Localization of Calcium-binding Proteins in Physiologically and Morphologically Characterized Interneurons of Monkey Dorsolateral Prefrontal Cortex

In the primate neocortex, little is known about the possible associations between functional subclasses of GABA neurons, their morphological properties and calcium-binding protein (CaBP) content. We used whole-cell current clamp recordings, combined with intracellular labeling and fluorescence immunohistochemistry, to determine these relationships for interneurons in layers 2–3 of monkey prefrontal cortex (PFC). Eighty-one interneurons were included in the analysis. Thirty-eight of these cells showed immunoreactivity for one of the three CaBPs tested. Co-localization of more than one CaBP was not observed in any of the interneurons examined. Interneurons with different CaBPs formed distinct populations with specific physiological membrane properties and morphological features. Parvalbumin (PV)-positive cells had the physiological properties characteristic of fast-spiking interneurons (FS) and the morphology of basket or chandelier neurons. Most calretinin (CR)-containing cells had the physiological properties ascribed to non-fast-spiking cells (non-FS) and a vertically oriented axonal morphology, similar to that of double bouquet cells. Calbindin (CB)-positive interneurons also had non-FS properties and included cells with double bouquet morphology or with a characteristic dense web of axonal collaterals in layer 1. Classification of the interneurons based on cluster analysis of multiple electrophysiological properties suggested the existence of at least two distinct groups of interneurons. The first group contained mainly PV-positive FS cells and the second group consisted predominantly of CR- and CB-positive non-FS interneurons. These findings may help to illuminate the functional roles of different groups of interneurons in primate PFC circuitry.

Keywords: calbindin, calretinin, fast-spiking, parvalbumin, regular-spiking

Introduction

In primates, the prefrontal cortex (PFC) occupies up to 30% of the total cortical volume (Fuster, 1997) and represents the largest neocortical area in the human brain. It is well established that the PFC mediates complex behaviors that are characteristic of primates generally, and of humans in particular (Wood and Grafman, 2003). Working memory, the ability to hold information in mind for a short period of time, is a critical cognitive process underlying complex behaviors in primates (Goldman-Rakic, 1995; Fuster, 1997; Baddeley, 2003). In vivo recordings in primates have suggested that GABA-mediated inhibition in the dorsolateral PFC (DLPFC) is an important element of the neuronal circuitry subserving working memory (Sawaguchi et al., 1988, 1989; Wilson et al., 1994; Rao et al., 1999, 2000, Constantinidis and Goldman-Rakic, 2002). For example, putative GABA interneurons recorded from the monkey DLPFC during a spatial working memory task displayed delay-period activity selective for memoranda in specific spatial locations (Wilson et al., 1994; Rao et al., 1999, 2000).

Cortical GABAergic neurons are a diverse population (Cauli et al., 1997; Gupta et al., 2000; McBain and Fisahn, 2001). Determining the properties that contribute to this diversity is an important step towards delineation of different types of interneurons and for understanding their distinctive contributions to cognitive processes. Studies in rodents indicate that certain functional properties of GABA neurons may be correlated with their content of calcium-binding proteins (CaBPs). For example, analysis of the expression of CaBPs in morphologically and physiologically characterized neurons has revealed distinct classes of interneurons, which appear to have specialized inhibitory functions in rodent hippocampus (Freund and Buzsaki, 1996; van Hooft et al., 2000) and neocortex (Cauli et al., 1997, 2000; Kawaguchi and Kubota, 1997).

However, direct transfer of these findings in rodents to the primate DLPFC is difficult because this region is not well developed in the neocortex of rodents (Preuss, 1995; Fuster, 1997). In addition, there are differences in the intrinsic circuitry of the primate DLPFC and its most homologous cortical region in rats, the medial PFC (Gabbott et al., 1997). Furthermore, cortical interneurons appear to have a different developmental origin in rodents and primates. For example, in rodents, most of the neocortical GABA cells originate in the subcortical ganglionic eminence of the telencephalon (Xu et al., 2004), whereas in humans more than half of the GABA neurons originate within the cortical subventricular zone (Letinic et al., 2002). Perhaps because of these differences in the developmental origin of GABA neurons, rodents and primates differ substantially in the proportions of neocortical interneurons immunoreactive for specific CaBPs. Whereas in the rat frontal cortex parvalbumin (PV)-positive interneurons are the prevalent interneuron subpopulation (Gabbott et al., 1997; Kawaguchi and Kubota, 1997), calretinin (CR)-positive interneurons predominate in the PFC of monkeys (Conde et al., 1994; Gabbott and Bacon, 1996b). In addition, a high degree of colocalization of different CaBPs in the same interneurons was observed in the rat frontal cortex (Kubota et al., 1994; Kawaguchi and Kubota, 1997), but not in the monkey DLPFC (Conde et al., 1994). Thus, it is unclear whether interneurons containing a given CaBP share the same physiological, morphological and functional properties across species. Answering this question is critical for our understanding of the pathophysiology that underlies alterations in a subclass of GABA neurons in human disease states, such as schizophrenia (Hashimoto et al., 2003), where the function of these neurons cannot be directly addressed.

Thus, in this study we used whole-cell current clamp recordings, combined with intracellular labeling and fluorescence immunohistochemistry, to determine the relationship between CaBP content and physiological and morphological properties of...
interneurons in the DLPFC of nonhuman primates. This experimental approach, combined with multivariate cluster analysis, allowed us to distinguish two major groups of interneurons. The first group was comprised of cells containing PV. PV-positive interneurons showed fast-spiking (FS) physiological properties and had the morphological features of spreading arbor (basket) cells or chandelier neurons. The second group consisted of CR or calbindin (CB)-positive non-FS interneurons that displayed a predominantly vertically oriented morphology.

Materials and Methods

Slice preparation

Tissue blocks containing a portion of DLPFC areas 9 and 46 were obtained from five young adult male cynomolgus monkeys (*Macaca fascicularis*) treated according to the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The procedure used to obtain tissue from the DLPFC has been described in detail in other studies that used tissue slices from the same animals (Gonzalez-Burgos et al., 2000, 2004).

The tissue blocks were placed in ice-cold Ringer's solution, containing (in mM): NaCl 126, KCl 2.5, NaH2PO4 1.25, CaCl2 2, MgSO4 1, NaHCO3 26 and dextrose 10, pH 7.4. perfused with a 95%O2/5%CO2 gas mixture. Multiple coronal (at least eight) 350 μm thick slices were cut from each block using a vibratome (VT 1000S Leica, Germany) and incubated for 1 h at 36°C and at room temperature thereafter, or at room temperature from the beginning. For recordings, slices were submerged in a chamber mounted on the microscope and perfused with Ringer solution at 32°C. Each experiment lasted up to 24 h as dictated by the survival of the slices. A total of 58 slices were used.

Electrophysiological Recordings

Interneurons of layers 2-3 were visualized using infrared DIC video microscopy and distinguished from pyramidal cells based on their small soma size, with round or oval shape and the absence of an apical dendrite. Patch electrodes with open-tip resistances of 7–15 MΩ were filled with a solution containing (in mM): potassium glutamate 114, KCl 0, ATP-Mg 4, GTP 0.3, HEPES 10 and 0.5% biocytin; pH 7.25 adjusted with KOH. Whole-cell current clamp recordings were performed after reaching seal resistance of at least 4–5 GΩ. Recording pipettes were withdrawn after a short recording period (5–7 min) to minimize dilution of the somatic concentration of CaBPs by the pipette solution. Voltages were amplified using Intracellular Electrometers IE-210 (Warner Instrument Corporation, Hamden, CT), operating in bridge-balance mode, were amplified using Intracellular Electrometers IE-210 (Warner Instrument Corporation, Hamden, CT), and filtered on line at 5 kHz and acquired on a personal computer at sampling rates of 20 kHz using Power 1401 interface and Signal 2 software program (CED, Cambridge, UK).

Electrophysiological Analysis

To characterize the intrinsic membrane properties of neurons, rectangular hyper- and depolarizing current pulses of 500 ms duration were applied in 10 pA increments at 0.2 Hz with two repetitions. Input resistance (*Ri*) was determined from the slope of the linear portion of the *I–V* curve (usually between -50 and -10 pA), as measured at the end of the 500 ms step. Membrane time constant (τ) was determined by fit of a single exponential to the average voltage response to hyperpolarizing current steps of -10 to -30 pA. Properties of single action potentials, including threshold (APT), duration (ADP), amplitude (APA) and amplitude of the afterhyperpolarization (AHPA), were measured using current steps close to the threshold of firing for each individual cell, which usually elicited either one or a few action potentials. Spike frequency adaptation (ARFP) was measured as the ratio between the first interspike interval to the last interspike interval, measured with depolarizing current steps of 60 pA above the threshold of firing.

Histological Processing

After recordings, slices were incubated for at least 20 min at 32–35°C to allow for good dye diffusion and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 12–24 h at 4°C. After the fixation, slices were rinsed in PB, serially sectioned at 50 μm, and processed for visualization of the CaBPs in recorded cells using triple immunofluorescent labeling. Sections were incubated for 3 days at 4°C in blocking serum (10% normal goat serum; 2% bovine serum albumin; 0.4% Triton X-100 in PB) containing streptavidin-Alexa Fluor 350 conjugate (Molecular Probes, dilution 1:500) and a mixture of antibodies against two of the three CaBPs (Table 1). After thorough rinsing, the sections were incubated in a mixture of two secondary fluorescently tagged antibodies (Alexa Fluor 594 conjugated goat anti-mouse IgG, 1:500 and Alexa Fluor 488 conjugated goat anti-rabbit IgG, 1:500, Molecular Probes) dilluted in the blocking serum. This procedure yielded differential fluorescent co-visualization of Alexa Fluor 350-biocytin-filled interneurons and Alexa Fluor Fluor 488- and 594-labeled CaBPs. In some cases, different combinations of Alexa Fluor Dyes were used. Rinsed sections were coverslipped and photographed on a Zeiss Axioskop microscope.

After analysis of the fluorescent signal, the sections were treated with 1% H2O2 for 2-3 h at room temperature and then were incubated with the avidin-biotin–peroxidase complex (1:100, Vector Laboratories, Burlingame, CA) in PB for 4 h. Sections were rinsed, stained with 3,3′-diaminobenzidine (DAB), mounted on gelatin-coated glass slides, dehydrated and coverslipped. Neurons were reconstructed using the NeuroLucida tracing system (MicroBrightField, Williston, VT).

Laminar distribution of CaBPs in DLPFC was assessed in sections obtained from the brain of another monkey, which had been perfusion-fixed with 4% paraformaldehyde. The sections were immunostained as described above and their laminar boundaries were verified using adjacent sections stained with Nissl.

Statistical Analysis

Unless otherwise stated, all data are reported as means, confidence limits (*P* = 0.95), and standard deviations. The statistical significance between group means was tested using ANOVA, followed by Fisher LSD post hoc tests (multiple comparison tests). To divide interneurons into groups based on electrophysiological properties, we employed cluster analysis (Johnson and Wichern, 1998), following Ward’s hierarchical clustering algorithm with Euclidean distance, which reduces cluster size by consecutively merging data points based on the least possible increase in the within-group sum of squared deviation. This method allowed us to classify the interneurons without *a priori* knowledge of the number of groups. Before performing the cluster analysis all variables were normalized to their *z*-scores. Statistical significance of the observed differences between cluster means was tested using two-sample *t*-tests, independently for each of the variables (see Table 3). All statistical tests were performed using Statistica 6.1 software (Statsoft Inc., Tulsa, OK).

Results

Calcium-binding Protein Content and Morphological Features of the Recorded Interneurons

To confirm the specificity of the antibodies and the quality of the immunostaining, we first performed immunofluorescent staining in DLPFC sections obtained from monkeys perfused with 4% paraformaldehyde. Analysis of the immunofluorescent signals showed that all three PV-, CR- and CB-positive populations of interneurons were present in layers 2–3 (Fig. 1A). The density of CB- and CR-immunoreactive interneurons was greater in these cortical layers than in deeper laminae, whereas...
PV-positive interneurons were encountered most often in the middle cortical layers. This pattern of staining corresponds well with the previous studies of CaBPs in monkey PFC (Conde et al., 1994; Dombrowski et al., 2001), suggesting an appropriate specificity of the immunostaining method employed here.

Eighty-one cells were morphologically identified as interneurons after electrophysiological recordings and subsequent development of biocytin using DAB-peroxidase histochemistry. In some cases, a detailed morphological identification of interneuronal types was not possible because of limited labeling of the axonal arborization. Nevertheless, all included cells clearly had a nonpyramidal morphology, including round or oval somata, aspiny or sparsely spiny dendrites and the absence of an apical dendrite.

Thirty-eight (46.9%) of these cells showed immunoreactivity (IR) for one of the three calcium-binding proteins tested. Of the 69 interneurons incubated with PV, 14 (20.3%) were PV-IR; of 56 interneurons incubated with CR, 16 (28.6%) were CR-IR; and of 32 interneurons incubated with CB, 8 (25%) were CB-IR (Fig. 1B). Co-localization of more than one CaBP was never observed in any of the interneurons studied.

The majority (11 of 14) of PV-positive cells (Fig. 2, PV1) morphologically resembled 'basket cells' described in other cortical regions (Jones and Hendry, 1984). However, in our sample the axons of these cells did not form well-defined pericellular baskets, and therefore we refer to them as spreading arbor cells. Similar to previous Golgi study of the DLPFC (Lund and Lewis, 1993), the axons of spreading arbor cells projected horizontally from 400 to 1000 μm and had a simple branching pattern, with a primary axon trunk that gave rise to a few main lateral branches. The latter were long, stout linear processes, giving off linear collaterals at right or oblique angles, which spread across several layers, but almost completely avoided layer 1. The somata of these multipolar neurons were relatively large, with dendrites that often spread in both horizontal and vertical directions, crossing several layers. Two PV-positive cells showed the morphology of chandelier neurons (Fig. 2, PV2). As previously described (Lewis and Lund, 1990), a prominent feature of these interneurons was the characteristic vertical arrangements of axonal terminals or cartridges. Labeling of the axonal arborizations in the one remaining PV-positive cell was not of sufficient quality to allow reliable morphological identification.

We obtained good morphological recovery after DAB-peroxidase staining in 11 out of 16 CR-positive interneurons. All of these neurons showed a predominantly vertical orientation of both dendritic and axonal arbors (Fig. 2, CR1-3). CR-positive neurons typically had a small, oval cell body, which was vertically oriented. Thick primary dendrites arose vertically from each pole of the soma and bifurcated close to their origin. Shortly after the bifurcation, each branch divided again, finally producing two tufts of beaded dendrites at each pole from the soma. The axons of the CR-positive neurons arose from one of the primary dendrites or from the proximal portion of a secondary dendrite. Along its invariably descending course, the main axon gave off multiple short and finely beaded branches, mostly at right angles. The axonal arbor of these cells had the appearance of a narrow 'bottlebrush' about 150 μm wide, which crossed several layers, sometimes reaching layer 6. The horizontal collaterals generally did not extend beyond the width of the dendritic arbor. CR-positive neurons were morphologically quite similar to the narrow arbor, vertically projecting, local circuit neurons identified in Golgi studies of monkey DLPFC (Lund and Lewis, 1993). The axons of the remaining five CR-positive interneurons were not sufficiently labeled by the DAB-peroxidase reaction to reliably identify their morphology.

Five out of eight CB-positive cells were morphologically reconstructed, and these neurons showed a predominantly vertical orientation of their axonal arbors. The main features of CB-positive interneurons were a dense web of recurrent axonal collaterals of fine appearance with numerous axonal branches in layer 1. Three of the reconstructed cells were located in layer 2 and had ascending axons (Fig. 2, CB1). The other two cells were...
also located in layer 2, but in contrast, had a predominantly descending axonal arbor and were similar to double bouquet cells (Fig. 2, CB2).

Physiological Properties of Interneurons Immunoreactive for Different Calcium-binding Proteins

We first examined the extent to which interneurons with different CaBP content have distinct electrophysiological properties (Table 2, Fig. 3). The input resistance of PV-positive cells was 2-fold lower than that of both CR- and CB-positive interneurons. In addition, both the membrane time constant and action potential duration were shorter in PV-positive cells than in the other two types of nonpyramidal cells. The majority of PV-positive cells had spike durations of \(<0.45\) ms, with a median of 0.31 ms, which is twice as fast as that of CR- and CB-IR interneurons (Fig. 3C). Trains of action potentials evoked by large suprathreshold currents exhibited relatively little spike frequency adaptation in PV-IR neurons as compared to the other two groups of interneurons. However, at near-threshold current levels, PV-positive interneurons displayed considerable variability in firing pattern. They could discharge either single spikes with a large afterhyperpolarization (AHP), or a number of spikes with irregular interspike intervals, or start firing after a prolonged quiescent period. The average action potential amplitude was smaller in PV-positive cells compared to CR-positive cells. In CB-positive cells, the action potential amplitude had intermediate values that did not differ statistically from spike amplitude in the other two groups. The majority of cells, independent of their CaBP content, had monophasic AHPs (Fig. 3A), but a few CR- and CB-positive cells exhibited biphasic AHPs. In spite of the large variability of AHP amplitude within subgroups of interneurons, the AHP amplitude of PV-positive cells was, on average, larger than that of CR-positive cells. The mean amplitude of the AHP in CB-positive cells did not differ statistically from the other two cell groups.

The membrane properties of CR- and CB-positive neurons appeared to be very similar to each other, showing no statistically significant differences. However, a large variability in some physiological measurements within the CR- and CB-positive cell groups (Fig. 3) suggested heterogeneity within these neuronal populations. For example, the input resistance ranged from 240 to 1160 MΩ, whereas the time constant had a minimum of 7 ms and a maximum of 38 ms. At near-threshold depolarizing current steps, these cells fired regular spikes usually during early parts of the current pulses. All but 4 CR- and CB-positive neurons had action potentials of relatively long duration (duration \(>0.55\) ms).

Cluster Analysis of Interneurons: Correlation between Expression of CaBP Proteins and Electrophysiological Properties

As shown in Table 2, when grouped based on CaBP content, interneurons of the monkey DLPFC displayed differences in several electrophysiological properties. An additional question is whether the grouping of cells based solely on electrophysiological properties would, in turn, show significant differences in CaBP content. If so, then the two independent approaches would indicate a strong correlation between electrophysiological properties and expression of CaBPs. To address this question, we employed cluster analysis as a multivariate exploratory technique. This approach has been demonstrated to be useful for unbiased delineation of physiological groups of interneurons based on their membrane properties (Cauli et al., 2000; Nowak et al., 2003). We adopted this approach to test the hypothesis that interneurons containing different CaBPs are found in distinct physiological clusters.

To perform cluster analysis based on electrophysiological variables, we included all but five of the recorded interneurons (\(n = 76\)). Two interneurons were excluded from the analysis because their time constants could not be described by a single exponential function; three other interneurons were excluded because an irregular firing pattern made the calculation of adaptation ratio difficult. The physiological classification of interneurons was performed using Ward’s hierarchical clustering algorithm. In order to delineate the physiological groups of interneurons, we used only variables previously reported to be distinctive of physiological subclasses of interneurons (Kawaguchi, 1995; Cauli et al., 1997, 2000; Gupta et al., 2000). As the results of cluster analysis can be dependent on the choice of variables analyzed, different combinations of physiological parameters were used to delineate groups: (i) \(R_i\), \(\tau\), APD (the parameters that showed the most significant differences between interneurons containing different CaBPs, \(P < 0.001\), see Table 2); (ii) \(R_i\), \(\tau\), APA, APD and AHPA (all the variables that showed significant differences at \(p < 0.05\)); (iii) \(R_i\), \(\tau\), APT, APA, APD, AHPA and AR60 (all the physiological parameters measured in our study). The results of all three attempts were very similar, indicating the validity and reliability of this approach. Here we report only the results of the third solution, which included all physiological parameters, and thus probably reflects
the most satisfactory physiological classification of interneurons.

The hierarchical tree plot (Fig. 4) clearly suggested the existence of at least two distinct groups of interneurons (with linkage distance of 35). The final cluster sizes of the two groups were 32 and 44. Compared to the second cluster, interneurons in the first cluster (Table 3) had lower input resistance and shorter time constant, thinner spikes with lower amplitudes, larger AHP amplitude and less spike frequency adaptation (larger ARδ0). Thus, these two clusters matched well previous descriptions of cell populations classically described as FS and the group of non-FS — regular spiking nonpyramidal cells in the rat (Kawaguchi, 1995; Karube et al., 2004). The first cluster mainly contained PV-positive interneurons and the second one consisted exclusively of CB- and CR-positive cells. Interneurons that did not show immunoreactivity for CaBPs were distributed equally between both clusters (Table 3).

Cluster analysis also demonstrated that the group of non-FS can be further subdivided into two subgroups (with linkage distance of 20). The larger subgroup included 27 cells, and the smaller included 17 interneurons. Both subgroups displayed typical characteristics of regular-spiking nonpyramidal cells, such as intermediate spike duration (0.69 ms and 0.68 ms, \( P > 0.10 \)) and adaptation ratio (0.56 and 0.65, \( P > 0.10 \)), but they differed in other physiological properties. The interneurons from the larger subgroup had smaller input resistance (518 MΩ versus 928 MΩ, \( P < 0.0001 \)), shorter time constant (13.7 ms versus 23.9 ms, \( P < 0.0001 \)), smaller AHPA (15.0 mV versus 21.0 mV, \( P < 0.01 \)), lower threshold of action potential (~46.6 mV versus ~37.2 mV, \( P < 0.0001 \)) and larger amplitude of action potential (70.1 mV versus 60.7 mV, \( P < 0.05 \)) as compared to the cells from the smaller subgroup. This larger subgroup (\( n = 27 \)) contained predominantly CR-positive cells (10 CR-, 2 CB-, and 15 cells that showed no CaBP signal). The smaller subgroup (\( n = 17 \)) contained nearly equal numbers of CR-IR and CB-IR interneurons (4 CB, 3 CR and 10 cells with no CaBPs). These findings support the notion that the electrophysiological group of non-FS cells may comprise at least two subgroups of interneurons, which predominantly contain CR or CB. Indeed, cluster analysis yielded statistically significant differences between the means of certain physiological parameters that were in agreement with the tendencies observed in Table 2.

### Discussion

We have analyzed the CaBP content in physiologically and morphologically identified interneurons from monkey DLPC areas 9 and 46. Previous studies showed significant differences in physiological subtypes and distribution of CaBPs across cortical layers (Kawaguchi and Kubota, 1993; Conde et al., 1994; Kawaguchi, 1995; Gabbott and Bacon, 1996b). In addition, interneurons of the superficial layers probably play a critical role in regulating the function of layer 3 pyramidal neurons (Lewis et al., 1999), which appear to play a critical role in the circuitry that subserves working memory (Fuster et al., 1985; Friedman and Goldman-Rakic, 1994). Thus, in this study we focused on neurons of layer 2–3.

We have demonstrated that interneurons with different CaBPs form distinct subpopulations with specific physiological and morphological properties. PV-positive cells had physiological properties of classical FS interneurons and the morphology of spreading arbor (basket) cells or chandelier neurons. CR- and CB-containing cells usually had non-FS-like properties and vertically oriented axonal morphology. CR-IR interneurons were similar to that of double bouquet cells and the main features of CB-positive interneurons were dense webs of recurrent axonal collaterals of fine appearance and with numerous axonal branches in layer 1.

### Methodological Considerations

There are several methodological limitations that could reduce the detectability of CaBPs in our study. First, the cells were tested simultaneously only for two CaBPs, or, in a few cases, only for one CaBP. Thus, the immunonegative interneurons simply could contain an untested CaBP. Second, there is a strong concentration gradient of CaBPs between the cytosol of the recorded interneurons and the internal solution of the recording electrodes. This gradient forces mobile cytosolic low-molecular weight proteins such as PV, CR and CB (Baimbridge et al., 1992; Plogmann and Celig, 1993) to quickly diffuse out of the cells (Kawaguchi, 1995). To minimize this effect, we used electrodes with relatively high resistance and we limited the recording time to ~5–7 min, since trial experiments showed a significant reduction of the fluorescent signal in immunopositive cells if they were recorded for >10 min. However, it cannot be ruled out that the recording procedure reduced the detectability of CaBPs, thus potentially leading to false negative results in some interneurons. Third, ~10% of cortical GABA neurons do not express detectable levels of any of the three CaBPs tested, as demonstrated by comparisons of the numbers of GABA-IR and CaBP-IR neurons (Conde et al., 1994). Finally, the selection of interneurons for the recordings could create a sampling bias. Interneurons with very small soma size (Conde et al., 1994; Gabbott and Bacon, 1996a) were more difficult to visualize in slices prepared from adult monkey brain. Therefore, despite our best efforts these cells could be underrepresented in our sample.

### Table 2

<table>
<thead>
<tr>
<th>Physiological parameters measured</th>
<th>Groups of CaBP-containing interneurons</th>
<th>Results of statistical analysis</th>
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<tr>
<td></td>
<td>PV</td>
<td>CR</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>235 ± 68 (117)*</td>
<td>586 ± 137 (256)</td>
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<tr>
<td>Time constant (ms)</td>
<td>0.35 ± 0.06 (0.10)</td>
<td>0.65 ± 0.12 (0.16)</td>
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<td>Adaptation ratio</td>
<td>0.80 ± 0.11 (0.20)</td>
<td>0.64 ± 0.12 (0.16)</td>
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<tr>
<td>Threshold of action potential (mV)</td>
<td>42 ± 4 (7)</td>
<td>46 ± 4 (8)</td>
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<tr>
<td>Duration of action potential (MS)</td>
<td>0.35 ± 0.06 (0.10)</td>
<td>0.65 ± 0.12 (0.16)</td>
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<tr>
<td>Amplitude of action potential (mV)</td>
<td>50 ± 8 (13)</td>
<td>67 ± 8 (15)</td>
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<tr>
<td>AHP amplitude (mV)</td>
<td>21 ± 3 (5)</td>
<td>15 ± 2 (4)</td>
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Means ± confidence limits (SD).

Groups of interneurons within parentheses did not differ significantly from each other according to post-hoc test.
Immunoreactivity for PV, CR or CB was detected in 20%, 29% and 25% of the tested interneurons, respectively. No colocalization of CaBPs was observed in any of the tested cells. This finding is supported by previous immunohistochemical observations, demonstrating that in the adult monkey PFC interneurons containing PV, CR or CB form three non-overlapping subpopulations accounting for 20–25%, 45–50% and 20–25% of interneurons, respectively (Conde et al., 1994; Gabbott and Bacon, 1996b). Only Cajal-Retzius cells in layer 1 (Conde et al., 1994) and interneurons in the immature neocortex have been found to contain more than one CaBP (Yan et al., 1995; Hof et al., 1999). Among PV- and CR-positive cells, we found morphological types similar to those previously described in monkey DLPFC (Conde et al., 1994) and other cortical regions (Gabbott and Bacon, 1996a; Meskenaite, 1997; DeFelipe, 1997). It appears that CR-positive cells of layers 2–3 form a homogeneous morphological group characterized by narrow and predominantly vertical axonal arbors that span several layers and reach as far as layer 6. In contrast, most PV-positive cells have predominantly a horizontal arborization, which tends to stay within one cortical layer. CB-positive cells appeared to be morphologically more diverse. Although consistent with previous reports (Gabbott and Bacon, 1996a; DeFelipe, 1997; DeFelipe et al., 1999), many of them also showed a predominantly vertical orientation of their axons and resembled double bouquet cells; their most distinctive feature seemed to be a very rich web-like local plexus of recurrent axonal collaterals with numerous axonal branches in layer 1. Interneurons with similar

**Figure 3.** Electrophysiological properties of interneurons from the monkey DLPFC. (A) Representative examples of responses to injection of hyperpolarizing and depolarizing current steps of three cells containing PV, CR and CB, respectively. Note the nonadapting spike train in PV-IR interneuron and spike frequency adaptation in CR- and CB-IR interneurons. (B) Slope of voltage deflection versus current step (I–V curves). The slope of the I–V curves depends on input resistance of cell. The slope of the I–V curve is considerably steeper in the PV-IR cells compared with that for CR- and CB-IR interneurons. Each point is the average of the voltage deflection for each group of interneurons for a given current intensity. Bars represent SE. (C) Distribution of membrane properties in cells containing different CaBPs. Square = mean; box range = 25, 75 percentile; line within box = median; box whiskers = 10, 90 percentile.

**Anatomical Properties of Interneurons Containing Different CaBPs**

Immunoreactivity for PV, CR or CB was detected in 20%, 29% and 25% of the tested interneurons, respectively. No colocalization of CaBPs was observed in any of the tested cells. This finding is supported by previous immunohistochemical observations, demonstrating that in the adult monkey PFC interneurons containing PV, CR or CB form three non-overlapping subpopulations accounting for 20–25%, 45–50% and 20–25% of interneurons, respectively (Conde et al., 1994; Gabbott and Bacon, 1996b). Only Cajal-Retzius cells in layer 1 (Conde et al., 1994) and interneurons in the immature neocortex have been found to contain more than one CaBP (Yan et al., 1995; Hof et al., 1999). Among PV- and CR-positive cells, we found morphological types similar to those previously described in monkey DLPFC (Conde et al., 1994) and other cortical regions (Gabbott and Bacon, 1996a; Meskenaite, 1997; DeFelipe, 1997). It appears that CR-positive cells of layers 2–3 form a homogeneous morphological group characterized by narrow and predominantly vertical axonal arbors that span several layers and reach as far as layer 6. In contrast, most PV-positive cells have predominantly a horizontal arborization, which tends to stay within one cortical layer. CB-positive cells appeared to be morphologically more diverse. Although consistent with previous reports (Gabbott and Bacon, 1996a; DeFelipe, 1997; DeFelipe et al., 1999), many of them also showed a predominantly vertical orientation of their axons and resembled double bouquet cells; their most distinctive feature seemed to be a very rich web-like local plexus of recurrent axonal collaterals with numerous axonal branches in layer 1. Interneurons with similar
physiological clusters of cells differed in their CaBP content. Taken together, this suggests a strong correlation between the expression of CaBPs and the electrophysiological properties of interneurons. By acting as intracellular calcium buffers, CaBPs may influence the firing pattern and other electrical properties of the cells, thus creating a biochemical and physiological basis for such correlation. For example, cerebellar granule cells of calretinin-deficient mice exhibit faster action potentials and show enhanced spike discharge in response to injected current (Gall et al., 2003).

With few exceptions, PV-positive cells formed a relatively homogeneous physiological population with characteristics typical of FS interneurons previously described in studies of rodent frontal cortex (Kawaguchi, 1995; Kawaguchi and Kubota, 1997) and other cortical regions (Reyes et al., 1998; Gibson et al., 1999; Galaretta and Hestrin, 2002), including hippocampus (Pawelzik et al., 2002). Distinctive features of this subgroup were a shorter duration of action potentials, smaller input resistance and a shorter time constant as compared to CR- and CB-positive cells. However, we have not observed bursting interneurons among PV-IR cells as shown recently in developing mouse neocortex (Blatow et al., 2003).

In our data, CR- and CB-positive cells had relatively long spike duration and obvious spike frequency adaptation and thus could be clearly distinguished from FS interneurons, similar to the CR- and CB-positive regular-spiking nonpyramidal cells in rodent frontal cortex (Kawaguchi and Kubota, 1997). However, within the large group of non-FS cells, individual cells displayed considerable variability in several electrophysiological parameters, so that CR-positive cells could not be clearly distinguished from CB-positive cells based on physiological parameters alone.

In addition, in rodent neocortex, burst spiking interneurons were often encountered among CR-positive neurons (Kawaguchi and Kubota, 1997; Cauli et al., 1997, 2000), especially those containing both CR and vasoactive intestinal polypeptide (VIP) (Porter et al., 1998). Although the majority (85%) of CR-positive neurons in layer 2–3 from monkey PFC also contain VIP (Gabbott and Bacon, 1997), we have not observed bursting interneurons in our sample. The potential correlation between subgroups of non-FS cells and neuropeptide content remains to be investigated.

It is also worth mentioning that among all recorded interneurons we did not find cells with typical low-threshold spiking firing patterns (Kawaguchi, 1995). Whether these discrepancies reflect differences between species, or differences between the age ranges that is typically studied in rodent models (<35 days) versus our young adult monkeys, remains to be investigated.

### Differential Roles of PV-, CR- and CB-positive Interneurons in Intrinsic Prefrontal Cortical Circuitry

The distinct subgroups of neurons described here may perform highly specialized and distinct roles during cognitive functions, including working memory. Our data show that PV-positive cells in monkey DLPFC form a homogeneous physiological group of FS interneurons, which share similar properties with PV-IR cells from rat cortex. PV-positive cells provide proximal inhibitory input to pyramidal neurons (Somogyi, 1977; DeFelipe et al., 1985; Lund and Lewis, 1993; Somogyi et al., 1998). In the rodent, these proximal inhibitory inputs elicit inhibitory postsynaptic potentials (IPSPs) or currents (IPSCs) with faster kinetics and usually

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**Table 3**

Properties of interneurons, according to statistical clustering

<table>
<thead>
<tr>
<th>Membrane properties</th>
<th>Cluster 1; n = 36</th>
<th>Cluster 2; n = 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input resistance (MΩ)</td>
<td>317 ± 46 (129)*</td>
<td>677 ± 104 (343)***</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>9.5 ± 1.1 (3.1)</td>
<td>17.6 ± 2.6 (8.7)***</td>
</tr>
<tr>
<td>Threshold of action potential (mV)</td>
<td>−43 ± 2 (6)</td>
<td>−43 ± 2 (6)</td>
</tr>
<tr>
<td>Amplitude of action potential (mV)</td>
<td>55 ± 5 (13)</td>
<td>67 ± 4 (13)***</td>
</tr>
<tr>
<td>Duration of action potential (ms)</td>
<td>0.39 ± 0.03 (0.10)</td>
<td>0.69 ± 0.04 (0.14)***</td>
</tr>
<tr>
<td>Amplitude of AHP (mV)</td>
<td>20.8 ± 1.6 (4.5)</td>
<td>17.4 ± 2.0 (6.5)**</td>
</tr>
<tr>
<td>Adaptation ratio</td>
<td>0.72 ± 0.07 (0.20)</td>
<td>0.60 ± 0.06 (0.18)**</td>
</tr>
<tr>
<td>CaBPs</td>
<td>negative for CBa, CR, CB, 15</td>
<td>negative for CaBPs</td>
</tr>
</tbody>
</table>

*aMeans ± confidence limits (SD).
*P < 0.05; **P < 0.01; ***P < 0.001.

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**Physiological Properties of Interneurons Containing Different CaBPs**

Monkey DLPFC interneurons exhibit a wide range of physiological properties (Table 2). Despite this heterogeneity, however, we found that cells with the same CaBP content shared similar physiological properties that differed from those containing another CaBP. Consistent with this observation, distinct morphological characteristics were described previously in rat frontal cortex as cells immunoreactive for CB and somatostatin (Kawaguchi and Kubota, 1997).

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**Figure 4.** Hierarchical tree plot illustrating the results of cluster analysis. There are two main branches corresponding to FS interneurons (FSI) and non-FS cells. The first cluster mainly contained PV-positive interneurons and the second one consisted exclusively of CB- and CR-positive cells.

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larger amplitudes at the soma, compared to distal inputs (Thomson et al., 1996; Tamás et al., 1997; Maccaferri et al., 2000; Beierlein et al., 2003). These properties provide both higher temporal fidelity and a more powerful inhibitory control over initiation of action potentials, which typically occurs in proximal compartments of the pyramidal cell membrane.

Functionally, both CB- and CR-immunopositive neurons shared firing characteristics of regular-spiking nonpyramidal cells. However, although these cells have similar physiological properties, they may differ in their connectivity with pre- and postsynaptic elements. For example, both CB- and CR-containing interneurons have vertically oriented dendritic and axonal arborizations and most likely serve inhibition within a cortical column; however, CB-positive cells often send numerous axonal branches to layer 1, suggesting that CB-containing interneurons preferentially target the tufts of pyramidal cells, as previously demonstrated (DeFelipe, 1997). This suggests that within a column CB-positive interneurons target the most distal compartments of pyramidal cell dendrites. Distal inhibition can strongly affect the ability of pyramidal cells to associate synaptic inputs arriving to upper and lower layers within a few milliseconds (Larkum et al., 1999). Thus, the CB cell subpopulation may be positioned to selectively modulate interactions between inputs arriving at different cortical layers.

In contrast, the axons of CR-positive neurons of layers 2–3 send few branches to layer 1. CR-positive cells are known to target the dendrites of other GABA neurons rather than pyramidal cells (Meskenaite, 1997; DeFelipe et al., 1999). Recently, it was also shown that CR-IR dendrites in the monkey DLPFC receive a lower density of excitatory synapses, compared to PV-IR dendrites (Melchitzky and Lewis, 2003). Taken together, this suggests that CR-IR neurons constitute an interneuronal system whose activity may mediate overall network disinhibition.

In monkey DLPFC the relative proportion of PV-IR interneurons among all GABA cells is only about half of that found in the rat frontal cortex (20–25% and 43–61%, respectively) (Kubota et al., 1994; Gabott and Bacon, 1996b). Thus, in monkey DLPFC, FS interneurons do not represent the dominant group of inhibitory cells as they do in rat cortex (Kawaguchi and Kubota, 1997; Gibson et al., 1999). GABA cells represent a greater percentage of all neurons in monkey cortex (24.9% in medial PFC (Gabott and Bacon, 1996b) than in rat medial frontal cortex (16.2%) (Gabott et al., 1997). This difference may reflect an increase in the absolute number of non-FS cells in monkey PFC.

The discrepancy in the proportions of FS and non-FS interneurons in rat and monkey PFC may reflect species differences in the organization of neocortical circuits. Moreover, this difference could be reflected in a relative shift in the postsynaptic location and type of inhibition. How network properties, and thus eventually cognitive operations, are differentially shaped by interneurons in the monkey DLPFC remains to be investigated.

Notes
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References