Diversity of GABAergic Interneurons in Layer VIa and VIb of Mouse Barrel Cortex

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Introduction

Information is processed in the neocortex by specific neuronal networks through distinct cortical layers (Petersen 2007; Aronoff and Petersen 2008). In sensory cortices, layer VI appears to influence the activity of thalamic neurons that relay sensory information (Zhang and Deschenes 1997; Jones 2009). Projections from layer VI target the primary and secondary thalamic nuclei as well as the inhibitory reticular nucleus (Zhang and Deschenes 1997) and dynamically sharpen the receptive fields of thalamocortical cells (Andolina et al. 2007) through modulatory connections (Liu et al. 1995; Liu and Jones 1999). In addition, layer VI of primary sensory cortices integrates the corticocortical inputs of secondary cortices enabling it to shape the thalamocortical transfer of sensory information according to higher order cortical processing (Sillito and Jones 2002). This activity is believed to set the precision and the rapidity of anticipated sensory responses (Sillito et al. 2006). Although the implication of layer VI in information processing is well accepted, the neuronal network underlying the cortical computations in this layer is not yet fully understood.

Layer VI subdivides into layer VIa, which represents its major fraction, and layer VIb, which constitutes a thin band of cells packed at the interface with the white matter (Allendoerfer and Shatz 1994; Thomson 2010). Glutamatergic neurons represent 85–90% of the neurons of layer VI. In layer VIa, glutamatergic neurons originate from the neocortical plate and can be subdivided into corticothalamic (CT) and corticocortical (CC) cells (Zhang and Deschenes 1997; Thomson 2010). These cells can be distinguished on the basis of their morphology and their electrophysiological properties (Merrick et al. 2005; Kumar and Ohana 2008). In layer VIb, glutamatergic neurons are believed to survive from the developmental substrate and their function in adult is currently unknown (Torres-Reveron and Friedlander 2007). γ-Aminobutyric acidergic (GABAergic) interneurons represent 10–15% of layer VI neurons and are present in both sublayers. In layer VIa, they seem to be preferentially contacted by local collaterals of CT cells (West et al. 2006), but some have also been shown to receive direct thalamocortical afferences (Beierlein and Connors 2002) as well as callosal inputs (Karayannis et al. 2007). A recent study suggested that CT cells are specifically silenced by local inhibitory cells during sensory stimulation in vivo (Zhou et al. 2010), illustrating the importance of GABAergic interneurons in the modulation of layer VI glutamatergic outputs. However, layer VI GABAergic cells have only been sparsely described at the present time (Thomson 2010). Hence, it appears that characterizing these interneurons is an important step toward understanding their implication in the modulation of the sensory information processing.

In the present study, the electrophysiological, molecular, and morphological properties of GABAergic interneurons of layer VIa and VIb have been characterized using whole-cell current-clamp recordings, single-cell reverse transcription-polymerase chain reaction (scRT-PCR), and biocytin labeling followed by Neurolucida reconstructions in acute slices of mouse somatosensory barrel cortex. This approach allowed the unsupervised identification of 4 types of GABAergic neurons with distinctive physiological, molecular, and morphological features. In addition, we have characterized the distributions of GABAergic cell types through the depth of layer VI by using immunohistochemical labeling of distinct chemical markers on GAD67-GFP knock-in mice (Tamamaki et al. 2003).

Materials and Methods

Slice Preparation for Electrophysiological Recordings

All experiments were performed in accordance with the guidelines of the European Community Council Directive of November 24, 1986 (86/609/EEC). Juvenile C57BL/6 mice (Janvier) aged postnatal day P14–P17 were deeply anesthetized with halothane and decapitated. Brains were quickly removed and immersed into an ice cold (~4 °C) slicing solution continuously aerated with carbogen (95%O2/5%CO2) (Air Liquide), containing (in mM): 110 choline chloride, 11.6 sodium...
ascorbate, 7 MgCl₂, 2.5 KCl, 1.25 NaH₂PO₄, 25 glucose, 25 NaHCO₃, and 3.1 sodium pyruvate (Bureau et al. 2006). Cooled brains were placed on the stage of a vibratome (VT-1200, Leica) and cut in 300 μm thick slices with a 30°–40° inclination from the sagittal plane so as to be perpendicular to the surface of the barrel cortex. Slices were subsequently transferred to a chamber containing warm (~33°C) slicing solution aerated with carbogen for 20 min. Slices were then maintained at room temperature in a holding chamber containing artificial cerebrospinal fluid (aCSF) aerated with carbogen, containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 20 glucose, 26 NaHCO₃. In order to avoid excitotoxicity, 1 mM of kynurenic acid (Sigma) was added to the mixture. Slices were allowed to incubate for 1 h at least, prior to recording.

**Whole-Cell Patch-Clamp Recordings**

Slices were submerged in a thermostated recording chamber, placed on the stage of an Axioskop 2FS microscope (Carl Zeiss), equipped with Diod gradient contrast optics (Luigs & Neumann) and a CoolSnap FX CCD camera (Roper Scientific), and visualized by under infrared (IR) illumination. The preparation was continuously superfused (~1 mL/min) with oxygenated aCSF warmed up at 30°C. Barrels were visualized in the absence of light condenser. Recordings were performed within the extent of the barrel field. Pipettes (~6 MΩ) were pulled from borosilicate capillaries and filled with 8 μL of autoclaved internal solution containing 144 mM K-gluconate, 3 M MgCl₂, 0.5 mM ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2 (285/295 mOsm), and 3 mg/mL biocytin (Sigma). Whole-cell recordings were performed at 30°C ± 1°C in the current-clamp mode of a MultiClamp 700B amplifier (Molecular Devices). Signals were filtered at 4 kHz and digitized at 50 kHz with an analog signal converter (Digidata 1322A; Molecular Devices).

**scRT-PCR Protocol**

At the end of recordings, cytoplasms were gently aspirated into the patch pipette. Pipettes were slowly removed from recorded cells in order to allow closure of the membrane. The content of pipettes was expelled into a test tube in which reverse transcription was performed as described previously (Lambrecht et al. 1992). Products of reverse transcription were stored at ~80°C until further processing was performed.

The scRT-PCR protocol was designed to detect the presence of messenger mRNAs coding for the vesicular glutamate transporter 1 (vGluT1), the 2 isoforms of glutamic acid decarboxylase (GAD65 and GAD67), the neuronal nitric oxide synthase (NOS-1), the calcium-binding proteins calbindin 28K (CB), calretinin (CR), and parvalbumin (PV), and the neuropeptides somatostatin (SOM), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), and cholecystokinin (CCK) (Table 1). Two successive rounds of amplification were performed using nested primer pairs (Cauli et al. 1997). All markers were first amplified simultaneously with a first set of primers (Table 1), undergoing 21 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C, 55 s) in a final volume of 100 μL. Each marker was then amplified individually using a second primer pair internal to the pair used in the first round (nested primer pairs) during 35 additional cycles of amplification. All primers (Table 1) were designed to be on 2 different exons of the target mRNA so as to differentiate actual transcripts from eventual genomic DNA contaminations. The presence of products of amplification was finally detected on a 2% agarose gel stained with ethidium bromide (Sigma). The size of the products was verified with a standard molecular weight marker (100 bp Ladder, Promega).

**Histochecmical Labeling and Reconstruction of the Arborizations of Recorded Neurons**

Recorded slices were fixed overnight at 4°C with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Slices were then kept in PB at 4°C until further processing was performed. Slices were then sequentially incubated in PB containing 1% H₂O₂ in order to quench endogenous peroxidase and in PB containing avidin biotin complex (Vector; 1:200) and Triton X-100 (0.5%). The arborization of the neurons was revealed with a solution of dianibenzidine nickel (DAB elite kit, Vector). Between each step slices were thoroughly rinsed in PB. Slices were finally mounted in Mowiol (Calbiochem). When the morphology of the recorded cells was clearly visible, their arborization was vectorized using a ×100 objective on the stage of a DMR microscope (Leica), equipped with a standard Neurolucida system (MBF Bioscience).

**Electrophysiological Analysis**

To describe the behavior of recorded neurons comprehensively, 28 electrophysiological parameters were measured on traces corresponding to the voltage responses induced by 800 ms hyperpolarizing and depolarizing current pulses. These parameters are following the Petilla nomenclature (Ascoli et al. 2008). (1) Resting potential (RMP) was measured immediately after electrical access to the intracellular compartment of the cell was achieved. (2) Input resistance (Rm), (3) membrane time constant (τm), and (4) membrane capacitance (Cm) were determined in response to hyperpolarizing

<table>
<thead>
<tr>
<th>Genes</th>
<th>Size</th>
<th>First PCR primers</th>
<th>Size</th>
<th>Second PCR primers</th>
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<tr>
<td>vGluT1</td>
<td>593</td>
<td>Sense, 124 CCACTGAGAAGCGAATGCCT</td>
<td>367</td>
<td>Sense, 148 CAGACAGCCTTGTGGCGTTC</td>
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<tr>
<td>NM_182993.1</td>
<td>375</td>
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<td>Sense, 219 CACCTGCAAGAAGCAATTCCT</td>
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<td>GAD65</td>
<td>253</td>
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<td>177</td>
<td>Sense, 447 GATTTCGCGTCGGTCCTCC</td>
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<tr>
<td>NM_008787.1</td>
<td>426</td>
<td>Sense, 83 ATGATCGTGGTCGCTGAAC</td>
<td>177</td>
<td>Sense, 159 CAAATGCTGTCGAGAATGGTC</td>
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<tr>
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<td>Sense, 314 GTTCTGCTCCCTGCTCCTAG</td>
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<tr>
<td>NM_008773.3</td>
<td>265</td>
<td>Sense, 327 CACGCCTTCTAANATCTAGG</td>
<td>151</td>
<td>Sense, 194 ATGAGCAAGAATGAGATGAAA</td>
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<td>NOS-1</td>
<td>257</td>
<td>Sense, 104 GCTCTAGAAGAAGGACCG</td>
<td>163</td>
<td>Sense, 485 TCCCACCTTCCATATTCTG</td>
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<tr>
<td>PV</td>
<td>275</td>
<td>Sense, 275 ATTTGCTGCGCTGACCTT</td>
<td>236</td>
<td>Sense, 141 CTCGAGAAGGCAAGAAGG</td>
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<tr>
<td>NM_013485.2</td>
<td>373</td>
<td>Sense, 236 GAGTTCTGACCCAAAAAAC</td>
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<td>Sense, 271 ATTCTTCTGCGTGGCGAGAT</td>
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<td>NMDA</td>
<td>294</td>
<td>Sense, 165 GCCATGGCGTCGGCTGGA</td>
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<td>NPY</td>
<td>250</td>
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<td>NM_002915.1</td>
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<td>Sense, 313 CCTCTGCTCTTCCCTCCTC</td>
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<td>VIP</td>
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<td>156</td>
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<td>202</td>
<td>Sense, 425 TCTCCTGCACTTCCTCCT</td>
<td>440</td>
<td>Sense, 440 TTCCTCACCTTCCTCCTC</td>
</tr>
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</table>

**Table 1**

PCR primer

current pulses eliciting voltage shifts whose amplitude was 10–15 mV negative to rest (Kawaguchi 1993). Rm was measured following Ohms law and tm was computed as the time constant of a single exponential fit of the voltage response from onset to maximum hyperpolarization. Cm was obtained following the formula \( Cm = \frac{tm}{Rm} \). Some neurons have been described to undergo a partial repolarization following a hyperpolarization peak in response to negative currents, reflecting the activation of the voltage-activated cationic current (Ih). In order to quantify this phenomenon, the maximal resistance Rmax was computed as the slope of a linear curve fitted to the current-voltage (IV) plot of the maximal voltage response of recorded neurons to hyperpolarizing current injections ranging from -100 to 0 pA with a 10 mV increment. Similarly, the steady state resistance Rssg was estimated as the slope of a linear curve fitted to the IV plot of the steady state voltage response to hyperpolarizing currents measured at the end of 800 ms pulses (Karagiannis et al. 2009). (5) Sag index was computed to correspond to Rssg expressed as a percentage of Rm. (6) Rheobase was computed as the minimal intensity of the 800-ms current pulse, which was sufficient to induce an action potential. As some neurons have been shown to display a delayed firing (Ascoli et al. 2008), (7) first spike latency, t1, was computed as the time needed to elicit a spike at the onset of a current pulse corresponding to the rheobase. Cortical interneurons have been described to exhibit a wide range of firing behaviors around threshold, some exhibiting bursting, adapting, stuttering (Gupta et al. 2000), or accelerating trains of action potentials. In order to describe this variety of behavior with quantitative parameters, the interspike intervals measured in response to the minimal current injection eliciting more than 3 action potentials were plotted and fitted to a linear curve. (8) Adaptation (mthreshold) was computed as the slope of the linear fit and (9) minimal steady state frequency (Fmin) was computed as its y-intercept (Fthreshold = mthreshold \( t + Fmin \)). In some neurons, a transient decrease of spike amplitude can occur during a spike train (Cauli et al. 2000). (10) Amplitude modulation (hump) was defined as the voltage difference between the peaks of the action potential having the smallest amplitude and the action potential having the maximal amplitude in the following spike train. At high firing frequencies, adaptation has been described to adopt a complex temporal pattern characterized by an early exponential decrease in spike frequency, followed by a subsequent linear decrease. In order to fully quantify this behavior, the interspike interval of the train of spike induced by the maximal depolarizing current injection that did not induce saturation was fitted to the addition of a decaying exponential function and of a linear function (Halabisky et al. 2006). (11) Amplitude of early adaptation (Asat). (12) time constant of early adaptation (tsat). (13) maximal steady state frequency (msat), and (14) late adaptation (Fmax) were defined such that \( Fsat = Fthreshold + \exp(-t/tsat) + msat + t/Fmax \). Spike waveforms have been described to vary between neuronal cell types (Cauli et al. 2000). In order to quantify these variations, 6 parameters were measured on the first and second spikes of traces corresponding to the minimal depolarizing current injection eliciting at least 3 action potentials. (15), (21) The amplitude of the first (A1) and second action potential (A2) was defined as the difference of membrane potential between its onset and its peak. (16), (22) Spike duration of the first (D1) and second spike (D2) was defined as the spike width at half amplitude. After hyperpolarization potentials (AHPs) are complex phenonmena that require the intervention of several cationic currents (Sah and Faber 2002). The amplitude and the temporal profile of these currents can vary significantly between cell types resulting in differences in AHP waveforms (Cauli et al. 2000; Halabisky et al. 2006). (18), (24) AHP maximums (AHP1m and AHP2m) were calculated using the membrane potential at the onset of the action potential as a reference. Comparatively, (19), (25) AHP latencies (tAHP1m and tAHP2m) were calculated as the time span between the AHP maximum and the onset of the action potential (Yucurovic et al. 2010). On some occurrences, AHPs have been reported to adopt a complex biphase shape, characterized by an initial fast AHP component and a slower AHP component separated by a transient after depolarization (ADP) (Karagiannis et al. 2009). In this case, (17), (23) the amplitude of the ADP (ADP1 and ADP2) was calculated using the peak of the fast AHP component as a reference. (19), (25) The latency of the ADP (tADP1 and tADP2) was computed using the onset of the spike as a reference. During a spike train, spikes can display variations in their amplitude and duration at half width. In order to quantify this phenomenon, (27) amplitude reduction (Ampl.Red) and (28) duration increase (Dur.Inc) were computed as (A1 – A2)/A1 and (D2 – D1)/D1, respectively (Cauli et al. 2000).

**Morphological Analysis**

In order to describe variations of morphology between recorded neurons, a total of 18 parameters related to features of the soma and dendrites were extracted. scRT-PCR requires harvesting of up to one-third of the cytoplasm of cells inducing variation in the shape of the soma. In order to avoid this potential drawback, morphologies of somata were reconstructed from IR pictures taken prior to whole-cell recording, while in the cell-attached configuration. In order to describe somata of reconstructed neurons, (1) the area of the contour and (2) their perimeter as well as the (3) maximal, (4) minimal diameter passing through the centroid were computed. (5) Elongation was defined as the ratio between the maximal and the minimal diameters (Karagiannis et al. 2009). The dendritic properties of vectorized neurons were extracted with Neurulucida explorer (MBF Bioscience) and Excel (Microsoft). In order to be able to describe differences in the local features of dendritic arborizations, we systematically quantified the following parameters: (6) number of primary dendrites, (7) total dendritic length, and (8) average of the length of segments (defined as the portion of dendrites between 2 nodes), (9) ratio of the total dendritic length to the total dendritic surface, (10) average tortuosity of dendritic segments (defined as the ratio of the length of segments to the straight line path between their extremities), (11) number of dendritic nodes, and (12) fractal index (calculated with the box counting method (Douketis et al. 1995)). Sholl analysis (Sholl 1953) has been widely used to describe neuronal arborizations (Gupta et al. 2000; Wang et al. 2002). (13) The length of the dendritic arborization enclosed within a 100 μm radius circle around the cell body, (14) between 100 and 200 μm radii, (15) between 200 and 300 μm radii, and (16) outside a 300 μm radius were systematically extracted and expressed as a fraction of the total dendritic length. In order to detect portions of the slice plane preferentially occupied by the dendrites of reconstructed neurons, a wedge analysis was performed (Dumitriu et al. 2007; Vucurovic et al. 2010). The volume around each reconstructed neuron was divided into 12 wedges centered on the soma, and the length of the portion of the dendritic tree that was enclosed within each wedge was extracted. (17) Vertical extent was defined as the fraction of the dendritic arborization enclosed in the 4 wedges closest to the radial cortical axis. (18) Horizontal extent was defined as the fraction of the dendritic arborization enclosed in the 4 wedges closest to the tangential cortical axis.

**Unsupervised Clustering**

In Ward’s method (Ward 1963), cells are grouped in clusters so as to minimize the variance within clusters. One new cluster is formed at each iteration until every individual is linked. Here, variances were estimated calculating the Euclidean distances between cells and cluster centroids in a parameter space. This unsupervised clustering algorithm has the advantage that it is not necessary to define the number of classes to be characterized in advance. However, a drawback of Ward’s method is that misassigned cells are not corrected during the iterative process. Clusters generated with Ward’s method were thus corrected using the Kmeans algorithm. This method starts with k initial arbitrary centroids and assigns each observation to one of k corresponding clusters by minimizing the distance of the observation to the centroids. The actual cluster centroids are subsequently computed, and the process is repeated until an optimum is reached. In the end, the observations are reclassified into k nonoverlapping groups. Here, initial centroids were chosen so as to correspond to the centroids of the clusters generated with Ward’s method. This combination allowed the generation of nonoverlapping clusters without making prior assumptions about the number of groups into which neurons should be separated. Electrophysiological parameters used for clustering were Rm, Rm, Cm, sag index, rheobase, hump, Asat, tsat, Csat, msat, A1, D1, AHP1max, tAHP1max, Ampl.Red, and Dur.Inc. Molecular parameters used
for clustering were vGlut1, GAD, NOS-1, CB, PV, CR, NPY, VIP, SOM, and CCK. Molecular parameters were computed as binary variables set to 1 if the marker was present and 0 if it was absent. GAD corresponds to the transcription of GAD65 or GAD67. For all parameters, distributions were centered and reduced prior to clustering. Finally for a subset of the GABAergic neurons, the morphological parameters soma area, soma elongation, number of primary dendrites, total dendritic length, average length of dendritic segments, and length/surface ratio, dendritic segments tortuosity, number of nodes, dendritic Sholl (0–100 μm), (100–200 μm), (200–500 μm), vertical extent, and horizontal extent were included in the clustering. As all neurons transcribed GAD in this subset, GAD was omitted. The quality of clusterings was assessed by comparison to clusterings generated with a similar approach on randomized databases (Karagiannis et al. 2009). In order to perform randomization, vectors representing variables in the data matrix were replaced by independent random permutations. This scrambling did not alter the distribution of parameters but disrupted the correlation between them. The clustering qualities were compared using silhouette analysis. In this method, the value $s(i)$ is computed for each data point as:

$$ s(i) = \frac{b(i) - a(i)}{\max\{a(i), b(i)\}$$

where, for a data point $i$, $a(i)$ corresponds to the average distance between $i$ and the points belonging to the same cluster and $b(i)$ corresponds to the average distance between $i$ and the points of the closest cluster. In our case, a positive silhouette value indicates that on average, the neuron is closer to the neurons of its own cluster than from the neurons belonging to other clusters in the parameter space. By contrast, a negative value indicates a potential misclassification. Thus, a decrease of the mean silhouette value of the cells following randomization was interpreted as a lower quality of clustering. Ward’s clustering was performed on the Statistica software (Statsoft). $K$-means clustering, principal component analysis, and silhouette analysis were performed within the Matlab environment (MathWorks).

### Statistical Analysis

The subdivision of interneurons proposed here corresponds to a polythetic classification scheme that intrinsically tolerates a certain degree of variability within classes (Karagiannis et al. 2009). For electrophysiological variables, differences between pairs of identified clusters were systematically tested using nonparametric Mann–Whitney $U$-tests. In order to test differences in the occurrence of molecular markers between clusters, we computed the statistic

$$|i| = |pa - pb|/\sqrt{(pq/na) + (pq/nb)}$$

where $pa$ and $pb$ represent the percentages of occurrence of the marker and na and nb the number of individuals in cluster $a$ and $b$. The percentages of occurrence and of absence of the marker in the overall population were represented by $p$ and $q = 1 - p$, respectively. $P$ values of the difference of expression were computed by plotting this statistic against a standard normal distribution (Karagiannis et al. 2009). Morphological variable differences were tested using Kruskal–Wallis nonparametric analyses of variance (ANOVA) followed by Mann–Whitney $U$-tests for pair comparisons. Variations in the density of labeled GABAergic cells between fractions of layer VI were tested using a one-way ANOVA on repeated measures test, followed by Student-Newman-Keuls tests for pair comparisons. Kruskal-Wallis ANOVA, one-way ANOVA on repeated measures, and Student-Newman-Keuls test were performed using Sigma Stat (LogiLabo). Mann–Whitney $U$-tests were performed within the Matlab environment (MathWorks).

### Immunobiochemistry

Four P17 GAD67-GFP knock-in (Aneo) transgenic mice (Tamamaki et al. 2003) maintained on a C57BL/6 genetic background were used for this study. Mice were kindly provided by Dr Y. Yanagawa. Mice were deeply anesthetized with an intraperitoneal injection of pentobarbital (150 mg/kg body weight) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 (PFA). Brains were dissected out, embedded in 3% agarose diluted in PBS, and cut coronally on a vibratome (Leica; VT1000S). Free-floating 45 μm-thick coronal sections were collected serially. Alternate sections were incubated for 48 h at 4 °C with one of the following antibodies diluted in PBS containing triton (0.2%; PBST): rabbit anti-PV (1:800; Swant PV28), rat anti-SOM (1:500; Millipore MAB354), rabbit anti-NPY (1:8000, Sigma N9528), or rabbit anti-VIP (1:500, ImmunoStar 20077). After washing in PBST, sections were incubated with AlexaFluor 568 goat anti-rabbit antibody or AlexaFluor 568 goat anti-rat antibody (1:300; Invitrogen). Sections were rinsed in PBST, mounted in Vectashield (Vector) containing Dapi. Confocal images were acquired with an SP5 confocal microscope (Nikon) for illustration.

### Results

In order to characterize the diversity of neocortical interneurons in layer VI, 117 neurons from layer Vla and 42 from layer Vlb ($n = 159$) were recorded in the barrel cortex of juvenile mice (P14–P17). These neurons were submitted to whole-cell current-clamp recordings coupled to the scRT-PCR protocol and filled with biocytin for morphological analysis.

Twenty-eight electrophysiological parameters (Tables 2–5) were measured from responses to 800 ms hyperpolarizing and depolarizing current pulses. In accordance with the Petilla terminology (Ascoli et al. 2008), these parameters take into account passive membrane properties (5 parameters, Table 2), the properties of the discharges at threshold (4 parameters, Table 3) and saturation (5 parameters, Table 4) and the properties of action potentials (14 parameters, Table 5). The scRT-PCR was designed to detect the presence of 11 molecular markers commonly used to define subpopulations of cortical interneurons: vesicular glutamate transporter 1 (vGluT1), glutamic acid decarboxylase 65k and 67k (GAD65 and GAD67), neuronal NO synthase (NOS-1), the calcium-binding proteins CB, PV, and CR, and the peptides NPY, VIP, SOM, and CCK (Table 6). Finally, for a subset of 85 GABAergic neurons, morphological variables were measured from infrared pictures of somata and from Neurolucida reconstructions of the dendritic trees. In many cases, we

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**Layer VI Interneurons**

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had difficulties in recovering axonal arborizations. Indeed, high
scRT-PCR efficiency requires the harvesting of up to one-third
of the cytoplasm (Tsuzuki et al. 2001), which is not optimal for
biocytin labeling (Karagiannis et al. 2009; Vucurovic et al.
2010). Nevertheless, axons were identified for representative
cells in our sample, and we focused our quantitative analysis on
the somatodendritic morphology of interneurons. Eighteen
morphometric parameters describing the soma (5 parameters,
Table 7), the local features of the dendrites (7 parameters,
Table 8), and the spatial organization of the dendritic arboriza-
tion (6 parameters Table 9) were extracted for each cell.

Identification of Classes amongst Layer VI GABAergic
Neurons
In order to identify classes of GABAergic interneurons in layer
VI, the sampled neurons were grouped using Ward’s unsupervised
cluster analysis, a method that has been widely used to
define neuronal classes based on multiple variables (see Materials
and Methods; Tamas et al. 1997; Cauli et al. 2000; Karube et al.
2004; Gallopini et al. 2006; Halabisky et al. 2006; David et al. 2007;
Dumitriu et al. 2007; Andjelic et al. 2009; Helmstaedter et al.
2009; Vucurovic et al. 2010). This multifactorial analysis was
performed on a subset of electrophysiological and molecular
parameters and primarily divides our sample into 2 clusters:
Cluster 1 and Cluster 2 (Fig. 1). All the neurons of Cluster 1
(n = 105) are GABAergic as shown by the systematic tran-
scription of at least one isoform of GAD. In contrast, neurons of
Cluster 2 (n = 54) transcribed vGlut1 but not GAD, except for
one cell cotranscribing both markers. Therefore, it appears that
in layer VI, GABAergic interneurons can be reliably separated
from glutamatergic cells by unsupervised methods, as already
observed in other neocortical layers in rat (Cauli et al. 2000;
Gallopini et al. 2006; Karagiannis et al. 2009). Cluster 1 contains
neurons transcribing all tested molecular markers in various
frequencies and combinations and displaying a wide variety of

| Table 2 |
| Passive membrane properties

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<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
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<td>(1) Resting potential (mV)</td>
<td>−67.9 ± 8.6</td>
<td>−64.1 ± 4.2</td>
<td>−65.8 ± 5.5</td>
<td>−64.8 ± 8.7</td>
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<tr>
<td>(2) Input resistance (MΩs)</td>
<td>247.4 ± 134.2</td>
<td>538.8 ± 251.8</td>
<td>459.9 ± 187.5</td>
<td>1212.0 ± 971.4</td>
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<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&lt; &lt; &lt; SOM</td>
<td>&gt; &gt; &gt; PV</td>
<td>&gt; &gt; &gt; PV</td>
<td>&gt; &gt; &gt; PV</td>
</tr>
<tr>
<td>(3) Membrane time constant (ms)</td>
<td>22.3 ± 12.4</td>
<td>65.7 ± 33.0</td>
<td>33.4 ± 14.3</td>
<td>44.9 ± 39.2</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&lt; &lt; &lt; SOM</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&gt; &gt; &gt; PV</td>
<td>&lt; &lt; &lt; PV</td>
</tr>
<tr>
<td>(4) Membrane capacitance (pF)</td>
<td>101.1 ± 55.7</td>
<td>136.0 ± 82.5</td>
<td>76.5 ± 24.8</td>
<td>37.7 ± 9.2</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&gt; &gt; &gt; SOM</td>
<td>&gt; &gt; &gt; VIP</td>
<td>&gt; &gt; &gt; SOM</td>
<td>&gt; &gt; &gt; SOM</td>
</tr>
<tr>
<td>(5) Sag index (%)</td>
<td>91.5 ± 5.6</td>
<td>81.7 ± 10.6</td>
<td>87.8 ± 5.3</td>
<td>97.6 ± 3.0</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&gt; &gt; &gt; SOM</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&gt; &lt; VIP</td>
<td>&gt; &gt; &gt; PV</td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; < < <, and < < <: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and > >, and > > >: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.

| Table 3 |
| Just above threshold properties

<table>
<thead>
<tr>
<th></th>
<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6) Rheobase (pA)</td>
<td>124.5 ± 82.6</td>
<td>33.6 ± 24.2</td>
<td>48.7 ± 26.7</td>
<td>20.0 ± 18.3</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&gt; &gt; &gt; SOM</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&gt; &gt; &gt; PV</td>
<td>&lt; &lt; &lt; PV</td>
</tr>
<tr>
<td>(7) First spike latency (ms)</td>
<td>325.9 ± 221.7</td>
<td>253.9 ± 151.3</td>
<td>390.0 ± 269.0</td>
<td>114.6 ± 82.1</td>
</tr>
<tr>
<td>ANOVA, NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) Adaptation (Hz/s)</td>
<td>102.4 ± 59.2</td>
<td>−8.4 ± 15.1</td>
<td>4.0 ± 15.2</td>
<td>−7.0 ± 6.6</td>
</tr>
<tr>
<td>ANOVA, NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9) Minimal steady state frequency (Hz)</td>
<td>19.9 ± 22.4</td>
<td>11.3 ± 6.7</td>
<td>6.2 ± 1.8</td>
<td>9.8 ± 4.2</td>
</tr>
<tr>
<td>ANOVA, P = 0.01</td>
<td>&gt; &lt; &lt; SOM</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&lt; &lt; &lt; SOM</td>
<td>&lt; &lt; &lt; SOM</td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; < < <, and < < <: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and > >, and > > >: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.

| Table 4 |
| Firing properties

<table>
<thead>
<tr>
<th></th>
<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10) Amplitude accommodation (mV)</td>
<td>0.3 ± 0.7</td>
<td>0.6 ± 1.5</td>
<td>4.9 ± 2.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&lt;&lt; &lt; NPY</td>
<td>&lt;&lt; &lt; NPY</td>
<td>&gt; &gt; &gt; SOM.VIP</td>
<td>&lt;&lt; &lt; NPY</td>
</tr>
<tr>
<td>(11) Amplitude of late adaptation (Hz)</td>
<td>50.6 ± 18.9</td>
<td>51.4 ± 23.8</td>
<td>97.1 ± 29.9</td>
<td>23.0 ± 10.0</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&gt; &gt; &gt; PV</td>
<td>&lt; &lt; &lt; PV</td>
</tr>
<tr>
<td>(12) Time constant of late adaptation (ms)</td>
<td>12.8 ± 10.1</td>
<td>41.3 ± 42.4</td>
<td>18.7 ± 6.1</td>
<td>47.2 ± 13.8</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&lt; &lt; &lt; SOM.VP</td>
<td>&lt; &lt; &lt; SOM.VP</td>
<td>&gt; &gt; &gt; SOM.VP</td>
<td>&gt; &gt; &gt; SOM</td>
</tr>
<tr>
<td>(13) Maximal steady state frequency (Hz)</td>
<td>169.9 ± 40.5</td>
<td>88.5 ± 36.1</td>
<td>80.1 ± 17.8</td>
<td>25.8 ± 10.3</td>
</tr>
<tr>
<td>ANOVA, P &lt; 0.001</td>
<td>&gt; &gt; &gt; SOM.NPY</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&gt; &gt; &gt; SOM.VP</td>
<td>&lt; &lt; &lt; NPY</td>
</tr>
<tr>
<td>(14) Late adaptation (Hz/s)</td>
<td>−32.5 ± 14.9</td>
<td>−22.7 ± 18.9</td>
<td>−21.5 ± 14.0</td>
<td>−11.5 ± 3.3</td>
</tr>
<tr>
<td>ANOVA, P = 0.005</td>
<td>&gt; &gt; &gt; SOM</td>
<td>&lt; &lt; &lt; NPY</td>
<td>&lt; &lt; &lt; SOM</td>
<td>&lt; &lt; &lt; SOM</td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; < < <, and < < <: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and > >, and > > >: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.
electrophysiological behaviors. This diversity indicates that the adaptation of firing frequency, broader action potentials, and sustained firing rates with little spike frequency adaptation. Accordingly, it is possible to confirm the presence of several homogeneous classes. Cluster 1 was further subdivided into Cluster 1.1 and Cluster 1.2. Cluster 1.1 is composed of neurons (97%) transcribing PV (77%) and exhibiting high and sustained firing rates with little spike frequency adaptation. This group was thus termed ‘‘Fast-spiking PV’’ (Fast-spiking PV). In contrast, neurons contained in Cluster 1.2 (n = 80) exhibit a marked adaptation of firing frequency, broader action potentials, and are unable to sustain high frequencies of repetitive discharges. However, they show heterogeneous electrophysiological features and transcribe multiple combinations of molecular markers. This cluster was thus further subdivided into Cluster 1.2.1 and Cluster 1.2.2. Cluster 1.2.1 is composed of neurons (93%) transcribing SOM (95%), NPY (67%), and VIP (4%). This group was thus termed ‘‘Adapting SOM’’ (Adapting SOM). Cluster 1.2.2 is composed of neurons (97%) transcribing VGLUT1 (92%), NOS-1 (60%), and PV (81%). This group was thus termed ‘‘Adapting VGLUT1’’ (Adapting VGLUT1). Table 5 summarizes the molecular characterization of the different clusters of neurons transcribing SOM, NPY, and VIP.

Table 5: Molecular characterization of Layer VI interneurons transcribing SOM, NPY, and VIP.

<table>
<thead>
<tr>
<th>Molecular Marker</th>
<th>Cluster 1.1</th>
<th>Cluster 1.1.1</th>
<th>Cluster 1.1.2</th>
<th>Cluster 1.2</th>
<th>Cluster 1.2.1</th>
<th>Cluster 1.2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOM</td>
<td>20%</td>
<td>60%</td>
<td>20%</td>
<td>10%</td>
<td>10%</td>
<td>30%</td>
</tr>
<tr>
<td>NPY</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>20%</td>
<td>10%</td>
</tr>
<tr>
<td>VIP</td>
<td>60%</td>
<td>20%</td>
<td>20%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
</tbody>
</table>

This table indicates that the diversity of molecular markers transcribed by neurons in Layer VI can be explained by the presence of several homogeneous classes. Cluster 1 was further subdivided into Cluster 1.1 and Cluster 1.2. Cluster 1.1 is composed of neurons (97%) transcribing PV (77%) and exhibiting high and sustained firing rates with little spike frequency adaptation. This group was thus termed ‘‘Fast-spiking PV’’ (Fast-spiking PV). In contrast, neurons contained in Cluster 1.2 (n = 80) exhibit a marked adaptation of firing frequency, broader action potentials, and are unable to sustain high frequencies of repetitive discharges. However, they show heterogeneous electrophysiological features and transcribe multiple combinations of molecular markers. This cluster was thus further subdivided into Cluster 1.2.1 and Cluster 1.2.2. Cluster 1.2.1 is composed of neurons (93%) transcribing SOM (95%), NPY (67%), and VIP (4%). This group was thus termed ‘‘Adapting SOM’’ (Adapting SOM). Cluster 1.2.2 is composed of neurons (97%) transcribing VGLUT1 (92%), NOS-1 (60%), and PV (81%). This group was thus termed ‘‘Adapting VGLUT1’’ (Adapting VGLUT1).
aggregation dendrogram. These subclusters were hence analyzed separately. Cluster 1.2.1.1 (n = 15) is composed of cells transcribing NPY (100%), rarely transcribing PV (7%), and never SOM (0%) or VIP (0%). This cluster was named the “Adapting NPY” (Adapt-NPY) cluster. Cluster 1.2.1.2 is composed of 4 cells transcribing VIP and was identified as “Adapting VIP” (Adapt-VIP).

Ward’s method has the advantage not to make any prior assumptions about the number of groups into which individual data points should be segregated. However, Ward’s algorithm does not correct for neurons, which are potentially misassigned during the iterative process. This drawback was corrected using the K-means algorithm initialized on Ward’s clusters centroids (see Materials and Methods). Following this correction, 14 cells were reassigned into a different cluster (Fig. 1f). Four cells originally assigned to the FS-PV group by the Ward clustering fell into the Adapt-SOM cluster and 2 cells into the Adapt-NPY cluster when using the K-means algorithm. In the same way, the Adapt-SOM group gave away 6 cells to the FS-PV group and 1 to the Adapt-NPY cluster. Finally the GABAergic cell, which remained in the glutamatergic group, was reassigned to the Adapt-SOM cluster.

### Table 7
Soma under IR microscopy

<table>
<thead>
<tr>
<th></th>
<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Area (μm²)</td>
<td>220.2 ± 117.5</td>
<td>168.1 ± 38.4</td>
<td>169.0 ± 131.6</td>
<td>98.6 ± 41.6</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Perimeter (Km)</td>
<td>55.9 ± 13.1</td>
<td>51.1 ± 8.1</td>
<td>48.8 ± 15.1</td>
<td>40.8 ± 11.0</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Maximal Diameter (μm)</td>
<td>20.4 ± 5.4</td>
<td>19.6 ± 4.0</td>
<td>19.4 ± 6.0</td>
<td>16.5 ± 5.5</td>
</tr>
<tr>
<td>ANOVA: NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Minimal Diameter (μm)</td>
<td>13.7 ± 3.0</td>
<td>11.4 ± 1.5</td>
<td>11.2 ± 3.4</td>
<td>8.0 ± 1.4</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5) Elongation</td>
<td>1.5 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td>1.7 ± 0.4</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>ANOVA: NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; <, <, and <<: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and >, >>, and >>>: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.

### Table 8
Local metric of dendrites

<table>
<thead>
<tr>
<th></th>
<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6) Number of primary dendrites</td>
<td>6.3 ± 1.5</td>
<td>4.7 ± 1.2</td>
<td>8.5 ± 2.7</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7) Total dendritic length (μm)</td>
<td>2080.1 ± 815.2</td>
<td>2327.4 ± 753.9</td>
<td>1815.5 ± 1013.0</td>
<td>1548.0 ± 548.5</td>
</tr>
<tr>
<td>ANOVA: P = 0.043</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8) Length of segments (average) (μm)</td>
<td>45.6 ± 13.9</td>
<td>56.6 ± 14.0</td>
<td>27.1 ± 7.7</td>
<td>48.2 ± 15.9</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9) Length/surface</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10) Dendritic segments tortuosity (average)</td>
<td>1.20 ± 0.05</td>
<td>1.21 ± 0.04</td>
<td>1.16 ± 0.03</td>
<td>1.29 ± 0.07</td>
</tr>
<tr>
<td>ANOVA: P = 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11) Number of nodes</td>
<td>20.7 ± 9.36</td>
<td>18.7 ± 7.52</td>
<td>28.45 ± 17.81</td>
<td>16.50 ± 11.39</td>
</tr>
<tr>
<td>ANOVA: P = 0.032</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12) Fractal index</td>
<td>&lt;NPY</td>
<td>&lt;NPY</td>
<td>&lt;NPY</td>
<td>&lt;NPY</td>
</tr>
<tr>
<td>ANOVA: P = 0.034</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; <, <, and <<: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and >, >>, and >>>: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.

### Table 9
Spatial distribution of the dendritic arbor

<table>
<thead>
<tr>
<th></th>
<th>Fast-spiking PV, n = 44</th>
<th>Adapting SOM, n = 22</th>
<th>Adapting NPY, n = 15</th>
<th>Adapting VIP, n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(13) Dendritic Sholl (0-100 μm) (%)</td>
<td>64.4 ± 14.7</td>
<td>48.0 ± 12.3</td>
<td>90.1 ± 10.1</td>
<td>62.4 ± 14.9</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(14) Dendritic Sholl (100-200 μm) (%)</td>
<td>28.2 ± 8.8</td>
<td>36.0 ± 7.1</td>
<td>9.0 ± 9.0</td>
<td>32.5 ± 7.9</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15) Dendritic Sholl (200-300 μm) (%)</td>
<td>6.4 ± 8.1</td>
<td>13.8 ± 8.8</td>
<td>0.9 ± 2.2</td>
<td>5.1 ± 7.6</td>
</tr>
<tr>
<td>ANOVA: P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16) Dendritic Sholl (&gt;300 μm) (%)</td>
<td>1.0 ± 2.4</td>
<td>2.2 ± 0.9</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>ANOVA: P = 0.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(17) Vertical extent (%)</td>
<td>39.6 ± 14.7</td>
<td>45.7 ± 22.7</td>
<td>35.7 ± 9.9</td>
<td>63.8 ± 15.9</td>
</tr>
<tr>
<td>ANOVA: P = 0.046</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(18) Horizontal extent (%)</td>
<td>29.7 ± 15.3</td>
<td>28.5 ± 22.0</td>
<td>31.2 ± 12.0</td>
<td>14.2 ± 9.7</td>
</tr>
<tr>
<td>ANOVA: NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: n: number of cells; NS: nonsignificant; <, <, and <<: inferior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively; and >, >>, and >>>: superior with P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively.
Characterization of Layer VI FS-PV Interneurons

FS-PV form the largest cluster of GABAergic interneurons in our sample (n = 44; Fig. 1C). These cells have the most distinctive electrophysiological properties. They are characterized by significantly higher maximal frequencies of discharge (Table 4), shorter spike durations (Table 5), and faster AHPs (Table 5 and Fig. 3: A1, A2 insets) than the neurons of other groups. When depolarized around threshold FS-PV typically fired clusters of 3-6 action potentials. Though interspike intervals were relatively invariant within spike clusters, the duration of the time interval separating consecutive clusters was less predictable (stuttering pattern, Gupta et al. 2000; Ascoli et al. 2008), resulting in highly variable measures of minimal steady state frequency (Table 3). In addition, these cells significantly differ
from other neurons on the basis of passive membrane properties, such as a hyperpolarized resting potential, a low input resistance, and a low time constant (Table 2).

The fundamental molecular characteristics of neurons in the FS-PV cluster are the high expression of PV mRNA (81.8%) (Table 6 and Fig. 3E1,E2,F1,F2), a feature that has been consistently reported in fast-spiking neurons of superficial cortical layers in the rodent (Kawaguchi and Kubota 1993; Caud et al. 1997; Kawaguchi and Kubota 1997; Toledo-Rodriguez et al. 2005). Interestingly, we found that FS-PV cells of layer VI

Table 10
Parameters contribution to K-means clustering (Fig. 2)

<table>
<thead>
<tr>
<th>Electrophysiological parameters</th>
<th>Silhouette loss (%)</th>
<th>Molecular parameters</th>
<th>Silhouette loss (%)</th>
<th>Morphological parameters</th>
<th>Silhouette loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrambled parameter</td>
<td></td>
<td>Scrambled parameter</td>
<td></td>
<td>Scrambled parameter</td>
<td></td>
</tr>
<tr>
<td>Total electrophysiology</td>
<td>55.0 ± 3.2</td>
<td>Total molecular expression</td>
<td>20.7 ± 4.4</td>
<td>Total morphology</td>
<td>22.7 ± 2.1</td>
</tr>
<tr>
<td>(20) First spike AHP max latency (ms)</td>
<td>9.9 ± 0.6</td>
<td>VIP</td>
<td>9.0 ± 0.9</td>
<td>(6) Number of primary dendrites</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>(16) First spike duration (ms)</td>
<td>8.7 ± 0.5</td>
<td>PV</td>
<td>6.0 ± 0.7</td>
<td>(14) Dendritic Sholl (200–300 μm) (%)</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>(13) Membrane steady state frequency (Hz)</td>
<td>9.6 ± 0.7</td>
<td>CA</td>
<td>5.2 ± 0.7</td>
<td>(13) Dendritic Sholl (100–200 μm) (%)</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>(3) Membrane time constant (ms)</td>
<td>8.4 ± 0.7</td>
<td>SOM</td>
<td>3.9 ± 0.9</td>
<td>(12) Dendritic Sholl (0–100 μm) (%)</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>(10) Amplitude accommodation (mV)</td>
<td>7.1 ± 0.6</td>
<td>vGluT1</td>
<td>2.1 ± 1.0</td>
<td>(9) Length/surface</td>
<td>2.6 ± 1.0</td>
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<td>(6) Rheobase (pA)</td>
<td>6.0 ± 0.4</td>
<td>NPY</td>
<td>1.7 ± 0.4</td>
<td>(8) Length of segments (average) (μm)</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>(11) Amplitude of late adaptation (Hz)</td>
<td>4.9 ± 0.6</td>
<td>CR</td>
<td>1.0 ± 0.5</td>
<td>(11) Number of nodes</td>
<td>1.9 ± 0.7</td>
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<tr>
<td>(5) Sag index (%)</td>
<td>4.3 ± 0.5</td>
<td>COX</td>
<td>0.9 ± 0.5</td>
<td>(10) Soma elongation</td>
<td>1.2 ± 1.0</td>
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<td>(2) Input resistance (MOhms)</td>
<td>4.1 ± 0.6</td>
<td>NOS-1</td>
<td>0.4 ± 0.6</td>
<td>(1) Soma area (μm²)</td>
<td>0.9 ± 0.7</td>
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<tr>
<td>(12) Time constant of late adaptation (ms)</td>
<td>3.7 ± 0.8</td>
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<td></td>
<td>(10) Segments tortuosity (average)</td>
<td>0.9 ± 0.8</td>
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<tr>
<td>(28) Duration increase (%)</td>
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<td></td>
<td></td>
<td>(16) Vertical extent (%)</td>
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<td>(1) Resting potential (mV)</td>
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<td></td>
<td>(17) Horizontal extent (%)</td>
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<td>(17) First spike ADP (mV)</td>
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<td></td>
<td>(7) Total dendritic length (μm)</td>
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<tr>
<td>(14) Late adaptation (Hz/s)</td>
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<td>(19) First spike ADP latency (ms)</td>
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<td>(4) Membrane capacitance (pF)</td>
<td>1.0 ± 0.4</td>
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<td>(15) First spike amplitude (mV)</td>
<td>0.8 ± 0.5</td>
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<tr>
<td>(27) Amplitude reduction (%)</td>
<td>0.8 ± 1.1</td>
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</table>

Figure 2. Clustering of layer VI interneurons including morphological parameters. (A) Ward clustering of 85 layer VI interneurons. Individual cells are represented along the x-axis. The y-axis represents the distance of aggregation (see Materials and Methods) in a space of 18 electrophysiological variables, 9 molecular markers, and 13 morphological parameters measured on the soma and dendrites. As in Figure 1, 4 clusters named FS-PV, Adapt-NPY, Adapt-VIP, and Adapt-SOM could be identified. (B) Matching table of the K-means clusters described in Figure 1 and of the clusters generated by the K-means algorithm with the subset of 85 layer VI interneurons and the parameters used in A. Note that the clusters are mostly overlapping. (C) Silhouette plot of the K-means clustering including morphology. The y-axis represents cells ranked in decreasing order of silhouette value within each cluster. The x-axis represents the silhouette value of individual neurons. Negative value indicates cells, which are closer from the cells of a neighboring cluster on average. (D) Scaled representative reconstruction of neurons in each cluster. The somatodendritic trees are in black. Axons are represented in gray. For each cluster except Adapt-VIP, a neuron of layer VIa and a neuron of layer VIb are represented. (E) Mean silhouette value of the full polythetic clustering and clustering generated following the scrambling of electrophysiological, molecular, or morphological parameters altogether or separately. Scrambling parameters result in a decrease of the mean silhouette value of the clusterings. Error bars correspond to standard deviation over 1000 independent randomizations.
displayed consistent transcription of NPY (65.9%) and SOM (43.2%).

FS-PV neurons display significantly larger somata than most other neurons (Table 7). They exhibit 5–7 primary dendrites branching infrequently and are characterized by large dendritic arborizations extending further than 300 μm away from the soma (Table 9).

The properties of cells in the FS-PV cluster were compared according to their sublaminar location in layer VIa (34 neurons) or VIb (10 neurons). In layer VIb, FS-PV cells display significantly distinct patterns of adaptation of firing frequency at saturation, lower minimal steady state frequencies, and shorter time constant (Table 9). The dendritic arborization of FS-PV cells of layer VIb appears to ramify significantly closer to the soma, as illustrated by Sholl analysis (Table 11). Finally in layer VIa and VIb, FS-PV tend to fit to the vertical extent of their sublayer of origin resulting in a flat appearance of the dendritic extent of FS-PV cells in layer VIb (Fig. 2C). Accordingly, cells in layer VIb extend significantly less in the vertical directions (Table 11).

The axons of 14 FS-PV cells of layer VIa and 2 FS-PV cells of layer VIb were reconstructed to a length exceeding 1 mm. The axons of FS-PV cells in layer VIb (2/2) and the majority of those in layer VIa (10/14) ramify primarily within their sublayer of origin. However, the axons of 4 cells in layer VIa display ascending arborization extending up to layer IV.

Figure 3. Multiparametric characterization of FS-PVs. The electrophysiological behavior (A), somatodendritic morphology (C), and scRT-PCR products (F) of representative cells of the FS-PV cluster in layer VIa (left (1)) and in layer VIb (right (2)) are illustrated. (A) Voltage responses induced by current injections (duration: 800 ms): bottom traces: –100 to 0 pA by increments of 10 pA and rheobase; top trace: last step of current before saturation; inset: AHP of the first spike at rheobase (rectangle). (B) IR videomicroscopic picture of the neuron whose trace is represented in A. (C) Three-dimensional reconstruction of the neuron whose trace is represented in A. (D) Wedge analysis of FS-PVs (Materials and Methods) of layer VIa (D1) and VIb (D2). (E): Frequencies of occurrence of molecular markers in FS-PVs of layer VIa (E1) and VIb (E2). Filled bars correspond to frequencies above 0.5 (dashed line). (F) Gel electrophoresis of scRT-PCR products of the cell represented in A. MW: molecular weigh marker.
Sublaminar specificity of layer VI interneurons

<table>
<thead>
<tr>
<th></th>
<th>Vla</th>
<th>Vlb</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>(3) Membrane time constant (ms)</td>
<td>24.2 ± 13.3</td>
<td>15.8 ± 4.8</td>
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<td>(9) Minimal steady state frequency (Hz)</td>
<td>23.8 ± 23.9</td>
<td>6.7 ± 7.4</td>
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<td>(12) Time constant of late adaptation (ms)</td>
<td>14.7 ± 16.7</td>
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<td>(14) Late adaptation (Hz/s)</td>
<td>−30.1 ± 15.5</td>
<td>−40.5 ± 9.6</td>
<td>0.018</td>
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<td>(12) Dendritic Sholl (0-100 μm) (%)</td>
<td>61.7 ± 14.0</td>
<td>73.0 ± 13.8</td>
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<td>(14) Dendritic Sholl (200-300 μm) (%)</td>
<td>7.6 ± 8.4</td>
<td>2.5 ± 5.7</td>
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<td>(16) Vertical extent (%)</td>
<td>41.5 ± 13.3</td>
<td>22.8 ± 18.1</td>
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<tr>
<td>Adapt-SOM</td>
<td>n = 16</td>
<td>n = 6</td>
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<td>(16) First spike duration (ms)</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.036</td>
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<td>(22) Second spike duration (ms)</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>(3) Membrane time constant (ms)</td>
<td>11.0 ± 1.4</td>
<td>12.5 ± 1.3</td>
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</tr>
<tr>
<td>(16) Vertical extent (%)</td>
<td>54.1 ± 18.6</td>
<td>23.5 ± 18.0</td>
<td>0.006</td>
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<tr>
<td>(17) Horizontal extent (%)</td>
<td>18.8 ± 13.3</td>
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<td>0.002</td>
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<tr>
<td>Adapt-NPY</td>
<td>n = 9</td>
<td>n = 6</td>
<td></td>
</tr>
<tr>
<td>(4) Minimal diameter (μm)</td>
<td>9.8 ± 1.3</td>
<td>13.3 ± 4.6</td>
<td>0.018</td>
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</table>

Characterization of Layer VI Adapt-NPY Interneurons

Adapt-NPY neurons form a relatively small cluster (n = 15, Fig. 2B). However, these cells share distinctive properties. On an electrophysiological basis, Adapt-NPY are most readily characterized by large monophasic AHPs, which are significantly longer for neurons of other groups (Table 5 and Fig. 5A1, A2) as well as by significantly larger accommodation humps occurring at the beginning of spike trains (illustrated by an asterisk in the upper trace of the Fig. 5A1, A2 and Table 4). Seven out of 15 Adapt-NPY interneurons display a marked delayed firing occurring at the top of a slow depolarizing ramp, characteristic of late-spiking neuronal firing (Kawaguchi 1995). Accordingly, Adapt-NPY cells have an accelerating firing pattern around threshold (Table 3). However, their firing pattern shows a marked adaptation around maximal firing frequency (Table 4).

All Adapt-NPY interneurons transcribe NPY but never SOM, CR, or CB (Table 6). By contrast, CCK and NOS-1 are transcribed by 46.7% and 40% of Adapt-NPY interneurons, respectively (Fig. 5E1, E2, F1, F2 and Table 6).

Adapt-NPY neurons display the most distinctive somatodendritic morphologies of our sample. They are characterized by somata exhibiting 7–9 primary dendrites (Table 8). Compared with the neurons of other groups, their dendritic arbor displays significantly thinner and more highly branched segments (Table 8) and ramifies significantly more within a radius of 100 μm around the cell body (Table 9). As a result, these cells form a dense plexus of dendrites around their somata (Table 9 and Figs 2D and 5G1, C1, C2) as indicated by their significantly higher fractal index (Table 8). The axons of one Adapt-NPY in layer Vla and one in layer Vlb have been reconstructed to a length in excess of 1 mm. In both layers, they appear to ramify densely around the soma (Fig. 1C). The combination of these electrophysiological, morphological, and molecular features makes Adapt-NPY very likely to correspond to neurogliaform interneurons (Jones 1984; Kawaguchi 1995). Interestingly, Adapt-NPY neurons of layer Vla (10 neurons) and of layer Vlb (6 neurons), only significantly differ regarding the extent of their minimal diameter (Table 11), among the 55 electrophysiological, molecular, and morphological parameters tested in this study.

Characterization of Layer VI Adapt-VIP Interneurons

Adapt-VIP form a small cluster of 4 cells, well segregated by Ward’s algorithm. One of these cells was recorded in layer Vlb. This sample is unlikely to be fully representative of layer VI VIP interneurons. However, VIP transcribing cells display consistent features that distinguished them from other GABAergic cells. Adapt-VIP neurons have characteristic passive membrane properties. They have significantly higher input resistances (Table 2) and lower membrane capacitances (Table 2) than other neurons, reminiscent of the properties of VIP-expressing cells in the superficial layers of the mouse neocortex (Vucurovic et al. 2010). They show a marked adaptation of firing frequency around threshold (Table 3) and saturation (Table 4), and their maximal firing frequency is significantly lower than for other groups of interneurons (Table 4). Three out of four cells exhibit a sharp monophasic AHP. The remaining cell displays a biphasic AHP with a high amplitude fast component and a low amplitude slow component (data not shown). Finally, Adapt-VIP neurons have a low sag index (Table 2).

In good agreement with previous reports performed in the superficial layers (Cauli et al. 1997, 2000, Kawaguchi and Kubota
Adapt-VIP neurons do not transcribe SOM, NPY, PV, or CB, but 3 of them transcribe CR (Table 6). Layer VI Adapt-VIP cells are characterized by small somata. Their number of primary dendrites range from 2 to 4 (Table 8) and their dendritic arbors display a wide variety of spatial organizations (Figs 2D and 6C). However, wedge analysis indicates that the somatodendritic arborizations of Adapt-VIP are preferentially orientated radially toward the pial surface (Fig. 6D).

Distribution of PV-, SOM-, NPY-, and VIP-Expressing GABAergic Neurons in Layer VI

Though powerful for classification, multiparametric analysis using whole-cell patch-clamp recordings on acute slices are not suited to estimate cell densities and repartition within brain regions because of the inherent sampling bias of this approach. Moreover, mRNA expression has been found to exhibit divergences with data based on immunodetection, probably resulting from translational regulations (Burkhalter 2008). In order to complete our description of layer VI interneurons, we examined the repartition of GABAergic interneurons expressing PV, SOM, VIP, and NPY proteins in layer VI using immunohistochemistry. In order to avoid underdetection of GAD-expressing cells, our analysis was performed on a strain of GAD67-GFP knock-in transgenic mice (Tamamaki et al. 2003), where GFP has been shown to be restricted to and expressed in all cortical GABAergic neurons. Mice were perfused at P17 in order to match the age of mice used to perform multiparametric...
All quantifications were performed in the region of the PMBSF (see Materials and Methods). Our protocol was designed to detect variations of cell densities according to cell location. The vertical extent of layer VI was divided into 8 equal bins (Fig. 7A and see Materials and Methods). The deepest bin (bin 8) was found to closely correspond to layer VIb (Fig. 7A). Labeled cells were counted in each bin, and the numbers of positive cells were normalized by areas so as to provide measures of density (expressed as cell/mm², Fig. 7B).

GABAergic cells in layer VI have an overall density of 428.2 ± 3.5 cell/mm² of tissue. The distribution of GABAergic cells appears to vary significantly across the vertical extent of layer VI (Fig. 7). Cell densities are highest in the superficial portion of layer VI (bins 1, 2, and 3 Fig. 7B) reaching a maximum in bin 2 with a density of 632.6 ± 84.0 cell/mm². Densities appear significantly less elevated in deeper layer VIa (bins 4, 5, 6, and 7) reaching a second maximum of 399.6 ± 23.9 cell/mm² in bin 5.

GABAergic cells appear to have the lowest concentration in layer VIb (bin 8, Fig. 7A, B) where their density was estimated as 258.5 ± 40.6 cell/mm².

PV-expressing GABAergic interneurons were the most densely distributed with an overall density of 163.8 ± 4.2 cell/mm². PV-expressing cells appear to be significantly more concentrated in upper layer VI (bins 1, 2, and 3, Fig. 8A–F). Their density peaks...
in bin 2 where it reached $362.5 \pm 16.9$ cell/mm$^2$ representing 57.3\% of GABAergic interneurons at this depth. PV-expressing interneurons are therefore largely responsible for the elevated concentration of GABAergic cells found in upper layer VIa. The density of PV-expressing cells diminishes significantly in lower layer VIa (bins 5, 6, and 7) and is sparse in layer VIb ($18.2 \pm 12.6$ cell/mm$^2$), representing only 7\% of GAD67:GFP-expressing neurons.

NPY was the second most densely expressed marker, among layer VI GABAergic cells, with a density of $134.3 \pm 29.3$ cell/mm$^2$. NPY-labeled GABAergic cells are moderately concentrated in the upper portion of layer VI. They reach their lowest density in bin 2 with a value of $78.5 \pm 41.3$ cell/mm$^2$ (Fig. 8B4). They are, however, significantly more concentrated in the lower fraction of layer VIa (bins 4, 5, 6, and 7) culminating in bin 6 (Fig. 8B4) with a density of $190.8 \pm 36.7$ cell/mm$^2$ (49.8\% of GABAergic cells). Interestingly, NPY-expressing interneurons represent 43.1\% of GABAergic cells in layer VIb.

SOM-immunoreactive GABAergic cells appeared homogeneously distributed through layer VI. Accordingly, there is no significant difference between the densities of SOM-expressing interneurons located at different depths of layer VI (Fig. 8C4). These cells have a mean density of $89.8 \pm 18.7$ cell/mm$^2$, representing 21\% of layer VI GABAergic cells.

VIP-labeled GABAergic interneurons are very sparse in layer VI (Fig. 8D4). Their density was estimated to be $24.1 \pm 3.2$ cell/mm$^2$ across layer VI. Our study therefore indicates that VIP-expressing cells represent only a minor proportion of layer VI GABAergic interneurons, explaining the paucity of VIP-expressing cells in the sample used in this study for multiparametric analysis.

**Discussion**

Layer VI GABAergic interneurons have only been partly described at the present time, limiting the understanding of their function in the neocortical network (Thomson 2010). Combining patch-clamp recordings, scRT-PCR, biocytin labeling, and unsupervised clustering, we identified 4 populations of GABAergic interneurons in cortical layer VIa and VIb. Additionally, using immunohistochemistry on GAD67-GFP knock-in mice (Tamura et al. 2003), we described the distribution of GABAergic cells expressing PV, SOM, VIP, and NPY in layer VI, demonstrating that PV- and NPY-expressing interneurons concentrate in the upper and the lower part of layer VI, respectively. This study provides the first multiparametric characterization of layer VI interneurons and indicates that neocortical layer VI comprises a complex network that may be subdivided into distinct functional territories.

**Identification of Classes of Layer VI Interneurons**

Neocortical interneurons constitute a highly heterogeneous population diversifying according to morphological, electrophysiological, and molecular characteristics. The technical difficulties of collecting all these features on single cells have often led researchers to focus only on the description of particular features. As a result, different laboratories tend to adopt distinct classifications that are sometimes hard to relate to each others. Following the Petilla Interneuron Nomenclature Group’ recommendations (Ascoli et al. 2008), a reliable characterization of neocortical interneurons should take physiological, morphological, and molecular features into account. In this study, these 3 main criteria were used to characterize layer
The results of unsupervised approaches are also highly dependent on the parameters, which are used for clustering. In this study, in order to gain insight about the relevance of parameters, the quality of the results was verified by comparison with clustering performed on randomized databases (see Materials and Methods; Karagiannis et al. 2009). Scrambling individual parameters systematically resulted in an average loss of clustering quality, indicating that each parameter was beneficial for clustering. The loss of quality induced by the scrambling of individual parameters also allowed to provide an estimate of their relevance as classifiers (Table 10). Randomizing parameters altogether, highly disrupted the quality of our results indicating that the classification proposed in our study arose from the correlation of electrophysiological, molecular, and electrophysiological features. Accordingly, very similar results were found with or without the inclusion of morphological parameters, as already observed for supragranular interneurons (Karagiannis et al. 2009). These observations indicate the robustness of our classification scheme. Thus, we are confident that the clustering proposed in this study truly discloses a fundamental aspect of the diversity of layer VI interneurons.

It is important to note, however, that the description provided in this study remains conditioned by the specificity of our technical approach. Firstly, as in many electrophysiological studies, our experiments were performed on P14--P--20 juvenile animals. Indeed, at this stage, the integrity of surviving cells, which is a prerequisite to quality recordings, is easier to obtain, and neocortical interneurons have acquired many of their adult characteristics (Minelli et al. 2003; Long et al. 2005). However, the neocortical network continues to mature until up to P90 (Uylings et al. 1990; Luhmann and Prince 1991; Dorn et al. 2010). Thus, it is likely that some of the properties of interneurons reported here evolve at more mature stages.

A second important specificity of our approach is the use of scRT-PCR. This technique is, to our knowledge, the sole way to test the presence of up to several dozens of markers on single neurons, which is of high interest to obtain robust multiparametric clusterings. However, this technique detects marker expressions at the mRNA level. Due to potential translational regulations, the transcription of mRNAs does not necessarily predict the expression of corresponding proteins. A second issue is that scRT-PCR, requires to harvest up 2 one-third of the cytoplasm of recorded neurons, which is not optimal for biocytin labeling. In our conditions, axonal arborizations, in particular, could not be acceptably recovered. Thus, we expect that the comprehensive multiparametric characterization of sampled neurons, provided in this study, will prove helpful to complement our description of layer VI interneurons, with approaches providing good quality morphological reconstructions even at the expense of a thorough molecular description. In that regard, our quantification of the contribution of each parameter to clustering (Table 10) might constitute a useful body of data for researchers willing to choose pertinent classifiers of interneurons.

Figure 7. GAD67 expression across the radial extent of layer VI. (A) Representative photomicrograph of DAPI staining (left) and GFP expression (right) in the primary somatosensory cortex of GAD67-GFP knock-in mice. Layers and sublayers (plain lines) as well as schematic bins of layer VI (dashed lines) are superimposed. Bins are numbered in increasing order, from the most superficial to the deepest. (B) Histogram of the densities of GFP-expressing cells in bins of the extent of layer VI from the lower border of layer V to white matter. The y-axis labels refer to the numbers attributed to the depth bins in A (n = 3 mice; error bars: standard deviation; < or > indicates significant difference with P \leq 0.05).

VI interneurons. The characteristics of sampled cells were quantified through a set of 56 parameters. Finally, so as to eliminate bias induced by personal interpretation, interneurons were classified with unsupervised methods.

Several parameters can affect the quality of unsupervised approaches. First, the results are dependent on the intrinsic characteristic and the limitation of clustering algorithms. The 2 techniques most commonly applied to the classification of cortical interneurons are Ward’s method (Tamas et al. 1997; Cauli et al. 2000; Dumitriu et al. 2007; Gallop et al. 2006; David et al. 2007; Chen et al. 2009) and the K-means algorithm (Karagiannis et al. 2009; McGarry et al. 2010; Tricoire et al. 2011). Due to its iterative nature, Ward’s method has the drawback that misassigned cells are not corrected. The K-means method, on the other hand, generates nonoverlapping clusters but requires the number of groups to be set in advance. In the study, we used a combination of both methods. This enabled us to eliminate the drawbacks of each technique by generating nonoverlapping clusters while making no assumption about the number of classes into which interneurons should be classified.
disclosed 4 distinct types of interneurons in layer VI: FS-PV, Adapt-SOM, Adapt-NPY, and Adapt-VIP. These groups are remarkably similar to classes identified using a similar approach in layers II–IV of the rat neocortex (Karagiannis et al. 2009), indicating that GABAergic interneurons form comparable populations in layer VI and in superficial layers of the neocortex.

Fast-spiking cells expressing PV have been well characterized in more superficial layers and correspond mainly to basket cells (Kawaguchi 1993; Wang et al. 2002) and to axo-axonic chandelier cells (Kawaguchi 1995; Szabadics et al. 2006; Woodruff et al. 2009). Although we have not identified their morphological subclass in the current study, layer VI FS-PV interneurons adopted similar features to superficial layer FS-PV cells, such as a low membrane resistance and a high frequency of discharge (McCormick et al. 1985). Agreeing with previous reports (Kumar and Ohana 2008), our study suggest that in addition to intralaminar neurons, layer VI FS-PV include translaminar neurons, projecting to layer IV.

Layer VI Adapt-SOM neurons had depolarized resting potentials, pronounced voltage sags, and frequently transcribed CB and NPY. This was reminiscent of the properties of SOM-expressing interneurons in superficial layers (Wang et al. 2004;
Halabisky et al. 2006; Uematsu et al. 2008; Karagiannis et al. 2009; McGarry et al. 2010). In superficial layers, SOM-expressing cells correspond mainly to layer I projecting Martinotti cells (Kawaguchi and Kubota 1996) and layer IV projecting neurons (Ma et al. 2006). None of the reconstructed cells in our sample was found to project to layer I. However, 50% were found to branch within layer IV confirming previous descriptions of layer VI interneurons (Kumar and Ohana 2008). Agreeing with previous studies (West et al. 2006), our results suggest that layer VI Adap-SOM also include intralaminar neurons. 

Layer VI Adap-NPY neurons shared most of the typical features of neuroglialform neurons, such as dense dendritic arborizations confined 200 μm around the soma, delayed firings, characteristic medium AHPs (Kawaguchi 1995; Tamas et al. 2003; Olah et al. 2009), marked frequency adaptations at high firing rates, and pronounced amplitude accommodations during action potential discharges (Karagiannis et al. 2009). Moreover, Adap-NPY interneurons presented a high transcription of NPY (Kawaguchi and Kubota 1997), but not SOM, VIP, or any calcium-binding protein (Karagiannis et al. 2009; Rancillac et al. 2010; Kubota et al. 2011).

Four VIP transcribing cells were segregated by the unsupervised clustering and exhibited typical properties of VIP-expressing interneurons described in layer II/III. They displayed a high membrane resistance, low membrane capacitance adapting firing patterns, and transcription of CR (Kawaguchi and Kubota 1997; CauI et al. 2000).

Finally, it is interesting to note that each cluster contained a substantial fraction of neurons expressing NOS-1. The properties of these neurons will be addressed specifically in a parallel study.

**Specific Distributions of GABAergic Neuron Subpopulations within Layer VI**

While patch-clamp recording permits interneuron subtypes to be identified through multiparametric analysis, it does not allow unbiased spatial distributions to be estimated. Immunodetection of PV, SOM, VIP, and NPY, on the other hand, labels specific interneurons subpopulations and allows a direct visualization of their dispersion (Gonchar et al. 2007; Xu et al. 2010). Thus, in order to complement our description, we used this approach to disclose how interneuron subgroups populate layer VI. Antibodies raised against PV, SOM, and VIP have been used and validated on adult mice of the same strain (Xu et al. 2010). The overall layer VI density of PV, SOM, and VIP was coherent between our study and that of Xu et al. (2010), indicating that the mature expression of these markers is already set at juvenile stages. By contrast, NPY expression appeared to be nearly twice as elevated in our study. This difference is likely due to the age of animals, since NPY expression has been shown to decrease in adults (Obst and Wahle 1997).

Our results indicate that PV, SOM, VIP, and NPY adopted distinct distributions in layer VI. PV-expressing cells accumulated in the upper layer VI, while NPY-expressing interneurons were more densely distributed in the lower parts. Though NPY can be immunolabeled with other markers, the immunodetection of PV, SOM, and VIP labels 3 distinct groups in rat (Gonchar and Burkhalter 1997; Kubota et al. 2011) and mouse (Gonchar et al. 2007; Xu et al. 2010). Therefore, our data strongly suggest that separate groups of interneurons accumulate in distinct parts of layer VI.

Discrepancies exist between data based on the detection of mRNAs and data relying on the immunodetection of proteins. In neocortical interneurons in particular, PV-immunolabeled neurons do not coexpress other calcium-binding protein or peptides at the protein level (Kubota and Kawaguchi 1994; Gonchar and Burkhalter 1997; Gonchar et al. 2007; Xu et al. 2010; Kubota et al. 2011), whereas the mRNA of PV has been consistently codetected with the mRNAs of SOM, NPY, and CB (Cauli et al. 1997; Gallop et al. 2006; Karagiannis et al. 2009; Lee et al. 2010; present study). In our study, primers pairs used in the PCRs were positioned on 2 different exons, allowing the detection of possible genomic DNA contaminations. Furthermore, the use of nested primers in the second round of PCR increased the specificity of cDNAs amplifications, making it extremely unlikely that these colocalizations were the results of false positivity. Quantitative estimates indicate that scRT-PCR is sensitive enough to detect the presence of single transcripts (Tsuzuki et al. 2001). Thus, the detection of unusual cotranscriptions is most likely to reflect the presence of transcripts expressed at low levels, as confirmed recently in hippocampal interneurons (Tricoire et al. 2011).

The functional impact of these rare mRNA species remains to be determined. Nevertheless, due to their presence, it is not totally straightforward to determine how populations defined by immunolabeling correspond to classes identified through multiparametric analysis. PV-labeled interneurons appeared significantly more concentrated in the upper parts of layer VI. PV immunodetection has been consistently associated with fast-spiking phenotypes (Kawaguchi and Kubota 1993; Kawaguchi and Kubota 1997; Uematsu et al. 2008). Therefore, our data suggest that at least a subset of the FS-PV interneurons concentrate in the upper layer Vla. By contrast, NPY-expressing interneurons appeared significantly more concentrated in the lower regions of layer VI. NPY mostly colocalizes with SOM at the protein level (Xu et al. 2010). In lower layer VI, the concentration of NPY-expressing interneurons exceeded the density of SOM-expressing interneurons, indicating an accumulation of GABAergic neurons expressing NPY only. As, layer VI Adap-NPY never transcribed SOM, our data suggest that neuroglialform neurons could accumulate in the deeper part of layer VI.

**Integration of GABAergic Interneurons in Layer Vla and Vlb**

Layer VI can be subdivided into layer Vla, which occupies the main part of layer VI, and layer Vlb, which constitutes a narrow band of cells densely packed at the interface of gray and white matters (Allendoerfer and Shatz 1994; Clancy et al. 2001). In our study, FS-PV and Adap-SOM neurons displayed specific electrophysiological, molecular, and morphological features in layer Vla and layer Vlb, strengthening the view that each sublayer constitutes a segregated functional network (Allendoerfer and Shatz 1994; Jones 2009).

Layer Vla constitutes a major source of CT efferents (Jones 2009) as well as a primary recipient of thalamocortical afferents (Petersen 2007; Meyer et al. 2010). Layer Vlb is predominantly composed of glutamatergic principal neurons that are believed to persist from the developmental subplate (Clancy et al. 2009; Friedlander and Torres-Reveron 2009; Luhmann et al. 2009;
Layer VI Interneurons


Kawaguchi Y, Kubota Y. 1996. Physiological and morphological identification of somatostatin- or vasoactive intestinal polypeptide-


