Title: Subtype-specific dendritic Ca\textsuperscript{2+} dynamics of inhibitory interneurons in the rat visual cortex

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ABSTRACT

The Ca^{2+} increase in dendrites that is evoked by the backpropagation of somatic action potentials (APs) is involved in the activity-dependent modulation of dendritic and synaptic functions that are location-dependent. In the present study, we investigated dendritic Ca^{2+} dynamics evoked by backpropagating APs (bAPs) in four subtypes of inhibitory interneurons classified by their spiking patterns: fast spiking (FS), late spiking (LS), burst spiking (BS) and regular-spiking nonpyramidal (RSNP) cells. Cluster analysis, single-cell RT-PCR, and immunohistochemistry confirmed the least-overlapping nature of the grouped cell populations. Somatic APs evoked dendritic Ca^{2+} transients in all subtypes of inhibitory interneurons with different spatial profiles along the tree: constantly linear in FS and LS cells, increasing to a plateau in BS cells and bell-shaped in RSNP cells. The increases in bAP-evoked dendritic Ca^{2+} transients brought about by the blocking of A-type K^{+} channels were similar in whole dendritic trees of each subtype of inhibitory interneurons. However, in RSNP cells, the increases in the distal dendrites were larger than those in the proximal dendrites. Upon cholinergic activation, nicotinic inhibition of bAP-evoked dendritic Ca^{2+} transients was observed only in BS cells expressing CCK and VIP mRNAs, with no muscarinic modulation in all subtypes of inhibitory interneurons. Cell subtype-
specific differential spatial profiles and their modulation in bAP-evoked dendritic Ca$^{2+}$
transients might be important for the domain-specific modulation of segregated inputs in inhibitory interneurons and differential control between the excitatory and inhibitory networks in the visual cortex.

Keywords: Inhibitory interneuron, Visual cortex, Dendritic calcium, Spatial profile, A-type K$^+$ channel, Nicotinic modulation.
INTRODUCTION

An increase in dendritic Ca\(^{2+}\) concentration is involved in dendritic excitability, synaptic plasticity, restoring intracellular Ca\(^{2+}\) stores, and intracellular metabolic processes (Egorov et al. 1999; Hausser et al. 2001; Magee and Johnston 1997; Williams and Stuart 2000; Zhou et al. 2005). In cortical pyramidal neurons, backpropagating action potentials (bAPs) evoke dendritic Ca\(^{2+}\) transients depending on the distance from the soma (Waters et al. 2005). Spatial profiles of bAP-evoked Ca\(^{2+}\) transients in cortical pyramidal neurons differ between apical (Spruston et al. 1995; Waters et al. 2003) and basal dendrites (Cho et al. 2006; Hoogland and Saggau 2004; Nevian et al. 2007). Because many synaptic activities are regulated by [Ca\(^{2+}\)]\(_i\), differential kinetics in bAP-evoked dendritic Ca\(^{2+}\) transients between restricted dendritic areas might be critical for local control of synaptic and dendritic activities (Koester and Sakmann 1998; Topolnik et al. 2009; Waters et al. 2003). Furthermore, a local change in dendritic activity might be involved in the pathway-specific regulation of synaptic inputs from different brain areas, which are relatively segregated and terminate in isolated dendritic areas (Binzegger et al. 2004; Feldmeyer et al. 2002; Petreanu et al. 2009).

The bAP-evoked dendritic Ca\(^{2+}\) transients are cell type-specific for respective brain
areas (see review by Waters et al. 2005). Furthermore, huge variation in dendritic arbors, active and passive membrane properties and endogenous Ca\textsuperscript{2+} buffers responsible for dendritic Ca\textsuperscript{2+} dynamics (for review see Ascoli et al. 2008) appear to be important in the subtype-specific function of inhibitory interneurons. An increase in dendritic Ca\textsuperscript{2+} causes EPSP depression (Zilberter et al. 1999), potentiation of L-type Ca\textsuperscript{2+} channels (Topolnik et al. 2009) and long-term synaptic potentiation (Laezza and Dingledine 2004) in a certain subtype of inhibitory interneurons. It has been reported that spatial profiles of bAP-evoked dendritic Ca\textsuperscript{2+} transients were not different among three subtypes of supragranular inhibitory interneurons from the mouse visual cortex (Goldberg et al. 2003a). However, they might be unable to resolve the differential spatial profile of dendritic Ca\textsuperscript{2+} transients in inhibitory interneurons by using a high concentration of Ca\textsuperscript{2+} dyes (Goldberg et al. 2004; Goldberg et al. 2003b).

In the present study, therefore, we characterized the subtypes of inhibitory interneurons grouped by a classification scheme for the rat visual cortical interneurons used in our previous study (Rhie et al. 2003) with single-cell RT-PCR and immunohistochemistry for neurochemicals. We investigated the spatial profiles of dendritic Ca\textsuperscript{2+} transients evoked by somatic APs and their modulation using a low concentration of a
high affinity Ca\textsuperscript{2+} dye in four subtypes of inhibitory interneurons: fast spiking (FS), late spiking (LS), burst spiking (BS) and regular spiking non-pyramidal (RSNP) cells. We found cell subtype-specific spatial profiles for dendritic Ca\textsuperscript{2+} dynamics and BS cell-specific nicotinic modulation, which might be important in the control of the dendritic function of inhibitory interneurons in cell subtype-specific and dendritic location-dependent manners.
MATERIALS AND METHODS

Slice preparation

Coronal slices of primary visual cortex were prepared from Sprague-Dawley (SD) rats (Orientbio Inc., Korea) on their postnatal days 21 to 27. Animal care and surgical procedures were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). After decapitation, the brains were quickly removed and then immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 1 CaCl2, 2 MgSO4 and 10 D-glucose and aerated with a mixture of 95% O2 and 5% CO2. Coronal sections (300-µm thick) containing the visual cortex were prepared with a vibroslicer (HM650V, Microm, Walldorf, Germany) and allowed to recover in a submerged slice chamber for 30 min at 37 °C. The slices were then maintained at room temperature (22-24°C) in the same ACSF before use. The slices were individually transferred to the recording chamber and superfused continuously (1-1.5 ml/min) with the same aerated solution, except for the addition of 2 mM CaCl2 and 1 mM MgSO4. The temperature of the bath solution in the recording chamber was maintained at
Whole-cell patch clamp

A standard whole-cell patch clamp technique with a bridge amplifier (BVC-700A, Dagan, Minneapolis, MN, USA) was used to record the membrane potential and to evoke somatic APs. The patch electrodes (4-6 MΩ) were pulled from borosilicate glass on a P-97 flaming-brown micropipette puller (Sutter Instrument Co., Novato, CA, USA). Patch pipettes were filled with a solution containing (in mM) 130 K-gluconate, 10 KCl, 3 Mg-ATP, 10 Na₂-phosphocreatine, 0.3 Na₃-GTP and 10 Hepes (pH 7.25/KOH), supplemented with 50 μM Oregon Green 488 BAPTA-1 (OGB-1; Kₐ = 170 nM; Molecular Probes, Eugene, OR, USA) as a Ca²⁺ indicator. Biocytin (0.5%) was added to the pipette solution when morphological reconstruction of the recorded cells was required. Under visual guidance, utilizing IR-DIC video-microscopy with an upright microscope (BX51-WI fitted with a 40×/0.80NA water immersion objective, Olympus, Tokyo, Japan), potential interneurons in the layer 2/3 of the primary visual cortex (Paxinos and Watson 1997) were distinguished from pyramidal neurons, according to bipolar or multipolar dendritic processes, as well as bipolar, oval or rounded somata. Typical access resistance was 15-20
MΩ. Electrophysiological parameters were measured as previously described (Rhie et al., 2003). Membrane potentials were not corrected for ~14-mV junction potential. Input resistance (IR) and time constant (τ) were measured with a hyperpolarization response of about 5-15 mV from the resting membrane potential (RMP) by negative step current injection into the soma (40-50 pA, 300 ms). The AP was generated with a graded step-current injection of 10-20 pA for 980 ms from the RMP. With the exception of AP adaptation, all the parameters were measured at the first AP evoked by minimal current amplitude. The AP amplitude was measured from the AP threshold to the peak level. The width of the AP was measured at its half amplitude as a time unit. Afterhyperpolarization (AHP) and P-T times were estimated from the AP threshold to the trough peak level of the AHP and from the AP peak to the trough peak. AP adaptation was calculated by dividing the fifth inter-spike interval by the third inter-spike interval, when at least six consecutive APs were generated with the minimum current injection. Command generation, data acquisition and analyses were performed using the pClamp 9.2 Suite software (Axon Instruments, Foster City, CA, USA). Data were filtered at 5 kHz, sampled at 20 kHz, and saved to a computer hard drive (Pentium PC).

When the effect of 4-aminopyridine (4-AP) was investigated, 6,7-
dinitroquinoxaline-2,3-dione (DNQX, 20 μM), D-amino-5-phosphonovaleric acid (D-AP5, 50 μM), and bicuculline (10 μM) were added to the bath solution throughout the experiment to prevent epileptic discharge. In our experiments, we found no changes in the bAP-evoked Ca$^{2+}$ transients with these blockers ($n = 5$, data not shown).

**Staining and confocal reconstruction**

Following recording, the slices containing biocytin-loaded cells were fixed overnight at 4 °C with 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4). After 3 washes with 10 mM sodium phosphate-buffered saline (PBS), the slices were incubated overnight at 4 °C with 0.5% Triton X-100 in PBS to permeabilize cell membranes. Subsequently, the slices reacted for 2 h at room temperature with Alexa Fluor 488-conjugated avidin (1 μg/ml in PBS, Molecular Probes) to detect intracellular biocytin. The slices were rinsed and mounted on glass slides using fluorescent mounting medium (DakoCytomation, Carpinteria, CA). The distribution of intracellular biocytin was then reconstructed under confocal microscopy (FV-300, Olympus).

**Ca$^{2+}$ imaging**
Fluorescence Ca\textsuperscript{2+} imaging was performed by laser-scanning confocal microscopy (FV-300, Olympus, Tokyo, Japan) at least 25 min after obtaining the cells. Light from an argon ion laser (488 nm) was used for illumination and the emitting fluorescence was filtered with a 510-nm long-pass filter. Dendrites were traced from the soma with the aid of the fluorescence signal of the calcium indicator. The laser intensity for excitation of the indicator was adjusted to minimize phototoxic damage. Dendritic Ca\textsuperscript{2+} transients evoked by bAPs, which were generated by a brief (5 ms) current pulse delivered by the recording pipette into the soma, were measured. The fluorescence signals were obtained using either the line-scan (every 1.4-1.6 ms) or area-scan mode (every 17-24 ms). Every 10 data points for the line-scanned data and dendritic areas of 10 \( \mu \)m in length for the area-scanned data, respectively, were averaged. The distance of the measured dendritic area from the soma was calculated from the center of the soma. Fluorescence signals were background-corrected and traces were expressed as the relative change in fluorescence \([\Delta F/F_0 = (F-F_0)/F_0]\), where \(F_0\) is background-corrected pre-stimulus fluorescence. The peak amplitude of the Ca\textsuperscript{2+} transients was determined at the largest \(\Delta F/F_0\) value of the transients. When recording the BS cells or bath application of 4-AP, hyperpolarizing currents were intercalated between depolarizing current injection for AP firing to restrict the additional AP or AP burst firing.
When a bath application of carbachol (CCh) depolarized cells (see supplemental Table S1),
the membrane depolarization was offset with hyperpolarizing current injection via the
recording pipette.

**Cytoplasm harvest and reverse transcription**

Cytoplasm harvest and reverse transcription (RT) were performed as previously
described with minor modification (Lambolez et al. 1992; Wang et al. 2004). In brief, patch
pipettes were loaded with a 6.5 μl autoclaved internal solution consisting (in mM) of 142
K-gluconate, 10 KCl, 3 MgCl₂, and Hepes (pH 7.25/KOH). Moreover, patch pipettes were
also autoclaved to inactivate RNases. After a brief electrophysiological recording (<5 min),
the cytoplasm of the neurons was aspirated into a patch pipette with gentle negative
pressure under visual control. The cytoplasm in the pipette was then expelled into a 0.2 ml
microtube containing 1.5 μl of nuclease-free water and 1 μl of RNaseOUT (40 U/μl), and
then immediately frozen in liquid nitrogen and stored at -80°C before use. After thawing of
a frozen cytoplasm on ice, the tube was heated to 65°C for 5 min and placed on ice. The
mixture of RT consisting of 2 μl of 10x RT buffer, 1 μl of mixed 10 mM deoxy NTPs, 4 μl
of 25 mM MgCl₂, 2 μl of 0.1 M DTT, 1 μl of random hexamer (50 ng/μl), and 1 μl of
SuperScript III reverse transcriptase was subsequently added and resulted in a total reaction volume of 20 μl. This RT mixture was incubated at 25°C for 10 min, followed by 50 min at 50°C. The reaction was terminated by heating at 85°C for 5 min, followed either by chilling on ice or storage at -20°C. All reagents for these RT reactions were purchased from Invitrogen (Eugene, OR, USA).

**Multiplex PCR**

The two steps of multiplex PCR were carried out as described elsewhere to amplify the cDNAs for neurochemical markers (Cauli et al. 1997). All of the resultant cDNA (20 μl) from the single-cell RT reactions were first simultaneously amplified with primers for two calcium binding proteins [calretinin (CR) and calbindin D28k (CB)], four neuropeptides [neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), somatostatin (SS), cholecystokinin (CCK)], three enzymes [glutamic acid decarboxylase 65 (GAD65), GAD67 and neuronal nitric oxide synthase (nNOS)], and house-keeping protein GAPDH (as a cytoplasm harvesting control). The PCR primers used are described by Cauli et al. (1997) with the exception of parvalbumin (PV) (Vysokanov et al. 1998), nNOS (Cauli et al. 2004), and GAPDH (Wang et al. 2004). The mixture of the PCR reaction was contained
with 1 \mu l of each primer (10 pmol/\mu l), 10 \mu l of 5x green GoTaq reaction buffer (Promega, Madison, WI, USA), 1 \mu l of mixed 10 mM deoxy NTPs, and 0.25 \mu l of GoTaq DNA polymerase (5 U/\mu l, Promega), in a final volume of 50 \mu l. Thermal cycling conditions were as follows: 5 min of initial denaturation at 94°C, 20 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 35 s, and 7 min of final elongation at 72°C. In a separate amplification, the calcium binding protein PV was co-amplified only with GAPDH under the same PCR conditions as above. A second round of PCR was performed using 3 \mu l of the first PCR products as an amplification template. In the second round of PCR, each cDNA was individually amplified using its specific primer pair in a new tube and 35 cycles of PCR under the same thermal conditions as above were run. All primers were obtained from Bioneer Co. (Daejeon, Korea). PCR products (10 \mu l) were run on a 1\% agarose gel, and stained with SYBR Safe DNA gel stain (Invitrogen) for visualization. The sensitivity of the single-cell RT-multiplex PCR was tested on 250 pg of total RNA without non-specific amplification. Total RNA was prepared from the visual cortex of SD rats using an RNA STAT-60™ (Tel-Test Inc. Friendswood, TX, USA) solution according to the manufacturer’s instructions. The predicted sizes for PCR fragments were 432 (CB), 309 (CR), 359 (NPY), 290 (VIP), 208 (SS), 216 (CCK), 391
(GAD65), 600 (GAD67), 548 (nNOS), 309 (PV), and 315 (GAPDH) bp. Intron-overspanning primer pairs and exclusion of the nucleus during cytoplasm harvesting eliminated contamination of genomic DNA in amplification. Complete removal of genomic DNA was confirmed by RT(-) control, which was performed in the absence of a reverse transcriptase enzyme during the normal RT processes. To rule out mRNA contamination from extracellular surrounding tissue, patch pipettes penetrated the slice without seal formation. After the release of positive pressure and the removal of the pipette from the slice, the pipette content was subjected to RT and multiplex PCR. No PCR product was obtained.

**Immunohistochemistry**

Following reconstruction of biocytin-loaded cells under confocal microscopy, slices were further processed for immunohistochemistry. After removal of the coverslips and thorough washing in PBS, the slices were incubated with PBS containing 1% bovine serum albumin (BSA) and 0.3% triton X-100 overnight, then were incubated with a mixture of mouse anti-PV (1:5000; Swant, Bellinzona, Switzerland) and goat anti-nNOS (Herbison et al., 1996) antibodies in the same BSA solution for 7 days at 20°C, followed by
incubation with Rhodamine Red-conjugated donkey anti-goat IgG (1:100; Invitrogen) and
Cy5-conjugated donkey anti-mouse IgG (1:200; Invitrogen) overnight. The slices were then
mounted in Vectashield (Vector Laboratories) and examined with a confocal laser-scanning
microscope (Leica TCS SP2). The long incubation period with primary antibodies was
essential to obtain signals in the deep part of the 300 μm-thick slices. The specificity of the
antibodies used was thoroughly confirmed elsewhere (Celio, 1990; Herbison et al., 1996;
Nomura et al., 1997; Fukuda and Kosaka, 2003). Moreover, slices containing RSNP cells
were used for negative controls in PV- and NOS-immunoreactivities.

Statistics

All data are expressed as the mean ± S.E. A Student’s t-test and analysis of
variance (ANOVA) with a post-hoc Tukey test were used for statistical comparisons. A P-
value of less than 0.05 was considered to be statistically significant. To classify visual
cortical neurons into groups based on electrophysiological properties, the cluster analysis
was used with nine electrophysiological variables (Table 1). Unsupervised clustering using
Ward’s linkage with Euclidean distance was employed (Cauli et al. 2000). Before
performing the cluster analysis all electrophysiological parameters were normalized to their
z-scores. Cluster analysis was performed using Systat 11 software (Systat Software Inc., Chicago, IL).

Drugs

The manufacturers of chemicals and enzymes for the single-cell RT-PCR, fluorescent indicators are indicated above. DNQX disodium salt, bicuculline methiodide, D-AP5, and mecamylamine hydrochloride were obtained from Tocris bioscience (Bristol, UK). CCh, atropine, 4-AP and all other chemicals were purchased from Sigma (St. Louis, MO).
RESULTS

Classification of inhibitory interneurons

We classified inhibitory interneurons primarily with spiking patterns evoked by step current injection, which should represent passive and active membrane properties—one of the most important factors to specify Ca\(^{2+}\) dynamics by the influx of extracellular Ca\(^{2+}\) (Fig. 1). The group of interneurons based on frequently encountered spiking patterns was named with reference to previous reports from other cortical areas (Kawaguchi 1997; Kawaguchi and Kubota 1997) and from our previous study (Rhie et al. 2003). Quantitative analysis for membrane parameters was performed on cells successful for Ca\(^{2+}\) imaging. FS cells were characterized by abrupt non-adapting fast spikes (> 40 Hz) after a slight depolarizing ramp by near-threshold current. The membrane time constant was short (7.2 ± 0.4 ms, \(n = 11\)) and the IR was low (114.1 ± 7.3 M\(\Omega\)). LS cells exhibited slow non-adapting spikes (8 – 20 Hz) with slow depolarizing ramp potential before spike generation with an intermediate membrane time constant (10.5 ± 0.5 ms, \(n = 28\)) and an IR value (172.2 ± 13.5 M\(\Omega\)). These LS cells constituted a large population in supragranular layers of rat visual cortex (32% out of all inhibitory interneurons in this experimental set, \(n = 86\)). BS cells were characterized by a burst of two or three spikes at high frequency on a slow
depolarizing hump at the beginning of plateau depolarization, which were followed by few
spikes, with a slow membrane time constant \((18.1 \pm 0.8 \text{ ms}, n = 32)\) and a high IR \((354.7 \pm
16.2 \text{ M}\Omega)\). RSNP cells showed a generation of adapting slow spikes similar to pyramidal
cells. They exhibited a similar membrane time constant \((18.8 \pm 1.4 \text{ ms}, n = 15)\) and a two-
fold higher IR \((285.1 \pm 28.1 \text{ M}\Omega)\), compared with pyramidal neurons \((16.0 \pm 0.7 \text{ ms and}
125.2 \pm 8.2 \text{ M}\Omega, \text{ respectively, } n = 22)\). We summarized all the analyzed membrane
parameters in Table 1, which were consistent with results found in our previous study (Rhie
et al. 2003). One difference was that the AP widths of the inhibitory interneurons in the
present study were a little longer than those found in the previous study. Because
capacitance compensation is critical for the recording of the fast events, individual variation
for visual compensation and response time of the amplifiers seemed to cause this difference.

We compared our grouping of inhibitory interneurons by characteristic spiking
patterns with groups segregated by unsupervised cluster analysis using all the
electrophysiological dataset in Table 1. BS cells were separated from the other cells,
because they were clearly identified as a non-overlapping group, and a variable for AP
adaptation was missing in this cell subtype. Although some exceptions remained (5 out of
108 cells), cluster analysis yielded well-suited segregation of inhibitory interneurons with
our classification (Fig. 2). Thus, grouping by spiking patterns is likely to be appropriate for studying cell subtype-specific properties of membrane excitability-related physiological events.

Neurochemical markers for inhibitory interneurons

In a previous report of the mouse supragranular visual cortex, LS cells were not identified (Goldberg et al. 2003a). However, in the present study of rat visual cortex, FS and LS cells were clearly distinguished with electrophysiological features, especially by spiking frequencies. Because FS and LS cells exhibited overlapping morphologic features, molecular markers were identified so they could be grouped as different cell subtypes with single-cell RT-PCR (Fig. 3). A different set of the recorded cells was used for this experiment. We also performed cluster analysis for quantitative electrophysiological parameters taken in these cells (supplemental Table S2) and found subtypes to be well segregated with only two exceptions among 63 cells (supplemental Fig. S1). In accordance with previous immunohistochemical staining (Kawaguchi and Kubota 1997), whereas all FS cells showed PV(+) (n = 9), all LS cells showed PV(-) (n = 10). Because PV is an invariable marker for FS cells, this finding confirmed that LS cells are different from FS
cells in supragranular layers of rat visual cortex. In contrast to pyramidal cells \(n = 8\), all
the non-pyramidal cells were positive for GAD65 and/or GAD67, confirming they are
GABA-containing inhibitory interneurons. The results for other Ca\(^{2+}\)-binding proteins and
neuropeptides are summarized in Fig. 3. Whereas VIP mRNA was positive only in BS cells
(100%, \(n = 10\)), CCK mRNA was present in ~85% of all inhibitory interneurons. SS mRNA
was also positive in ~40% of pyramidal cells and in ~20% of FS and LS cells. Moreover, it
was particularly noteworthy that 40% of LS cells expressed nNOS-specific mRNAs, which
were found exclusively in LS cells. The nNOS mRNAs have also been found in some
perivascular RSNP and LS cells in frontoparietal and somatosensory cortices (Cauli et al.
2004).

Although it has been suggested that \(\alpha\)-actinin-2 is a specific chemical marker for
LS cells in the rat frontal cortex (Uematsu et al. 2008), we tested the possibility that nNOS
could be used as another specific marker to identify LS cells in histological specimens.
Slices containing either LS or FS cells were double-immunostained for nNOS and PV in
another set of cells, which were patch-clamped for more than 1 hr (Fig.4). Consistent with
multiplex RT-PCR, half of the LS cells (8 out of 15 cells) exhibited weak, but clearly
identifiable, immunoreactivity for nNOS. The occurrence of a neuronal population
characterized by weak immunoreactivity for nNOS was further confirmed by observations of the tissues surrounding the recorded cells (Fig. 4C, arrows). These cells had small, round soma and were scattered mainly in supragranular layers. Similar weakly nNOS-positive neurons have been described in the mouse visual cortex (Lee and Jeon 2005), which might correspond to a subpopulation of LS cells as demonstrated here in the rat visual cortex. As seen in Fig. 4C, there was another type of nNOS neurons that had a much larger soma and exhibited intense nNOS labeling. We could not obtain these cells in the present study, most likely because this subtype makes up a very small portion (0.5%) of GABAergic neurons in the rat visual cortex (Gonchar and Burkhalter 1997). Thus, we concluded that weakly nNOS-immunoreactive non-pyramidal neurons having small soma represent a part of the LS cell population in the rat visual cortex. By contrast, PV-immunoreactivity was found only in FS cells (3 out of 8 cells), whereas the vast majority of LS cells (14 out of 15 cells) were PV-negative. Negative PV-immunoreactivity in some FS cells might be due to the free diffusion of PV into the pipette during long recording sessions (Schmidt et al. 2007).

In addition to cluster analysis (Fig. 2 and supplemental Fig. S1), these results from single-cell RT-PCR and immunohistochemical staining for neurochemicals verify that inhibitory interneurons in supragranular layers of the rat primary visual cortex can be
classified by the spiking patterns into four main subtypes despite some overlapping
electrophysiological and morphological features.

Subtype-specific spatial profiles of bAP-evoked dendritic Ca\textsuperscript{2+} transients

To study the spatial profiles of dendritic Ca\textsuperscript{2+} transients evoked by somatic APs in
four subtypes of inhibitory interneurons, we used a low concentration (50 μM) of high-
affinity Ca\textsuperscript{2+} dye, OGB-1 (Fig. 5). Single or burst APs at 20 Hz were evoked by step current
injection (5-10 ms, 200-900 pA) into the soma. FS cells showed only small Ca\textsuperscript{2+} transients
with a similar amplitude in all measured dendritic arbors up to 105 μm from the soma (n =
11 cells) (Fig. 5A). These Ca\textsuperscript{2+} transients increased with the number of somatic APs up to
ten APs (20 Hz). Although the length of targeted dendrites in FS cells was 118 ± 12 μm (n =
13), we could obtain dendritic Ca\textsuperscript{2+} transients up to 72 ± 6 μm from the soma, because of
the low fluorescent signal in thin distal dendrites. LS cells showed a profile of dendritic
Ca\textsuperscript{2+} transients that was similar to that of FS cells, with the exception of the amplitude: LS
cells exhibited larger bAP-evoked Ca\textsuperscript{2+} transients of similar amplitude along the dendritic
tree up to ~120 μm from the soma, which increased linearly with the number of somatic
APs (n = 23 cells).
Peak amplitude of bAP-evoked dendritic Ca\textsuperscript{2+} transients in BS cells increased as distance from the soma increased (n = 32 cells), and reached a plateau at about 50 - 100 \( \mu \)m from the soma. A single exponential function analysis of peak amplitude to the dendritic distance yielded increasing length constants (the distance at 63% of maximal amplitude) with the number of APs (16.3 \( \pm \) 7.7 \( \mu \)m, 24.8 \( \pm \) 8.1 \( \mu \)m, and 30.1 \( \pm \) 11.5 \( \mu \)m from the soma to single, three and five APs, respectively). Because we could find no difference in the spatial profiles between apical and basal dendrites, we pooled the data in this analysis. A previous study has reported widespread but decreased AP-evoked Ca\textsuperscript{2+} transients in the distal dendrites of bitufted interneurons in the somatosensory cortex (Kaiser et al. 2001). Although the bipolar nature of bitufted cells corresponds to that of the BS cells in the present study, they have no burst firing with current injection, which is one of electrophysiological characteristics of BS cells. Based on the regular spiking pattern of bitufted cells in previous studies (Kaiser et al. 2001; Reyes et al. 1998), they might be a subset of RSNP cells in the present study. The relatively younger age of animals in their study (P14 – 16) might be responsible for the discrepancy in AP firing and bAP-evoked Ca\textsuperscript{2+} transients (Okyaty et al. 2009). RSNP cells exhibited a bell-shaped profile in bAP-evoked Ca\textsuperscript{2+} transients along the
dendritic length up to ~180 μm from the soma (n = 19 cells). Interestingly, the spatial profile of the RSNP cells was similar to that of pyramidal cells, as shown in our previous study (Cho et al. 2008; Cho et al. 2006), as well as in the study of others’ (Waters et al. 2003). However, the amplitude of the Ca²⁺ transients in RSNP cells was smaller than that of the pyramidal cells: the largest peak amplitude of ΔF/F₀ of Ca²⁺ transients evoked by five APs at 20 Hz from the baseline level was about 2.7 in pyramidal neurons of our previous study (Cho et al. 2008), and it was about 1.8 in RSNP cells in the present study.

Collectively, the dendritic Ca²⁺ transients evoked by bAPs were lower in inhibitory interneurons than in pyramidal neurons. When decay time constants of Ca²⁺ transients evoked by five APs were analyzed from single exponential fits in whole dendritic trees, the decay time constant of LS cells showed the smallest value (341 ± 28 ms). FS and BS cells followed (406 ± 38 ms and 478 ± 31 ms, respectively) and RSNP cells showed the largest decay time constant (576 ± 57 ms). These values were larger than those found in pyramidal cells (256 ± 11 ms, p < 0.05). These results support that the presence of calcium-binding proteins such as PV, calbindin and calretinin, might be responsible for the lower increase in Ca²⁺ in interneurons (Goldberg et al. 2003a; Kaiser et al. 2001). In addition, the lower increases might involve the smaller amplitude and shorter duration of APs in inhibitory
interneurons (Table 1) as well as differential expression of dendritic Ca\(^{2+}\) channels (Vinet
and Sik 2006).

Based on our visual inspection of the data, the amplitude of Ca\(^{2+}\) transients along
the dendritic tree were fitted with the line function for FS and LS cells, with the exponential
function for BS cells, and with the Gaussian function for RSNP cells (Fig. 5). We assessed
the variability of the grouped interneurons in the present study by comparing the variability
of the Ca\(^{2+}\) transients in each subtype of inhibitory interneurons to that of pyramidal
neurons. Because average values were different in each dendritic location, we analyzed
mean squared errors (MSE) of individual Ca\(^{2+}\) transients with respect to the fitted values
obtained at the same dendritic location from the soma. Whereas MSE values of Ca\(^{2+}\)
transients in apical dendrites of pyramidal neurons analyzed from our previous data (Cho et
al. 2006) were 0.106, 0.221, and 0.369 for single, three, and five APs, respectively (from 82
data points), MSE values in all subtypes of inhibitory interneurons in the present study
(data not shown) were less than the respective values in pyramidal neurons. The normalized
MSE to the mean was 6-13% in FS cells, 6-10% in LS cells, 8-10% in BS cells, and 6-10%
in RSNP cells. These values were similar or less than those of pyramidal cells (10-18%).
This finding implies that each grouped interneuron showed a relatively homogeneous
population, which was comparable to pyramidal neurons.

These results clearly demonstrate that the spatial profiles of bAP-evoked Ca\textsuperscript{2+} transients along the dendritic trees in different interneuron subtypes have distinct characteristics. The cell subtype-specific location-dependent increase in dendritic [Ca\textsuperscript{2+}], might be involved in many cellular functions, endowing cell-specific function and modulation in the cortical circuit.

**Inhibition of A-type K\textsuperscript{+} channels on bAP-evoked dendritic Ca\textsuperscript{2+} transients**

Transient A-type K\textsuperscript{+} channels restrict invasion of APs into dendrites, and, thus, limit the influx of Ca\textsuperscript{2+} into dendrites, which is significant for distal dendrites in hippocampal (Hoffman et al. 1997) and neocortical pyramidal neurons (Cho et al. 2008) and in neocortical inhibitory interneurons (Goldberg et al. 2003a). Thus, we investigated the effect of the inhibition of A-type K\textsuperscript{+} channels on dendritic Ca\textsuperscript{2+} transients evoked by bAPs to characterize further cell subtype-specific dendritic Ca\textsuperscript{2+} dynamics (Fig. 6). Upon the bath application of 4-AP (1-2 mM), bAP-evoked Ca\textsuperscript{2+} transients were increased in all dendritic areas. However, whereas distal dendrites (154.3 ± 17.9% from the control) showed significantly larger increases in Ca\textsuperscript{2+} transients than proximal dendrites (58.5 ± 9.8%
from the control) in RSNP cells ($n = 5, p < 0.01$), FS ($n = 7$), LS ($n = 7$) and BS cells ($n = 4$) showed a similar increase in Ca$^{2+}$ transients between proximal and distal dendrites (Fig. 6).

Thus, we clearly demonstrated that A-type K$^+$ channels significantly restricted the backpropagation of bAP into dendritic trees in all subtypes of interneurons with a greater effect in the distal dendrites of RSNP cells.

In this experiment, AP amplitude and width were increased markedly in all subtypes of inhibitory interneurons with the application of 1-2 mM 4-AP (Fig. 7A). Both A-type K$^+$ channels and fast-activating delayed rectifier K$^+$ channels assembled from Kv3.1 and Kv3.2 subunits are preferentially inhibited by 4-AP at this concentration. Because it has been reported that the blocking of fast-activating delayed rectifier K$^+$ channels increases the AP amplitude and width in fast-spiking interneurons of mouse somatosensory cortex (Erisir et al. 1999), we addressed this issue further by examining the effect of a low concentration of 4-AP (0.1 mM). The amplitude and width of somatic APs were not changed significantly, except for a slight increase in the AP width of RSNP cells ($122.6 \pm 3.2\%$ of the control, $n = 3, p < 0.05$). However, a low concentration of 4-AP did not increase bAP-evoked dendritic Ca$^{2+}$ transients in all subtypes of inhibitory interneurons (Fig. 7D). A low concentration of TEA (1 mM), which blocks > 80% of Kv3.1 and Kv3.2 channels (Erisir et al. 1999), did not
increase bAP-evoked dendritic Ca$^{2+}$ transients, while both AP amplitudes and widths in FS, BS and RSNP cells were slightly increased. These results suggest that fast-activating delayed rectifier K$^+$ channels are minimally involved in the shaping of APs in visual cortical interneurons, which is consistent with the finding of 1 mM TEA by Goldberg et al. (2003a). Thus, bAP-evoked dendritic Ca$^{2+}$ transients were increased by the blocking of A-type, but not by fast-activating delayed rectifier K$^+$ channels in inhibitory interneurons of the rat primary visual cortex. Our results also indicate that A-type K$^+$ channels significantly limit the propagation of somatic APs into distal dendrites in all interneuron classes, and exhibit a greater effect in distal dendrites of RSNP cells. In addition, an increase in dendritic Ca$^{2+}$ transients by the blocking of A-type K$^+$ channels appears to be caused partly by an increase in the amplitude and width of somatic AP in inhibitory interneurons.

Nicotinic modulation of bAP-evoked dendritic Ca$^{2+}$ transients in BS cells

Balance between excitatory and inhibitory activities in cortical circuits is critical for proper functioning of the cortex. Both muscarinic and nicotinic receptors regulate excitability of inhibitory interneurons. Although a substantial amount of information on cholinergic modulation of dendritic Ca$^{2+}$ transients in pyramidal neurons has been reported
(Cho et al. 2008; Larkum et al. 2003; Nakamura et al. 2000), relatively little is known about the cholinergic modulation of dendritic Ca\textsuperscript{2+} transients in neocortical inhibitory interneurons. Thus, we investigated the cell subtype-specific modulation of dendritic Ca\textsuperscript{2+} transients evoked by AP backpropagation. Cholinergic agonist CCh (20 μM) in the bath (for 5 min at a flow rate 1 ml/min) activates muscarinic receptors, resulting in secondary Ca\textsuperscript{2+} release from the store via an IP\textsubscript{3}-dependent mechanisms in pyramidal neurons, as shown by the present experiment (Fig. 8A, Pyr), and in other reports (Cho et al. 2008; Nakamura et al. 2000). In contrast to excitatory pyramidal neurons, CCh failed to increase dendritic Ca\textsuperscript{2+} transients evoked by bAP in all subtypes of inhibitory interneurons (Fig. 8A). Rather, one subtype of inhibitory interneurons, BS cells (18 out of 21 cells), exhibited a constant decrease in Ca\textsuperscript{2+} transients along the dendritic tree (single AP: 72.6 ± 2.9% of the control; three APs: 70.7 ± 2.2% of the control, n = 18 cells, p < 0.01 each) (Fig. 8B). Although changes in RMP were offset by negative current injection to the initial level after CCh application in this experiment, the decrease in Ca\textsuperscript{2+} transients might have resulted from the elevated baseline Ca\textsuperscript{2+} concentration by the nicotinic stimulation of BS cells in the present study (supplemental Table S1\textsuperscript{1}), which was also reported previously (Gulledge et al. 2007).

Thus, we addressed this issue in another set of experiments. We found no changes in
baseline Ca$^{2+}$ levels after CCh application in BS cells, while bAP-evoked Ca$^{2+}$ transients were attenuated with the application of CCh and recovered with a wash-out of CCh ($n = 5$, supplemental Fig. S2^4). We next examined which subtype of acetylcholine receptors is involved in the cholinergic decrease of dendritic Ca$^{2+}$ transients in BS cells. As shown in Fig. 8C, the application of mecamylamine (30 μM), a nicotinic receptor antagonist, abolished the CCh-induced decrease in bAP-evoked dendritic Ca$^{2+}$ transients (100.6 ± 2.8% of the control in proximal dendrites, $p < 0.05$ vs. CCh only; 96.4 ± 2.1% of the control in distal dendrites, $p < 0.05$ vs. CCh only; $n = 4$), while atropine (10 μM), a muscarinic receptor antagonist, did not (66.1 ± 3.1% of the control in proximal dendrites, $p = 0.20$ vs. CCh only; 71.5 ± 7.7% of the control in distal dendrites, $p = 0.26$ vs. CCh only; $n = 5$). The block by mecamylamine was not different between the proximal and distal dendrites of BS cells. CCh application did not change the shape or parameters of somatic APs (AP threshold: -37.4 ± 1.7 mV before CCh, -37.1 ± 1.9 mV after CCh, $p = 0.59$; AP amplitude: 49.6 ± 2.3 mV before CCh, 49.4 ± 2.4 mV after CCh, $p = 0.78$; AP width at half maximal amplitude: 1.0 ± 0.1 ms before CCh, 1.0 ± 0.1 ms after CCh, $p = 0.31$; $n = 22$). In accordance with nicotinic effect on Ca$^{2+}$ transients, CCh-induced depolarization was also blocked by a pre-
application of mecamylamine ($n = 2$), but not atropine ($n = 2$), in BS cells (data not shown).

Thus, these results indicate that cholinergic activation increases bAP-evoked dendritic $Ca^{2+}$
transients via muscarinic receptors only in excitatory pyramidal neurons, but decrease them
via nicotinic receptors only in BS inhibitory interneurons. These results suggest that
cholinergic activation might change the network function with differential action on the
$Ca^{2+}$-mediated membrane excitability between excitatory and a subset of inhibitory
interneurons.
DISCUSSION

Dendritic Ca\(^{2+}\) dynamics were investigated in four subtypes of inhibitory interneurons (FS, LS, BS, and RSNP cells) in the supragranular layers of the rat primary visual cortex. There are three major aspects of the present study: 1) cell subtype-specific spatial profiles of dendritic Ca\(^{2+}\) transients evoked by bAPs; 2) differential involvement of A-type K\(^+\) channels in the backpropagation of somatic APs into distal dendrites among subtypes of inhibitory interneurons; and, 3) nicotinic inhibition of bAP-evoked dendritic Ca\(^{2+}\) transients in BS cells.

Subtypes of inhibitory interneurons in supragranular layers of the rat primary visual cortex

In the present study, we did not find low-threshold spiking (LTS) cells in supragranular layers of rat primary visual cortex. Other studies have found LTS cells present as a small population in the supragranular layers of the agranular frontal cortex (Kawaguchi 1995) and abundantly present in layer IV (Beierlein et al. 2000; Gibson et al. 1999) and in the infragranular cortex (Bacci et al. 2003; Goldberg et al. 2004; Xiang et al. 1998). In those studies, some LTS cells showed burst spiking and bitufted dendritic arbors.
In the present study, BS cells, which appeared frequently in rat primary visual cortex, had a distinct bipolar shape of the soma, bitufted dendritic arbor, and VIP as a specific neurochemical marker. Thus, in the present study, BS cells constituted a different subset from LTS cells.

In a previous report on dendritic Ca$^{2+}$ dynamics of inhibitory interneurons in the supragranular layers of the mouse primary visual cortex, three subtypes were studied: FS, irregular spiking, and adapting cells (Goldberg et al. 2003a). Whereas we found LS cells more frequently than FS cells in the same region of rats, this aforementioned study did not report LS cells having slow non-adapting spikes. It seems that LS cells are the major population of interneurons in layer 1 (Chu et al. 2003; Gulledge et al. 2007). However, Gulledge et al. (2007) also found LS cells in layer 2/3 in the rat neocortex. LS cells have clearly been distinguished from FS cells by their spiking frequencies and characteristic neuronal markers in previous studies (Kawaguchi and Kubota 1996; Uematsu et al. 2008), as well as in the present study. Although a large proportion of LS cells in the present study might be due to the relatively large soma, it is likely that they constitute a major population of inhibitory interneurons in the rat visual cortex. This appears to be an apparent difference in subtypes of inhibitory interneurons among species (Zaitsev et al. 2004).
Non-FS cells are generally subdivided into BS, RSNP and irregular spiking cells. BS cells appear to be a relatively homogeneous population because of the characteristic bursting spikes on the hump and bipolar dendritic arbors. The bipolar dendritic morphology and bursting nature of irregular spiking cells of previous reports (Cauli et al. 1997; Goldberg et al. 2003a) appear to correspond to similar instances of BS cells in the present study. However, RSNP cells in the present study appeared to be relatively heterogeneous, and at least two subgroups could be distinguished (Fig. 2): one having adapting spikes with an initial slow burst and the other without the initial burst. Thus, a subset of RSNP cells in the present study might also have been included in the irregular spiking cells in the Goldberg et al. study.

Spatial profiles of bAP-evoked dendritic Ca\(^{2+}\) transients

Individual synapses at different dendritic locations might be modulated with a differential amount of Ca\(^{2+}\) influx. In a previous study, dendritic Ca\(^{2+}\) accumulation was studied in three subtypes of interneurons in the mouse visual cortex (Goldberg et al. 2003a). They found barely detectable single AP-evoked Ca\(^{2+}\) transients even in proximal dendrites and no difference in high-frequency AP-evoked Ca\(^{2+}\) accumulation among different
subtypes of interneurons, probably by using a high concentration of Ca\(^{2+}\) dye (200 \(\mu\)M calcium green or 400 \(\mu\)M Fluo-4). In their following study, they found global dendritic Ca\(^{2+}\) spikes in layer 5 LTS interneurons by using a lower concentration (100 \(\mu\)M) of Fluo-4 (Goldberg et al. 2004). In the present study, however, we clearly found subtype-specific spatial profiles of bAP-evoked dendritic Ca\(^{2+}\) transients by using a high-affinity dye at low concentration (50 \(\mu\)M OGB-1). Again, the discrepancy might have been due to the different ages of the animals (Isomura et al. 1999; Okaty et al. 2009). It is noteworthy that Ca\(^{2+}\) transients did not experience a decrease in distal dendrites in FS and LS cells, but instead increase in BS cells, which differs from the apical dendrites of pyramidal cells. The spatial profiles were similar to those of basal dendrites from pyramidal cells (Cho et al. 2008; Cho et al. 2006). These results imply that the distal synapses in most inhibitory interneurons, with the exception of RSNP cells, might be regulated tightly as a single compartment, which is different from pyramidal neurons with multiple compartments (Antic 2003). Increases in bAP-evoked Ca\(^{2+}\) transients that were larger in distal, than in proximal, dendrites of BS cells might have resulted from a higher surface-to-volume ratio in distal fine dendrites (Schiller et al. 1995), less attenuated propagation of somatic AP into the dendritic tree by a high IR for BS cells (Table 1) and a differential expression of various
voltage-gated ion channels along the dendrites (Migliore and Shepherd 2002), which requires further investigation.

**Dendritic propagation of somatic APs in inhibitory interneurons**

A-type K$^+$ channels critically control the propagation of somatic APs into dendrites in cortical pyramidal neurons (Hoffman et al. 1997). In a previous study, bAP-evoked dendritic Ca$^{2+}$ transients were increased by the blocking of Ia-type K$^+$ channels, but not by the blocking of fast-activating delayed rectifier Kv3-type K$^+$ channels (Goldberg et al. 2003a). In the present study, the blocking of Kv3-type current with 0.1 mM 4-AP or 1 mM TEA did not increase dendritic Ca$^{2+}$ transients, confirming the previous findings by Goldberg et al. (2003a). High expression of fast-activating K$^+$ channels (Kv3.1 and Kv3.2) endows the fast firing of brief APs in interneurons (Erisir et al. 1999; Martina et al. 1998). In the present study, however, we observed no increase in dendritic Ca$^{2+}$ transients following the application of a low concentration of TEA in inhibitory interneurons, despite a small increase in the amplitude and duration of APs. Thus, a small increase in the amplitude and width of somatic APs by fast-activating K$^+$ channels was not enough to modulate the bAP-evoked dendritic Ca$^{2+}$ transients in visual cortical interneurons. The
increase in amplitude and the broadening of somatic APs were much larger with the application of 1 mM 4-AP than with the application of 0.1 mM 4-AP in all inhibitory interneurons in the present study. Goldberg et al. (2003a) concluded that A-type K$^+$ channels restrict the propagation of somatic APs into dendrites in interneurons, because the increase in bAP-evoked Ca$^{2+}$ transients in distal dendrites was larger than that in apical dendrites with blocking A-type K$^+$ channels. In the present study, however, we observed a larger increase in Ca$^{2+}$ transients in distal dendrites than in proximal dendrites only in RSNP cells. Therefore, we suggest that A-type K$^+$ channels in distal dendrites control critically the propagation of somatic APs to a greater extent in RSNP cells. In other subtypes of inhibitory interneurons, A-type K$^+$ channels appear to control the propagation of somatic APs into dendritic trees, acting primarily on the proximal compartments including the soma.

Nicotinic modulation of bAP-evoked dendritic Ca$^{2+}$ transients

While it is well known that muscarinic activation increases dendritic Ca$^{2+}$ transients via IP$_3$-dependent Ca$^{2+}$-induced Ca$^{2+}$ release in pyramidal cells (Cho et al. 2008; Nakamura et al. 2000), cholinergic modulation of bAP-evoked dendritic Ca$^{2+}$ transients in
inhibitory interneurons is still lacking. In previous studies, FS cells were nonresponsive to the application of cholinergic agonists (Gulledge et al. 2007; Kawaguchi 1997). On the other hand, phasic nicotinic AChR-mediated excitation was found only in non-FS cells expressing VIP and CCK in layer 2/3 (Gulledge et al. 2007; Porter et al. 1999), LTS cells in layer 5 (Xiang et al. 1998) and layer 1 interneurons of the neocortex (Christophe et al. 2002). In accordance with the previous electrophysiological findings, we have found no cholinergic modulation on dendritic Ca^{2+} transients in FS cells in the present study. We have also found CCh-induced depolarization (25-30 mV) (supplemental Table S1)\(^1\) and spontaneous firing (data not shown) only in BS cells that expressed both VIP (10 out of 10 cells) and CCK (9 out of 10 cells). Thus, the subset of non-FS inhibitory interneurons exhibiting excitatory response to nicotinic AChR activation is identical to the BS cells in the present study. However, in the present study, bAP-evoked dendritic Ca^{2+} transients were inhibited by tonic nicotinic activation, which was in contrast to the stimulatory effect on electrical excitability in these BS cells. A leaky membrane with a decreased IR (\(~70\) MΩ in the present study) might dampen the propagation of bAP into dendritic trees in BS cells. Although we observed no changes in the baseline dendritic Ca^{2+} levels throughout the CCh application, additional factors might not be excluded, such as the desensitization of
dendritic calcium channels by local dendritic depolarization. In addition, although Gulledge et al. (2007) reported nicotinic excitation of two LS cells in layer 2/3, we observed no changes in Ca\(^{2+}\) transients in LS cells with tonic CCh application \((n = 4)\). The lack of cholinergic modulation on dendritic Ca\(^{2+}\) transients in LS cells might be due to rapid receptor desensitization in the LS cells (Gulledge et al. 2007) or to the difference between electrical potential of the soma and the Ca\(^{2+}\) transients in dendrites. Taken together, we confirmed the previous findings on cholinergic modulation of interneurons and clarified that VIP(+) and CCK(+) BS cells were the major subset of inhibitory interneurons modulated by nicotinic activation in the visual cortex.

**Functional implication of cell subtype-specific dendritic Ca\(^{2+}\) dynamics**

The variability of inhibitory interneurons is very important in that they endow various functions to the cortical network. Subsets of inhibitory interneurons characterized by their electrophysiological, morphological and molecular nature exert specific functions of the cortical network. Cell subtype-specific profiles of bAP-evoked dendritic Ca\(^{2+}\) transients in inhibitory interneurons in this study might be important for the domain-specific modulation of segregated input from different sources of input (Petreanu et al. 2007).
neocortex is modulated in a state-dependent manner by neuromodulators. In addition to cholinergic modulation of the excitatory pathway (Hasselmo 2006), cholinergic modulation of dendritic Ca\(^{2+}\) dynamics in a subtype of inhibitory interneurons could be involved in certain cortical functions.
ACKNOWLEDGEMENTS

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GRANTS

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FOOTNOTES

1 The online version of this article contains supplemental Table S1.

2 The online version of this article contains supplemental Table S2.

3 The online version of this article contains supplemental Fig. S1.

4 The online version of this article contains supplemental Fig. S2.
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Table 1. Electrophysiological properties of layer 2/3 visual cortical neurons

<table>
<thead>
<tr>
<th>Cell Subtype</th>
<th>Pyr ($n = 22$)</th>
<th>FS ($n = 11$)</th>
<th>LS ($n = 28$)</th>
<th>BS ($n = 32$)</th>
<th>RSNP ($n = 15$)</th>
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<tr>
<td>RMP (mV)</td>
<td>-78.56 ± 1.34&lt;sup&gt;de&lt;/sup&gt;</td>
<td>-72.32 ± 2.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-69.59 ± 1.29&lt;sup&gt;ae&lt;/sup&gt;</td>
<td>-69.55 ± 1.19&lt;sup&gt;ae&lt;/sup&gt;</td>
<td>-63.87 ± 1.08&lt;sup&gt;abcd&lt;/sup&gt;</td>
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<tr>
<td>IR (MΩ)</td>
<td>125.2 ± 8.2&lt;sup&gt;de&lt;/sup&gt;</td>
<td>114.1 ± 7.3&lt;sup&gt;de&lt;/sup&gt;</td>
<td>172.2 ± 13.5&lt;sup&gt;de&lt;/sup&gt;</td>
<td>354.7 ± 16.2&lt;sup&gt;abce&lt;/sup&gt;</td>
<td>285.1 ± 28.1&lt;sup&gt;abcd&lt;/sup&gt;</td>
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<tr>
<td>τ (ms)</td>
<td>16.0 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.0 ± 0.4&lt;sup&gt;ade&lt;/sup&gt;</td>
<td>10.5 ± 0.5&lt;sup&gt;ade&lt;/sup&gt;</td>
<td>18.1 ± 0.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>18.8 ± 1.4&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>AP threshold (mV)</td>
<td>-37.7 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-30.8 ± 2.1&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>-34.2 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-38.5 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>-35.2 ± 1.3</td>
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<td>AP amplitude (mV)</td>
<td>67.8 ± 1.8&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>38.3 ± 2.0&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>52.4 ± 1.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>45.9 ± 1.3&lt;sup&gt;abc&lt;/sup&gt;</td>
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<td>AP width (ms)</td>
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<td>0.61 ± 0.04&lt;sup&gt;bcde&lt;/sup&gt;</td>
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<td>0.99 ± 0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.04 ± 0.08&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>AHP (mV)</td>
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<td>P-T time (ms)</td>
<td>55.3 ± 2.7&lt;sup&gt;bcde&lt;/sup&gt;</td>
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<td>AP adaptation</td>
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<td>NA</td>
<td>1.76 ± 0.22&lt;sup&gt;abc&lt;/sup&gt;</td>
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a, vs. Pyr; b, vs. FS; c, vs. LS; d, vs. BS; e, vs. RSNP. ANOVA with Tukey test were used for statistical comparison. A $p$-value less than 0.05 was considered to be statistically significant. NA, not applicable.
FIGURE LEGENDS

FIG. 1. Spiking patterns and dendritic morphology of supragranular inhibitory interneurons in the rat primary visual cortex.

Representative spiking patterns evoked by positive step current injection into the soma is shown in the upper two rows for fast spiking (FS), late spiking (LS), burst spiking (BS) and regular spiking non-pyramidal (RSNP) cells. Current steps injected for 980 ms are shown in the third row. Spikes generated by the largest current injection are shown in the first row for easy identification. Reconstructed cellular morphology obtained by biocytin staining is shown on the bottom row. Scale bars represent 100 μm.
FIG. 2. Cluster analysis of cortical cells in layer 2/3 of the rat primary visual cortex.

A: nine electrophysiological variables in Table 1 were used for unsupervised cluster analysis. Two FS cells (indicated with filled squares), two LS cells (indicated with open triangles) and one RSNP cell (indicated with filled triangle) were clustered to LS, RSNP and pyramidal (Pyr) cells, respectively. B: BS cells were separated from the other cells before the analysis.
FIG. 3. Single-cell RT-PCR of cortical cells in layer 2/3 of the rat primary visual cortex.

A: a different set of cells was subjected to single-cell RT-PCR for Ca\(^{2+}\)-binding proteins (calbindin D28k: CB, parvalbumin: PV, and calretinin: CR), neurochemicals (neuropeptide Y: NPY, vasoactive intestinal peptide: VIP, somatostatin: SS, and cholecystokinin: CCK), two glutamic acid decarboxylase (GAD65 and GAD67), neuronal nitric oxide synthase (nNOS) and GAPDH. Percent expression of neurochemical markers for each subtype are plotted in the right panel. Expression of PV was investigated at a separate amplification set with GAPDH (B) in each cell subtype and is included in the right panel histogram. B: the RT-PCR for the PV of representative cells for each cell subtype is shown.
FIG. 4. Immunohistochemical staining for PV and nNOS in supragranular layers of the visual cortex.

A: a FS cell (green arrow) clearly shows PV immunoreactivity but it lacks nNOS immunoreactivity. Red arrows indicate weak nNOS-labeling in surrounding small cells. B: an LS cell (green arrow) is weakly nNOS-positive but is negative for PV. C: two types of nNOS immunoreactivity. In addition to a large, intensely stained neuron (arrowhead), more lightly stained cells with small somata (arrows) are scattered. Scale bars: 20 μm.
FIG. 5. Spatial profiles of dendritic Ca\textsuperscript{2+} transients evoked by somatic APs in inhibitory interneurons.

Dendritic Ca\textsuperscript{2+} transients evoked by somatic APs were measured in inhibitory interneurons, which were grouped into FS (A), LS (B), BS (C) and RSNP (D) cells primarily by spiking patterns generated by step current injection into the soma just after obtaining cells. *Left panels* show the representative traces of Ca\textsuperscript{2+} transients at the numbered dendritic areas of the reconstructed cells with 100 μm scale bar insets. Ca\textsuperscript{2+} transients were evoked by single or bursts of three or five APs at 20 Hz, shown in the *bottom rows* for each cell. An additional 10 APs at 20 Hz were applied to the FS cells. The *right panels* show the spatial profiles of dendritic Ca\textsuperscript{2+} transients evoked by single APs and bursts of five APs at 20 Hz for each cell subtype. Filled squares on the x-axis represent the length of the targeted dendrite, which was measured after the experiment.
FIG. 6. Effect of inhibition of A-type K⁺ channels on bAP-evoked dendritic Ca²⁺ transients in inhibitory interneurons.

To investigate the blocking effect of the A-type K⁺ channel on the dendritic backpropagation of somatic APs, 4-AP (1 mM) was added in ACSF after control imaging for single APs and a burst of three APs at 20 Hz. Ca²⁺ transients measured at each numbered location are specified in the representative reconstructed image in FS (A), LS (B), BS (C) and RSNP (D) cells. E: summary of the percent increase of three APs-evoked Ca²⁺ transients by the application of 4-AP in inhibitory interneurons. Proximal and distal dendrites were set at half distances of the mean dendritic length in each subtype (FS: 59 μm, LS: 48 μm, BS: 76 μm and RSNP: 88 μm). The number in parenthesis is the number of measured dendritic locations. *: p < 0.05, vs. proximal dendrites.
FIG. 7. Effect of inhibition of K⁺ channel blockers on AP shape and dendritic Ca²⁺ transients in inhibitory interneurons.

A: effect of 4-AP at high concentration (1 mM) on AP amplitude and width in inhibitory interneurons. AP amplitude was measured from the threshold to the peak of voltage deflection. AP width was measured at half amplitude of AP. The left panel shows the representative recordings in a FS cell. The right panel summarizes the effect of 1 mM 4-AP application on AP shape. B: effect of 4-AP at low concentration (0.1 mM) on AP amplitude and width in inhibitory interneurons. The left panel shows the representative recordings in a FS cell. The right panel summarizes the effect following the application of 4-AP. C: effect of TEA (1 mM) on AP amplitude and width in inhibitory interneurons. The left panel shows the representative recording in a FS cell. The right panel summarizes the effect by the application of TEA. D: effect of 4-AP (0.1 mM) and TEA (1 mM) on dendritic Ca²⁺ transients evoked by three APs in inhibitory interneurons. The left panel shows representative dendritic Ca²⁺ transients in a FS cell. The right panel summarizes the increase in Ca²⁺ transients from control by the application of 4-AP and TEA in four subtypes of inhibitory interneurons. *: p < 0.05, **: p < 0.01, vs. control.
FIG. 8. Nicotinic modulation of bAP-evoked dendritic Ca\(^{2+}\) transients in BS cells.

A: cholinergic modulation of bAP-evoked dendritic Ca\(^{2+}\) transients in pyramidal cells (Pyr) and inhibitory interneurons. After obtaining Ca\(^{2+}\) transients evoked by single APs (left column) and a burst of five APs at 20 Hz (right column), carbachol (CCh, 20 μM) was bath applied. OGB-1 (50 μM) was used as a Ca\(^{2+}\) dye in all measurements. Ca\(^{2+}\) transients in FS cells were evoked by five (left column) and ten APs (right column). B: representative bAP-evoked dendritic Ca\(^{2+}\) transients decreased by CCh (20 μM) application in a BS cell. The left panel shows the reconstructed image of a BS cell. The scale bar represents 100 μm. The right panel shows Ca\(^{2+}\) transients measured at the numbered dendritic locations in the left panel. Hyperpolarizing currents were injected after depolarizing current steps to suppress additional AP generation. C: nicotinic modulation of dendritic Ca\(^{2+}\) transients in BS cells. Data were analyzed in proximal (≤ 76 μm from the soma) and distal (> 76 μm from the soma) dendrites. Either atropine (10 μM, \(n = 5\)) or mecamylamine (30 μM, \(n = 4\)) was co-applied with CCh after measuring Ca\(^{2+}\) transients in the presence of CCh. Wash-out effect (\(n = 4\)) was obtained at the end of the experiment in some cells. **: \(p < 0.01\), ***: \(p < 0.001\), vs. control.