Transgenic Mice Expressing a Fluorescent In Vivo Label in a Distinct Subpopulation of Neocortical Layer 5 Pyramidal Cells

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ABSTRACT

The neuronal components of cortical circuits have been characterized on the basis of their morphological and functional properties, and further refined by correlation of marker proteins with particular cell types. This latter approach has been very fruitful for GABA-containing neurons, but comparable diagnostic markers for subpopulations of excitatory pyramidal cells have been more elusive. An emerging new approach consists of transgenic mice that express fluorescent proteins under the control of promoters that are active in specific cell types. Here, we analyzed a line of transgenic mice that carries a transgene consisting of regulatory sequences of the potassium channel Kv3.1 and enhanced yellow fluorescent protein (EYFP). In these mice, a set of neurons in neocortical layer 5 expresses high levels of the transgenic marker protein. EYFP-expressing, and nonexpressing layer 5 cells were easily identified in living tissue under conditions suitable for patch-clamp electrophysiology. By using immunolabeling, retrograde Fast Blue labeling and electrophysiological recordings with biocytin injections, we identified the fluorescent neurons as a population of pyramidal cells with distinct morphological and electrophysiological properties when compared with nonfluorescent neighboring layer 5 pyramidal cells. The most prominent morphological difference between these two populations was a much smaller number of apical oblique dendrites in EYFP-positive as compared with the EYFP-negative cells. The most prominent electrophysiological feature was a steady spike frequency adaptation in EYFP-positive cells, whereas EYFP-negative cells responded to a depolarizing current injection with a closely spaced spike doublet followed by constant frequency firing. The in vivo labeled transgenic mice provide an experimental tool for further functional differentiation of these populations of layer 5 pyramidal cells. J. Comp. Neurol. 480:72–88, 2004.

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A rational classification of neocortical cell types is essential to understanding cortical organization and function. Dramatic progress in this direction has been made for γ-aminobutyric acid (GABA)-containing neurons by immunohistochemical demonstration of marker proteins, such as calcium-binding proteins and neuropeptides, in relation to morphologically and connectionally specific types of neurons (Celio and Heizmann, 1981; Hendry et al., 1984; DeFelipe, 1993). More recently, this effort has been extended to gene expression patterns, demonstrated by single cell reverse transcriptase-polymerase chain reaction (RT-PCR; Lambolez et al., 1992; Cauli et al., 1997). For glutamatergic layer 5 pyramidal neurons, early studies distinguished two broad populations on the basis of...
dendritic architecture and intrinsic firing properties (McCormick et al., 1985; Chagnac-Amiatii et al., 1990; Mason and Larkman, 1990; Kasper et al., 1994a,b). The combination of this approach with quantitative analysis has raised the possibility of finer subdivisions (Tsiola et al., 2003).

In contrast to GABA-containing neurons, for pyramidal cell types, diagnostic markers, including single-cell PCR products, have been much less successful. An emerging new approach consists of transgenic mice that express fluorescent proteins under control of promoters that are active in specific cell types. This approach has led to the in vivo labeling and identification of a variety of GABA-containing neurons (Oliva et al., 2000; Meyer et al., 2002; Tamamaki et al., 2003). Here, we analyzed transgenic mice expressing an enhanced yellow fluorescent protein (EYFP) under the control of upstream regulatory sequences of the potassium channel Kv3.1 (Metzger et al., 2002). These mice express high levels of EYFP in several regions and, in some cases, in defined neuronal subsets, including cerebellar granule cells and neurons in the hippocampus and cortex (see Table 1 in Metzger et al., 2002). In three of four mouse lines carrying this transgene, cortical neurons, appearing to be pyramidal neurons, are densely labeled in layer 5 of the motor and somatosensory cortices (Metzger et al., 2002).

Cortical layer 5 EYFP-positive cells can be readily identified in living brain slices, fixed slices, and histological preparations. We characterized the layer 5 EYFP-positive cells by comparing their morphological and electrophysiological features with those of neighboring nonfluorescent pyramidal cells. We found that EYFP-positive and EYFP-negative cells, although both having apical tufts extending to or into layer 1, comprise distinct subgroups of pyramidal cells. The differentiation between these two subgroups overlaps with previous classification schemes of layer 5 pyramidal cells.

**MATERIALS AND METHODS**

We used Kv3.1-EYFP transgenic mice carrying the gene for enhanced yellow-fluorescent protein under the control of regulatory sequences of the Kv3.1 potassium channel (line 27, according to Metzger et al., 2002). A total of 18 adult (> 7 weeks) mice were used for immunohistochemistry, 15 adult (> 6 weeks) mice were used for Fast Blue injections, and 20 (20–29 days old) mice were used for combined electrophysiology and intracellular fills. All groups included homo- and heterozygous individuals. All experimental protocols were approved by the Experimental Animal Committee of the RIKEN Institute and performed in accordance with the Guidelines for the Use of Animals in Neuroscience Research (The Society for Neuroscience, Washington, DC). Kv3.1-EYFP transgenic mice can be provided upon request addressed to the authors.

**Fixation and tissue preparation for immunofluorescence**

Mice were anesthetized with Nembutal (100 mg/kg) and perfused transcardially, in sequence, with saline (2 minutes) and 4% paraformaldehyde (5 minutes) in 0.1 M phosphate buffer (PB, pH 8). For two animals used for GABA immunohistochemistry, 0.1% glutaraldehyde was added to the fixative. The brains were post-fixed in the same fixative for 2.5 hours. After fixation, the brains were placed in 30% sucrose, and after sinking, were cut into 40-µm coronal sections on a freezing microtome.

**Immunofluorescence**

Free-floating sections were preincubated for 1 hour with 0.1 M phosphate-buffered saline (PBS, pH 8) containing 0.5% Triton X-100 and 5% normal goat serum (PBS-TG) at room temperature. Sections were incubated for 40–48 hours at 4°C in antibodies raised from different animals as follows: anti–glutamate receptor 2 and 3 (GluR2/3) polyclonal rabbit antibody (Chemicon, Temecula, CA; 1:100), anti–nonphosphorylated neurofilament (SMI-32) monoclonal mouse antibody (Sternerberger Monoclonals, Lutheville, MD; 1:1,500), anti–GABA mouse polyclonal antibody (Sigma, St. Louis, MO; 1:100), anti–calbindin D-28k (CB) monoclonal mouse antibody (Swant, Bellinzona, Switzerland; 1:1,000), anti-parvalbumin (PV) monoclonal mouse antibody (Swant; 1:5,000), anti–Kv3.1b polyclonal rabbit antibody (Chemicon; 1:600). After rinsing, the sections were incubated for 1.5 hours in PBS-TG containing the appropriate fluorescent-conjugated secondary antibodies: Cy5-conjugated anti-rabbit IgG polyclonal rabbit antibody (Jackson ImmunoResearch, West Grove, PA; 1:200) for Glur2/3; Cy5-conjugated anti-mouse IgG polyclonal goat antibody (Jackson ImmunoResearch; 1:200) for GABA; Alexa Fluor 594–conjugated anti-mouse IgG polyclonal goat antibody (Molecular Probes, Eugene, OR; 1:200) for CB and PV; Alexa Fluor 594–conjugated anti-rabbit IgG polyclonal goat antibody (Molecular Probes; 1:200) for SMI-32. For controls, one of the primary antibodies was omitted. No immunofluorescence was detected for the antibody under these circumstances.

**Retrograde Fast Blue labeling**

The retrograde tracer Fast Blue (FB; Dr. Illing Plastics GmbH, Breuberg, Germany) was injected into several cortical areas, including primary somatosensory, primary motor, primary visual, perirhinal, and entorhinal cortical areas, and several different subcortical targets, including the posterior nucleus of the thalamus, striatum, superior colliculus, and pontine nuclei. Mice were anesthetized by Nembutal (100 mg/kg). The cortical and subcortical areas of interest were localized with reference to an atlas of mouse brains (Hof et al., 2000). Subsequent to craniotomy and durotomies, injections were made by pressure through a 10.0-µl Hamilton syringe, on the tip of which we glued a glass pipette (diameter, 70–100 µm). FB was diluted to 2–3% in 0.1 M PBS, and 0.2–0.5 µl was delivered per injection site. To achieve smaller injection sites, 0.15 µl volume was injected in motor and primary somatosensory cortices. To avoid tissue damage and tracking of FB through the white matter (in deep injections), we injected slowly by using a stereotoxic injector (Model 310, Muro-machi Kakai Co., Ltd., Tokyo, Japan). Two cortical injections were ≤ 300–350 µm, but other cortical injections were larger, approximately 400–520 µm. The subcortical injections were ≤ 800–900 µm. After a survival time of 5–7 days, animals were reanesthetized with Nembutal (100 mg/kg) and perfused transcardially, in sequence, with saline and 4% paraformaldehyde. The brains were cut into 40-µm coronal sections on a freezing microtome and mounted immediately onto subbed slides.
Electrophysiological recording

After decapitation, brains were quickly removed and transferred into ice-cold artificial cerebrospinal fluid (ACSF) composed of the following (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃ and 20 D-glucose, and oxygenated with carbogen (95% O₂, 5% CO₂) to obtain pH 7.4. A series of 350-μm-thick coronal sections were cut with a Vibratome (Leica VT1000S, Leica Microsystems, Nussloch, Germany) from areas containing motor and primary somatosensory cortices (assignment according to Hof et al., 2000). The slices were kept in oxygenated ACSF at 20–24°C for 1 up to 7 hours before being used in experiments. Slices were transferred into a recording chamber mounted on a Leica DMLFSA microscope equipped for epifluorescence (Till Photonics, Gräfelfing, Germany) and laser-scan imaging (Leica TCS SP 2, Leica Microsystems, Mannheim, Germany) and were superfused with oxygen-saturated ACSF at 2.5 ml/min flow rate. The majority of recordings were performed at a temperature of 23–25°C, whereas a subset of recordings were performed at 34 ± 1°C. Patch-clamp electrodes were pulled from borosilicate glass and had resistances of 4–7 MΩ when filled with the following (in mM): 132 K-glucuronate, 6 Na-glucuronate, 4 MgCl₂, 0.2 ethyleneglycoltetraacetic acid, 10 HEPES, 4 Na₂ATP, 0.3 Na₃GTP, 10 di-tris-creatine-phosphate and 8 biocytin (0.3%, e-biotinyl-L-lysine). Whole-cell recordings from the somata of EYFP-positive and -negative cells were performed with an Axoclamp-200B amplifier (Axon Instruments, Union City, CA) in current-clamp mode. A small hyperpolarizing holding current (≥ −30 pA) was used to obtain a holding potential of −70 mV at the cell soma. Membrane properties were probed with hyperpolarizing and depolarizing current steps of 100 and 500 msec duration, and the resulting voltage transients were low-pass filtered (5 kHz) and sampled at 20- to 100-μsec intervals. The whole-cell configuration was kept for 15 minutes to ensure sufficient biocytin fill of the cells by diffusive loading through the patch pipette. The electrode was then gently redrawn from the cell body. The slices were kept for at least 2 hours in ACSF to provide additional time for complete loading by biocytin of distal dendrites and axons and then fixed in 4% paraformaldehyde in 0.1 M PBS (pH 8) at 4°C for 12 hours.

Diaminobenzidine histochemistry for biocytin

After fixation, the slices were washed with PBS overnight. After rinsing, endogenous peroxidases were quenched by a 5-minute incubation with 1% H₂O₂. Tissue was reacted for 20–24 hours in avidin–biotin complex (ABC Elite kit; Vector Laboratories, Burlingame, CA) at 4°C. Biocytin was finally demonstrated by 3,3’-diaminobenzidine tetrahydrochloride histochemistry with the addition of 0.03% nickel–ammonium sulfate.

Data analysis

To assess whether EYFP-positive neurons were labeled with various immunohistochemical markers, five sections were selected and 10 fields through portions of primary somatosensory and motor cortex were analyzed at 200× (field width 640 × 500 μm with Olympus BX60 microscope) or 400× (field 230 × 230 μm with Zeiss LSM5 Pascal confocal microscope) magnification. To evaluate the density of neurons double-labeled for EYFP and FB, four sections were selected and 16 fields through primary somatosensory or primary motor cortex were analyzed at 200× (field width 200 × 200 μm) magnification.

For the quantification of morphological parameters, neurons were reconstructed by using a camera lucida microscope attachment. Standard features of dendritic and axonal architecture were evaluated. Oblique dendrites were scored at the point where they exit the apical dendrite. In the few cases where these bifurcated immediately at the exit point, they were counted as two separate dendrites. Apical dendritic tufts were analyzed in terms of number of total branches.

Electrophysiological data were analyzed using Clampfit 9.0 software (Axon Instruments). For each neuron, membrane time constant and input resistance were obtained from an exponential fit to the initial voltage transient evoked by a −50 pA current step. Hyperpolarization-activated rectification was evaluated from the sag in the voltage response evoked by a −100 pA, 500-msec step. The sag was quantified by the difference between the minimum and steady state voltages divided by the initial maximum voltage deflection. Action potential waveforms were analyzed by using the first spike in the train of action potentials evoked by near threshold current injection. Action potential widths were measured at half amplitude. All measured parameters are given as means ± SEM. Differences between EYFP-positive and -negative populations (designated as EYFP(+) cells and EYFP(−) cells, respectively) were tested for statistical significance by unpaired t tests.

Fluorescence images of neurons in fixed tissue were recorded by using Olympus BX60 and Fluoview FV500 microscopes. Fluorescent photomicrographs for sections with Cy5 labeling were obtained with a Zeiss LSM5 Pascal confocal microscope. Images obtained with different immunostains and EYFP were merged with software of this confocal system. Fluorescent photomicrographs for sections with Alexa Fluor 594 labeling were obtained with an Olympus DP50 digital camera mounted on an Olympus BX60 microscope, and images with different fluorochromes were merged with Adobe Photoshop 6.0 software. Photomicrographs of neurons filled with biocytin were captured by using a DP50 digital camera mounted on an Olympus BX60 microscope. All images were saved in TIFF format and imported into Adobe Photoshop 6.0. Image brightness, contrast, and color were adjusted if necessary to reproduce the original histological data.

RESULTS

Inspection of tissue sections prepared from Kv3.1-EYFP transgenic mice, by epifluorescence or laser scanning confocal microscopy, revealed a dense band of brightly labeled cells in layer 5 in almost all areas of the neocortex except for the entorhinal cortex. Labeled cells also occurred in the entorhinal cortex. Labeled cells were scored at the point where they exit the apical dendrite. In the few cases where these bifurcated immediately at the exit point, they were counted as two separate dendrites. Apical dendritic tufts were analyzed in terms of number of total branches.

Electrophysiological recording

To assess whether EYFP-positive neurons were labeled with various immunohistochemical markers, five sections were selected and 10 fields through portions of primary somatosensory and motor cortex were analyzed at 200× (field width 640 × 500 μm with Olympus BX60 microscope) or 400× (field 230 × 230 μm with Zeiss LSM5 Pascal confocal microscope) magnification. To evaluate the density of neurons double-labeled for EYFP and FB, four sections were selected and 16 fields through primary somatosensory or primary motor cortex were analyzed at 200× (field width 200 × 200 μm) magnification.

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Fig. 1. Photomicrographs (A–C) and confocal images (D–G) of enhanced yellow fluorescent protein (EYFP) fluorescence from the primary somatosensory cortex of Kv3.1-EYFP mice (fixed coronal sections, 150 μm thickness). Note that EYFP-expressing cell bodies appear pyramidal in shape, with distinct apical dendrites. The brightest fluorescence is observed from neurons located in layer 5 (L5).

A: Fluorescence in cortical layers 1 to 6 (L1–L6) and white matter (WM). B,C: Higher-magnification images showing apical dendrites (arrowheads in A–C) emanating from cell bodies in layer 5. Arrowheads in A and B point to same dendrite.

D–F: Confocal images of neurons in layer 5. Arrowheads in E, F point to basal dendrites.

G: Branching of a distal apical tuft (arrowheads). Scale bars = 200 μm in A; 20 μm in B–G.
most of these cells, a thin dendritic process, resembling a pyramidal cell apical dendrite, could be followed toward layer 1 (Fig. 1). For some cells, basal dendrites (Fig. 1F) and apical tufts (Fig. 1G), both standard pyramidal cell features, could also be identified. To confirm the impression, based on dendritic morphology, that the EYFP(+/H11001) cells were pyramidal neurons, we carried out immunochemistry with various cell-type specific markers.

**Immunohistochemical characterization**

As markers for pyramidal cells, we used antibodies against GluR2/3 and SMI-32 (Gutierrez-Ibarluzea et al., 1997; Kirkcaldie et al., 2002). When sections containing EYFP(+) neurons were processed for GluR2/3, double-labeled neurons were apparent (Fig. 2A–C). We ascertained that 16.5% of EYFP(+) cells were GluR2/3-positive. Similarly, neurons could be found that were double-labeled for SMI-32 and EYFP (Fig. 2D–F). These were fewer (1.6%) than for GluR2/3, probably because SMI-32 labels an overall smaller subpopulation of pyramidal neurons. As markers for GABAergic cells, we used antibodies against GABA, CB, and PV along with an antibody against the potassium channel subunit Kv3.1b (Kubota et al., 1994; Weiser et al., 1995; Fig. 3). Inspection of stained tissue containing EYFP(+/H11001) neurons failed to demonstrate double-labeled neurons. The neurons whose cell bodies were positive for GABA, CB, PV, or Kv3.1b, had short dendrites, contrasted with the long apical dendrites of EYFP(+) neurons.

**Morphology of biocytin-filled neurons**

EYFP(+) and EYFP(−) cells in layer 5 of neocortex were easily identified by combining transmission and epifluorescence images of acutely prepared neocortical slices mounted in a patch-clamp setup (Fig. 4). By using this in vivo label system, we performed targeted patch-clamp recordings with biocytin-containing patch pipettes to characterize the population of layer 5 pyramidal cells electrophysiologically and as to their dendritic branching pattern. Inspection of the morphology of biocytin-filled cells (n = 38 EYFP(+) cells and n = 20 EYFP(−) cells) confirmed our conclusion that EYFP(+) neurons were pyramidal (Fig. 5). The long and transverse axis of the soma of the patched (and fixed) EYFP(+) neurons (15.6 ± 0.5 μm and 10.2 ± 0.2 μm; n = 30) were smaller than those of EYFP(−) neurons (17.6 ± 0.7 μm; P < 0.02 and 13.6 ± 0.4 μm; P < 0.001; n = 19). The most striking morphological difference between EYFP(+) and EYFP(−) cells was that EYFP(+) cells had a smaller number of oblique dendrites branching off from the ascending apical trunk (Fig. 5).

Quantitative analysis was carried out with a sample of cells that excluded neurons where the staining contrast was considered not optimal, or where dendrites appeared to have been cut during sectioning. For EYFP(+) neurons, the mean number of oblique dendrites branching off from the ascending apical trunk was 3.6 ± 0.4 (n = 25), whereas for the EYFP(−) group, the mean number of oblique dendrites was 10.9 ± 1.4 (n = 12; P < 0.0001;
In contrast, the number of branches at the apical tufts did not differ between the two populations (Fig. 6B).

The number and arrangement of the oblique dendrites for the two populations are represented graphically in Figure 6C. To examine the vertical distribution of the

Fig. 3. Enhanced yellow fluorescent protein (EYFP) -expressing neurons do not colocalize with interneuron markers. A–C: EYFP fluorescence (A), γ-aminobutyric acid (GABA) immunoreactivity (B), and the merged image (C). D–F: EYFP fluorescence (D), calbindin (CB) immunoreactivity (E), and merged image (F). G–I: EYFP fluorescence (G), parvalbumin (PV) immunoreactivity (H), and merged image (I). J–L: EYFP fluorescence (J), Kv3.1b immunoreactivity (K), and the merged image (L). Note that EYFP-expressing neurons are not labeled by GABA, CB, PV, or Kv3.1b antibodies. Examples are taken from the primary motor cortex (A–C and J–L), from the secondary somatosensory cortex (D–F) and from the perirhinal cortex (G–I). Scale bar = 50 μm in A (applies to A–L).
oblique dendrites, we measured the distance of each dendrite from the cell body. The number of primary branches within a 100 μm proximal territory was significantly smaller for EYFP(+) (2.3 ± 0.3; n = 17) than for EYFP(−) (6.4 ± 0.9; n = 9; P < 0.0001) neurons. No significant difference was found between the fraction of oblique dendrites in the proximal zone and the total number of oblique dendrites (72 ± 7% for EYFP(+); 59 ± 5% for EYFP(−); P > 0.17; see Schaefer et al., 2003).

We also examined several other morphometric parameters. No significant difference could be determined in the organization of the basal dendritic tree between the two groups, although this parameter was more difficult to evaluate because some basal trees were very dense (in both groups) and others had obviously been cut during the slice preparation. The mean length of the longest basal dendrite, however, was found to differ significantly between EYFP(+) (254 ± 17 μm, n = 23) and EYFP(−) neurons (182 ± 20 μm, n = 10; P < 0.05). The mean diameter of the proximal apical dendritic trunk was also significantly different between EYFP(+) (2.1 ± 0.2 μm, n = 21) and EYFP(−) neurons (3.0 ± 0.2 μm, n = 10; P < 0.0001).

Various studies have noted differences in the diameter and width of the apical dendritic tuft between subtypes of pyramidal neurons (Chagnac-Amitai et al., 1990; Hefti and Smith, 2000; Tsiola et al., 2003). In our material, there was no significant difference in the width of the apical tuft (144 ± 15 μm for EYFP(+), n = 27; 162 ± 35 μm for EYFP(−), n = 9) or the number of tuft branches (Fig. 6B) between EYFP(+) and EYFP(−) cells.

**Electrophysiological characterization**

Fluorescent and nonfluorescent layer 5 pyramidal cells were patch-clamped in the whole cell configuration with pipettes that, in the majority of cases, contained biocytin (73 of 90 cells). Thus, the same cells that were analyzed morphologically were also tested in current-clamp mode for their responses to hyperpolarizing and depolarizing currents injected by means of the patch-clamp electrode (with the exception of four EYFP(+) neurons and two EYFP(−) neurons, which were excluded from electrophysiological analysis because of excess current (<−30 pA) required to hold these cells at rest). Figure 7 illustrates the typical response pattern of EYFP(+) (Fig. 7A–C) and EYFP(−) (Fig. 7D–F) cells. Figure 7A shows the response of a EYFP(+) neuron to intrasomatic injection of a depolarizing current that amounted ~2 times the threshold for action potential induction. The induced train of action potentials was characterized by a gradual increase in interspike interval. The response of a EYFP(−) neuron to a corresponding stimulus, by contrast, consisted of two closely spaced action potentials at the beginning of the spike train followed by action potential discharges with longer, but constant, interspike intervals (Fig. 7D). These firing patterns persisted when the temperature was raised to 34°C (Fig. 7B,E). For stimulus currents close to threshold, the differences between EYFP(+) and EYFP(−) neurons were more subtle but still noticeable (Fig. 7C,F). During a negative current step, the initial membrane hyperpolarization relaxed toward more positive steady-state voltages in both the EYFP(+) and EYFP(−) neurons. This voltage sag is indicative of the activation of I_h (Williams and Stuart, 2000).

We analyzed 34 (9) EYFP(+) and 25 (17) EYFP(−) cells at 23–25°C (34 ± 1°C) with regard to their current-to-frequency relationship and to their spike frequency adaptation (Fig. 8). The mean spike frequencies attained during 500-msec current steps were substantially higher for EYFP(+) pyramidal cells than for EYFP(−) cells at 23–25°C as well as at 34°C (Fig. 8A,B). For EYFP(+) cells, the relationship between mean firing rate and amplitude of injected current (50 to 300 pA) is sublinear. For the EYFP(−) population, on the other hand, the mean firing rate increased linearly as a function of the current amplitude in the range 100 to 300 pA (23–25°C) and 150 to 300 pA (34°C). Of interest, for the same current amplitude, EYFP(−) cells at 34°C fired with lower frequency than those recorded at 23–25°C, whereas EYFP(+) cells respond with almost the same firing frequency irrespective of temperature. Spike frequency adaptation was evaluated by plotting the instantaneous frequency (i.e., the inverse of the interspike interval) for each spike in a train of action potentials against the spike interval count (Fig. 8C,D).
Fig. 5. Morphology of biocytin-filled neurons in layer 5. A,B: enhanced yellow fluorescent protein-positive (EYFP(+) neurons in the secondary motor (M2, A) and primary somatosensory cortices (S1, B). C,D: EYFP(−) neurons in the primary motor (M1, C) and primary somatosensory cortices (S1, D). EYFP(−) neurons have a larger number of oblique dendrites than the EYFP(+) neurons. Arrowheads point to portions of labeled axons. Scale bar = 100 μm in A (applies to A–D).
The current amplitude used to evoke the spike train was normalized to the measured capacitance of each cell to 1.8 ± 0.3 A/F to account for the variations in input resistance between neurons. Spike frequency adaptation strikingly differed between EYFP(+) and EYFP(−) cells (Fig. 8C, D). While EYFP(+) cells exhibited a modest but progressive frequency adaptation, EYFP(−) cells responded with a closely spaced spike doublet followed by regular firing with little spike frequency adaptation. These distinctive firing patterns were apparent in recordings at 23–25°C and at 34°C (Fig. 8C, D). Of 17 EYFP(−) neurons tested at 34°, 2 exhibited two or three initial spike doublets followed by constant-frequency spike firing. Because we observed this response pattern only in 2 of 17 neurons, these two cases were excluded from the data averages shown in Figure 8D.

Analysis of membrane capacitance and input resistance yielded relatively broad distributions (Fig. 9). However, mean values for both parameters differed clearly between the EYFP(+) and EYFP(−) cells. At 23–25°C, capacitances averaged 56 ± 4 pF for EYFP(+) and 162 ± 12 pF for EYFP(−) cells (P < 0.0001) and mean input resistance was 344 ± 18 MΩ for EYFP(+) and 162 ± 19 MΩ for EYFP(−) cells (P < 0.0001). At 34°C, mean values for the capacitance were 63 ± 4 pF and 173 ± 17 pF (P < 0.0002) and mean values for the input resistance were 316 ± 32 MΩ and 79 ± 8 MΩ (P < 0.0001) for EYFP(+) and EYFP(−) cells, respectively. The threefold difference in capacitance is in agreement with the, on average, slightly smaller diameters of cell bodies, thinner apical trunks, and smaller number of oblique dendrites of EYFP(+) compared with EYFP(−) neurons (Fig. 6A). The membrane time constants did not differ significantly between the two populations at 23–25°C (19.2 ± 1.3 msec for EYFP(+); 22.5 ± 1.5 msec for EYFP(−); P > 0.1) but differed significantly when recordings were performed at 34°C (21 ± 2 msec for EYFP(+); 12 ± 1 msec for EYFP(−); P < 0.0005).

The sag of the hyperpolarization-induced voltage transient, only slightly differed between the two populations at 23–25°C (14 ± 2% for EYFP(+), n = 27; 20 ± 2.0% for EYFP(−), n = 25; P < 0.02), whereas no significant difference was found at 34°C (14 ± 3% for EYFP(+), n = 8; 17 ± 2% for EYFP(−), n = 15; P > 0.3).

Finally, we assessed the waveform of action potentials (Fig. 10). We found that action potential widths were slightly broader in EYFP(+) cells (1.38 ± 0.04 msec) compared with EYFP(−) cells (1.21 ± 0.04 msec; P < 0.01; Fig. 10A, B). Consistent with this difference in action potential
widths, the maximum slope depolarization during upstroke of the action potential was slightly slower in EYFP(+) cells (279 ± 11 mV/msec) than in EYFP(−) cells (310 ± 15 mV/msec; P = 0.07; Fig. 10C), whereas the maximum slope of repolarization was not different (EYFP(+) cells, −64 ± 3 mV/msec; EYFP(−) cells, −65 ± 3 mV/msec; P > 0.7). Raising the recording temperature to 34°C decreased action potentials widths considerably (0.83 ± 0.06 msec for EYFP(+), n = 9; 0.75 ± 0.04 msec for EYFP(−), n = 17) and accelerated the maximal slopes of depolarization during action potential generation (426 ± 21 mV/msec for EYFP(+); 441 ± 29 mV/msec for EYFP(−)) and action potential repolarization (−95 ± 7 mV/msec for EYFP(+); −99 ± 6 mV/msec for EYFP(−)). At 34°C, none of the differences in action potential shape parameters between the samples of EYFP(+) and EYFP(−) neurons reached statistical significance, unlike for the larger data set obtained at lower temperature.

Fig. 7. Electrophysiological characterization of layer 5 pyramidal cells. Left column (A–C) and right column (D–F) illustrate recordings from enhanced yellow fluorescent protein-positive (EYFP(+)) cells and EYFP(−) cells in layer 5 of somatosensory cortex, respectively. A: Action potential firing induced by a depolarizing intrasomatic current injection (100 pA) of a EYFP(+) cell at 25°C. B: Response to depolarization at 34°C (75 pA; different EYFP(+) cell from that in A). C: Membrane voltage responses evoked by a series of intracellular current injections into a EYFP(+) cell at 25°C (same cell as in A). The applied current steps (−100, −50, +50 pA; 500 msec) are shown at the bottom. D–F: Action potential firing induced in a EYFP(−) cell at 25°C (D and F) and at 34°C (E). Current steps are 400 pA (D), 350 pA (E), and −100, −50, 250 pA (F).
Table 1 summarizes the morphological and electrophysiological parameters measured from EYFP(+) and EYFP(-) layer 5 pyramidal cells.

Axonal projections

In addition to dendritic architecture and intrinsic firing properties, layer 5 pyramidal cells may be classified by their axonal arborization (Wang and McCormick, 1993; Kasper et al., 1994a,b). In our material, neurons in both EYFP(+) and EYFP(-) populations had collaterals mainly in the deeper layers or in both the superficial and the deeper layers. For EYFP(+) neurons with exclusively deeper layer collaterals (n = 16), one example had an axon that could be followed into the white matter. For those with both superficial and deep collaterals (n = 13), five axons entered the white matter but could not be followed further. For EYFP(-) neurons with only deeper layer collaterals (n = 7), two examples had an axon that entered into the white matter. For those with both superficial and deep collaterals (n = 6), one axon appeared to continue into the white matter.

To more conclusively characterize the axonal projections of EYFP(+) layer 5 neurons, we carried out surgical procedures to inject the retrograde tracer Fast Blue (FB) into several cortical areas, including primary somatosensory, primary motor, primary visual, perirhinal, and entorhinal cortical areas, and several different subcortical targets, including the posterior nucleus of the thalamus, striatum, superior colliculus, and pontine nuclei (n = 15). Injections resulted in FB-labeled neurons as expected from previous studies. Callosally projecting neurons were particularly dense in layers 2, 3 and 5; extrinsic cortico-cortically projecting neurons, in layers 2, 3, 5, and 6; corticopontine and corticotectal neurons, in layer 5; corticostriatal neurons, in layers 5 and 3; and corticothalamic neurons, in layers 5 and 6. For callosal connections, doubled-labeled (EYFP and FB) neurons could only very occasionally be found, and none were found for long distance ipsilateral corticocortical projections. No EYFP(+) neurons were double labeled for FB after any of the subcortical injections (Fig. 11).

To evaluate local connectivity, two smaller cortical injections were suitable (three others were very large and used only for evaluation of contralateral projections). These smaller cortical injections resulted in abundant FB-labeled neurons in all layers nearby the injection (up to...
600 μm from the injection center). The least label was in layer 4 (of primary somatosensory cortex), and the most was in layers 3 and 5. In layer 5, neurons double-labeled for EYFP and FB were easily apparent after the small injections of both motor (n = 11; Fig. 12) and somatosensory cortex (n = 1). Quantification of these results revealed that 17.3% of EYFP(+) cells were FB-labeled neurons, whereas 9.8% of FB-labeled neurons were EYFP(+) cells in the primary somatosensory cortex. In the primary motor cortex, 20.9% of EYFP(+) cells were FB-labeled neurons, whereas 10.6% of FB-labeled neurons were EYFP(+) cells. Thus, we conclude that EYFP(+) neurons give rise to long-distance subcortical projections and that EYFP(+) neurons are involved in local cortical connections.

DISCUSSION

We present evidence for a grouping of layer 5 pyramidal cells in mouse motor and somatosensory cortices based on morphological and electrophysiological criteria and sorted by the expression of a recombinant fluorescent marker protein. EYFP(+) pyramidal cells differ morphologically from neighboring EYFP(−) cells in soma size, thickness of the apical dendrite, and the number of oblique dendrites that emerge from the ascending apical trunk. Electrophysiologically, EYFP(+) cells are characterized by a modest but progressive spike frequency adaptation, whereas EYFP(−) cells exhibit a short first interspike interval followed by regular firing with little spike frequency adaptation. Action potentials of EYFP(+) cells are slightly broader than those of EYFP(−) cells.

EYFP expression under the Kv3.1 promoter

Originally, Kv3.1 potassium channel subunits were thought to be preferentially expressed in fast spiking GABAergic cells (Weiser et al., 1995; Du et al., 1996), but at least a subpopulation of regularly spiking pyramidal neurons in the hippocampus also express Kv3.1 mRNA (Weiser et al., 1994; Martina et al., 1998). At present, we do not have positive evidence for the possibility that the EYFP(+) cells described here express one or several splice variants of the Kv3.1 gene. The relatively broad action potential of the EYFP(+) cells suggests that they do not
Physiological parameters at 23–25°C

EYFP(+) recorded from enhanced yellow fluorescent protein-positive (EYFP(+), solid line) and EYFP(−) (dashed line) layer 5 pyramidal cells. B, C: Distributions of action potential width (full widths at half maximum, B) and the maximal rate of depolarization during the upstroke of the action potential (C). The means of the distributions are indicated as vertical bars on top of the figures. The P values (t tests) are indicated.

Table 1. Morphometric and Physiological Properties of EYFP(+) and EYFP(−) Layer 5 Pyramidal Neurons

<table>
<thead>
<tr>
<th>Morphological parameters</th>
<th>EYFP(+) cells</th>
<th>EYFP(−) cells</th>
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<tbody>
<tr>
<td>Distributions of action potential width (full widths at half maximum) (ms)</td>
<td>1.38 ± 0.04*** (n = 33)</td>
<td>1.21 ± 0.04 (n = 25)</td>
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<tr>
<td>Action potential, halfwidth (ms)</td>
<td>19.2 ± 1.3</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>Time constant (msec)</td>
<td>3.6 ± 0.4*** (n = 25)</td>
<td>10.9 ± 1.4 (n = 12)</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>344 ± 18***</td>
<td>162 ± 19</td>
</tr>
<tr>
<td>Input resistance (pF)</td>
<td>10.2 ± 0.2***</td>
<td>13.6 ± 0.4</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>56 ± 4*** (n = 33)</td>
<td>162 ± 12 ± 25</td>
</tr>
<tr>
<td>Time constant (msec)</td>
<td>444 ± 16***</td>
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1Data are given as mean ± SEM. Asterisks denote significant differences (P < 0.1, *, P < 0.01, **, P < 0.001, ***).
2Maximal slope during action potential firing.
3Voltage sag evaluated for a current injection of −100 pA.

Express significant amounts of Kv3.1 in their somatic membrane.

There are two technical issues that should be discussed.

First, it should be noted that the fluorescence of EYFPs is modulated by local pH and Cl− concentration, and this property has in fact been exploited for functional imaging experiments (Metzger et al., 2002; Slemmer et al., 2004). Because the pH and Cl− concentrations are constant in fixed tissue, the environmental sensitivity of EYFP is not relevant for the morphological differentiations presented here but may afford opportunities for future studies of the EYFP(+) pyramidal cells in living slices. Second, the transgenic expression of EYFP might raise concerns of possible cell-toxic effects of the recombinant protein, but we believe that this possibility is unlikely. As reported for other lines of transgenic mice, which express high levels of EYFP in subsets of neurons (Feng et al., 2000), our Kv3.1-EYFP mice are indistinguishable from wild-type mice regarding behavior, body weight, and fertility. Moreover, differences between EYFP(+) and EYFP(−) pyramidal cells are consistent with descriptions of similar functional and morphometric phenotypes in wild type rodents, as we discuss below.

Comparisons with previously presented classification schemes

Classic work in guinea pig and rodents distinguished two major subpopulations of layer 5 pyramidal neurons. Physiologically, responses to direct current injections were characterized as intrinsically bursting (IB) and reg-
of currents that generate intrinsic bursting. Taking together with the morphological characteristics of larger cell bodies, thick apical dendrites, numerous oblique dendritic branches and subcortical axonal projections, the majority, if not all, of the recorded EYFP(−) cells can be identified as the IB type of layer 5 pyramidal cells. In turn, EYFP(+) pyramidal cells most likely correspond to RS cells and the subtype that was termed “slender L5” (Mason and Larkman, 1990; Larkman and Mason, 1990) or “group I” (Kim and Connors, 1993).

In the somatosensory cortex, RS layer 5 pyramidal cells were further subdivided into slow-adapting (RS1/SA) and...
fast-adapting (RS2/FA) subtypes according to their spike frequency adaptation (Chagnac-Amitai and Connors, 1989; Chagnac-Amitai et al., 1990; Agmon and Connors, 1992; Schwindt et al., 1997). We have no clear evidence demonstrating that EYFP expression differentiates between (RS1/SA) and (RS2/FA) types of RS cells; but if this were the case, the observed spike frequency adaptation characteristics might be seen as suggesting that EYFP(+) correspond to the more abundant RS1/SA subgroup. Action potentials in this latter cell type are broader than in the case of IB (and RS2/FA cells; Schwindt et al., 1997; Franceschetti et al., 1998), consistent with our observations.

Tsiola et al. (2003) recently presented a classification based on a principal component and cluster analysis of morphological variables of layer 5 neurons in mouse primary visual cortex. Their analysis revealed two principal groups of pyramidal neurons (“tall” and “narrow”) with apical tufted dendrites reaching layer 1 and giving rise to different numbers of oblique branches (10.2 and 3.9, respectively). Both groups contained identifiable subtypes (“tall”, 1A, 1B1, 1B2; “narrow”, 3A, 3B). The physiological profile of these subtypes and whether similar subtypes occur in the S1 and M1/M2 areas is still unknown. Our results give no indication of distinct subtypes within the EYFP(+) group, although we cannot exclude this possibility.

**Functional implications**

Somatic action potentials actively backpropagate into the distal dendrites of layer 5 pyramidal cells where they can either directly elicit Ca\(^{2+}\) action potentials or gate the induction of Ca\(^{2+}\) action potentials by excitatory synaptic inputs (Stuart et al., 1997; Larkum et al., 1999a,b; Vetter et al., 2001). Recent studies emphasized the role of the proximal apical dendrite in mediating the coupling between the axosomatic and distal-dendritic compartments (Larkum et al., 2001; Rhodes and Llinas, 2001; Schaefer et al., 2003). Neuronal simulations of reconstructed rat layer 5 pyramidal neurons suggested that the efficiency of backpropagation activated Ca\(^{2+}\) spike firing (BAC firing) in these neurons correlates with a purely morphometric parameter, defined as the fraction of oblique branches within a distance of 140 \(\mu\)m from the soma and the total number of oblique dendrites (Schaefer et al., 2003). Our data are only partially consistent with this proposal. By using either the same (140 \(\mu\)m) or a shorter cutoff distance (100 \(\mu\)m, to take into account the thinner mouse cortex), we found that EYFP(+) cells have, indeed, fewer proximal oblique branches than EYFP(-) cells. However, the fraction of proximal branches, calculated for both cutoff distances, does not significantly differ between the two groups.
Circuitries and neuronal diversity

Functional, connectional, and morphological features all indicate some degree of heterogeneity of cortical pyramidal cell types (DeFelipe and Farinas, 1992; DeFelipe et al., 2002). Although there are several known organizing principles for GABAergic interneurons and their synaptic connectivity (Cauli et al., 1997; Kawaguchi and Kubota, 1997; Somogyi et al., 1998; Gupta et al., 2000), there are fewer available comparable data for evaluating pyramidal cell diversity and the consequent functional range within the cortical microcircuitry (Markram et al., 1997; Williams and Stuart, 1999; Kozloski et al., 2001; Thomson et al., 2002; Angulo et al., 2003).

Our data demonstrate that EYFP(+) layer 5 pyramidal cells project mainly locally and few of them callosally, whereas EYFP(−) cells are retrogradely labeled with subcortical marker injections. The subcortical projections of IB are well documented in previous work (Wang and McCormick, 1993; Kasper et al., 1994a,b), whereas intracortical local and interhemispheric projections are described for cells similar to EYFP(+) cells (Kasper et al., 1994a,b).

As to local circuitries, the recent data from Angulo et al. (2003) raise the possibility that EYFP(+) and EYFP(−) may differentially participate in the local circuitry. That is, these authors described one subtype of layer 5 pyramidal cells that was characterized by a connection, formed with interneurons, that exhibited paired-pulse depression and had a sparse distribution of oblique dendrites. A second subtype exhibited paired pulse facilitation and had a more dense distribution of oblique dendrites. Interestingly, the morphological and physiological characterization of “depressing” and “facilitating” layer 5 pyramids correlates well with EYFP(+) and EYFP(−) cells, respectively.

In summary, our results contribute to the classification and characterization of neocortical cell types. These findings, along with the availability of mice carrying an in vivo label of a defined subpopulation of layer 5 pyramidal neurons, may serve as a basis for further studies on the involvement of distinct pyramidal cell populations in defined cortical circuits.

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LITERATURE CITED


