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Functional columns in the primate prefrontal cortex revealed by optical imaging *in vitro*

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Abstract

Although the functional column has been implicated in the dorsolateral prefrontal cortex (DLPFC) of primates, its dynamics and even existence are still uncertain. We performed optical recording with a voltage-sensitive dye (RH482) in brain slices obtained from the principal sulcal region (area 46) of macaque monkeys. Columnar activity was evoked by electrical stimulation of the middle layer (lower layer III or layer IV); this activity consisted of two components: probably action potentials and excitatory postsynaptic potentials. The width of the columnar activity was saturated when the current intensity of stimulation exceeded a certain level, that is, approximately 900 μ m at the peak with this intensity. The stimulation of different sites within the same slice activated different columnar activities with only slight overlaps. Furthermore, similar columnar activity appeared when a different site within the area with columnar activity was stimulated. These findings suggest that the primate DLPFC consists of functional columns formed by excitatory synaptic connections.

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Keywords: Functional column; Optical recording; Prefrontal cortex; Primate; Voltage-sensitive dye

1. Introduction

In primates, the dorsolateral prefrontal cortex (DLPFC) plays important roles in various cognitive functions such as thinking, planning, and decision making (Fuster, 1997). Moreover, neuroanatomical studies have demonstrated that the DLPFC has a columnar organization (Goldman-Rakic and Schwartz, 1982; Arikuni et al., 1988; Watanabe-Sawaguchi et al., 1991), and the column in the DLPFC has been considered a functional unit, representing various cognitive functions mediated by the DLPFC (Goldman-Rakic, 1995; Mountcastle, 1997). Consistent with this premise, studies applying the 2-deoxyglucose (2DG) method have demonstrated that local cerebral glucose use shows patch- or column-like bands in the principal sulcal region during working memory tasks (Matsunami and Kubota, 1983; Friedman and Goldman-Rakic, 1994). Indirect evidence for functional columns/modules in the DLPFC has also been obtained by a single-neuron study (Constantinidis et al., 2001) and pharmacological inactivation studies (Sawaguchi and Goldman-Rakic, 1994; Sawaguchi and Iba, 2001). However, the existence and dynamics of functional columns in the DLPFC is still uncertain.

A useful methodology for examining functional columns in the cerebral cortex is optical recording (Grinvald, 1985; Momose-Sato et al., 1999; Petersen and Sakmann, 2001). In particular, optical recording with voltage-sensitive dyes in brain slices is suitable for revealing the existence and dynamics of functional columns (Petersen and Sakmann, 2001). Therefore, we performed optical recording with a voltage-sensitive dye in brain slices from the principal sulcal region (area 46) of macaque monkey DLPFC. We obtained evidence that the primate DLPFC consists of functional columns, formed by excitatory synaptic connections.

2. Materials and methods

2.1. Slice preparation and experimental procedures

Animals were treated in accordance with the guide for care and use of laboratory animals (National Institutes of Health, Bethesda, MD, USA) and the guide for our institute (Hokkaido University, Japan).

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Six adult Japanese monkeys (Macaca fuscata, both sexes) were used. Each monkey was treated with ketamine hydrochloride (10 mg/kg, i.m.) and then deeply anesthetized with sodium pentobarbital (approximately 25 mg/kg, i.v.). A craniotomy was performed over the prefrontal cortex. Tissue blocks were then obtained from the principal sulcal region (area 46; Walker, 1940) of the DLPFC under aseptic conditions (Fig. 1A). As soon as possible, the brain tissue was cut coronally into 400-µm thick slices using a microslicer (DSK-3000W; Dosaka, Kyoto, Japan). Each coronal slice was incubated in artificial cerebrospinal fluid (ACSF, containing 125 mM NaCl, 26 mM NaHCO₃, 5 mM KCl, 1.2 mM KH₂PO₄, 1 mM MgSO₄, 2 mM CaCl₂, and 10 mM glucose, with a mix of 95% O₂/5% CO₂, pH 7.4) for at least 2 h at room temperature (approximately 25 °C) and then stained with the voltage-sensitive dve RH482 (NK3630, 0.1 mg/ml in ACSF) for 20 min. To wash out unbound dye, stained slices were perfused with ACSF for >30 min before starting the optical recording. The slice was mounted on a microscope (BX51WI; Olympus, Tokyo, Japan), and cortical layers (usually lower layer III or layer IV) of the principal sulcus area were stimulated electrically (single pulse, 0.3 ms in duration) using a bipolar tungsten electrode (100 µm outer diameter, <50 µm sharp tip). We identified each layer visually through the optics and were able to discriminate layer IV. The intensity of stimulation was usually 300 μ A because this was sufficient to induce significant changes in the optical signal (see Section 3). The perfused ACSF was warmed between 31 and 33 °C for recording sessions.

A 150-W tungsten-halogen lamp was lit using a stabilized DC power supply. Heat absorption and band-pass (700 \pm 30 nm) filters and a mechanical shutter were inserted in the light path between the source and a condenser lens. The neural activities elicited by electrical stimulation were converted to absorbance changes associated with membrane potential changes and were detected with metal-oxide semiconductor (MOS) image sensors (9 mm × 9 mm, 128 × 128 pixels) at a sampling rate of 1700 Hz (0.6 ms/frame) using an optical recording system (SD 1001; Fuji Film Microdevices, Tokyo, Japan). Images were obtained through a 4× objective lens (XLFLUOR 4×/340, NA = 0.28) and 0.6× relay lens. To reduce noise, eight consecutive stimuli were averaged, with each stimulus separated by an interval of 5 s. Each image (*I*) was subtracted from an initial control image (*I*₀) in each pixel. This difference image ($\Delta I = I - I_0$) was amplified 800 times by the optical recording system. The optical signal was converted into an 8-bit numerical value (i.e., 0–255) for quantitative analysis.

In some recording sessions (n = 5), the excitatory amino acid antagonist, kynurenic acid (KYN, 10 mM) or the sodium channel blocker tetrodotoxin



Fig. 1. Experimental procedures—(A) coronal slices (400- μ m thick) were obtained from the principal sulcal region (area 46) of the dorsolateral prefrontal cortex (DLPFC) of macaque monkeys. Each slice was stained with a voltage-sensitive dye (RH482). Cortical layers (usually, layer III or IV) were electrically stimulated. The optical signal was detected through an optical filter by an optical recording system with a 128 × 128 pixels MOS image sensor covering an area of 3.75 mm × 3.75 mm. (B) The method used to detect significant changes in the optical signal (absorbance change). An optical signal was detected by the image sensor. The mean and S.D. of the optical signal (8-bit value) for each area/pixel were calculated at a sampling rate of 0.6 ms for about 20 ms before the onset of stimulation as a control. When the optical signal at a given area at a given time (e.g., red closed square in the upper-left figure) after the onset of stimulation exceeded ± 2 S.D. of the control level, the optical signal was considered to have changed significantly. Different levels of change in the optical signal are indicated by different colors. PS, principal sulcus and WM, white matter.

(TTX, 1 μ M) was bath-applied to the brain slice to confirm that the optical signal represented excitatory neural activity. KYN is an ionotropic-glutamate receptor antagonist that nonspecifically blocks excitatory synaptic transmission mediated by excitatory amino acids in the mammalian cerebral cortex.

After optical recording, each slice was fixed with 2.5% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) and stored at 4 °C for at least 2 days. Each slice was mounted on a gelatin-coated glass slide and dried naturally at room temperature. After defatting, stimulation sites were confirmed under a stereoscopic microscope (SZX 12; Olympus). Images of recording sites were then recorded with a color CCD camera (CoolSNAP; Roper Scientific, Trenton, NJ, USA) and collected on a personal computer for quantitative analysis.

2.2. Data analysis

To quantify significant changes in the optical signals following electrical stimulation, the mean and standard deviation (S.D.) of the averaged measurement at each area (approximately 30 $\mu m \times$ 30 $\mu m)$ were calculated for approximately 20 ms before the onset of electrical stimulation as a control. When the optical signal (absorbance change) at a given area at a given time after the onset of stimulation exceeded ± 2 S.D. of the control level, the optical signal was considered to have changed significantly (Fig. 1B). When an optical signal associated with neuronal activation in a given area at a given time after stimulation exceeded 2 S.D. (i.e., P < 0.0456), neurons in that area were considered to be significantly activated. In the optical image, different colors indicate different levels of change in the optical signal. A significant increase in the optical signal associated with neuronal activation (i.e., >2 S.D.) was indicated by magenta and lighter colors (i.e., yellow for >4 S.D. and white for >6 S.D.), whereas a significant decrease in the optical signal was indicated by blue (<-2 S.D.) and black (<-4 S.D.). An insignificant change (between -2 S.D. and 2 S.D.) was indicated by green. Furthermore, to evaluate the adequacy of this statistical methodology using S.D.s, we used the paired t-test and the Mann-Whitney U-test; the measurement (the 8-bit value, 0-255) of a given area at a given time after stimulation was compared to the measurement of that area during the control period trial-by-trial. We confirmed that an optical signal that exceeded ± 2 S.D. of the control level was always statistically significant (P < 0.01). Therefore, only data from the 2 S.D.-method are described below. The width of the activation area was examined for peak activation after stimulation (approximately 20 ms after stimulation) and on the horizontal plane of the strongest activation region (usually middle layer III). In such a horizontal plane, the range that continuously exceeded 2 S.D. was defined as the width of the activation (see Fig. 4A).

3. Results

3.1. Evoked columnar optical signals in DLPFC slices

In total, 63 slices were obtained from the principal sulcal region (area 46) of both hemispheres of six macaque monkeys. When the middle layer (lower layer III or layer IV) of a slice was stimulated electrically, the evoked optical signals spread in a columnar fashion within the cortex (n = 25 sites in 20 slices). The stimulation of each layer in 43 slices evoked no clear optical signals (see Section 4); data from these slices were excluded from analysis.

In many cases (n = 20/25, 80%), the columnar signal spread into layers II and V/VI. In the remaining cases (n = 5/25, 20%), the signal spread ended within the upper layer III for the upward spread. The downward spread always reached at least the middle layer V. The signal showed two peaks: the first at approximately 6 ms after the onset of stimulation and the second at approximately 20 ms (see Fig. 1B). The signal was approximately 900 μ m in wide at the second peak and

was sustained for a relatively long period (>100 ms; Fig. 1B). The signal then gradually faded away and returned to the control level approximately 100–150 ms after stimulation. During this gradual fading of the optical signal, local and modular activation persisted in layer II/III and was sustained for a relatively long period (approximately 120 ms).

An example of the columnar spread following the stimulation of the middle layer (layer IV in this case) is shown in Fig. 2A. The optical signal spread in a columnar fashion from the stimulus site toward the cortical surface to the white matter, reaching layer II and lower layer V within ~ 10 ms after the stimulation. The optical signal faded gradually, accompanying a long-lasting modular signal in layer II/III. The horizontal spread was limited within a range of $\sim 900 \,\mu$ m during the signal spread, which was biased toward the ventral side of the stimulation site (i.e., the right side Fig. 2A).

When layers other than the middle layer (i.e., layers I, II, upper/middle layer III, layers V, VI or the white matter just below layer VI) were stimulated, the signals were less columnar compared to those following the stimulation at the middle layers. This was evident when the same intensity of stimulation (300 µA) was applied at layer II, upper/middle layers III-VI or the white matter just below layer VI in a similar vertical domain in the same slice preparation (Fig. 2B). Stimulation at layer IV induced a clear columnar signal that reached layer II and lower layer V with a width of ~ 1.2 mm in this case. In contrast, the stimulation of layer II, upper/middle layer III, and layer V resulted in the columnar spread of the signal, whereas its vertical spread was less than that with stimulation at layer IV. Stimulation at layer VI resulted in a horizontally diffused spread of the signal. Therefore, the columnar spread was most evident and greatest when the middle layer was stimulated. Thus, we focused on the data from such stimulations.

Referencing previous studies (Tanifuji et al., 1994; Sekino et al., 1997; Momose-Sato et al., 1999), the columnar optical signal detected here was considered to be mediated by action potentials and excitatory postsynaptic potentials. To confirm this, an excitatory amino acid antagonist, KYN was bathapplied to a brain slice in which a columnar optical signal had been clearly detected. Bath application of KYN (10 mM, for 10 min) completely suppressed the second peak of the columnar optical signal, although the first peak remained. When the slice was washed with ACSF, the columnar optical signal recovered gradually after 15 min and recovered completely after approximately 1 h (Fig. 3). Further, the first and second peaks of the optical signals disappeared during application of the sodium channel blocker TTX $(1 \mu M, data not shown)$. These results indicate that the columnar spread of the optical signal was mediated by both stimulus-induced action potentials and excitatory postsynaptic potentials in the DLPFC slice. Therefore, the columnar optical signal was mediated by the excitatory neural transmission; we define this as "columnar activity." In the vicinity of the fundus of the principal sulcus, evoked optical signals appeared in an oval or circular fashion, covering

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Fig. 2. Columnar spread of excitation following electrical stimulation in a coronal slice of the monkey DLPFC. (A) The slice was obtained from the principal sulcal area and the change in neuronal activity following stimulation was recorded optically in a 3.75 mm \times 3.75 mm square area of the slice (inset: L, lateral; M, medial; PS, principal sulcus; WM, white matter). Stimulation (300 μ A intensity) was applied to layer IV (red star) and changes in the optical signal relative to the average signal during the control period before the onset of the stimulation are indicated sequentially in 1.8 ms increments, from the onset of stimulation to 27 ms later. Different levels of changes in the optical signal are indicated by different colors: significant excitatory activity is indicated by cyan and lighter colors (i.e., yellow and white). Excitatory activity spread in a columnar fashion from the stimulated site to the surface and base of the cortex after stimulation. (B) The spread of activity at peak activation (18 ms after stimulation) following stimulation of different layers in the same slice. The slice was obtained from the principal sulcal area (inset: PS, principal sulcus; WM, white matter). Stimulation (300 μ A intensity) was applied to layer II, middle layer III, layers IV–VI, and the white matter. Clear columnar activity was induced when layer IV was stimulated. The right side of this figure shows a Nissl image. The black arrows indicate the position of the electrode used for stimulation. Stars indicate the sites of stimulation.

almost all of the cortical layers (see Fig. 6B; n = 4). This phenomenon appears to be consistent with previous anatomical findings for columns in this particular region (Goldman-Rakic and Schwartz, 1982), and we included such oval activity in our analysis of "columnar activity".

3.2. Columnar/unitary properties of columnar activity in the DLPFC

The columnar activity spread vertically toward the cortical surface and the white matter. At the second peak of activity, the

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Fig. 3. The effect of an excitatory amino acid antagonist, kynurenic acid (KYN, 10 mM), on columnar activity. KYN was bath-applied to a brain slice in which columnar activity had been clearly detected. The spread of activity at the second peak activation (18 ms after stimulation) following stimulation (300 μ A) of layer IV is shown before (pre), 10 min after application of KYN, and 15 min after washing with ACSF. The optical signal, which was obtained from four adjacent pixels of layer III in each condition, is shown at the bottom right. Con (black line), control condition; KYN (red line), the condition with KYN; wash (light blue line), the condition after washout of KYN. PS, principal sulcus; WM, white matter.

horizontal spread of the columnar activity appeared to be maximal. If the horizontal spread of columnar activity is limited as a "functional column", its horizontal spread should be saturated when the stimulus intensity exceeds a certain level. To examine this, different intensities of stimulation were applied at the same site (n = 4 sites in four slices) at which the stimulation induced columnar activities. We measured the horizontal spread of the columnar activity at the second peak (i.e., ~ 20 ms after stimulation) in lower layer III because the horizontal spread and amplitude of activity were usually the largest in this layer at the second peak (Fig. 4A). The horizontal spread increased when the stimulus intensity increased from 0 to 300 μ A and was saturated when the stimulus intensity exceeded 300 µA (Fig. 4B). Indeed, no significant difference in the average width was found between 300 and 700 µA stimulus intensity at four sites examined with these intensities (mean \pm S.D., $1237 \pm 236 \,\mu\text{m}$ vs. $1260 \pm 182 \,\mu\text{m}, n = 4$, paired *t*-test, t = 0.531, P = 0.632, NS; Fig. 4B). With a stimulus intensity of 300 µA at 25 stimulation sites, the mean width of columnar activity at its peak was $887 \pm 258 \ \mu\text{m}$. When "oval" columnar activity in the vicinity of the fundus of the principal sulcus was excluded, the mean width was $825 \pm 223 \ \mu m \ (n = 21)$.

The columnar activity observed here might be 'created' artificially by the electrical stimulation. To clarify this, we examined the relative horizontal distance of the stimulation site from the boundary of columnar activity, which was saturated at a stimulus intensity of 300 μ A (Fig. 5A). In this examination,

the horizontal distance of either the left or right border (closer to a stimulation site) of a column from its center was normalized as 1: when a stimulation site was at the center or a border of a column, its relative distance was 0 or 1, respectively. Thus, if columnar activity was created artificially by electrical stimulation, the stimulation site should always be near the center of the columnar activity, and the relative distance of the stimulation site should converge in the vicinity of 0 in any columnar activity. The relative distance, however, varied considerably among columnar activities (Fig. 5B). When the relative distance among columnar activities was arbitrarily divided into five ranges, no significant difference was found in the distribution of stimulation sites across these five ranges $(\chi^2$ -test, $\chi^2 = 5.498$, d.f. = 4, P = 0.240, NS; Fig. 5C). These results suggest that the columnar activity is of an actual column, rather than an artificial one created by electrical stimulation.

To further examine whether the columnar activity reflected the activity of an actual column, we applied the stimulation (300 μ A) at different sites in the middle layer (lower layer III or layer IV) in the same slice (n = 3 slices). If the columnar activity is of an actual column, different columnar activities should result when the stimulation is applied beyond the boundary of a certain columnar activity. We found that this was the case (Fig. 6A(1and 2/3), and C(1–3)), although different columnar activities in the same slice had slight overlaps. In contrast, when stimulation was applied at a different site within a column, similar columnar activity was observed at each



Fig. 4. Evidence of limited width and vertical boundaries of columnar activity. (A) Columnar activity at the peak of activation (18 ms after stimulation) and spatial spread of activity in horizontal and vertical planes. The significance level (i.e., $>\pm 2$ S.D.) at each pixel along the plane is indicated by the green line. (B) Saturation of columnar activation with high current intensities. The width of columnar activation at the peak of activation (18 ms after stimulation) following stimulation with different current intensities is plotted against the current intensity. The stimulation was applied at the same site in the same slice. The width of the columnar activity was saturated at about 1200 μ m when the current intensity exceeded a certain level (i.e., >300 μ A). NS, not significant.

stimulation site (Fig. 6A(2 and 3) and B(1 and 2)), also providing evidence that the columnar activity reflected an actual functional column.

4. Discussion

4.1. Methodological considerations

We applied optical recording with a voltage-sensitive dye (RH482) to brain slices of the monkey DLPFC to examine population activities of neurons in this area *in vitro*. With this methodology, we succeeded in visualizing the columnar activity in the principal sulcal cortex (area 46), which was evoked by the electrical stimulation of the middle layer (lower layer III or layer IV) and spread into layers II and V/VI in most



Fig. 5. The relative horizontal distance of each stimulation site from the dorsal perpendicular boundary of columnar activity. (A) The method for calculating the relative horizontal distance of the stimulation site in each column. The center of a column was set as 0 and the border that was closest to the stimulation site as 1. (B) The relative distances of stimulation sites from the center of a column (n = 25). (C) The distribution of stimulation sites across seven ranges of the relative horizontal distances; the relative horizontal distance was arbitrarily divided into seven ranges. The distribution was not significantly different across these ranges (χ^2 -test, $\chi^2 = 5.498$, d.f. = 4, P = 0.240, NS).

cases. To our knowledge, this is the first *in vitro* optical recording of monkey neocortical areas using a voltage-sensitive dye, except for brief reports (e.g., Nakamura and Sawaguchi, 1995). However, the number of successfully imaged slices was small (20 of 59 slices, 34%). Possible reasons for this are (1) intrinsic connections within the cortex may have been damaged when the brain tissue block was cut; (2) the incubation medium and condition may not have been suitable for the slices; (3) the time of incubation before the start of recording may have been too long. However, these problems do not seem to be critical because significant optical signals were detected in any slices that had been obtained from any tissue blocks. Therefore, we could not clarify the major reason(s) for the low success of imaging. There are relatively few reports of voltage-sensitive dye imaging in monkey brain slices, and we had difficulty in



Fig. 6. Separation and boundaries of columnar activity. Stimulation (300 μ A intensity) was applied to two or three different sites in the same slice, and the activity at the peak (18 ms after stimulation) following stimulation is shown. (A) The spread of activity following stimulation at three different sites. Superimposed contours of the activation area for the different stimulation sites are illustrated in (4). Stars indicate the sites of stimulation, and the numbers (1–3) and colors of stars correspond to those in the contour of the activation area shown in (4). The arrow indicates the shade of the electrode. (B) When stimulation was applied at a different site within the column, the same columnar activity was observed. (C) When stimulation was applied at different sites outside a column, different columnar activity was observed at each stimulation site. PS, principal sulcus; WM, white matter.

obtaining and maintaining the slices. Further studies are required to develop methodological techniques for obtaining and maintaining monkey brain slices in which significant optical signals can always be detected.

A number of imaging techniques have enabled the visualization of the functional organization or architecture of the cerebral cortex of non-human primates. One useful technique is intrinsic imaging *in vivo*, which has been used to elucidate the functional anatomy of the primate visual system (Vnek et al., 1999; Tsunoda et al., 2001; Heider et al., 2005). This methodology is fundamentally different from the voltage-sensitive dye method; intrinsic imaging detects light scattering,

reflecting changes in capillary blood flow with neural activity, whereas voltage-sensitive dye imaging detects membrane potential changes of neurons. Therefore, voltage-sensitive dye imaging is linked to neural activity and is suitable to determine not only the cortical functional organization, but also the spatiotemporal dynamics of neuronal activity in the cerebral cortex.

In non-primate mammals, voltage-sensitive dye imaging has been used widely to determine the spatiotemporal dynamics of neuronal activity in the cerebral cortex (Tanifuji et al., 1994; Kubota et al., 1997; Sekino et al., 1997; Petersen and Sakmann, 2001). Our methodology was similar to that of these previous

studies, although there were some differences from it. First, the spatial resolution of our recording was relatively low $(3.75 \text{ mm} \times 3.75 \text{ mm})$ because of the relatively low magnification objective lens (4× objective lens, NA = 0.28; $0.6 \times$ relay lens). However, this resolution was suitable for recording population activity from large regions in the cortical slice and for examining the spatiotemporal dynamics of possible activity in monkey DLPFC columns with relative large dimensions (>100-µm wide; Goldman-Rakic and Schwartz, 1982; Arikuni et al., 1988; Watanabe-Sawaguchi et al., 1991). Second, we recognized that the evoked optical signals had a relatively low signal-to-noise ratio compared to some previous studies with cortical slices and RH482 (Tanifuji et al., 1994; Kubota et al., 1997; Sekino et al., 1997; Petersen and Sakmann, 2001). This was because of limitations of our technique, the subject used (macaque monkeys), and/or the brain area examined (the DLPFC). However, this did not pose a problem because we used the "2 S.D. method", which allowed us to detect significant optical signals at a <5% significance level (i.e., P < 0.0456). Third, the stimulation intensity that induced clear and sufficient change in optical signals (i.e., 300 µA) was large compared to that applied in some previous studies (10-100 µA; Sugitani et al., 1994; Takagi et al., 2002). This was also not important because the spatial spread of the optical signal gradually increased with the stimulation intensity. In addition, it eventually saturated at a certain width (i.e., approximately 900 μ m) when the intensity exceeded 300 μ A. Moreover, the spatial spread with a 300-µA stimulus was columnar (except some cases in the vicinity of the fundus of the principal sulcus), rather than circular, although the current field spread would be circular, and the profile/shape of the spatial spread of optical signal differed by layer when stimulated with 300 μ A.

Previous studies with voltage-sensitive dyes have demonstrated that stimulus-induced changes in optical signals in cortical slices have two components that are mediated by action potentials and excitatory postsynaptic potentials (Kojima et al., 1999; Momose-Sato et al., 1999). In our study, the stimulusevoked optical signals also consisted of two components. The findings with bath-applications of KYN and TTX were also similar to those of previous studies, indicating that these two components were mediated by stimulus-induced action potentials and excitatory postsynaptic potentials.

Therefore, our methodology was sufficient and effective for examining population activity of neurons in the monkey DLPFC. We conclude that the electrical stimulation in the middle layers (lower layer III or IV) of the monkey DLPFC slice induced columnar activity consisting of action potentials and excitatory postsynaptic potentials.

4.2. Columnar activity as a unitary activity of a "functional column"

The columnar activity found here is suggested to represent the unitary activity of a population of DLPFC neurons, based on the following findings. First, the width of the columnar activity was saturated when the stimulus intensity exceeded a certain current intensity (i.e., 300μ A). Second, the relative horizontal distance of the stimulation site from the perpendicular boundary of the columnar activity varied by site. Third, different columnar activities with only slight overlaps were induced by stimulation at different sites in the same slice. Fourth, similar columnar activity appeared when a different site within the area with columnar activity was stimulated. Further, clear columnar activity was evoked by stimulating the middle layer (lower layer III or IV) in the DLPFC, which consisted of action potentials and excitatory postsynaptic potentials (see above). Given that the electrical stimulation corresponds to an excitatory input to the cortical layer, columnar activity would be activated by excitatory input to the middle layer in the DLPFC in vivo. Therefore, the columnar activity appears to be a unitary activity mediated by excitatory synaptic transmission of a population of neurons, and this population may form a "functional column" that is activated by an excitatory input to the middle layer.

The columnar activity did not spread horizontally beyond a certain width, even with strong electrical stimulation (e.g., 700 μ A, which was more than twice the intensity sufficient to induce columnar activity). This is evidence of the unit-nature of the columnar activity (see above), but might be inconsistent with the anatomical finding that neurons in the monkey DLPFC have abundant horizontal collaterals (Melchitzky et al., 1998, 2001). A simple possible interpretation for this discrepancy is that horizontal collaterals were cut or damaged during slice preparation. However, this is unlikely because the stimulation of layer VI induced substantial horizontal spread of activity (see Fig. 2B), the width of horizontal spread of activity varied by stimulation site in different layers along a similar perpendicular axis (see Fig. 2B), and two or three columnar activities with similar widths for which the perpendicular boundaries overlap slightly could be induced by stimulating the same slice (Fig. 6A and C). It is difficult to imagine that collaterals were cut or damaged just along such boundaries. This notion is also strengthened by the study of Tanifuji et al. (1994), who used voltage-sensitive dye imaging in visual cortical slices of rats. Electrical stimulation of the slice induced vertical/columnar activity with a limited horizontal spread, similar to our results, but remarkable horizontal spread of activity appeared when GABAA receptor-mediated inhibition was blocked (Tanifuji et al., 1994). Similar blocking of the extended spread of horizontal excitation by the GABA_A receptor-mediated inhibition has been observed in slices of rat barrel cortex in a voltage-sensitive dye imaging study (Petersen and Sakmann, 2001). In a preliminary study, we also obtained similar data in monkey DLPFC slices; substantial horizontal spread of activity appeared following the bath-application of a \mbox{GABA}_A receptor antagonist (Hirata and Sawaguchi, 2004). Therefore, the horizontal collaterals are considered to be maintained in our recording condition, and the limitation of horizontal spread of activity is difficult to explain by their being cut or damaged. Rather, it is more likely that GABA-ergic inhibition limits the horizontal spread of activity (see Petersen and Sakmann, 2001), and lateral inhibition by GABA-ergic interneurons may work between neighboring functional columns in the DLPFC.

Our findings seem to be consistent with anatomical findings for the columnar organization of corticocortical connections in the primate DLPFC (Goldman-Rakic and Schwartz, 1982; Bugbee and Goldman-Rakic, 1983; Arikuni et al., 1988; Watanabe-Sawaguchi et al., 1991; Levitt et al., 1993). The dimensions of columnar activity in our study (approximately 900-µm wide or approximately 800 µm in wide excluding data around the fundus of the principal sulcus) was comparable to that for cortical columns, including association and callosal columns, detected using anatomical methods in coronal sections of the DLPFC (about 600-800 µm in width; Goldman-Rakic and Schwartz, 1982; Bugbee and Goldman-Rakic, 1983; Arikuni et al., 1988; Watanabe-Sawaguchi et al., 1991). The association and callosal columns in the principal sulcus are interdigitated (Goldman-Rakic and Schwartz, 1982), and their features are comparable to those of the columnar activity in the same region. Furthermore, columnar activity in the vicinity of the fundus of the principal sulcus occurred in a relatively wide oval, which is also consistent with the anatomical finding that cortical columns in these regions are oval and relatively wide (Goldman-Rakic and Schwartz, 1982). Therefore, it is likely that the columnar activity is that of cortical columns, and they correspond to functional columns in the DLPFC, although we could not clarify the anatomical substrate involved in the columnar activity.

4.3. Possible activity of functional columns in the DLPFC

As described above, clear large columnar activity appeared when stimulation was applied at the middle layers (lower layer III or layer IV); the vertical spread of activity following the stimulation of other layers was narrower/less. This is likely due to the major input system in the DLPFC. The DLPFC receives dense afferents from sensory association cortices (e.g., the posterior parietal and inferotemporal cortices), which terminate predominantly in the middle layer (lower layer III or layer IV; Felleman and Van Essen, 1991). Furthermore, the middle layer of the DLPFC receives dense afferents from the mediodorsal thalamus (Giguere and Goldman-Rakic, 1988; Erickson and Lewis, 2004). These afferents into the middle layer would induce excitatory columnar activity expanding to superficial (layer II/III) and deep (mainly layer V) layers. Efferent connections of the DLPFC with other cortical areas, such as the premotor cortex and frontal eye field originate predominantly from pyramidal neurons in the superficial (mainly layer II/III) and deep (mainly layer V) layers (Arikuni et al., 1988; Watanabe-Sawaguchi et al., 1991), and those with subcortical structures, such as the mediodorsal thalamus and caudate nucleus, originate mainly from neurons in the deep layers (mainly layer V; Arikuni and Kubota, 1986; Giguere and Goldman-Rakic, 1988; Yeterian and Pandya, 1994; Zikopopulos and Barbas, 2007). Therefore, a major input into the middle layer would activate fully functional columns in the DLPFC to send outputs to other cortical areas and subcortical structures, whereas input into other layers less activates them to send outputs to more restricted brain regions. Such arrivinginput-dependent operation of the functional columns in the DLPFC could serve as a neural substrate for various or multiple cognitive functions mediated by the DLPFC.

In summary, our findings provide novel evidence of functional columns in the DLPFC, as well as some clues to their operation in relation to the cognitive functions and neural dynamics of this area. However, several issues remain to be addressed, particularly the anatomical substrate of the columnar activity. Finally, the present methodology used for the monkey DLPFC may be applicable for the examination of other issues such as the roles of transmitters and receptors in functional columns (Hirata and Sawaguchi, 2002, 2004) and their developmental processes and mechanisms (Emoto and Sawaguchi, 2005) in the primate DLPFC.

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