Interneuron Diversity in Layers 2-3 of Monkey Prefrontal Cortex

The heterogeneity of γ-aminobutyric acid interneurons in the rodent neocortex is well-established, but their classification into distinct subtypes remains a matter of debate. The classification of interneurons in the primate neocortex is further complicated by a less extensive database of the features of these neurons and by reported interspecies differences. Consequently, in this study we characterized 8 different morphological types of interneurons from monkey prefrontal cortex, 4 of which have not been previously classified. These interneuron types differed in their expression of molecular markers and clustered into 3 different electrophysiological classes. The first class consisted of fast-spiking parvalbumin-positive chandelier and linear arbor cells. The second class comprised 5 different morphological types of continuous-adapting calretinin- or calbindin-positive interneurons that had the lowest level of firing duration, which is not typical for rodent adapting cells. Neurogliaform cells (NGFCs), which coexpressed calbindin and neuropeptide Y, formed the third class, characterized by strong initial adaptation. They did not exhibit the delayed spikes seen in rodent NGFCs. These results indicate that primate interneurons have some specific properties; consequently, direct translation of classification schemes developed from studies in rodents to primates might be inappropriate.

Keywords: cerebral cortex, fast-spiking cells, GABA cells, inhibition, parvalbumin, patch clamp

Introduction

Cortical γ-aminobutyric acid (GABA) interneurons are heterogeneous, with subpopulations distinguished by particular combinations of morphological, physiological, and molecular attributes (Cauli et al. 1997; Kawaguchi and Kubota 1997; McBain and Fisahn 2001) and with variance in these attributes within subpopulations (Soltész 2006). In addition, abnormalities of specific types of cortical interneurons are thought to be critical components of the pathophysiological mechanisms underlying human brain disorders such as epilepsy and schizophrenia (DeFelipe 1999; Lewis et al. 2005). However, relatively few studies characterizing the properties of cortical GABA neurons have been performed in monkeys or humans (e.g., Krimer et al. 2005; Szabadi et al. 2006). Consequently, the proper translation of the extensive and growing database of rodent cortical interneurons to an understanding of these human disorders requires the ability to identify similar types of interneurons across species.

Although homologous types of interneurons have been reported in multiple species, differences have been observed even between phylogenetically close species. For instance, in rat cortex parvalbumin (PV), somatostatin (SST), and calretinin (CR) interneurons constitute primarily nonoverlapping subpopulations (Gonchar and Burkhalter 1997; Kawaguchi and Kubota 1997), whereas in the mouse cortex, a large subpopulation of interneurons coexpresses CR and SST (Xu et al. 2006). Furthermore, compared with rodent neocortex, in the primate neocortex 1) the percentage of cortical neurons that are GABAergic is larger (Gabbott and Bacon 1996; Gabbott et al. 1997), 2) interneurons characterized by a vertical bundling of axons are much more common (Yanez et al. 2005), 3) the developmental origin of at least some interneurons appears to differ (Letinic et al. 2002; Molyneaux et al. 2007), and 4) the relative proportions of chemically identified subtypes of interneurons are dissimilar (Conde et al. 1994; Kawaguchi and Kubota 1997). Moreover, interneurons with firing properties unusual for rodents have been found in monkey prefrontal cortex (Krimer et al. 2005; Povysheva et al. 2007). Thus, a robust and reliable classification of different interneuron types in the primate neocortex is critically needed.

Recently, we attempted to functionally categorize interneurons in monkey dorsolateral prefrontal cortex (DLPFC) by correlating their electrophysiological properties either with morphological types (Krimer et al. 2005) or with calcium-binding protein (CaBP) content (Zaitsev et al. 2005). By using cluster analysis, we demonstrated that monkey interneurons form distinct physiological groupings. However, the physiological-based clusters obtained in these studies appeared to contain heterogeneous morphological and molecular types, and thus a different approach for classification is needed.

In order to address this issue, in this study we used morphological criteria as a starting point for identifying subsets of interneurons in monkey DLPFC. Of the 8 morphological types of layer 2-3 interneurons identified, the electrophysiological and molecular properties of 4 types have not been previously described in primates. We found that monkey interneurons of the same morphological type exhibited similar electrophysiological and molecular attributes; at least some morphological types of monkey interneurons demonstrated different membrane properties from those for homologous morphological types described in rat. We did not observe interneurons, exhibiting late-spiking, stuttering, or bursting firing patterns, which are typical for some types of rodent interneurons, while we detected some firing patterns that are unusual for rats. These findings indicate that direct translation of classification schemes developed from studies in rodents to primates might be inappropriate.

Materials and Methods

Slice Preparation

Seventeen experimentally naive young adult (3.5–6 kg, 3.5–4 years old) male long-tailed macaque monkeys (Macaca fascicularis) were used in this study. Animals were 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 DLPIFC has been previously described in detail (Gonzalez-Burgos et al. 2004). Briefly, animals were treated with ketamine hydrochloride (25 mg/kg, intramuscular [im]), demethano-
sone phosphate (0.5 mg/kg, im), and atropine sulfate (0.05 mg/kg, subcutaneously); an endotrachial tube was inserted, and anesthesia was maintained with 1% halothane in a 28% O2-air mixture. Monkeys were placed in a stereotaxic apparatus, and a craniotomy was performed over the DLPIFC in one hemisphere. The dura was removed in a location determined by stereotactic coordinates and by the position of relevant sulcal landmarks, and a small block of tissue was excised containing both the medial and lateral banks of the principal sulcus (area 46) as well as a small adjacent portion of dorsal area 9. After the surgery, the animals were treated with an antibiotic (chloramphenicol, 15 mg/kg, im) and an analgesic (hydromorphone, 0.02 mg/kg, im) 3 times a day for 3 days. All animals recovered quickly with no impairments in eating or drinking and no overt behavioral deficits. In most cases, the animals underwent the same procedure 2–4 weeks later to obtain tissue from the opposite hemisphere. During the second procedure, after the craniotomy, the animal was given an overdose of pentobarbital (30 mg/kg) and was perfused through the heart with ice-cold modified artificial cerebrospinal fluid. A tissue block containing portions of areas 46 and 9 from a nonhomotopic portion of the contralateral hemisphere was quickly excised. Subsequent treatment of the tissue was the same for both procedures. The tissue blocks were placed in ice-cold Ringer solution, containing (in mM): NaCl 120, 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. Coronial 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. Twenty-one electrophysiological parameters were measured as follows:

1. RMP (in mV): resting membrane potential, the stable membrane potential (no holding current applied) reached a few minutes after breaking the membrane.
2. R in (MΩ): input resistance, the slope of the regression line fitted to the I-V curve (usually between -50 and -10 pA), as measured at the end of the 500-ms voltage responses.
3. τ (in ms): membrane time constant, determined from the monoexponential curve best fitting to the average voltage response to hyperpolarizing current steps of -10 to -50 pA.
4. Rb (in pA): rheobase, the intercept of the extrapolated F-I curve with the current axis.
5. Sag (dimensionless): sag being the difference between the most negative membrane potential during a 500-ms hyperpolarizing current step and the membrane potential at the end of the step. For analysis, we graded sag into 3 intensities: "0," if sag amplitude has been less than 20% of total voltage step at the end of 500 ms hyperpolarizing current step; "1," if more than 20%, but less than 50%; "2," if more than 50%.
6. Hump (dimensionless): hump being the difference between the most positive membrane potential during a 500-ms depolarizing current step and the membrane potential at the end of the step. We used the same grades as for Sag.
7. RD (dimensionless): rebound depolarization (or spike/s), measured as positive voltage deflection above RMP after the offset of hyperpolarizing current. For analysis, we distinguished 3 grades of RD intensity: "0," if RD amplitude has been less than 20% of total voltage step at the end of 500 ms hyperpolarizing current step; "1," if more than 20%, but did not evoke rebound spike; "2," if evoked rebound spikes.
8. Sum (dimensionless): the sum of grades for Sag, RD, and Hump.

Electrophysiological Analysis

Interneurons in layers 2-3 were visualized using infrared differential interference contrast videomicroscopy and distinguished from pyramidal cells based on their small round, or oval soma and the absence of an apical dendrite. Patch electrodes with open-tip resistances of 5–10 MΩ were filled with a solution containing (in mM): potassium glucose 114, KCl 6, ATP-Mg 4, GTP 0.3, Hepes 10 (i.e. [2-hydroxyethyl]-L-
pi-perazineethanesulfonic acid), 0.5% biocytin, and pH 7.25 adjusted with KOH. Whole-cell current clamp recordings were performed after reaching seal resistance of at least 4–5 GΩ. Voltages were amplified using Intracellular Electrometers IE-210 (Warner Instrument Corporation, Hamden, CT) or MultiClamp 700A (Axon Instruments, Union City, CA), operating in a bridge-balance mode; filtered on line at 4-5 kHz and acquired on a personal computer at a sampling rate of 20 kHz using Power 1401 interface and Signal 2 or Signal 3 software program (CED, Cambridge, UK). To characterize the intrinsic membrane properties of neurons, hyper- and depolarizing current steps of 500 ms duration were applied in 5–10 pA increments at 0.2 Hz with 2 repetitions.

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Action potential (AP) properties (minimal suprathreshold current were applied)

9. DAP (in ms): time to first spike from the beginning of stimulation.
10. APV (in mV): action potential threshold, the membrane potential at the point at which the interpolated rate of voltage rise (dV/dt) reached >10 mV/1 ms.
11. APA (in mV): action potential amplitude, measured from the threshold to the peak.
12. APD (in ms): action potential duration, the spike width at its half-amplitude.
13. AHP (in mV): amplitude of the fast component of the afterhyperpolarization (AHP), measured from the AP toward the voltage drop to <5 mV/1 ms.
14. AHP (in mV): amplitude of medium component of the AHP was measured from the end of the time interval between the fastest action potential and the afterhyperpolarization peak.
15. AHP (in ms): AHP latency, the time interval from onset of AHP and the afterhyperpolarization peak.

Firing pattern properties (2×Rb current was applied)

16. Fr (in Hz): steady-state frequency, the reciprocal of the average of the 4–9 interspike intervals (ISIs), measured within the last 250 ms of the response to depolarizing current pulses where firing frequency remained relatively stable.
17. Vmean (dimensionless): coefficient of variance of ISIs measured within the last 250 ms of the response to depolarizing current pulses.
18. Fr (in %): long-term frequency adaptation, percentage of decrease in the frequency from onset (reciprocal to the first ISI) to steady-state frequency.
19. Fr (in %): frequency adaptation, percentage of decrease in frequency, measured as the reciprocal of first and second ISIs.
20. APA (in %): percentage change in AP amplitude between the first and last APs.
21. APA (in %): percentage change in total AHP amplitude between the first and last APs.

Principal Component Factor Analysis of Electrophysiological Parameters

To compress the variability of these electrophysiological parameters into a smaller number of variables, principal component factor analysis was conducted using multiple R-square algorithms. In this method,
prior to factoring the diagonal of the correlation matrix (communalities) is computed as the multiple R-square of the respective variable with all other variables. According to the Kaiser criterion, we retained 3 factors with eigenvalues greater than 1. Then a ‘varimax’ rotation of the factor loadings was performed. This rotation is aimed at maximizing the variances of the squared raw factor loadings across variables for each factor; this is equivalent to maximizing the variances in the columns of the matrix of the squared raw factor loadings. Interpretation of factors was done according to their factor loadings.

Phase plots show the rate of change in the membrane potential dV/dt (velocity) against the instantaneous membrane potential V(t). Differentiation of the membrane potential was done with Signal 3 software; each data point was replaced with the difference between that point and the previous point and divided the result by the sample interval; the first data point was set to zero. Sample interval (Δt) was equal to 0.05 μs.

These plots provide additional information about ionic currents during AP (Bean 2007). The net ionic current is proportional to velocity (\(I_{\text{ionic}} = -GDV/dt\), where \(I_{\text{ionic}}\) is the net ionic current, G is the cell capacitance). Positive velocity means inward current and the negative velocity outward current. In phase plots, an AP is represented by a loop (Fig. 8). Start point of the loop represents the APT. From this point, the velocity rapidly increases during depolarizing phase of AP from about 0 V s\(^{-1}\) to 200–500 V s\(^{-1}\) and then decreases, crossing 0 V s\(^{-1}\) at the AP peak values. During repolarizing, phase velocity has negative values and gets 0 V s\(^{-1}\) at the peak of fast hyperpolarization. Shift in voltage between start point and end of the loop represents fast component of AHPA.

**Histological Processing and Morphological Analysis**

After recordings, slices were immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 24–72 h at 4 °C and then cryoprotected (35% glycerol, 5% ethylene glycol, in 0.1 M phosphate-buffered saline [PBS]) and stored at −80 °C. To visualize biocytin, slices were incubated with streptavidin-Alexa Fluor 633 conjugate (Invitrogen, Carlsbad, CA, dilution 1:500) for 24–48 h at 4 °C in PB, containing 0.4% Triton X-100. Interneurons were completely reconstructed in 3 dimensions using an Olympus Fluoview 500 confocal laser scanning microscope (Olympus America Inc., Center Valley, PA) equipped with a ×20/0.8 N.A. oil immersion objective. After the confocal reconstruction of the recorded interneurons, 106 with sufficiently extensive axonal arbors were processed for visualization of combinations PV/CR/calbindin D28k (CB)/neuropeptide Y (NPY)/somatostatin (SST) immunoreactivity using triple immunofluorescent labeling (Zaitsev et al. 2005). Slices were serially resected at 40–50 μm and then incubated for 2–3 days at 4 °C in blocking serum (10% normal goat serum, 2% bovine serum albumin in PB) containing a mixture of 2 antibodies raised in the different hosts (Table 1). After thorough rinsing, the sections were incubated for 24–48 h at 4 °C in PB, containing 1% H2O2 and coated glass slides. To visualize biocytin, slices were incubated with 1% DAB and 3,3′-diaminobenzidine (DAB) in PBS for 4 h at room temperature. Sections were rinsed, stained with 3,3′-diaminobenzidine (DAB), mounted on gelatin coated glass slides, dehydrated, and coverslipped.

In addition to this, new set of 106 confocally reconstructed interneurons, 88 physiologically and morphologically characterized interneurons from our previous studies (Krimmer et al. 2005; Zaitsev et al. 2005) were included; 50 of these cells were tested for CaBPs (Zaitsev et al. 2005), whereas the remaining 38 interneurons were not tested for any molecular markers. Biocytin was visualized with DAB chromogen as described above. Some (n = 16) of these 88 neurons were digitally reconstructed using the Neuro lucida tracing system (MicroBrightField, Williston, VT). Their morphological and physiological properties were reanalysed for the present study.

The horizontal and vertical extent of axons was measured as the mean distance between the 5 most distal axonal endings on each side from the soma of individual interneurons. Somal sizes were estimated from confocal images; cell bodies were approximated by an ellipse, and the area was calculated by an equation:

$$ A = \frac{1}{2} \pi D_a D_w $$

where \(D_a\) and \(D_w\) are the radial and tangential axes, respectively, of the somata.

**Statistical Analysis**

All statistical tests were performed using Statistica 6.1 software (Statsoft Inc., Tulsa, OK). Unless otherwise stated, all data are reported as means and standard deviations. The statistical significance between group means was tested using analysis of variance (ANOVA), followed by Fisher’s Least Significant Difference (LSD) post hoc tests (multiple comparison tests).

**Results**

**Morphological Diversity of the Recorded Interneurons and Their Classification**

Layer 2–3 interneurons (n = 194) that retained a sufficiently extensive axonal tree within the slice to warrant a detailed reconstruction were included in this study. To differentiate morphological groups of interneurons, we modified the original classification of interneurons in the monkey DLPFC based on Golgi impregnations (Lund and Lewis 1993). In the classification scheme presented here (Fig. 1), we used 2 readily observed morphological criteria: 1) the distribution of axonal arbors across layers, which reflects the predominant laminar targets of a neuron; and 2) a combination of axonal arborization patterns and terminal branch properties, which may reflect the targeting of different subdomains of cortical neurons.

Using the first criterion, we distinguished three groups of interneurons. The first group of cells projected their axons toward layer 1, forming relatively wide axonal plexi in that layer, and thus provided inhibitory inputs to the tufts of pyramidal cells. Cells with a similar axonal projection in rodents were recognized as Martinotti cells (MCS) (Kawaguchi and Kubota 1997; Wang et al. 2004; Ma et al. 2006; Silberberg and Markram 2007).

The second group of cells formed relatively narrow, vertically oriented projections to the deep layers (Fig. 2). Although all these interlaminar–intracolumnar cells connect upper layers with deep layers, they have different morphological appearances. The axon of one type formed basket-like structures and, most likely, specialized in innervating the

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perisomatic regions of other neurons. We classified these as "vertically oriented cells with baskets" (VOBCs). The remaining vertically oriented cells may target mostly distal parts of pyramidal cells dendrites as they resemble double bouquet cells (DBC), described in multiple species (Somogyi and Cowey 1981; Kawaguchi 1995).

The third group of interneurons appeared to be the most numerous and morphologically heterogeneous. Axons of cells

Figure 1. Flow chart for morphological identification of layer 2-3 DLPFC monkey interneurons. All interneurons were 3-dimensional computer reconstructed using the Neurolucida tracing system. Interneuron somata and dendrites are drawn in red and the axons in blue. Scale bar = 100 μm. Layers are represented with Arabic figures.
in this group were mostly distributed in layers 2–3, although a few axonal branches projected to deeper layers or to layer 1, but without clustering. According to their pattern of arborization and terminal branch properties, we distinguished 5 different morphological types within this group (Figs 3 and 4). Two of these morphological types, namely, neurogliaform cells (NGFCS) (Kawaguchi and Kubota 1997; Povysheva et al. 2007) and chandelier cells (ChCs) (DeFelipe 1999), are widely recognized in different species. The other 3 morphological types resemble different types of basket cells described in many species; however, only one type of these cells actually formed basket or “claw”-like structures around potential postsynaptic cells, whereas the other 2 types did not.

I. Interlaminar Cells Projecting to Layer 1

The MCs

All cells recognized as MCs (n = 14) had a multipolar somata that gave rise to 3–7 primary dendrites, each of which frequently branched forming an elaborate, generally vertically oriented, dendritic tree. The majority of MCs possessed spines

Figure 2. Morphological varieties of interneurons projecting to deep layers. (A) Confocal reconstruction of DBC. Confocal (B1) and Neurolucida (B2) reconstructions of the same VOBC. At the Neurolucida drawing soma and dendrites of VOBC are designated in red and the axons in blue. Potential postsynaptic cells for VOBC are shown in orange. Scale bars for reconstructed cells = 50 μm. (C) Axon terminals of VOBC formed appositions on unstained somata of potential postsynaptic cells, which were observed with differential interference contrast. Scale bar = 20 μm.
on their nonbeaded dendrites. MCs formed the widest axonal arbor (up to 800 \text{\mu m}) in layer 1 (Fig. 5, Supplementary Fig. 1) and less prominent axonal clusters around their somata. Axonal branches were coarse, bearing large numerous beads, especially in layer 1. Some of the MCs sent a few axonal collaterals down to deeper layers.

II. Interlaminar Cells Projecting to Deep Layers

**The VOBCs**

These neurons \((n = 13)\) were easily distinguished by their characteristic thick and smooth descending axonal trunks, which often increased in diameter during descent and could be followed to layer 6 or even the white matter. Each cell had 1–2 (rarely 3) main axonal trunks. Remarkably, the main trunks gave off short, curving, and robustly beaded collaterals, which formed claw-like or basket-like configurations around cells of different sizes and shapes. In layers 2–3, the neurons enclosed in the "baskets" usually had small round bodies and presumably were interneurons. In the deeper layers, both pyramidal and nonpyramidal somata were surrounded by the claws (Fig. 2).

VOBCs had oval or in a few cases multipolar somata. 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 2 tufts of dendrites extending from the pia to layer 4 in a narrow column.

The morphological and physiological properties of these cells have not been described before in prefrontal cortex, although they resembled previously reported interneurons from monkey striate cortex ^4A-Base 3B Variety 8 local circuit.
neurons" (Lund and Yoshioka 1991) and "the first subtype" of CR-containing cells (Meskenaite 1997). VOBCs probably correspond to descending basket cells described in rats (Karube et al. 2004).

The DBCs
This group of cells (n = 21) included interneurons with fine descending (and ascending in some cells) axonal collaterals. Usually these interneurons did not form prominent main trunks. Slightly beaded axon collaterals of these cells tended to travel in isolation, branching rarely and did not form claw-like configurations around neurons. However, some cells included in this group had a web of fine recurrent collaterals of variable density.

III. Intralaminar Cells
These interneurons had very heterogeneous branching within layers 2–3 and seemed to specialize in targeting different domains of other neurons. Due to their distinct specialization, terminal branches in some types of these cells had a very specific morphological appearance and higher density of boutons. In the other cells, terminal parts of axons were indistinguishable from more proximal parts.

III. 1. Intralaminar Cells with Specialized Terminal Branches

The ChCs (n = 13). These relatively small cells (axonal width 280 ± 80 µm) were recognized based on their characteristic axonal terminals, which formed short vertical arrangements of boutons, termed axon cartridges (Fig. 3, Supplementary Fig. 1), known to target the axon initial segment of pyramidal cells (DeFelipe 1999). The axonal arbor of ChCs was formed by extensive branching at shallow angles. Somata of these cells had a multipolar or bipolar shape. Few primary dendrites arose from the cell body and rarely branched into vertically oriented thick processes, which were slightly beaded.

Local arbor pericellular basket cells (n = 14). These tiny cells (Fig. 3, Supplementary Fig. 1) were recognized by their dense, "curvy," complex and very compact axonal arbor on both radial (220 ± 70 µm) and tangential dimensions (180 ± 40 µm). However, as an exception, some of these cells had a few relatively long descending axonal branches with numerous boutons. The pattern of arborization resembled that of ChCs, but prominently beaded terminal portions of axons did not form vertical arrays (Lund and Lewis 1993). Instead, they often formed claw-like pericellular structures, similar to VOBCs. The cells enclosed in these structures had small round somata and presumably were nonpyramidal neurons.

Somata of local arbor pericellular basket cells (LPBCs) had round or slightly elongated shape. Dendrites were aspiny and slightly beaded, some spread farther than the axonal arbor. Similar cells were described in cat visual cortex as clutch cells (Kisvarday et al. 1985) and as small basket cells in rodent neocortex (Kawaguchi and Kubota 1996; Karube et al. 2004; Markram et al. 2004).

III. 2. Intralaminar Cells without Specialized Terminal Branches
These cells were characterized by a relatively random distribution of boutons along the axon length without a visible increase in bouton density at the terminal parts of axons. Two main patterns of axonal arborization were frequently observed (Fig. 4). The first pattern had a curvy arborization with bended axonal segments between branching points that formed an axonal mesh of variable density. The second pattern, consisted of "straight" arborization, characterized by relatively long and straight segments that bifurcated at right or oblique angles and, in general, headed away from the soma.

The NGFCs. NGFCs (n = 19) were readily identified based on their distinctive morphological features (Kawaguchi and Kubota 1997; Tamas et al. 2003; Povysheva et al. 2007). NGFCs had a small and almost perfectly round somata that gave rise to numerous radially distributed dendrites that formed a highly symmetrical, spherical structure (Supplementary Fig. 1). The shafts of the dendrites were fine, aspiny, and slightly beaded. NGFCs exhibited curvy axonal arborization, their axons arose either from the soma or from a primary dendrite, and almost immediately started to branch forming a dense, intertwined axonal mesh. The axonal collaterals were very thin and sparsely studded with fine beads. The zone occupied by the axons had a volume several times larger than that of the dendrites, but was still mainly confined within layers 2–3.

Curved arbor cells. This group of cells (n = 41) was very heterogeneous because they consisted of the remaining, yet unclassified, cells with curving axon trajectory (Supplementary Fig. 1). The density of arborization within this cell group varied: smaller cells usually had denser and more curving arborization, whereas cells with larger axon spread had somewhat looser and more irregular axon mesh. There seemed to be a continuum between these cell subtypes. Axons of these cells could extend into layer 1, but in contrast with MCs, did not form a significant arbor in this layer. Some cells also had a few axonal
processes descending to deeper layers. The dendrites spread predominantly in the vertical dimension, but some of them traveled horizontally, thus forming a dendritic tree with no particular pattern. The dendrites usually were sparsely spiny, but in some cells they were smooth. These cells resembled neurons with local beaded axons and simple beaded axons, described previously in monkey DL-PFC (Lund and Lewis 1993) or nest basket cells described in rat neocortex (Wang et al. 2002).

**Linear arbor cells.** These cells represent a large population in our sample (n = 59). Linear arbor cells (LACs) were recognized by their linear course of axonal branches (Supplementary Fig. 1). The primary axon gave rise to a few long and stout main lateral branches. The latter gave rise to linear collaterals at right or oblique angles. The collaterals spread across several layers, but almost never reached layer 1. This cell type included wide, medium, and local arbor morphological varieties, according to their horizontal axonal span (Lund and Lewis 1993; Krimer et al. 2005).

The somato-dendritic component was often multipolar, but could be bitufted. The bulk of dendrites ascended parallel to each other in layers 1–3, forming a narrow columnar structure. The descending dendrites crossed layers 3–5 and could spread more horizontally than vertically. Dendrites were usually slightly beaded and aspiny. These cells morphologically resembled “large basket cells” described in other cortical regions and species (Jones and Hendry 1984; Markram et al. 2004); however, in our sample, the axons of these cells did not form well-defined pericellular baskets.

**Quantitative Morphological Characteristics of Interneuron Subtypes**

To assess the validity of the morphological classification of interneurons, we investigated whether the morphological groups identified above differed in certain quantitative morphological parameters. We measured somal size, number of primary dendrites, and horizontal and vertical axonal spreads for 95 neurons (Figs 5 and 6). According to a one-way ANOVA and post hoc tests, all 4 parameters differed among the 8 morphological groups in a statistically significant manner (P < 0.01), supporting our classification scheme. By somal size, interneurons were divided into 3 partially overlapping subgroups: 1) small—ChCs (74 ± 8 μm²) and LPBCs (81 ± 10 μm²); 2) medium—MCs (93 ± 14 μm²), LACs (104 ± 24 μm²), NGFCs (105 ± 30 μm²), DBCs (106 ± 26 μm²), and 3) large—VOBCs (112 ± 21 μm²) and curved arbor cells (CACs) (117 ± 27 μm²) (Fig. 5A). The shape and orientation of somata varied greatly across different types of interneurons. In the majority of interneurons, the main axis of somata was perpendicular (radial) to the pial surface and, respectively, the ratio between radial and tangential axes of the somata (Drad/Dirad ratio) was > 1 (Fig. 6A). This ratio varied from 1.05 ± 0.16 in NGFCs that have almost round somata to 1.34 ± 0.24 in VOBCs with fusiform somata.

The number of primary dendrites ranged from 2 to 16 and NGFCs had the largest number of primary dendrites (10.9 ± 2.8), consistent with this characteristic feature of the NGFC type (Figs 5B and 6B). Both types of vertically oriented cells had the smallest number of primary dendrites (2.8–3.8), whereas the average number of dendrites varied from 4.2 to 6.0 for all other morphological subtypes.

**Figure 6.** Quantitative morphological parameters of different types of interneurons. (A) The ratio between radial and tangential extends of the somata. (B) Number of primary dendrites. (C) Vertical and horizontal axonal spans of different types of interneurons (n = 85 interneurons). Circles represent mean values and bars are standard deviations. Note that LPBCs, ChCs, DBCs, and VOBCs usually do not exceed one cortical column, whereas NGFCs, MCs, CACs, and LACs innervate adjacent columns as well.

Monkey interneurons had different horizontal (tangential) and vertical (radial) spreads of axons (Fig. 6C). The smallest vertical axonal extent (201 ± 69 μm) was detected for LPBCs; in these tiny cells, the axon arbors were mostly restricted to the layer of origin. Vertical spread of other intralaminar interneurons (ChCs, NGFCs, CACs, and LACs) was larger and varied from 450 to 550 μm. Ascending interlaminar MCs had only slightly larger vertical span (603 ± 230 μm) than intralaminar cells, whereas vertical axonal length was 2–3 times larger in descending VOBCs (1543 ± 835 μm) and DBCs (1125 ± 181 μm). By horizontal axonal spread, interneuronal morphological types formed 2 groups. The first group consisted of cells with narrow axonal spread (190–277 μm) and included LPBCs (191 ± 56 μm), VOBCs (209 ± 67 μm), DBCs (261 ± 77 μm), and ChCs (277 ± 75 μm). Thus, these cells may be considered intracolumnar cells. The other 4 morphological types had
a broader axonal spread (NGFCs [439 ± 128 μm], CACs [545 ± 257 μm], MCs [581 ± 89 μm], LACs [661 ± 340 μm]) sufficient to innervate adjacent columns.

Next, we investigated whether the combination of these 4 types of measures (soma size, number of primary dendrites, vertical and horizontal axonal spreads) would statistically distinguish interneuron morphological types. To answer this question, we applied a multivariate design of ANOVA and found that all 4 commonly used multivariate tests Wilks’ Lambda (=0.059), Pillai’s trace (=1.76), Hotelling-Lawley trace (=5.42), and Roy’s largest root (=3.64) indicate a significant difference (P<0.001) among morphological groups. These results strongly support the suggested morphological classification.

**Molecular Markers of Different Morphological Interneuron Subtypes**

CaBPs and several neuropeptides tend to be expressed in different subpopulations of interneurons (Conde et al. 1994; CauI et al. 1997; Kawaguchi and Kondo 2002). Although molecular markers do not map perfectly to different interneuron subtypes, their expression is an important general correlate of anatomical and electrophysiological attributes (Markram et al. 2004; Zaitsev et al. 2005). Therefore, to compare our suggested morphological classification with the previous schemes based on molecular markers, we tested a large set of interneurons for expression of three CaBPs (PV, CR, and CB) and 2 neuropeptides (SST and NPY). Each interneuron was usually tested for 2 markers (Fig. 7, Supplementary Fig. 2).

Our results established a correlation between the morphological types and the biochemical markers (Table 2), which is consistent with previous reports (Conde et al. 1994; CauI et al. 1997; Kawaguchi and Kondo 2002). It is worth mentioning that LACs and CACs were perfectly segregated by this criterion: more than half of tested LACs expressed PV, which is considered to be a marker for soma-targeting fast-spiking (FS) basket cells (Kawaguchi and Kondo 2002; Freund and Katona 2007), whereas none of the CACs expressed PV. Instead, CACs contained CB, with the exception of a small population that expressed either CR or SST. Both varieties of vertically oriented cells usually expressed CR. LPBCs contained CB.

As in our previous study (Zaitsev et al. 2005), we did not observe more than one CaBP in any of the tested interneurons. However, colocalization of a CB with a neuropeptide was frequently detected in MCs (CB with SST) and in NGFCs (CB with NPY).

**Electrophysiological Diversity of Monkey DLPFC Interneurons**

Our previous study was primarily based on electrophysiological membrane properties of interneurons and has demonstrated...
that they are not a continuum, but consist of 3 unique physiological clusters (class) of neurons (Krimer et al. 2005). However, when morphology was included in the classification, the 3 basic electrophysiological clusters each included several morphological cell types. Therefore, the physiological types of interneurons obtained via cluster analysis combined functionally heterogeneous populations of interneurons. Here, we used morphological criteria as a starting point for the classification of interneurons. Eight morphological types of layer 2–3 interneurons have been identified, including 4 types that were not previously identified and thus had not been physiologically characterized. We believe that this approach allowed us to distinguish functionally more uniform groups of interneurons in the circuitry. An important question was if these different morphological cell types were distinct in their electrophysiological properties or not. To address this issue in detail, we measured a total of 21 different electrophysiological parameters from each neuron, including subthreshold and suprathreshold voltage responses to current injection (see Materials and Methods). All the cells included in the analysis had stable resting potentials that were more negative than −60 mV and overshooting APs.

We found that all parameters except for RMP and DAP differed between groups in a statistically significant manner, according to a one-way ANOVA (Table 3), indicating that each morphological type had a specific combination of intrinsic electrical properties. Thus, we performed additional analyses in which the RMP and DAP were excluded. To determine how the electrophysiological properties differed between these eight morphological types, we used a post hoc Fisher’s LSD test (Table 4). This paired comparison analysis revealed that there were no morphological groups with an absolutely similar set of intrinsic properties, although the number of significantly different parameters between different groups varied. Some morphological varieties displayed many similarities in electrophysiological properties. For instance, CACs and MCs differed only in one out of 19 parameters, whereas CACs and LACs differed in 18 parameters. Below we briefly describe the most different electrophysiological properties between the morphological groups.

### Subthreshold Membrane Properties of Different Morphological Types of Interneurons

We found several significant differences in the responses of cells of different morphological types to hyperpolarizing and subthreshold depolarizing current steps. For example, Fisher’s LSD test revealed that according to \( R_n \) interneuron types could be subdivided into 3 different groups: cells with low \( R_n \) (LACs and ChCs), with high \( R_n \) (DBC), and with intermediate \( R_n \) (the remaining 5 morphological types). Shorter \( \tau \) were found in LACs, ChCs, and NGFCs, than in other morphological types (Table 3).

Some interneurons exhibited a time-dependent rectification in response to hyperpolarizing current steps (Fig. 8). This rectification was characterized by a delayed depolarizing drift or “sag” that shifted the membrane potential toward the RMP and could be observed when the cells were hyperpolarized more than 10–20 mV relative to RMP. Such sag most likely was produced by the \( I_h \) current (Pape 1996; Robinson 2003). In the majority of interneurons that exhibited profound depolarizing sag, subsequent return to RMP caused a transient RD, which sometimes triggered a few spikes. Although the ionic mechanisms of such RD usually are associated with a low-threshold calcium current, \( I_h \) current can contribute to the RD as well.

### Table 2

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<tr>
<th>Cell type</th>
<th>PV</th>
<th>CR</th>
<th>CB</th>
<th>SS</th>
<th>NPY</th>
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<tr>
<td>MCs</td>
<td>0/1</td>
<td>n/a</td>
<td>3/4</td>
<td>4/5</td>
<td>n/a</td>
</tr>
<tr>
<td>ChCs</td>
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<td>0/3</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<td>2/3</td>
<td>1/6</td>
<td>0/4</td>
<td>n/a</td>
</tr>
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<td>0/2</td>
<td>8/11</td>
<td>0/4</td>
<td>4/10</td>
</tr>
<tr>
<td>VDBC</td>
<td>0/6</td>
<td>5/8</td>
<td>0/2</td>
<td>0/1</td>
<td>n/a</td>
</tr>
<tr>
<td>DBC</td>
<td>0/12</td>
<td>10/12</td>
<td>1/6</td>
<td>0/5</td>
<td>n/a</td>
</tr>
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<td>LAC</td>
<td>14/24</td>
<td>4/9</td>
<td>1/8</td>
<td>0/8</td>
<td>0/2</td>
</tr>
<tr>
<td>CAC</td>
<td>0/13</td>
<td>1/15</td>
<td>4/12</td>
<td>1/13</td>
<td>0/2</td>
</tr>
</tbody>
</table>

Note: n/a, not applicable.

### Table 3

<table>
<thead>
<tr>
<th>LAC, ( n = 59 )</th>
<th>ChC, ( n = 13 )</th>
<th>CAC, ( n = 41 )</th>
<th>DBC, ( n = 21 )</th>
<th>MC, ( n = 14 )</th>
<th>VDBC, ( n = 13 )</th>
<th>LPBC, ( n = 14 )</th>
<th>NGFC, ( n = 19 )</th>
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</thead>
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<tr>
<td>RMP, mV</td>
<td>–70 ± 7</td>
<td>–65 ± 8</td>
<td>–67 ± 9</td>
<td>–70 ± 7</td>
<td>–68 ± 9</td>
<td>–71 ± 9</td>
<td>–68 ± 8</td>
</tr>
<tr>
<td>( R_n ), Ω</td>
<td>215 ± 91</td>
<td>330 ± 144</td>
<td>437 ± 178</td>
<td>665 ± 430</td>
<td>430 ± 207</td>
<td>483 ± 189</td>
<td>458 ± 220</td>
</tr>
<tr>
<td>( \tau ), ms</td>
<td>10.9 ± 3.9</td>
<td>10.1 ± 23</td>
<td>18.4 ± 5.3</td>
<td>16.5 ± 6.8</td>
<td>15.8 ± 4.9</td>
<td>15.7 ± 5.1</td>
<td>15 ± 4.5</td>
</tr>
<tr>
<td>RD, pA</td>
<td>59 ± 34</td>
<td>40 ± 27</td>
<td>23 ± 15</td>
<td>23 ± 19</td>
<td>24 ± 9</td>
<td>25 ± 18</td>
<td>25 ± 18</td>
</tr>
<tr>
<td>Sum</td>
<td>1.1 ± 1.1</td>
<td>1.3 ± 1.5</td>
<td>2 ± 2</td>
<td>2.4 ± 1.8</td>
<td>3.1 ± 1.7</td>
<td>2.3 ± 1.8</td>
<td>3.8 ± 1.9</td>
</tr>
<tr>
<td>DAP, ms</td>
<td>38 ± 113</td>
<td>145 ± 176</td>
<td>100 ± 98</td>
<td>68 ± 77</td>
<td>53 ± 27</td>
<td>72 ± 57</td>
<td>64 ± 68</td>
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<td>AM, pA</td>
<td>0.5 ± 0.6</td>
<td>0.5 ± 0.6</td>
<td>0.5 ± 0.7</td>
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<td>AHP, ms</td>
<td>60 ± 12</td>
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<td>59 ± 12</td>
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<td>68 ± 17</td>
<td>65 ± 9</td>
<td>55 ± 10</td>
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<td>APA, mV</td>
<td>0.3 ± 0.11</td>
<td>0.32 ± 0.06</td>
<td>0.68 ± 0.12</td>
<td>0.74 ± 0.19</td>
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<td>0.53 ± 0.12</td>
<td>0.52 ± 0.09</td>
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<td>AHPA, mV</td>
<td>21.8 ± 4.4</td>
<td>17.9 ± 4.2</td>
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<td>13.1 ± 5.2</td>
<td>14 ± 4.4</td>
<td>18.1 ± 3.4</td>
<td>13.7 ± 5.9</td>
</tr>
<tr>
<td>AHPAn, mV</td>
<td>1.3 ± 1.6</td>
<td>1.1 ± 1.8</td>
<td>2.6 ± 2.8</td>
<td>2.2 ± 1.7</td>
<td>1.9 ± 1.4</td>
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<tr>
<td>( I_h ), ms</td>
<td>2.9 ± 2.8</td>
<td>2.9 ± 3.5</td>
<td>7.8 ± 8.4</td>
<td>6 ± 4.4</td>
<td>9.8 ± 9.7</td>
<td>4.7 ± 5</td>
<td>6.8 ± 5.9</td>
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<tr>
<td>Fr, Hz</td>
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<td>86 ± 29</td>
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<td>43 ± 13</td>
<td>34 ± 11</td>
<td>44 ± 11</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>Rd, pA</td>
<td>1.9 ± 9.9</td>
<td>56 ± 14</td>
<td>115 ± 12.8</td>
<td>7.2 ± 11.3</td>
<td>4.8 ± 21.2</td>
<td>–0.9 ± 24.1</td>
<td>–3.5 ± 18.2</td>
</tr>
<tr>
<td>FrA1, %</td>
<td>17 ± 20</td>
<td>11 ± 20</td>
<td>34 ± 19</td>
<td>25 ± 22</td>
<td>36 ± 19</td>
<td>18 ± 28</td>
<td>25 ± 19</td>
</tr>
<tr>
<td>FrA2, %</td>
<td>16 ± 15</td>
<td>5.7 ± 15</td>
<td>25 ± 24</td>
<td>21 ± 20</td>
<td>35 ± 18</td>
<td>19 ± 22</td>
<td>26 ± 20</td>
</tr>
<tr>
<td>( \Delta A / \Delta P ), %</td>
<td>–0.5 ± 3.8</td>
<td>–2.5 ± 6.2</td>
<td>–7.3 ± 8.8</td>
<td>–9.8 ± 5.1</td>
<td>–11.4 ± 8.9</td>
<td>–9.1 ± 5.5</td>
<td>–11 ± 7.4</td>
</tr>
<tr>
<td>( \Delta A ) max, %</td>
<td>3.9 ± 13.2</td>
<td>5.6 ± 22.9</td>
<td>30.4 ± 42.5</td>
<td>27.6 ± 27.1</td>
<td>21.1 ± 40.3</td>
<td>8.7 ± 21.9</td>
<td>14.3 ± 22.2</td>
</tr>
</tbody>
</table>
In agreement with this, we found a significant correlation (0.76) between sag and RD amplitudes.

In response to subthreshold depolarizing current steps, a few interneurons \((n = 8)\), which were morphologically defined as ChCs \((n = 4)\), CACs \((n = 2)\), MC \((n = 1)\), and DBC \((n = 1)\), generated a depolarizing ramp (Fig. 8A). However, most ChCs \((9\) out of \(13)\) did not exhibit any ramp depolarization. More often, monkey interneurons showed hump-like upward voltage deflection at the beginning of the responses to the current injections (Fig. 8). The magnitude of the hump was also correlated with the amplitude of sag \((0.51)\) and of the RD \((0.53)\). Because of these correlations, additional comparisons of different morphological cell types included a compound parameter that we termed "Sum," which represented the sum of Sag, RD, and Hump amplitudes.

Responses to subthreshold depolarizing and hyperpolarizing current steps appeared to be cell-type specific. For example, majority of NGFCs (Fig. 8B) did not exhibit any or only a little sag or a hump; NGFCs did not exhibit RD as well. In contrast, practically all MCs, LPBCs, DBCs, VOBCs, and many of CACs exhibited more or less significant sag, RD, and hump. Moreover, in some of the MCs and LPBCs, rebound spikes were observed. LACs typically did not exhibit profound time-dependent rectification or RD, whereas they might display hump-like depolarization at the current level close to Rb.

**Single Spike Properties**

Among the parameters describing AP properties, spike duration was the most cell-type specific. Three different groups of cells were distinguished by this parameter. The shortest spike duration was observed in ChCs \((0.32 \pm 0.06\) ms) and LACs \((0.38 \pm 0.11\) ms); these values were typical for monkey FS cells (Krimer et al. 2005). Relatively brief spike duration was also registered for VOBCs \((0.53 \pm 0.12\) ms) and LPBCs \((0.52 \pm 0.09\) ms). The duration of spikes in the 4 other morphological types was longer; the average values varied from 0.62 to 0.74 ms.

Spike duration depends on many factors, among which is expression of different types of potassium channels. For example, narrow spike is associated with expression of Kv3-family channels (Rudy et al. 1999; Lau et al. 2000). Expression of different channels with distinct properties would also affect the shape of AP. Fast kinetics of AP precludes direct comparison and the analysis of AP shapes; however, there is a simple and informative way of examining the properties of APs by using phase plots (for details, see Materials and Methods). The phase plot for a membrane AP gives a direct read out of net ionic current as a function of voltage during the various phase of the AP (Bean 2007). In phase plots, AP is represented by a loop (Fig. 9). We observed 3 main types of the loop shapes, which were strongly associated with spike duration.

Spike durations among the cell types are shown in Table 1.

---

**Table 4**

Results of Fisher’s LSD test

<table>
<thead>
<tr>
<th></th>
<th>LAC</th>
<th>ChC</th>
<th>CAC</th>
<th>MC</th>
<th>DBC</th>
<th>VOBC</th>
<th>LPBC</th>
<th>NGFC</th>
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<tr>
<td>LAC</td>
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<td>5</td>
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<td>LPBC</td>
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<td>2</td>
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<tr>
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<td>13</td>
<td>9</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

Note: Number of physiological parameters (out of 19), which were statistically different between morphological groups.

---

Figure 8. Representative examples of subthreshold responses in monkey interneurons. (A) Example of ramp depolarization observed in ChC. (B) Subthreshold responses of NGFC. (C and D) Subthreshold responses in CAC (C) demonstrated smaller amplitude of hump (arrow), sag (arrowhead), and RD (asterisk) than in LPBC (D). APs were truncated at (A and D). (E) Bar graph demonstrating averaged components of Sum values for different morphological types of interneurons; ANOVA revealed significant differences between morphological types by all 4 parameters \((P < 0.01)\).
membrane voltage, and thus, the outward current was relatively constant during repolarizing phase. The latter might indicate an involvement of several types of potassium channels with different kinetics.

The third type of loops resembled in appearance a vertically oriented egg and was typical for interneurons with longer spike duration (CACs, MCs, DBCs, and NGFCs). This was the most symmetrical loop because the maximum of net inward and outward current was reached approximately at half-height of AP, that is at negative membrane potential, and the voltage shift between starting and ending points of the loop was relatively small. Therefore, the repolarization was achieved via potassium channels that were activated at membrane potentials more negative than required for activation of Kv3 channels.

Spikes of LACs and ChCs were followed by large monophasic AHPs of the shortest latency (about 3 ms) (Fig. 10). These properties of AHP are typical for FS cells (Kawaguchi 1993) and may indicate a contribution of Kv3 channels, which are very fast deactivating channels (Rudy et al. 1999). VOBCs had AHP with a shape similar to LACs and ChCs, but with longer latency of the former. LPBCs, CACs, MCs, and DBCs exhibited two-component AHP, which indicates involvement of several types of potassium channels with different kinetics. Total amplitude of AHP in these cells was about 15–17 mV. Amplitudes of an early, fast- and of delayed, and medium-duration components of AHP had a relative ratio of ~6:1. Finally, distinct AHP pattern was observed in NGFCs. In these cells AHP also could be divided into 2 components, but the medium component had almost the same amplitude as the fast component and represented about 40% of the total AHP amplitude. This was in striking contrast with the other morphological types of interneurons. AHP in NGFCs also had the longest latency (13.5 ± 4.7 ms).

### Firing Pattern Properties

To characterize the structure of firing pattern, we analysed the percentages of initial and late spike frequency adaptation. According to these parameters, we could distinguish 3 main types of temporal structure of firing (Fig. 11). Cells with nonadapting firing pattern exhibited very little, if any, adaptation (<25%). This pattern was typical for LACs and ChCs.

The firing frequency in CACs, MCs, LPBCs, VOBCs, and DBCs decreased gradually along the trains. The level of spike frequency adaptation did not change significantly within the range of stimulation currents used. This pattern was consistent with that of previously described as classic accommodating (Markram et al. 2004)/adapting (Gonzalez-Burgos et al. 2004) or regular spiking nonpyramidal cells (Kawaguchi and Kubota 1997). In some LPBCs and VOBCs, we observed a variation of this pattern (Krimer et al. 2005). In these cells, the first spike in the train had an enlarged AHP and the first ISI was much longer than the second one. The latter was followed by a typical adapting pattern.

Finally, NGFCs exhibited a unique firing pattern, with a temporal structure strongly dependent on stimulation current intensity. With near threshold depolarizing current steps, NGFCs displayed nonadapting properties of firing. However, an increase in stimulation current intensity strongly enhanced spike frequency adaptation. The changes in the level of adaptation were accompanied by a significant reduction in amplitude of the medium component AHP after the first spikes in trains (Fig. 12). In contrast to gradual firing adaptation in the...
AHP in NGF cells was almost 5 times longer than in LACs or ChCs. With larger amplitude of medium component AHP exhibited longer AHP. Latency of steady-state phase. In majority of cells (166 out of 189) and the VO cells had the largest firing variance (13.8 ± 20.5%). Note that impact of medium component to the total AHPA was relatively small in amplitude of fast and medium components of AHP in different types of interneurons. Other cell types demonstrated intermediate firing frequency in a range of 30–44 Hz. Other cell types demonstrated intermediate firing frequency in a range of 30–44 Hz.

To mathematically describe the irregularity of firing, we calculated coefficient of variance of ISIs ($k_{ISIs}$) during the steady-state phase. In majority of cells (166 out of 189) $k_{ISIs}$ was less than 10%, indicating a high level of regularity in the firing pattern. Only 4 cells in our sample had $k_{ISIs}$ >20%, which would classify them as irregular spiking cells. Morphologically, these cells included 2 DBCs, 1 MC, and 1 CAC. LACs and ChCs exhibited the lowest $k_{ISIs}$ (4.0±2.2 and 4.6±2.1%, respectively), and the VO cells had the largest firing variance (13.8 ± 20.5%). This is in agreement with data from rat neocortex (Cauli et al. 1997).

Not only the AP frequency changed during trains but also the shape of the spikes also underwent substantial changes. Therefore, we measured the percent of change in APA and AHPA from the first to the last spike in the train (for details, see Materials and Methods). We did not detect any changes in APA for LACs, ChCs, and NGFCS, whereas in the other morphological groups decline of amplitude was about 6–12%. Total AHP almost did not change in ChC or LAC, whereas it increased on 10–30% in adapting cells and even more (65%) in initial-adapting NGFCs.

To detect if other properties of spike shape were changing during the sweeps, we plotted the spike trains on the phase diagrams (Fig. 13). AP trains in ChC and LACs formed a set of identical superimposed loops, and the first AP in the train was indistinguishable from the rest. This might indicate that voltage gated ionic channels, involved in generation of APs, were completely recovering from spike to spike and that no additional types of channels were added to spike generation during the trains.

APs within the trains from NGFCs were also identically shaped, but in contrast to LACs and ChCs, the first spike was always slightly shifted to the left on the phase diagram. In the 5 other cell types, we usually observed a trend for a decrease in the loops’ area during trains, which translated to decrease in maximal amplitude of the underlying inward and outward currents. Such decrease leads to an increase in APD during trains (Fig. 13), which may significantly affect properties of synaptic transmission during trains of APs (Geiger and Jonas 2000).

**The Morphological Types of Interneurons in the Monkey DLPFC Are Assembled into 3 Groups That Share Physiologically Relevant Electrical Properties**

We identified 8 morphological types of monkey DLPFC interneurons. However, whereas electrophysiological differences were robust between some morphological types, the similarities between other morphological types in intrinsic membrane properties outweighed the differences. Electrophysiological diversity of interneurons results from the combined activity of different ion channels and from the morphology of the neurons. Nowadays, many types of ion channels underlie electrophysiological properties of interneurons, recent studies indicate that the channels are frequently coexpressed together in only a few specific combinations (Toledo-Rodriguez et al. 2004, 2005). Thus, these specific combinations of channels can give rise to a finite number of distinct electrical classes of interneurons with particular combinations of membrane properties.

Statistically, specific combinations of variables can be revealed using factor analysis. For this purpose, we performed principal factor analysis of the electrophysiological data (see for details, Materials and Methods). A total of 162 interneurons were included in this analysis. In factor model, we retained 3 factors with eigenvalues greater than 1. These 3 factors accounted for 45% of the total variance. Interpretation of the factors was done according to their factor loadings (Table 5).

The first factor was marked by high loadings on the parameters that associated with firing frequency. Cells with negative scores for this factor exhibited high frequency firing pattern with narrow spikes, followed by a deep monophasic
AHP. Compared with other cells, these neurons had smaller $R_{\text{in}}$ and time constant. In contrast, cells with a positive score for the first factor exhibited low-frequency firing and broader spikes followed by prolonged AHP with profound medium component. The second factor was marked by parameters describing mostly cells’ subthreshold properties. Interneurons with positive scores for this factor were more excitable, as they demonstrated low level of firing threshold and $R_b$, high $R_{\text{in}}$, profound sag, RD, and hump. These properties also correlated with larger AP amplitude, which had a tendency to decrease during the trains. The third factor was marked by parameters describing firing frequency adaptation. Cells with positive scores exhibited strong firing frequency adaptation and small amplitude of fast component AHP, whereas their total amplitude of AHP significantly increased during the trains.

Thus, 3 associations of electrophysiological parameters in monkey interneurons were discovered, and they were represented by 3 independent factors, which we named based on their key physiological properties as: factor of firing frequency, factor of interneuron excitability, and factor of firing frequency adaptation. According to a one-way ANOVA, all 3 factors were significantly different between distinct morphological types ($F_{7,154}$ were 15.2, 34.0, 8.0 for firing frequency, interneuron excitability, and firing frequency adaptation, respectively, and for each of them $P < 0.01$). However, a post hoc Fisher’s LSD test revealed that some morphological types could not be distinguished by their factor scores ($P < 0.01$). We combined such morphological types together and found 3 clusters of

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**Figure 11.** Interneurons of different morphological types exhibited specific firing patterns. Firing patterns from ChC, VOBC, CAC, and NGFC represented the firing patterns that were typically observed in monkey interneurons. (A) Overlapped first (gray) and last (black) APs from the train. Note that amplitude of spikes and their duration did not change in ChC and NGFC during train, whereas in VOBC and CAC they changed. (B) Sweeps with the responses to the first suprathreshold current step and to the 2× threshold current step. Compare the shape of AHP after first spike and after other spikes in the train in different morphological types: first AHP was enlarged in VOBC, whereas in NGFC it was reduced. (C) Plots of instantaneous frequency against ISI number, calculated from subsequent sweeps with the responses to increasing suprathreshold current steps (step = 10 pA). Note the absence of significant spike frequency adaptation on ChC at any stimulation current steps, short initial facilitation and then moderate adaptation in VOBC, and different level of initial adaptation in CAC and NGFC.

**Figure 12.** Shape of AHP of first spike in the trains was dependent on stimulation current in NGFC. Medium component of AHP was significantly reduced in amplitude with increase of depolarizing current. Note the appearance of an additional depolarizing component (arrow) with the increasing stimulation currents that led to significant reduction of total AHP and to the reduction of the first ISI. APs are truncated. Light gray line shows the level of AHP, gray line the fast component of AHP, and dark gray line the most negative membrane potential after first spike.
morphological types, which shared the most similar combinations of physiological properties. These associations of morphological types can be easily observed in the 3-dimensional plot (Fig. 14). Our interpretation of these results is that monkey interneurons form 3 different physiological classes.

The first class consisted of ChCs and LACs and, according to their factor scores (high frequency non-adapting firing pattern, low level of excitability), these cells can be recognized as nonadapting FS cells, described in different species (Foehring et al. 1991; Kawaguchi and Kubota 1997; Gonzalez-Burgos et al. 2005).

The second physiological class consists of only NGFCs, which are i-Ad cells with a high-threshold of firing and the lowest firing frequency. Although NGFCs in rodent neocortex also constitute a separate electrophysiological class (Kawaguchi and Kubota 1997), their membrane properties are quite different from those in monkey, and the commonly used term “late-spiking interneurons” does not fit the physiological properties of monkey NGFCs (Povysheva et al. 2007).

The third physiological class was formed by CACs, MCs, DBCs, VOBCs, and LPBCs, each of which had positive scores for the factor of excitability and thus had the lowest firing threshold and Rb and the largest R in. Cells of this class exhibited intermediate values of firing frequency and level of adaptation. In contrast to NGFCs that adapted extremely fast, the firing frequency of these interneurons decreased gradually along the train and, thus, they might be best described as continuous-adapting (c-Ad) cells. Within this physiological class, LPBCs and VOBCs had lower scores on the factor of firing frequency adaptation as compared with CACs, MCs, DBCs (difference is significant for $P < 0.1$). Because LPBCs and VOBCs also exhibited significantly shorter spike duration and had different shape of AP at “phase plot” diagram than other c-Ad cells, we delineated monkey c-Ad interneurons into 2 physiological subclasses, c-Ad1 and c-Ad2. The c-Ad1 subclass includes interneurons with typical c-Ad firing pattern and is represented morphologically by CACs, MCs, and DBCs, whereas the c-Ad2 subclass includes c-Ad interneurons with less adaptation and shorter spike duration. Morphologically they belong to LPBCs and VOBCs.

**Discussion**

In this study, we delineated 8 morphological types of monkey interneurons based on 1) the distribution of axonal arbors between layers and 2) the overall pattern of axonal arborization and terminal branching. We characterized the physiological and molecular properties of these 8 morphological types, 4 of which had not been previously characterized electrophysiologically in primates. For this physiological classification, we employed principal factor analysis and defined 3 factors, generally describing interneuron excitability, firing frequency, and level of adaptation of the latter. Monkey interneurons exhibited 3 basic combinations of factor scores, which were recognized as three distinct electrophysiological classes. The main results of this study are summarized in Table 6.

**Morphology-Based Classification of Interneurons in Monkey DLPFC**

Inhibitory interneurons vary greatly in their morphology; however, the axonal arborization can reveal the anatomical identity of an interneuron because interneurons seem to be

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**Table 5**

Factor loading coefficients after varimax rotation for each of the 3 factors

<table>
<thead>
<tr>
<th>Physiological parameters</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_t$</td>
<td>-0.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_i$</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_{PD}$</td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t$</td>
<td>0.63</td>
<td>-0.40</td>
<td></td>
</tr>
<tr>
<td>$A_{HPA_M}$</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_{AP}$</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_{HPA}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_{AP}$</td>
<td>-0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A$</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_{PT}$</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta_{APA}$</td>
<td>0.65</td>
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<td></td>
</tr>
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<td>$A_{HPA_I}$</td>
<td>0.37</td>
<td>-0.57</td>
<td></td>
</tr>
<tr>
<td>$F_{R_I}$</td>
<td></td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>$F_{R_P}$</td>
<td></td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>$\Delta_{APA}$</td>
<td></td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 13.** Spike shape was preserved during trains in LAC and ChC, whereas in other morphological types it was changed. (A) Illustration of APs trains and their derivations from ChC and DBC. Note the difference in spike shapes of DBC during train was more obvious at the voltage derivation graph. V is membrane voltage, and $dV/dt$ is the time derivative of membrane voltage. (B) Phase diagram of subsequent APs in the trains. First AP is shown in red. Note that shape of the first spike was undistinguishable from subsequent spikes in the train in ChC and LAC, slightly shifted to the left in NGFC. In other cell types, subsequent spikes had smaller area.
particularly specialized to target different domains of the postsynaptic cell membrane, different layers of a column, and different columns (Markram et al. 2004). Four morphological types (NGFCs, ChCs, MCs, and DBCs) described here are commonly recognized in different species and have very distinctive morphological characteristics. We found these types to express specific molecular markers in agreement with previously published data (Conde et al. 1994; DeFelipe 1997; Ma et al. 2006). Four other delineated morphological types are not conventionally accepted. Although we tried to find the closest morphological analogues to them from other cortical regions of monkey and other species, some differences with previously described types were presented.

We have described for the first time in monkey cortex CR-positive vertically oriented interneurons forming basket-like structures around postsynaptic cells. Although we do not have evidence that these cells actually form synaptic contacts on the cell bodies of other neurons, in some studies (Markram et al. 1997; Karube et al. 2004), a high probability of finding a synapse at the electron microscopy level for similar structures was noted. The first factor described properties, associated with frequency of firing, the second cell excitability, and the third adaptation. Morphological types formed 3 clusters, which shared the most similar combinations of physiological properties or formed 3 different physiological classes. First class consisted of ChCs and LACs, and according to their factor scores (high-frequency nonadapting firing pattern, low level of excitability), they could be recognized as FS cells. Second physiological class consisted of only NGFCs, which were i-Ad cells with high threshold of firing and the lowest firing frequency. Third class of c-Ad cells was formed by CACs, MCs, DBCs, and VOBCs, which all had positive scores for factor of excitability, they could be recognized as FS cells. Second physiological class consisted of only NGFCs, which were i-Ad cells with high threshold of firing and the lowest firing frequency. Third class of c-Ad cells was formed by CACs, MCs, DBCs, and VOBCs, which all had positive scores for factor of excitability, distinguished them from all other morphological types. Cells of that class exhibited the moderate level of firing frequency and its adaptation.

Figure 14. The graph of the averaged factor scores of different morphological types. The first factor described properties, associated with frequency of firing, the second cell excitability, and the third adaptation. Morphological types formed 3 clusters, which shared the most similar combinations of physiological properties or formed 3 different physiological classes. First class consisted of ChCs and LACs, and according to their factor scores (high-frequency nonadapting firing pattern, low level of excitability), they could be recognized as FS cells. Second physiological class consisted of only NGFCs, which were i-Ad cells with high threshold of firing and the lowest firing frequency. Third class of c-Ad cells was formed by CACs, MCs, DBCs, and VOBCs, which all had positive scores for factor of excitability, distinguished them from all other morphological types. Cells of that class exhibited the moderate level of firing frequency and its adaptation.

Table 6
Summary of interneuron diversity in layers 2-3 of monkey DLPFC

<table>
<thead>
<tr>
<th>Morphological type</th>
<th>Unique morphological features</th>
<th>Molecular markers</th>
<th>Shape of AP at phase plot diagram</th>
<th>Physiological class</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC</td>
<td>“Straight” pattern of axonal arborization</td>
<td>PV</td>
<td>Rocking chair</td>
<td>FS</td>
</tr>
<tr>
<td>ChC</td>
<td>Axon cartridges</td>
<td>PV</td>
<td>Rocking chair</td>
<td>FS</td>
</tr>
<tr>
<td>NGFC</td>
<td>“Curvy” arborization; numerous thin radially distributed dendrites</td>
<td>CB (and NPY)</td>
<td>Vertically oriented egg</td>
<td>i-Ad</td>
</tr>
<tr>
<td>MC</td>
<td>Dense axonal cluster in layer 1</td>
<td>SS (and CR)</td>
<td>Vertically oriented egg</td>
<td>c-Ad1</td>
</tr>
<tr>
<td>CAC</td>
<td>Curvy arborization; few predominantly vertically distributed dendrites</td>
<td>CB or CR in some cells</td>
<td>Vertically oriented egg</td>
<td>c-Ad1</td>
</tr>
<tr>
<td>DBC</td>
<td>Thin vertically oriented axon collaterals with minimal branching</td>
<td>CR (in some cells)</td>
<td>Vertically oriented egg</td>
<td>c-Ad1</td>
</tr>
<tr>
<td>VOBC</td>
<td>Thick smooth vertically oriented axon trunks with short beaded curving horizontal collaterals</td>
<td>CR</td>
<td>Snail</td>
<td>c-Ad2</td>
</tr>
<tr>
<td>LPBC</td>
<td>Tiny cells; axon terminals form “claw”-like structures</td>
<td>CR (in some cells)</td>
<td>Snail</td>
<td>c-Ad2</td>
</tr>
</tbody>
</table>

Electrophysiological Classes of Monkey DLPFC Interneurons

Currently, several different classification schemes to distinguish physiological groups of interneurons have recognized 2-15 different classes of interneurons (Kawaguchi and Kubota 1997; Gibson et al. 1999; Beierlein et al. 2000; Gupta et al. 2000; Bacci et al. 2003). These classification schemes are convenient for electrophysiological studies in the same species because they operate with only a few electrophysiological parameters; however, they do not work well across species. For example, NGFCs from monkey DLPFC do not exhibit late-spiking.
properties (Povysheva et al. 2007), which is a distinct feature of rat NGFCs (Kawaguchi and Kubota 1997). Therefore, a more complete combination of electrophysiological parameters is required for accurate cross-species correlations of interneurons.

In this study, we employed principal factor analysis, which allowed us to define 3 complex variables (factors), generally describing interneuron excitability, firing frequency, and a level of firing frequency adaptation. Monkey interneurons exhibited 3 basic combinations of factor scores, which were recognized as 3 distinct electrophysiological classes: nonadapting fast spiking, c-Ad, and i-Ad cells. Although these suggested terms reflect mostly difference between interneuron classes in firing frequency adaptation, other electrophysiological properties were dissimilar between these classes as well.

Some evidence suggests that the described electrophysiological factors are not just a convenient classification tool, but that they may reflect the level of expression of specific sets of ion channels in different types of interneurons (Toledo-Rodriguez et al. 2004; Sugino et al. 2006). For example, expression of a "PV cluster" of genes, contained HCN2, Kv3.1, Kv1.2, Kv1.6, PV, Kv 3.2, HCN1, Kvβ1, and Cas1A was correlated with high frequency of firing, narrow spike, large AHP, and low R∞ (Toledo-Rodriguez et al. 2004). All these physiological parameters load factor 1 and would then explain why PV-positive LACs and ChCs were significantly different by this factor from all the other non-PV types of interneurons.

Of note, we observed important differences in the electrophysiological features of interneurons from those reported in rodent studies. We did not observe stuttering or bursting cells and only a few irregular-spiking cells in monkey layers 2–3 DLPFC, although these cells were consistently recognized in rodents (Kawaguchi 1995; Cauli et al. 1997; Wang et al. 2002). In addition NGFCs exhibited firing properties different from those described in rodents. An important question is whether these differences reflect differences between species, cortical regions, or developmental stage. Such question is particularly relevant in the case of the primate DLPFC, an area apparently absent in the neocortex of rodents (Preuss 1995). Whether the appearance of specialized areas during evolution of the primate neocortex (Krubitzer 2007) is associated with acquisition of GABA neuron classes that allow specialized forms of information processing in cortical microcircuits needs further investigation.

Although the accurate matching of different mammalian species by a certain phase of their brain development is a challenging task (Clancy et al. 2001), we do not think that differences in developmental stage account for the observed species differences in membrane properties for the following reasons. First, membrane properties of rat neocortical neurons have been shown to undergo changes through the time span of postnatal development and seem to achieve a mature state by the third to fourth postnatal week in different cortical regions (Maravall et al. 2004; Zhang 2004; Povysheva et al. 2007; Oswald and Reyes 2008) and in many classification studies 3- to 4-week-old rats were used. Second, recently we directly compared the membrane properties of NGFCs from young (P19–P28) and adult rats (P56–P135) with NGFCs from young adult monkeys (Povysheva et al. 2007) and found significant interspecies differences, whereas almost all sub- and suprathreshold properties of rat NGFCs in the young rats were similar to those found in adults.

**Functional Implications**

In this study, we defined 8 morphologically distinct groups of monkey interneurons, which were validated by their different intrinsic physiological and molecular properties (Table 6) that possibly play distinct roles in the neocortical circuitry. Insight into these roles can be inferred from the different lateral extent of their axonal arbor. For example, DBCs, VOBs, LPBCs, and ChCs have a narrow axonal arbor, suggesting they contribute to information processing within the elementary cortical functional column. In contrast, the other cell types have a wider axonal arbor spread, suggesting they mediate lateral interactions between the neighboring columns.

Functions of monkey ChCs and LACs (FS PV-positive cells) may be similar to those of rodents. For example, they may exert strong perisomatic inhibition on pyramidal cells and be involved in regulation of synchronous and oscillatory activity of large populations of pyramidal cells (Freund 2003; Buzsaki and Draguhn 2004; Freund and Katona 2007). Monkey FS interneurons like those of rodents may also provide feed-forward inhibition of pyramidal cells (Pouille and Scanziani 2001; Povysheva et al. 2007). Importantly, in striking contrast to rodents, these cells in primates are not the predominant interneuron subpopulation (Conde et al. 1994; Kawaguchi and Kubota 1997), which may reflect species differences in the organization of neocortex.

We found that NGFCs in monkey DLPFC constitute a separate physiological class of i-Ad cells with very low firing frequency. In rodent, these cells have been shown to have a unique function in neocortical circuitry because they provide long-lasting inhibition of pyramidal cells via synapses containing both GABAa and GABAb receptors. Thus, NGFCs appear to be specialized for sparse temporal operations tuned for long-lasting metabotropic effects, which, in turn, may result in sustained modulation of cortical excitability (Tamas et al. 2003).

Although c-Ad cells had many common physiological properties, they still can play a distinct role in neocortical circuitry because they target different domains of pyramidal cells. For example, MCs were shown to innervate distal tufts of pyramidal cells’ apical dendrites in layer 1 and to mediate disynaptic inhibition between neocortical pyramidal cells (Wang et al. 2004; Kapfer et al. 2007; Silberberg and Markram 2007). DBCs also target mostly distal compartments of pyramidal cells but in deep cortical layers (DeFelipe 1997). Specific targets of CACs may be similar to the targets of nest basket cells from rat neocortex, which were reported to innervate mostly perisomatic regions of pyramidal cells (Wang et al. 2002). Physiological properties of c-Ad cells, such as low level of firing threshold, high R∞, and facilitating synaptic inputs to and outputs from pyramidal cells (Thomson and Deuchars 1997; Reyes et al. 1998; Gonzalez-Burgos et al. 2004), result in a reliable excitation of these interneurons by weak repetitive excitatory inputs, which puts c-Ad cells in a position to provide an effective feedback inhibition to pyramidal cells, preventing their overactivation. The functional significance of VOBs and LPBCs remains to be determined. These 2 cell types have unusually fast spikes for adapting cells described in rats. Morphological properties of their axons, forming claw-like structures around somata of postsynaptic cells, point to perisomatic inhibition of pyramidal cells and interneurons within a cortical column.
Supplementary Material

Supplementary material can be found at http://www.cercor.oxfordjournals.org/.

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