiles have revealed inhibitory cells with a broad range of receptive field properties similar to those of excitatory cells, including sharply tuned inhibitory cells with simple receptive fields, and broadly tuned inhibitory cells with complex receptive fields (Cardin et al., 2007; Hirsch et al., 2003; Nowak et al., 2008). In mouse V1, however, despite the fact that the receptive field selectivity in excitatory cells rivals those of higher mammals (Niell and Stryker, 2008), genetically marked GAD67+ cells in GAD67-GFP (Δneo) knockin mice show poor selectivity for orientation (Liu et al., 2009; Sohya et al., 2007), a finding that we have replicated (Figure S2). Importantly, the GAD67-GFP (Δneo) knockin mice develop with lower expression levels of GABA (Tamamaki et al., 2003), and even such a partial knockdown of GAD67 expression in PV+ cells has been shown to drastically alter their connectivity, reducing the number of perisomatic boutons and the number of axonal branches (Chattopadhyaya et al., 2007). This deficiency in the construction of inhibitory circuits would be expected to have significant effects on the development and plasticity of cortical neuron properties (Hensch, 2004; Yazaki-Sugiyama et al., 2009). Furthermore, specific alterations in GABA-mediated transmission in the hippocampus of GAD67-GFP (Δneo) mice have been described (Lu et al., 2010). Still, functional differences in the GAD67-GFP (Δneo) mice could be more pronounced in visual cortex than elsewhere (Gentet et al., 2010). Another possibility is that GFP is not expressed uniformly in all inhibitory cells in GAD67-GFP (Δneo) mice. Indeed, some cells are considerably brighter than other nearby GFP+ cells (data not shown). If the highly tuned population of PV+ cells that we have recorded expresses lower levels of GFP, they may be overlooked in visually targeted recording methods.

Another study in the mouse visual cortex found that a portion of fast-spiking (presumed inhibitory) cells were highly orientation-
selective but that the proportion of such cells was lower than for neurons with larger spike widths (Niell and Stryker, 2008). At first glance, this study might appear to contradict our present results. However, it is likely that the populations of neurons sampled with these two approaches do not completely overlap—not all fast-spiking neurons are inhibitory, not all fast-spiking neurons are PV+, and not all PV+ cells are fast-spiking (reviewed in Markram et al., 2004). Furthermore, our recordings were targeted to layers 2/3. By focusing on a very specific population—superficial PV+ inhibitory interneurons—we have been able to draw attention to a subtype of PV+ neurons with the highest response selectivity.

Our demonstration that many PV+ neurons have highly selective properties suggests that rather than simply dampening activity nonspecifically (Wang et al., 2004), perisomatic inhibition provided by these cells encodes specific visual information that may sharpen representations within the network. Indeed, not only the inputs but also the targets of individual PV+ cells seem to be highly specific. Despite the huge axonal fields of these cells, their targeting is nonrandom, and in cat visual cortex, they specifically target postsynaptic partners with iso-orientation preferences locally and cross-orientation preferences distally (Kisvárday et al., 2002; Wang et al., 2002). Individual layer 2/3 fast-spiking cells belong to precise microneurons, where they interconnect preferentially with specific pyramidal cells with which they share common inputs (Yoshimura and Callaway, 2005). Our data provide additional evidence of this highly specific interconnectivity, the computational utility of which is particularly striking in mouse V1, where large domains devoted to single orientations do not exist and adjacent cells frequently vary widely in orientation preference (as at pinwheel centers in cat or ferret V1). Here, orientation-specific connections cannot simply rely on map-based spatial relationships (Ohki and Reid, 2007). Indeed, the functional heterogeneity of PV+ neurons may result from a unique cell-specific connectivity, including the possibility that sharply tuned inhibitory neurons coexist in local networks with other cells with sharp tuning. An important future goal will be to understand how the properties of these cortical interneurons correspond with their connectivity profiles and the properties of the cells whose outputs they modulate. As well, our study was conducted in anesthetized mice, and because brain states affect the response properties of visual cortical neurons such as peak firing rates (Niell and Stryker, 2010) and spatial integration (Roberts et al., 2007), in future studies it will be important to determine the function of PV+ neurons in awake behaving animals. Furthermore, given the role of acetylcholine in the modulation of attention (Herrero et al., 2008) and the high expression levels of muscarinic acetylcholine receptors in PV+ neurons (Disney and Aoki, 2008), future studies should explore the functional roles of PV+ inhibitory neurons in attention-mediated influences on visual processing.

Our results highlight the issues with defining inhibitory neuronal subclasses along single dimensions, even by the expression of a specific protein marker. The idea of PV expression defining a homogeneous cell class was enticing: a large proportion of these cells are fast-spiking, they primarily include basket cells, they do not express other interneuron marker proteins such as somatostatin or calretinin, and, importantly, they primarily provide perisomatic inhibition (Gonchar et al., 2007; Markram et al., 2004; Zaitsev et al., 2005). However, recent studies have revealed diversity in gene expression, morphology, and postsynaptic type within the PV+ population (Burkhalter, 2008; Gupta et al., 2000; Markram et al., 2004; Wang et al., 2002), and additional subtypes of PV+ inhibitory interneurons have also been reported, including multipolar bursting cells, a superficial population with regular-spiking properties and nonbasket morphology (Blatow et al., 2003). These overlapping layers of diversity suggest that PV+ neurons are multidimensional, and need to be defined by a combination of features including structure, connections, gene expression profiles, electrophysiological properties, and response patterns (Parra et al., 1998).

**EXPERIMENTAL PROCEDURES**

**Mice**

Experiments were carried out in mice under protocols approved by MIT’s Animal Care and Use Committee and conformed to NIH guidelines. Heterozygous PV-Cre knockin driver mice, which express Cre in over 90% of PV+ neurons, were backcrossed into a C57BL/6 line (Hippemeyer et al., 2005). Mice heterozygous for the GAD67-GFP (Aneo) allele (Tamamaki et al., 2003) were maintained on a C57BL/6 background; only mice older than 8 weeks were used.

**Viral Construct and Injection**

RFP was expressed specifically in parvalbumin+ (PV+) interneurons in the visual cortex of PV-Cre mice by infection of the viral construct shown in Figure 1A. The LS3-L-RFP construct (Figure 1A) was packaged into adenovirus-associated virus (AAV, serotype 2/9) as described previously (Kuhlman and Huang, 2008). Six-week-old PV-Cre mice were initially anesthetized with 4% isoflurane in oxygen, and maintained on 2% isoflurane. The skull was thinned along a 1 mm line at the rostral edge of V1, and the remaining skull and dura were carefully punctured using a glass micropipette filled with the virus. Two injections were made at each site, one at 500 μm below the cortical surface, and one at a depth of 250 μm. A volume of 0.25 μl of virus was injected at 10 nl/min at each depth. After each injection, the pipette was held in place for 5 min prior to retraction to prevent leakage.

**Animal Preparation**

Two weeks postinjection, mice were anesthetized with a cocktail containing fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidine (0.5 mg/kg), supplemented with isoflurane. The eyes were protected with ophthalmic ointment during the surgery and moistened afterward with saline. A metal headplate was attached to the skull using superglue and dental acrylic, and one at a depth of 250 μm. A volume of 0.25 μl of virus was injected at 10 nl/min at each depth. After each injection, the pipette was held in place for 5 min prior to retraction to prevent leakage.

**Two-Photon Microscopy**

The microscope was made from a modified Fluoview confocal scanhead (Olympus Optical) and a titanium:sapphire laser providing 100 fs pulses at 80 Hz (Tsunami; Spectra-Physics, Menlo Park, CA) pumped by a 10 W solid-state source (Millenia; Spectra-Physics). Emitted fluorescence was detected using photomultiplier tubes (HC125-02; Hamamatsu, Shizuoka, Japan).
Imaging was performed through a 20×, 0.95 NA lens (IR2, Olympus Optical) using Fluoview software. We collected images at a 1 Hz frame-rate, at depths between 130 and 300 μm below the cortical surface.

**Targeted Loose-Patch Recording**
Glass pipettes with ~1.5 μm tip size and 3–7 MΩ resistance were filled with Alexa Fluor 488 (5 μM, in saline, Molecular Probes, Eugene, OR) and introduced to the pia above the viral injection site at a 21° angle using a micro-manipulator (Sutter, MP-285) under visual guidance with epifluorescence. Upon entering the brain, the pipette was guided toward RFP+ cells in superficial layer 2/3 of primary visual cortex under two-photon guidance. The laser was tuned to 920 nm, which allowed excitation of both the Alexa 488 and RFP fluorophores simultaneously. The pipette was targeted to RFP+ cells while applying constant positive pressure (0.2 psi), which was monitored with a digital pressure gauge (General Tools). When the pipette was just touching the cell surface, positive pressure was released, and sustained negative pressure immediately applied (0.2–0.6 psi) to obtain a loose seal (Joshi and Hawken, 2008). If well-isolated spikes were detected during the display of a drifting grating that randomly changed orientation and direction at 8 Hz, then the cell’s receptive field was assessed. Subsequently, current pulses (35 ms, 900–2000 nA) were delivered for 15 Hz for 30–60 s to fill the recorded cell. Only RFP+ cells that were distinctly filled during this procedure were included for analysis. In the same experiments, RFP− cells were targeted blindly. As the tip was slowly advanced through the cortex, ~0.5 nA current pulses were delivered for 6.3 ms at 55 Hz, and the tip resistance was monitored. As the tip resistance increased substantially, positive pressure was released, and negative pressure immediately applied, as above. The location of the tip was still monitored under two-photon guidance; all RFP− cells were released, and negative pressure immediately applied, as above. The location pulses were delivered for 6.3 ms at .55 Hz, and the tip resistance was monitored. Imaging was performed through a 20×, 0.95 NA lens (IR2, Olympus Optical) using Fluoview software. We collected images at a 1 Hz frame-rate, at depths between 130 and 300 μm below the cortical surface.

**Calcium Imaging**
A glass pipette filled with Oregon Green Bapta-1 AM (OGB1-AM, 1.0 mM, Molecular Probes, Eugene, OR) and Alexa Fluor 594 (100 μM, Molecular Probes, Eugene, OR) was visually guided into the brain and lowered to a depth between 100 and 200 μm below the pial surface, and a small amount of dye was released using a picospritzer. The laser was tuned to 810 nm, and 1 hr later, fluorescence changes in response to visual stimulation were monitored.

**Visual Stimulation**
Visual stimuli were displayed on a 17 inch LCD monitor placed 15 cm from the eyes. Stimuli were generated in Matlab (Mathworks, Natick, MA) using the PsychoPhysics Toolbox (Braddick, 1997). Square wave drifting gratings with 100% contrast were used to test orientation, direction, and spatial frequency tuning. Test stimuli were episodically presented, equally alternating with a blank grey screen with 8 or 16 cycles. For calcium imaging experiments, orientation and direction selectivity was measured with oriented gratings presented at a spatial frequency of 0.05 cycles per degree (cpd), and a temporal frequency of 3 cycles per second (cps). Spatial frequency selectivity was measured at several random orientations (changing at 4 Hz) and temporal frequency of 3 cps. For loose-patch recordings, attempts were made to optimize the spatial frequency and temporal frequency parameters of the oriented gratings for each cell’s preference, ranging in spatial frequency from 0.01 to 0.05 cpd and in temporal frequency from 1 to 3 cps. Receptive field locations were determined with vertical and horizontal bars that drifted periodically across the screen at 18 s intervals; each bar was 5° wide, moved in steps of 1.7°, and contained a checkerboard pattern of white and black squares that reversed sign at 10 Hz.

**Data Analysis**
Analysis of electrophysiological data was carried out with custom-written Matlab scripts. Time traces were imported into Matlab, smoothed with a Gaussian kernel, and spikes were detected offline with custom routines. Spikes were identified by detection of events based on the derivative of the voltage traces. The spontaneous firing rate of each neuron was measured for 10 s preceding visual stimulation for each trial. A neuron was considered visually responsive if its firing rate at the preferred orientation was significantly higher than its spontaneous firing rate, determined with a t test. The spontaneous firing rate was then subtracted from the response to each orientation, the mean firing rate across the entire 4 s stimulus interval.

Calcium imaging data were also imported into Matlab, where the change in fluorescence from baseline was calculated for each pixel. First, the unstimulated PMT fluorescence intensity was subtracted from the image series, the fluorescence time series was smoothed with a Gaussian kernel, and the change in fluorescence normalized by the baseline fluorescence (ΔF/F) was calculated for each pixel. The baseline fluorescence was defined as the pixel’s fluorescence intensity during the previous blank frame, so that the ΔF/F for a particular trial was equal to the mean fluorescence intensity during the stimulus presentation minus the baseline, divided by the baseline. This method is thus insensitive to changes in baseline fluorescence intensity that may occur during the course of a particular experiment. For receptive field mapping, stimuli were not episodically presented, and a fixed percentile of fluorescence intensity rather than the baseline intensity was subtracted. Individual neurons were circled manually, and astrocytes were not included. Astrocytes can be easily recognized from neurons in calcium images by their bright, irregular cell bodies, which we have confirmed in other experiments by labeling with the marker sulforhodamine 101 (data not shown). The ΔF/F of the surrounding neuropil was subtracted from the ΔF/F of each neuron. The ΔF/F was then calculated as the mean ΔF/F within each neuron. Only neurons that were visually responsive, defined as having response values above the surrounding neuropil response for at least 50% of trials, were further considered.

The responses, firing rates or ΔF/Fs, were then fit to Gaussian functions (orientation, receptive field size) or a difference-of-Gaussians (DOG), spatial frequency. Two measures of orientation selectivity were used: the orientation selectivity index (OSI) was calculated from the ΔF/F responses or firing rates as the vector average in the preferred direction, and the tuning width was taken as the half-width at half-height from the Gaussian fit (untuned cells that could not be fit with a Gaussian were assigned tuning widths of 90°). The direction selectivity index (DSI) was also calculated from the Gaussian fit as the difference of the response amplitude in each direction of the preferred orientation, divided by their sum. The half-width at half-height of the receptive field Gaussian was used as a measure of receptive field size. Finally, the preferred spatial frequency and spatial frequency tuning bandwidth were determined from the DOG function. The bandwidth was defined as the ratio of spatial frequencies yielding the half-maximal response (Neill and Stryker, 2008). The inverse Fourier transform of the excitatory and inhibitory components of the DOG then provided an estimate of the relationship between the center excitation and surround inhibition, as shown in the supplementary information (Enroth-Cugell and Robson, 1966, 1984; Shapley and Lennie, 1985).

Statistical comparisons were carried out using the Wilcoxon test, two-tailed t test, and the Kolmogorov-Smirnov test and yielded indistinguishable results. Values from the Wilcoxon test are reported under Results.

**Immunohistochemistry**
Immediately after physiology experiments were completed, mice were overdosed with pentobarbitol, and perfused transcardially with saline followed by 4% paraformaldehyde, cryoprotected in 30% sucrose, and 40 μm sections were thaw-mounted and then immunostained for PV or GABA. Sections were blocked in 10% normal goat serum with 0.1% triton in PBS for 1 hr, incubated overnight in either mouse anti-PV (1:250, Chemicon, MAB1572), or guinea pig anti-GABA (1:250, Millipore, AB175), then incubated in Alexa Fluor 633 goat anti-mouse or Alexa Fluor 488 goat anti-guinea-pig (1:200, Molecular Probes, A20152, A11073), and coveredslipped with Vectashield Hardset mounting media with DAPI (Vector Labs). Using a confocal microscope (Zeiss LSM 5 Pascal Exciter), z-stacks were obtained through the extent of RFP-labeled cells on sections that had been stained for PV or GABA respectively and these cells were then analyzed for colocalization with either marker. Counts of RFP+/PV+, RFP+/PV−, RFP−/GABA+, RFP+/GABA+, and RFP−/GABA− cells were made from these image stacks using ImageJ software (National Institutes of Health).
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Discussion and four figures and can be found with this article online at doi:10.1016/j.neuron.2010.08.006.

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