Distinct Subtypes of Somatostatin-Containing Neocortical Interneurons Revealed in Transgenic Mice

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GABA-releasing inhibitory interneurons in the cerebral cortex can be classified by their neurochemical content, firing patterns, or axonal targets, to name the most common criteria, but whether classifications using different criteria converge on the same neuronal subtypes, and how many such subtypes exist, is a matter of much current interest and considerable debate. To address these issues, we generated transgenic mice expressing green fluorescent protein (GFP) under control of the GAD67 promoter. In two of these lines, named X94 and X98, GFP expression was found in cortex, restricted to subsets of somatostatin-containing (SOM+) GABAergic interneurons, similar to the previously reported “GIN” line (Oliva et al., 2000), but the laminar distributions of GFP-expressing (GFP+) cell bodies in the X94, X98, and GIN lines were distinct and nearly complementary. We compared neurochemical content and axonal distribution patterns of GFP+ neurons among the three lines and analyzed in detail electrophysiological properties in a dataset of 150 neurons recorded in whole-cell, current-clamp mode. By all criteria, there was nearly perfect segregation of X94 and X98 GFP+ neurons, whereas GIN GFP+ neurons exhibited intermediate properties. In the X98 line, GFP expression was found in infragranular, calbindin-containing, layer 1-targeting (“Martinotti”) cells that had a propensity to fire low-threshold calcium spikes, whereas X94 GFP+ cells were stuttering interneurons with quasi fast-spiking properties, residing in and targeting the thalamo-recipient neocortical layers. We conclude that much of the variability previously attributed to neocortical SOM+ interneurons can be accounted for by their natural grouping into distinct subtypes.

Key words: neocortex; GABAergic; somatostatin; transgenic mice; inhibitory interneuron; barrel cortex

Introduction

Inhibitory, GABA-releasing interneurons are a minority of all cortical neurons but are crucially important for damping cortical excitability, imposing temporal precision on cortical firing, enhancing the saliency of sensory inputs, and promoting long-range synchrony (McBain and Fisahn, 2001; Whittington and Traub, 2003). Moreover, abnormalities in GABAergic neurons are implicated as a major factor in brain disorders ranging from epilepsy to autism and schizophrenia (DeFelipe, 1999; Levitt et al., 2004; Lewis et al., 2005). Such diverse functions are unlikely to be performed by a homogeneous population of neurons. Indeed, as studies during the last two decades have amply revealed, cortical interneurons display highly diverse anatomical, electrophysiological, and molecular properties (Freund and Buzsaki, 1996; Kawaguchi and Kubota, 1997; Markram et al., 2004). Electrophysiologically, interneurons can be described by their firing patterns as fast-spiking (FS), irregular-spiking nonpyramidal, low-threshold spiking (LTS), or stuttering. Based on their axonal targets, soma-prefering (basket), axon-prefering (chandelier), or dendrite-prefering interneurons are recognized. Neurochemically, interneurons may express parvalbumin (PV), somatostatin (SOM), cholecystokinin, or calretinin (CR). Unfortunately, classification schemes based on any individual criterion do not correlate with each other in a simple way, and different categories of properties appear at times to vary independently, leading some researchers to conclude that the number of potential GABAergic subtypes is very high (Gupta et al., 2000) or even that each interneuron is unique (Mott et al., 1997; Parra et al., 1998). This lack of consensus on the classification (and the classifiability) of interneurons has greatly hindered progress on deciphering their role in cortical circuitry (Yuste, 2005).

At the heart of the problem is the uncertainty in identifying the same subsets of neurons in different studies using different techniques. Recently, transgenic mice have been generated in which specific subsets of interneurons belonging to a single neurochemical class express green fluorescent protein (GFP) (Oliva et al., 2000; Meyer et al., 2002; Chattopadhyaya et al., 2004). In a few cases, their detailed analysis revealed novel subtypes within this class. A good example is the PV+ subset, which is probably the best studied neurochemical class of interneurons: initially, all PV+ interneurons were considered fast spiking, but studies using transgenic mice revealed a novel electrophysiological phenotype of bursting PV+ cells (Meyer et al., 2002).

Neocortical SOM+ interneurons appear considerably more
variable than PV+ interneurons in their neurochemical, morphological, and electrophysiological phenotypes (Kawaguchi and Shindou, 1998; Wang et al., 2004), but so far, no distinct SOM+ subtypes have been described based on a combination of multiple criteria. To use a recently proposed nomenclature (Soltesz, 2005), it is not known whether SOM+ interneuron heterogeneity reflects variability (within-group differences) or diversity (the existence of multiple subtypes). Here, we used novel lines of transgenic mice, with GFP-tagged SOM+ interneurons, to show that neocortical SOM+ interneurons belong to (at least) two morphologically, neurochemically and electrophysiologically distinct “species,” suggesting that SOM+ interneurons may be less variable, but more diverse, than previously recognized.

Materials and Methods

Generation of GAD67–GFP transgenic mice. The DNA construct used for pronuclear injection was derived from our previously described GAD67–GFP vector (Jin et al., 2001, 2003). The vector contained 10.3 kb of the mouse GAD67 promoter (Szabo et al., 1996; Katarova et al., 1998), including exon 1, intron 1, and a small portion of exon 2, fused in frame at the BamHI site to the enhanced GFP (EGFP) coding region in the pEGFP-1 vector (Clontech, Mountain View, CA). To isolate the fragment for injection using the upstream HindIII site and an AflII site present in the pEGFP-1 vector, we first removed the AflII site present in the GAD67 promoter by end-filling and religating the plasmid. The linearized fragment was injected into fertilized C57BL/6 × SJL hybrid eggs under contract with DNX (now Xenogen, Cranbury, NJ). Of 11 founder mice (strain FVB/N), 3 founders were isolated, Carlsbad, CA) in vehicle (1% NGS plus 0.1% TX in Tris) and gently agitated for 36 h at 4°C. After three rinses in PBS, sections were transferred into vehicle containing secondary antibody (1:1000 Alexa 546 goat anti-rabbit or anti-mouse; Invitrogen), rotated for 2 h at room temperature, rinsed three times, mounted in hard-set Vectashield (Vector Laboratories) on glass slides, and cover-slipped. Sections were imaged with a Zeiss (Thornext, NY) LSM510 confocal microscope, using nonsimultaneous excitation of GFP with the 488 nm line of the argon laser, and of Alexa 546 with the 543 nm line of the HeNe laser.

Cell counts. To quantify laminar distributions of GFP+ neurons, six coronal sections, from two different rostrocaudal levels through the barrel cortex, were selected for each line. Strips delineating the barrel region were outlined, and all DAB-labeled somatic profiles within each strip were marked under a 10× objective. Neuroexplorer software (MicroBrightField, Williston, VT) was then used to determine the orthogonal distance between each marked neuron and the pial surface. The fraction of SOM+ interneurons expressing GFP, and vice versa, was estimated from confocal projections of double-labeled sections from barrel cortex.

Electrophysiological recordings. Whole-cell recordings and intracellular staining were performed in juvenile animals (postnatal day 16 (P16) to P24), an age range easily amenable to patch-clamp recordings and comparable with the age used in many previous studies of cortical interneurons in vitro. Cortical inhibitory neurons have acquired many of their adult electrophysiological, morphological and molecular characteristics by the beginning of the third postnatal week (McDonald et al., 1982; Müller, 1986a; Edie et al., 1987; Minelli et al., 2003; Long et al., 2005). For preparation of brain slices, mice were anesthetized deeply with isoflurane (Abbott Laboratories, Abbott Park, IL) and decapitated. The brains were removed and submerged in ice-cold artificial CSF (ACSF). Using a Vibriosclicer (World Precision Instruments, Sarasota, FL), 300-μm-thick slices were cut and transferred into a holding chamber filled with recirculated ACSF at room temperature, incubated for at least 1 h, and then transferred to the recording chamber and continuously superfused with 32°C ACSF at a rate of 2–3 ml/min. ACSF was saturated with 95% O2/5% CO2 and contained the following (in mM): 126 NaCl, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 20 d-glucose.

For recording, barrel cortex GFP+ neurons were selected under fluorescence illumination using an Olympus BX50WI microscope equipped with a 40× water immersion objective and a Hamamatsu (Bridgewater, NJ) ORCA II CD camera linked to a PC and serviced by SimplePCI image analysis software (Cranberg Systems, Cranberry Township, PA). The selected cells were then visualized with differential interference contrast optics and targeted by patch pipettes for recordings. Just before seal formation, GFP expression in the targeted cell was verified by fluorescence. Whole-cell responses were recorded in true current-clamp mode with a patch-clamp amplifier (Axoclamp 2B; Molecular Devices, Sunnyvale, CA). Patch pipettes (5–7 MΩ resistance) were pulled from high lead content glass capillaries (PG52165; World Precision Instruments) and filled with intracellular solution containing (in mM): 122 KMeSO4, 10 KCl, 2 MgCl2, 0.1 CaCl2, 1.1 EGTA, 10 HEPES, 4 ATP-Mg, 0.3 GTP-Na, and 8 Phosphocreatine-Tris, pH 7.25, 285–295 mOsM; 2 mg/ml biocytin (Sigma) was routinely included in the pipette solution. Current pulse protocols were created with the Master-8 pulse generator (A.M.P.I., Jerusalem, Israel); injected current pulses were routinely 600 ms long. The recorded signals were filtered at 3 kHz and digitized at 10 kHz with a National Instruments (Austin, TX) ADC board. All reported membrane potentials are positively biased by 8 mV, which was the measured liquid junction potential in our experiments.

Electrophysiological parameter definitions (in alphabetical order). Afterhyperpolarization (AHP; in millivolts): spike afterhyperpolarization, the difference between threshold and the most negative membrane potential after the spike; measured on the response to the smallest current step evoking an action potential.
Adaptation ratio (dimensionless): the ratio of \( F_{\text{max}} \), steady state to \( F_{\text{max}} \), initial.

\( F-I \) slope (in hertz/picoampere): frequency–current slope, the slope of the regression line fitted to the initial, steeper portion of the \( F-I \) plot. Frequency was defined as the reciprocal of the first interspike interval (ISI).

\( F_{\text{max}}, \text{initial (in hertz)} \): the reciprocal of the first ISI, measured at the maximal current step applied before spike inactivation became evident.

\( F_{\text{max}}, \text{steady state (in hertz)} \): the reciprocal of the average of the last four ISIs, measured at the maximal current step applied before spike inactivation became evident.

\( F_{\text{max}}, \text{stuttering (in hertz)} \): for stuttering cells, the steady-state firing rate at the highest current level at which stuttering was evident (measured as the reciprocal of the average of the last four ISIs during the last firing epoch). For nonstuttering cells, the value of this parameter was defined as zero.

Rate of rise (in volts per second): maximal voltage slope (\( dV/dt \)) during action potential upswing.

Rate of fall (in volts per second): absolute value of maximal voltage slope (\( dV/dt \)) during action potential downswing.

Rheobase (in picoamperes): the intercet of the extrapolated \( F-I \) curve with the current axis.

\( R_m \) (in megaohms): input resistance, the slope of the regression line fitted to the \( V-I \) curve, measured from the response to small current steps resulting in deflections of \( \pm 10 \) mV on both sides of resting potential.

\( S \)-\( V \) slope (dimensionless): the slope of the regression line fitted to the plot of sag versus membrane potential, sag being the difference between the most negative membrane potential during a 600 ms hyperpolarizing current step and the membrane potential at the end of the step.

Spike height (in millivolts): the difference between threshold and voltage at peak of action potential.

Spike width (in milliseconds): measured at half height between threshold and peak action potential.

Susttering range (dimensionless): ratio of \( F_{\text{max}} \), stutting to \( F_{\text{max}} \), steady state.

\( \tau_m \) (in milliseconds): membrane time constant, determined from the monoexponential curve best fitting the rising or falling phase of the response to a small hyperpolarizing current step (\( \pm 10 \) mV from resting potential).

Threshold (in millivolts): the membrane potential at the point at which the interpolated rate of rise (\( dV/dt \)) of the action potential equaled 5 V/s, measured on the response to the smallest current step evoking an action potential.

\( V_{\text{rest}} \) (in millivolts): resting potential, the stable membrane potential reached a few minutes after breaking the seal, with no current applied.

Morphological reconstruction of recorded neurons. For morphological reconstruction, slices were fixed overnight in 4% paraformaldehyde in 0.1 M PBS with 0.6% TX. After three additional rinses in 0.1 M PBS, slices were incubated overnight at 4°C in ABC solution in 0.1 M PBS with 0.6% TX. After three additional rinses in PBS, slices were rinsed for 10 min in 0.05 M Tris buffer, pH 7.6, incubated in 0.06% DAB in Tris for an additional 15 min, and then the reaction was initiated by adding 0.006% \( H_2O_2 \). When the cell body and dendritic processes were clearly visible, the DAB reaction was stopped with cold PBS solution. Slices were mounted in PBS/glycerol, coverslipped, and sealed with nail polish. Biocytin-filled neurons were visualized, traced, and digitally reconstructed using the Neurolucida system (MicroBrightField) with a 60× water-immersion objective (Olympus) with a working distance of \( \approx 130 \) μm.

Multivariate analysis. Multivariate analysis followed Manly (2005). Each cell was represented as a vector in a multidimensional parameter space of \( p \) dimensions [\( p = 9 \) for principal component analysis (PCA) and 11 for discriminant function analysis (DFA)]. The full dataset was represented by a “total” matrix \( T \) of \( r = 150 \) rows (cells) and \( p \) columns (parameters), with values in each column shifted by the grand average of the corresponding parameter so as to have a mean of 0, and by a similarly sized “within” matrix, \( W \), in which the values in each column were

shifted independently for each group by the group average of the corresponding parameter. A “between” matrix was defined as \( B = T - W \). From \( T \), a “normalized total” matrix \( NT \) was constructed by scaling each column to have an SD of 1, and from \( W \), a “normalized within” matrix \( NW \) was constructed by scaling each group independently to have an SD of 1.

From \( T \), \( W \), and \( B \), three \( p \times p \) variance–covariance (VCV) matrices were computed (the superscript “T” denotes a transposed matrix) as follows: total VCV matrix, \( TV = 1/r(T^T \times T) \); (pooled) VCV matrix, \( WV = 1/r(W^T \times W) \); between VCV matrix, \( BV = 1/r(B^T \times B) \). The within VCV matrix was the average of the three group-wise VCV matrices, weighted by the number of cases in each group. The diagonal elements of \( TV \), \( WV \), and \( BV \) represented the total, within-group, and between-group variance in each parameter. The fraction of between-group variance (\( \eta^2 \)) for parameter \( i \) was therefore \( \eta^2_i = BV_{ii}/TV_{ii} \).

From \( NT \) and \( NW \), two \( p \times p \) correlation matrices were computed: total correlation matrix, \( TC = 1/r(NT^T \times NT) \); (pooled) correlation matrix, \( WC = 1/r(NW^T \times NW) \). The within correlation matrix was the average of the three group-wise correlation matrices, weighted by the number of cases in each group. Matrices \( TC \) and \( WC \) are shown graphically in Figure 7, E and F, respectively. The average correlation within each matrix was defined as the cosine of the average arccosine of the absolute values of the correlation coefficients, calculated over the lower triangle of the matrix.

For PCA, the eigenvalues of \( WC \) were calculated, and the two eigenvectors corresponding to the two largest eigenvalues, \( EVEC1 \) and \( EVEC2 \), used to define the two principal components, by the following:

\( \text{PC1} = NT \times EVEC1 \) and \( \text{PC2} = NT \times EVEC2 \).

For DFA, the eigenvalues of \( WV^{-1/2} \times BV \) were calculated, and the two eigenvectors corresponding to the two largest eigenvalues, \( EVEC1 \) and \( EVEC2 \) (not to be confused with the eigenvectors of WC above), used to define the two canonical discriminant functions, by the following:

\( \text{CDF1} = T \times EVEC1 \) and \( \text{CDF2} = T \times EVEC2 \).

Statistics. Unless noted otherwise, statistical significance values (\( p \) values) were computed numerically, by performing 10,000 random permutations of the dataset and computing the probability for occurrence of values as or more extreme than the experimental result (Good, 1999). Where 10,000 permutations yielded no more extreme values, this is indicated as \( p < 10^{-4} \). All tests were two-tailed unless noted otherwise. Some data are presented as mean ± SD.

Software. Data acquisition and processing were done with home-made software written in the LabView environment (National Instruments). Data were tabulated in Excel (Microsoft, Redmond, WA), which was also used to generate scatterplots. Multivariate analysis, correlation matrices, density plots, and permutation tests were programmed in MathCad (MathSoft, Cambridge, MA). Final graphics were prepared with CorelDraw (Corel, Ottawa, Ontario, Canada).

Results

We generated five transgenic mouse lines with GFP expression under control of the GAD67 promoter. Although all five lines were generated by in ovo injection of the same GAD67–GFPS DNA construct (see Materials and Methods), the spatial distribution of GFP+ neurons in the brain was distinct in each line. The present study is based on two of these novel transgenic lines, named X94 and X98, in which GFP expression in the cerebral cortex was clearly restricted to nonpyramidal cells, and on the previously published transgenic mouse line GIN in which GFP expression is also driven by the GAD67 promoter, albeit by a shorter segment of the promoter than used by us (Oliva et al., 2000). For convenience, we refer to GFP+ neurons in the X94, X98, and GIN lines as “X94 neurons,” “X98 neurons,” and “GIN neurons,” respectively. It should be understood, though, that we consider GFP+ neurons in these mice to be representative of homologous subsets of neurons that are presumed to exist in wild-type animals and, except for GFP expression, to display the same properties.
Laminar distribution of GFP+ neurons

In the neocortex of adult X94, X98, and GIN mice, GFP was expressed only in nonpyramidal, putative GABAergic neurons (Fig. 1). Because there were some interareal variations in the detailed cortical expression patterns within each line, we focused on the somatosensory barrel cortex, which provided an easily identifiable cortical region for interline comparisons and for which the composition and properties of GABAergic interneurons are already known in some detail (Staiger et al., 1996; Porter et al., 2001; Wang et al., 2002, 2004; Beierlein et al., 2003). Qualitative examination of the barrel cortex of adult (2–3 months of age) X94, X98, and GIN mice revealed a distinct laminar distribution pattern of GFP+ neurons in each line. X94 neurons were found mostly in layers 4 and 5B, with a small number in layers 2/3, 5A, and upper 6 (Fig. 1A,D). X98 neurons resided mostly in layers 5B and 6 (Fig. 1B,E), with a minor population in layers 2/3 (see Fig. 3B). In the X98 line, low-level expression of GFP was also found in many small cells with glial morphology (faintly visible in Fig. 1B,E), which formed a remarkably regular lattice and which will not be considered here further. GIN neurons were found mainly in layers 2/3 and 5A, with a smaller number of cells within layer 4 (Fig. 1C,F), consistent with their original description (Oliva et al., 2000). Thus, the laminar distributions of GFP+ neurons in the three lines were nearly complementary.

The qualitative impression of complementary distribution patterns was substantiated by counting cells in radial strips through the barrel cortex of the three lines (Fig. 1A–C; histograms at the left end of each panel). The interline differences were most striking in layer 4. In the X94 line, layer 4 contained a high density of GFP-containing neuropil, often segregated into clusters suggestive of barrel boundaries (Fig. 1A,D). Many labeled cell bodies were located at the lower border of layer 4 or within layer 5A immediately below this border; layer 5A was otherwise very lightly labeled. A second, lower tier of GFP+ cell bodies was found in layers 5B (with a small number in upper layer 6). Thus, GFP expression in the X94 cortex closely followed the laminar distribution pattern of thalamocortical afferents (Agmon et al., 1993). In the X98 line, in contrast, GFP expression in layer 4 was totally absent (Fig. 1B). In the GIN line, there was a dense expression of GFP in cell bodies and neuropil immediately above and below layer 4, but in layer 4 there were only a small number of GFP-containing cell bodies and very little labeled neuropil (Fig. 1C).

Because electrophysiological recordings are often done in brain slices from juvenile mice (as were our recordings), we also examined GFP expression patterns in tissue from 2- to 3-week-old mice (data not shown). GFP expression levels were somewhat lower in juveniles, but expression patterns were overall similar to the adult ones, with a few noteworthy exceptions. In juvenile X98 cortex, strongly labeled GFP+ neurons in the supragranular layers were considerably more numerous than in adults. In juvenile GIN mice, there was an apparently ectopic expression of GFP in a subset of pyramidal neurons in the deep cortical layers. GFP expression in 2- to 3-week-old X94 mice was qualitatively very similar to the adult pattern, but in X94 pups younger than 2 weeks, expression was very weak.

Neurochemical characterization of GFP+ neurons

Being nonpyramidal, GFP+ neurons in our transgenic lines were, most likely, GABAergic interneurons. Cortical interneurons fall into several distinct neurochemical classes (Kubota et al., 1994; Kawaguchi and Kondo, 2002). To determine whether GFP+ neurons in our lines belonged to a specific neurochemical class, we stained sections from adult brains (two animals per line) with antibodies to PV and to SOM (Fig. 2A–D) and quantified the fraction of GFP+ cells that were also immunopositive for each marker and the fraction of marker immunopositive cells that were also GFP+ (Table 1). Virtually all GFP+ neurons in both X94 and X98 lines (95 and 96%, respectively) were SOM+ (Fig. 2A,B), and all GFP+ neurons in both lines were PV immunonegative (Fig. 2C,D) (note that yellow regions in Fig. 2C are the result of overlap of red cell bodies and green neuropil, not colocalization of the labels). We also confirmed that nearly all GIN neurons (97%) were SOM immunopositive (data not shown), as reported previously (Oliva et al., 2000). GFP+ neurons were clearly only a partial subset of all SOM+ interneurons in their vicinity (Table 1). Even in the layers in which they were most numerous, layers 4, 5/6, and 2/3 for X94, X98, and GIN, respectively, GFP+ neurons accounted for only 41, 20, and 35% of all SOM+ neurons, respectively. In total, and assuming that X94, X98, and GIN neurons consist of nonoverlapping populations, GFP+ neurons in the three lines accounted for slightly more than one-half of all SOM+ neurons.

Several neurochemicals are known to be expressed in subsets...
of SOM+ interneurons and could potentially be used as markers for subtypes within the SOM+ population. We tested GFP+ interneurons in our mice for expression of two of these molecules, CB and NPY (two animals per line). X94 neurons never stained for either marker (Fig. 2 E, G). In contrast, virtually all X98 cells (96%) and about one-third of all GIN cells were immunopositive for CB; ~40% of all X98 neurons and 27% of all supragranular GIN cells were immunopositive for NPY (Fig. 2 F, H, Table 1). Thus, X94, X98, and GIN neurons had distinct neurochemical profiles. Moreover, these results indicate that even where they overlap in laminar position, e.g., in layer 5B, X94 and X98 neurons belong to distinct populations.

Axonal morphologies of GFP+ neurons

Confocal imaging of fixed brain sections from the three lines revealed strong GFP fluorescence in cortical layer 1 of X98 and GIN mice, seemingly emanating from GFP-containing axonal bundles running below the pial surface (Fig. 3 B, C); in contrast, layer 1 in X94 cortex was nearly devoid of fluorescence (Fig. 3 A). An axonal projection to layer 1 is the defining feature of Martinotti cells (DeFelipe and Jones, 1988), a morphological class of neocortical interneurons known to express SOM (Wahle, 1993; Kawaguchi and Kubota, 1996; Shlosberg et al., 2003), but it is not known whether all SOM+ cortical interneurons are Martinotti cells. To further examine this question at a single-cell resolution, we filled GFP+ neurons in the three lines with biocytin during electrophysiological recordings and reconstructed their dendritic and axonal trees using the Neurolucida system. From 230 recorded and filled cells, 69 neurons (35 X94, 19 X98, and 15 GIN cells) retained a sufficiently extensive axonal tree within the slice to warrant a detailed computer-assisted reconstruction; 12 representative reconstructions are illustrated in Figure 4.

In all three lines, the axon (Fig. 4, labeled red) almost always emerged from the upper aspect of the cell body or from an ascending dendritic trunk and, at least initially, extended toward the pial surface. Layer 4 X94 neurons (n = 27) (Fig. 4 A–D) had compact axonal trees that remained within layer 4 or (in a few cases) extended into layer 3 and formed a tight cluster that appeared restricted to a single barrel or barrel column. Following the “blueprint” of the barrel cortex (Woolsey et al., 1975; Lorente de Nó, 1992), X94 cells often extended highly asymmetric dendritic and axonal trees toward the presumed center of their barrel (Fig. 4 A–C). In six reconstructed cells, the main axon extended to upper layer 2/3 or even lower layer 1, where it made a sharp loop and descended back toward layer 4, in which it made its terminal arbor (Fig. 4 B–D, turning point indicated by arrows). Only one X94 neuron had an axon that branched within layer 1 (data not shown). Infragranular X94 neurons (n = 8) (Fig. 4 E, F) emitted a local axonal arbor in the vicinity of the cell body, but in all cases, one or more axonal branches entered layer 4 (and in some cases also layer 3) and formed a second arbor there, usually restricted to the same radial column, but in one case (data not shown) extending within layer 4 into adjacent columns. In all cases, by far the most extensive target of X94 neurons was layer 4, never layer 1, confirming the observations from GFP fluorescence (Fig. 3 A). Therefore, X94 neurons cannot be considered to be Martinotti cells; rather, they appear to constitute a separate morphological subtype within the SOM+ GABAergic subclass.

X98 axons always ramified immediately above the cell body, usually in upper layer 6 and layer 5. Invariably, however, the main axon continued upwards. This ascending trunk was unfortunately truncated in many cells at the cut surface of the slice, but in the cases in which it was retained within the slice (n = 11), it always reached layer 1. In seven of these cases (examples shown in Fig. 4 J–L), the axon ascended as multiple parallel branches through layers 4 and 2/3 before entering layer 1, where at least some of the branches made a 90° turn and coursed for some distance parallel to the pial surface, occasionally sending out short terminal side branches. By this pattern of axonal arborizations, which is consistent with the observations of GFP fluorescence.
Firing patterns of GFP+ interneurons

Previous studies reported considerable heterogeneity in the electrophysiological and morphological properties of SOM+ cortical interneurons (Wang et al., 2004), but it remains unclear whether they comprise several distinct subtypes. To address this question, we recorded from GFP+ neurons in the current-clamp, whole-cell mode and compared intracellularly elicited voltage responses between the three lines. This analysis was performed on a dataset of 150 barrel cortex neurons recorded at 32°C, including 58 X94, 59 X98, and 33 GIN neurons, from 71 juvenile mice (age range, P16–P24; average age, P18.9; no statistical difference in age between lines). All the cells included in this analysis had stable resting potentials more negative than $-60 \text{ mV}$ (uncorrected for junction potential) and overshooting action potentials. Although each of the three lines showed some degree of GFP expression in several cortical layers (Fig. 1), we selected our sample from the major concentration of GFP+ neurons in each line: layers 4 ($n = 32$) and 5B ($n = 26$) X94 neurons, infragranular X98 neurons, and supragranular GIN neurons.

When injected with sufficiently large depolarizing current steps, all three lines fired trains of action potentials that exhibited cence in layer 1 (Fig. 3B). X98 cells fit the classical definition of Martinotti cells (De-Felipe and Jones, 1988). None of the reconstructed X98 cells in our sample was found to branch within layer 4 to any noticeable degree. Finally, all supragranular GIN cells (examples in Fig. 4G,H), and the one reconstructed layer 5A GIN cell (Fig. 4I), had multiple ascending axonal branches that branched extensively within layer 1, consistent with our confocal images (Fig. 3C); GIN neurons were therefore supragranular Martinotti cells.

### Table 1. Overlap between GFP and SOM, CB, and NPY expression, by line and by laminar position

<table>
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<tr>
<th>Line</th>
<th>Layer</th>
<th>SOM+ n</th>
<th>% DL</th>
<th>GFP+ n</th>
<th>% DL</th>
<th>CB+ n</th>
<th>% DL</th>
<th>GFP+ n</th>
<th>% DL</th>
<th>NPY+ n</th>
<th>% DL</th>
<th>GFP- n</th>
<th>% DL</th>
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<td>X94</td>
<td>2–3</td>
<td>382</td>
<td>5.9</td>
<td>23</td>
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<td>408</td>
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<td>348</td>
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<td>473</td>
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<td>151</td>
<td>92.7</td>
<td>425</td>
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<td>177</td>
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For each marker, both the percentage of marker-immunopositive cells expressing GFP and the percentage of GFP-expressing cells immunopositive for the marker are indicated. DL, Double-labeled cells.
followed by a sequence of an early fast AHP followed by a delayed “slow” AHP (Zhang and McBain, 1995), which together formed a characteristic triphasic waveform (Fig. 5Ah,Ai, arrowheads). Only 14% of all X94 cells displayed a triphasic AHP; interestingly, all eight cells that did had input resistances above the median for their group (p = 0.007; Fisher’s exact test).

X94 neurons were distinctive in another respect; in most cells, spike trains were interrupted at seemingly random intervals for seemingly random periods, often replaced by subthreshold oscillations (Fig. 5Ad, asterisks). Most stuttering cells did so only at the lower range of firing frequencies (Fig. 5, compare Aa, Ad), but 11 X94 cells stuttered at all current levels. We quantified the stuttering range of a given cell as the ratio between the highest steady-state firing frequency at which stuttering still occurred to the maximal steady-state firing frequency in the same cell and defined as “robustly stuttering” cells that stuttered through at least the lower one-third of their frequency range (i.e., stuttering range ≥ 0.33). Overall, 58.6% of X94 cells robustly stuttered, but only 3.3% of X98 and 12.1% of GIN neurons did, a highly significant difference between X94 and the remaining groups (p = 2 × 10^{-12}; Fisher’s exact test). Layer 4 and layer 5 X94 neurons differed in their propensity to stutter: 75% of all layer 4 X94 cells robustly stuttered, but only 38.5% of layer 5 cells did, a highly significant difference between layers (p = 0.005; Fisher’s exact test). The probability of stuttering was correlated with a low input resistance: 36.7% of robustly stuttering X94 neurons were in the lower quartile of input resistances for their group, nearly 1.5 times the expected fraction (p = 0.01; Fisher’s exact test).

In response to hyperpolarizing currents, all three cell groups displayed a sag (Fig. 5Ba–Bc, asterisks), likely to be mediated by the hyperpolarization-activated cationic current I_{h} (Lupica et al., 2001). All three lines also displayed a depolarizing “rebound” after recovery from the hyperpolarizing step (Fig. 5Ba,Bc, open arrowheads). In X94 and GIN neurons, this rebound did not trigger spikes, or rarely (in one X94 cell and 18% of GIN neurons) triggered one action potential. In contrast, in 37% of X98 neurons recovery from hyperpolarization evoked at least one action potential; 25% of all X98 cells fired a burst of two to three spikes (Fig. 5Bb, right filled arrowhead). The difference in rebound spiking probability between X98 cells and the remaining groups was highly significant (p = 10^{-5}; Fisher’s exact test). Some X98 cells fired a burst also after depolarization directly from resting potential (Fig. 5Bb, left filled arrowhead). The probability of firing a rebound spike or burst was

Figure 5. Suprathreshold and subthreshold responses of GBP^+ neurons. A, Spike trains in response to current steps of increasing amplitudes. Aa–Ac, Ad–Af, and Ag–Ai are responses to high, medium, and low current levels, respectively; the three current steps for each neuron are shown superimposed in Aj–Al. The asterisks in Ad denote interruptions in firing characteristic of stuttering X94 neurons; some GIN neurons also stuttered, but X98 neurons never did. The insets in Ag–Ai are the first action potential from the corresponding trace, shown at half the vertical scale and at a 100-fold expanded horizontal scale; note the pronounced difference in spike widths between lines. The arrowheads in Ah and Ai point to the characteristic triphasic AHP in X98 and GIN neurons. Calibration: 40 mV (Aa–Al), 1000 pA (Aj–Al), 200 ms. B, Superimposed voltage responses (Ba–Bc) to the hyperpolarizing and small depolarizing current steps shown in Bd–Bf, in three other neurons. Note the very low input resistance of the X94 neuron compared with that of the X98 and GIN neurons (much larger current steps required to elicit similar voltage changes). The asterisks in Ba–Bc indicate the sag attributable to the hyperpolarization-activated cationic current I_{h}. The open arrowheads in Ba and Bc denote a depolarizing rebound, also attributable to I_{h}. The filled arrowheads in Bb point to bursts of action potentials riding on low-threshold Ca^{2+} spikes. Calibration: 20 mV (Ba–Bc), 300 pA (Bd–Bf), 200 ms.
correlated with a high input resistance: 45% of X98 neurons displaying a rebound spike or burst were in the upper quartile of input resistances for their group, nearly twice the expected fraction (p = 0.004; Fisher’s exact test). This correlation held also for the X94 and GIN samples: one-half the GIN neurons that fired a single rebound spike were in the upper quartile of R_{in} for their group, and the one X94 neuron with a rebound spike had the highest input resistance in its group.

The rebound burst in X98 cells was likely to be triggered by an LTS, mediated by the calcium current I_{Ca} (Goldberg et al., 2004). However, the coexistence of a prominent I_{h} in the same cells raised the possibility that an I_{h}-mediated rebound could have contributed to the bursting in X98 neurons. Several lines of evidence suggested that this was not the case. First, the amplitude of the I_{h}-mediated sag, as measured by the slope of the sag versus voltage curve, was not significantly different between lines (Table 2). Also, there was no significant correlation between sag amplitude and the number of rebound spikes fired by X98 cells (r^2 = 0.03). To further isolate the contributions of I_{h} and I_{f}, we used CsCl to block I_{h} channels (Maccaferri and McBain, 1996) and mibefradil to block T-type calcium channels (Yunke, 2003). CsCl (3 mm) totally or nearly totally blocked the sag and the rebound depolarization in X94 and GIN neurons (n = 4 cells from each line) (Fig. 6A,B, open arrowheads), consistent with both being I_{h}-mediated. CsCl also strongly blocked the hyperpolarization-induced sag in X98 cells (Fig. 6C, open arrowhead), but in most (five of seven) cells, it did not block the rebound single spike or burst (Fig. 6C, filled arrowhead). In contrast, 10 μM mibefradil had no observable effect on the hyperpolarization-induced sag (Fig. 6D, open arrowhead) but totally blocked the rebound burst in two of three cells, even when the hyperpolarizing step was increased (Fig. 6D, filled arrowhead), and reduced the burst to a single spike in the third case. We conclude that X98 cells, but not X94 or GIN neurons, have a propensity to fire low-threshold Ca^{2+} spikes mediated by I_{f} and can be classified as LTS cells.

**Multivariate analysis of electrophysiological parameters**

We used the subthreshold and suprathreshold voltage responses to calculate 15 basic electrophysiological parameters for each neuron (Table 2) (see Materials and Methods for definitions of parameters).

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**Table 2. Values for 15 electrophysiological parameters analyzed for each neuron**

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<th>X98 (n = 59)</th>
<th>GIN (n = 33)</th>
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<td>0.29</td>
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Table continues...
For each parameter, we quantified its overall variance in the sample, by calculating the total sample coefficient of variation (CV; i.e., SD/mean) and its $\eta^2$ value (i.e., the fraction of the total variance attributable to differences between, rather than within, the three groups) (see Materials and Methods). When $\eta^2$ was plotted against the CV for each parameter (Fig. 7A), there was a clear clustering of parameters into two groups. Six parameters (Fig. 7A, inside dotted half box) had low between-group variance ($\eta^2 < 0.1$) and low to intermediate total variance ($0.05 < CV < 0.35$), and seven parameters (Fig. 7A, numbered 1–3, 5–8) had high between-group variance ($\eta^2 > 0.55$, up to 0.72 for spike width) and intermediate to high total variance ($0.25 < CV \leq 1$). The remaining two parameters (#4 and #9) had intermediate values for both CV and $\eta^2$. This correlation between CV and $\eta^2$ suggested that most of the variance in the electrophysiological parameters of the neurons in our sample could be accounted for by their natural segregation into three groups.

The six parameters with low $\eta^2$ values (resting potential, threshold, spike height, sag–$V$ slope, adaptation ratio, and $F$–$I$ slope) were clearly not good indicators of group membership and were not analyzed further. The remaining nine parameters had $\eta^2 > 0.1$ (indeed, seven had $\eta^2 \approx 0.55$), values that were very significantly different from 0 ($p < 0.0001$, Table 2). Thus, these parameters were very good predictors of group membership. Figure 7B shows bygroup density plots for these nine parameters, computed with a Gaussian kernel (Hand et al., 2001). In nearly all plots, X94 and X98 neurons formed clearly separate peaks with very little overlap, whereas GIN neurons occupied intermediate positions, overlapping with both other groups. The clear separation between X94 and X98 can also be appreciated from Table 2: for the seven parameters with $\eta^2 \geq 0.55$, the 10–90th percentile ranges of the X94 and X98 samples were totally nonoverlapping (see also Table 3). Some of the pairwise differences between means for X98 and X94 parameters were quite large; for example, the average initial and steady-state $F_{\text{max}}$ values were twofold smaller, and the average $R_{\text{in}}$ and $\tau_m$ values fourfold larger, in X98 compared with X94.

Given the differences in stuttering range between layer 4 and layer 5 X94 cells, we also compared their electrophysiological parameters. Compared with layer 4 cells, layer 5 X94 neurons had higher $R_{\text{in}}$ (163 ± 62 vs 109 ± 41 MΩ), slower $\tau_m$ (12.3 ± 4.8 vs 7.9 ± 2.1 ms), and lower rheobase (166 ± 85 vs 231 ± 95 pA); these differences were highly significant ($p < 0.0001, p < 0.0001, p < 0.0001$, respectively). The remaining parameters were not significantly different between the two X94 subgroups.

Given the strong dependence of parameters 1–8 on group membership (Fig. 7B), we expected them to correlate with each other, as was indeed the case (Fig. 7C). However, such correlation could also indicate that the parameters in question were not independent (i.e., that they represented different manifestations of the same basic biophysical properties). To identify such intrinsic correlations between parameters, we compared the total sample correlation matrix (Fig. 7C) to the pooled within-group correlation matrix (Fig. 7D) (see Materials and Methods for definition of the two matrices), reasoning that correlations based on shared biophysical mechanisms should be evident also within each group, whereas correlations based solely on cosegregation into different groups should disappear. Indeed, the average correlation coefficient was reduced from 0.58 in the total-sample matrix to 0.34 in the within-group matrix, suggesting that ~60% of the total sample correlation reflected shared biophysical mechanisms between parameters. Specifically, the within-group correlation matrix (Fig. 7D) suggested that parameters 1–3, representing passive membrane properties, were correlated with each other and that parameters 4–9, representing action potential
properties, were correlated with each other, but that these two parameter clusters were not correlated with each other. Because information conveyed by intrinsically correlated parameters is redundant, we conducted PCA, a method that reduces such redundancy by redistributing the variance in the dataset among the smallest possible number of independent linear combinations of parameters (Manly, 2005). The two principal components with the largest variances (PC1 and PC2) accounted for 65% of the total variance in our sample and are plotted against each other in Figure 7E. PC1 and PC2 were correlated, respectively, with the active and passive parameter clusters identified above (correlation coefficients of 0.41 and 0.50, respectively). In the PC1–PC2 plane, X94 and X98 data points were almost completely segregated, whereas GIN data points occupied the intermediate region and were intermixed, to some degree, with the other two groups. As expected from the differences in passive parameters between layer 4 and layer 5 X94 cells, the latter (Fig. 7E, open red squares) had lower values of PC2 and accounted for most of the overlap between X94 and GIN data points.

PCA maximizes the overall variance per component but does not necessarily improve the discriminability between groups. To determine how well the electrophysiological parameters discriminate between the three subsets, we conducted DFA (see Materials and Methods). This method maximizes the separation between \( n \) groups (i.e., maximizes \( \eta^2 \)) along \( n - 1 \) independent linear combinations of parameters (Manly, 2005). In this analysis, we included the parameters 1–9 above, as well as the stuttering range and the number of rebound spikes fired by each neuron after recovery from a maximal hyperpolarizing step. The resulting two canonical discriminant functions (CDF1 and CDF2) are plotted against each other in Figure 7F. Overall, between-group variance accounted for 87% of the total variance in CDF1 and for 41% of the variance in CDF2. Clearly, in the CDF1–CDF2 plane, there was not only a near-perfect separation of X94 and X98 data points along CDF1 but also a very good separation of GIN data points from the other two groups along CDF2. The two intersecting lines in Figure 7F divide the CDF plane into three regions, with X94, X98, and GIN data points mostly segregated into separate regions, indicating that the electrophysiological parameters alone, even without neurochemical or morphological criteria, could be used to classify SOM+ neurons into one of these three groups.

**Age-dependent changes in electrophysiological parameters**

Although there were no statistically significant differences in age between the three samples, it was still possible that the large between-group differences we observed were related to differential maturation of cells in different cortical layers and that these differences would disappear by adulthood. This was a priori unlikely, because maturation of cortical layers proceeds in an inside-out gradient, whereas electrophysiological properties did not display a pia-to-white matter gradient (e.g., GIN neurons, in the upper layers, had electrophysiological parameters intermediate between X94 cells in the middle layers and X98 cells in the deep layers). Nevertheless, we tested this possibility by performing regression analysis of the two major principal components (PC1 and PC2) against age, by group. PC2 values did not change significantly with age; however, PC1 values showed a weak negative correlation with age, although this effect was statistically significant only in X98 and GIN neurons (\( r^2 = 0.15 \) and 0.16, respectively. The same effect could be seen in the individual active parameters that correlated with PC1; for example, spikes of X98 neurons narrowed slightly with age, with the trend line dropping by 0.15 ms through the age range of our experiments. To test how this small age-dependent change in the active parameters may have affected the estimated degree of difference between groups, we divided our sample into three age ranges, P16–P17 (\( n = 43 \)), P18–P19 (\( n = 59 \)), and P20–P24 (\( n = 48 \)), and computed the \( \eta^2 \) values of PC1 separately for each range. \( \eta^2 \) values were 0.78, 0.67, and 0.66 for the three age ranges, respectively, compared with 0.68 for the total sample. We conclude that the contribution of between-group variance to the total variance was somewhat higher at the lower range of ages in our sample but that it stabilized from P18 onwards, probably reaching its mature value, and that the higher \( \eta^2 \) at the younger ages did not bias our conclusions to any significant degree.

**Discussion**

We compared two novel and one published transgenic mouse lines in which GFP expression is driven by the GAD67 promoter. Although GAD67 is expressed by all GABAAergic interneurons (Mugnaini and Oertel, 1985), cortical GFP expression in all three lines was restricted to the SOM+ neurochemical class. Moreover, each line expressed GFP in a strikingly different subset of SOM+ interneurons, distinct in laminar location, neurochemical markers, axonal morphologies, and electrophysiological properties. Differences in these properties were most pronounced between X94 and X98 neurons, which segregated almost perfectly by all lines in which GFP expression is driven by the GAD67 promoter. Although GAD67 is expressed by all GABAAergic interneurons (Mugnaini and Oertel, 1985), cortical GFP expression in all three lines was restricted to the SOM+ neurochemical class. Moreover, each line expressed GFP in a strikingly different subset of SOM+ interneurons, distinct in laminar location, neurochemical markers, axonal morphologies, and electrophysiological properties. Differences in these properties were most pronounced between X94 and X98 neurons, which segregated almost perfectly by all lines in which GFP expression is driven by the GAD67 promoter.
ously to be electrophysiologically, morphologically, and chemically heterogeneous (Wang et al., 2004), but no clear correlations between these different categories of properties were noted. That we observed such correlations is no doubt attributable to our use of transgenic animals, allowing us to target our experiments to well defined and reproducible subsets of neurons identified by genetically encoded vital markers. The identifiability of these subsets means that our results could easily be extended in future studies targeting the same subtypes with additional techniques, e.g., ultrastructural characterization of their synaptic targets (Gulyas et al., 1993; Thomson et al., 1996) or microarray analysis of their gene expression profiles (Lobo et al., 2006; Sugino et al., 2006).

Low-threshold bursts in layer 1-targeting, infragranular SOM+ interneurons

X98 neurons resided in the infragranular layers, could generate low-threshold calcium spikes, and sent an ascending projection to layer 1. X98 neurons were therefore similar to LTS Martinotti cells described previously in rodent infragranular cortex (Kawaguchi, 1993, 1995; Deuchars and Thomson, 1995; Goldberg et al., 2004). All X98 cells expressed calbindin, also observed previously in Martinotti neurons (Kawaguchi and Kubota, 1996; Gabbott et al., 1997). As noted previously (Goldberg et al., 2004), SOM+ interneurons in supra-granular or granular cortical layers never fired low-threshold bursts, and referring (as is often done) to all putative SOM+ interneurons as LTS cells is incorrect.

Low-threshold bursts are thought to be mediated by T-type calcium channels of the CaV3.X gene family (Perez-Reyes, 2003). We confirmed this pharmacologically by showing that the T-channel-preferring blocker mibefradil (Yunker, 2003) blocked the low-threshold bursts. Intriguingly, the occurrence of low-threshold bursts or single rebound spikes, which were observed in 40% of all X98 neurons, correlated with high input resistance. This raises the possibility that a sufficiently high input resistance is required for a cell to generate an LTS and that bursting and nonbursting X98 neurons differed in their input resistance, rather than in the expression of CaV3.X channels.

Stuttering and quasi-fast spiking in layer 4-targeting SOM+ interneurons

X94 cells appear to be a novel subtype of SOM+ interneurons. They fired in a stut-
Table 3. Summary of the phenotypic differences between X94 and X98 neurons

<table>
<thead>
<tr>
<th>Phenotypic property</th>
<th>X94</th>
<th>X98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell body position</td>
<td>Layers 4 and 5B</td>
<td>Layers 5B and 6</td>
</tr>
<tr>
<td>Layer 1 axonal arborizations</td>
<td>Dense</td>
<td>None or sparse</td>
</tr>
<tr>
<td>Layer 2 axonal arborizations</td>
<td>None or sparse</td>
<td>Dense</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>Calbindin</td>
<td>None</td>
<td>All</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>None</td>
<td>~40%</td>
</tr>
<tr>
<td>Input resistance</td>
<td>&lt;250 MOe</td>
<td>&gt;300 MOe</td>
</tr>
<tr>
<td>Time constant</td>
<td>&lt;20 ms</td>
<td>&gt;20 ms</td>
</tr>
<tr>
<td>Spike width</td>
<td>≥0.5 ms</td>
<td>≥0.6 ms</td>
</tr>
<tr>
<td>r_max, initial</td>
<td>&gt;200 Hz</td>
<td>&lt;200 Hz</td>
</tr>
<tr>
<td>r_max, steady-state</td>
<td>&gt;100 Hz</td>
<td>&lt;100 Hz</td>
</tr>
<tr>
<td>Robust stuttering</td>
<td>~60%</td>
<td>~3%</td>
</tr>
<tr>
<td>Low-threshold spikes</td>
<td>~2%</td>
<td>~4%</td>
</tr>
</tbody>
</table>

Phenotypic properties are arranged in five groups: laminar position, axonal distributions, neurochemical content, electrophysiological parameters, and firing properties. Inequalities in the electrophysiological parameters group apply to at least 90% of each population.

Just as taxonomical separation between biological species reflects phylogenetic distances from a common ancestor, taxonomical separation between neuronal species should reflect ontogenetic distances from a common precursor (Soltész, 2005). That different neurochemical classes of GABAergic interneurons diverge relatively early in development follows from the findings that they originate from distinct progenitor zones in the ventral telencephalon (Xu et al., 2003, 2004). How then does intraclass diversity arise? Very recently, a fate-mapping study in vivo (Butt et al., 2005) revealed that late-born CR+ interneurons show more phenotypic diversity than early born CR+ interneurons, suggesting that intraclass diversity may be generated by temporal, rather than spatial, gradients. Similar experiments have not yet been done on SOM+ interneurons; nevertheless, our finding of distinct subtypes of SOM+ interneurons in different cortical layers, together with the pattern of inside-out layering of GABAergic cortical neurons according to age (Miller, 1986b; Valcănic and Tan, 2003), are consistent with a model by which SOM+ interneurons born at different developmental ages are committed to different phenotypes, possibly in response to a changing constellation of transcription factors in the progenitor population (Butt et al., 2005).

The mature phenotype of each interneuron will depend not only on its “genetics,” (i.e., the genes it expresses), but also on its local cortical environment. For example, X94 axons seem to respond positively to developmental cues emanating from layer 4 and negatively to cues emanating from layer 1, whereas X98 and GIN axons seem to do the opposite (Katz and Callaway, 1992; Castellani and Bolz, 1997; Castellani et al., 1998). Thus, although the capacity to respond to local molecular cues is likely to be genetically programmed, the detailed morphology of a given neuron is not, accounting for the considerable morphological variability within interneuronal subtypes.

Concluding remarks

In the past two decades, in vitro electrophysiological studies, together with immunocytochemistry, electron microscopy, computerized morphological reconstruction, and single-cell genomics and proteomics, have greatly expanded the compendium of known properties of neocortical interneurons (Markram et al., 2004). That a unified classification scheme has been slow to emerge is primarily attributable to the inherent uncertainty in the “identity” of cortical neurons, making it difficult to correlate data acquired by different laboratories using different techniques. What has emerged are parallel systems of technique-specific, or even practitioner-specific, classifications, each based on a limited set of features, and which are nearly impossible to reconcile with each other (Soltész, 2005). The advent of transgenic animals with genetically encoded vital markers has made it possible to identify visually the same subsets of neurons in different animals, provid-
ing, for the first time, a means to correlate results across tech-
niques, investigators, and studies and integrate them to a holistic
portrait of neuronal subtypes, as a necessary step toward the ul-
timate goal of deciphering the cortical circuit.

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