Odor-Driven Activity in the Olfactory Cortex of an In Vitro Isolated Guinea Pig Whole Brain With Olfactory Epithelium

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Ishikawa, Takahiro, Takaaki Sato, Akira Shimizu, Ken-Ichiro Tsutsui, Marco de Curtis, and Toshio Iijima. Odor-driven activity in the olfactory cortex of an in vitro isolated guinea pig whole brain with olfactory epithelium. J Neurophysiol 97: 670–679, 2007. First published July 26, 2006; doi:10.1152/jn.01366.2005. We developed a new technique to isolate a whole guinea pig brain with an intact olfactory epithelium (OE) that enables us to access the ventral surface of the brain including olfactory areas with ease during natural odor stimulation. We applied odors to OE and confirmed that odor-induced local field potentials (LFPs) could be induced in olfactory areas. In the olfactory bulb (OB) and the piriform cortex (PC), odor-induced LFPs consisted of a phasic initial component followed by a fast activity oscillation in the beta range (20 Hz). To understand the neural mechanisms of odor-induced responses especially in the anterior PC, we analyzed odor-induced LFPs, together with unit activity data. We confirmed that the initial component of odor-induced response has a characteristic temporal pattern, generated by a relatively weak direct afferent input, followed by an intra-cortical associative response, which was associated with a phasic inhibition. The beta oscillation might be formed by the repetition of these network activities. These electrophysiological data were consistent with the results of previous studies that used slice or in vivo preparations, suggesting that the olfactory neural network and activities of the brain are preserved in our new in vitro preparation. This study provides the basis for clarifying the sequence of neural activities underlying odor information processing in the brain in vitro following natural olfactory stimulation.

INTRODUCTION

The isolated and perfused whole brain preparation has been developed by the group of Rodolfo Llinas to investigate complex multisynaptic neuronal networks in vitro, which is difficult to perform with slice preparations (Llinas and Muhlethaler 1988a,b; Llinas et al. 1981; Muhlethaler et al. 1993). As this preparation enables the direct access to the ventral surface of the brain, it has been used for investigation of olfactory systems in vitro with electrophysiological (Biella et al. 1996a,b; de Curtis et al. 1991; Gnثkovsky and de Curtis 2006; Uva and de Curtis 2003, 2005) and optical recordings (Biella et al. 2003; de Curtis et al. 1999). However, in isolated whole brain preparation used in these previous studies, all sensory organs were removed in the process of isolation, making it impossible to record neural responses evoked by natural sensory stimulation. To record natural odor-induced response in this preparation, we developed a new technique to prepare an isolated whole brain preserving the olfactory epithelium (OE).

The primary olfactory cortex, particularly the piriform cortex (PC), is the major target of the fiber projection from the olfactory bulb (OB) principal cells. PC has a clear three-layer structure that can be well discriminated by cytoarchitecture and fiber connections (Neville and Haberly 2004). In layer I, incoming fibers form synapses on dendrites ascending from pyramidal cells in lower layers. Layer I can be further divided into two sublayers (Ia and Ib). In layer Ia, afferent OB fibers of the lateral olfactory tract (LOT) form excitatory synapses on ascending dendrites of pyramidal cells and inhibitory interneurons, which in turn form synapses with pyramidal cells (feed-forward inhibition) (Kapur et al. 1997). In layer Ib, association fibers from pyramidal cells within PC and those from other olfactory or nonolfactory areas form excitatory synapses on ascending dendrites of pyramidal cells. Layer II and III are compact layers of pyramidal cell bodies. The PC displays a gradient of cell density in its structure from surface to depth. In layer II, inhibitory interneurons receive excitatory input and form synapses with cell bodies of pyramidal cells (feedback inhibition) (Biedenbach and Stevens 1969; Haberly 1973; Satou et al. 1983). This highly ordered neuronal system allows the visualization of spatial and temporal sequences of extracellular current sinks and sources associated with membrane currents in dendrites by current source-density (CSD) analysis of laminar field potential profiles (Ketchum and Haberly 1993b; Mitzdorf 1985).

Neural activity in the mammalian olfactory system is known to be modulated by two types of fast oscillations, defined by their frequencies as gamma (50–100 Hz) and beta (15–40 Hz). The gamma oscillation has been described in field potential recordings from the OB and PC of awake animals (Boeijinga and Lopes da Silva 1988; Bressler 1984; Freeman 1959, 1978; Kay and Freeman 1998) and of animals anesthetized with urethan (Adrian 1950; Kashiwadani et al. 1999; Mori et al. 1992). The amplitude and frequency of this oscillation may reflect previous olfactory experience and the behavior of the animal (Boeijinga and Lopes da Silva 1988; Bressler 1984; Chabaud et al. 2000; Freeman 1960; Freeman and Schneider 1982; Kay and Freeman 1998). Early reports refer to another oscillation, at about half the frequency of the gamma oscillation, that is more prominent in PC than in OB (Bressler 1984; Freeman 1959). Recently, a 15- to 35-Hz beta oscillation has
been described in OB, PC, entorhinal cortex, and dentate gyrus. Such activity can be induced by olfactory stimulation with certain organic solvents or components of predator secretions (Chapman et al. 1998; Vanderwolf 1992; Zibrowski and Vanderwolf 1997). On the other hand, Neville and Haberly (2003) revealed that the gamma and beta oscillations could be induced by odorants in a concentration-dependent manner. Although the precise neural mechanism of both of these oscillations is unknown, oscillatory neural activity is thought to be essential for coding or processing of olfactory information.

Here we examine the potentials of the isolated whole brain preparation with OE by analyzing the neural activity induced by natural odor stimulation in the olfactory processing areas, especially in the anterior PC (APC). Taking advantage of this in vitro isolated whole brain preparation, which enables stable extracellular recording, we present a detailed description of odor-induced local field potential in PC and discuss possible neural mechanisms underlying the response.

**METHODS**

**Isolation of the whole brain with the olfactory epithelium preserved**

The technique of the isolated whole brain preparation with OE preserved is a modification of that previously developed by Llinas and his colleague (Llinas et al. 1981; Llinas and Muhlethaler 1988a;b; Muhlethaler et al. 1993; de Curtis et al. 1998). To maintain OE, the procedure of isolation of the brain was partly changed from the original method. Experiments were performed on young male Hartley guinea pigs (160–250g, postnatal days 14–28). The care and treatment of the animals were in accordance with National Institutes of Health guidelines and the Guidelines for Animal Care and Use published by our institute. After Nembutal injection (pentobarbital, 40 – 60 mg/kg ip), the thorax was opened quickly and cold artificial plasma solution [pH 7.4; containing (in mM) 88 NaCl, 2.3 KCl, 26 NaHCO₃, 1.3 MgSO₄, 2.4 CaCl₂, 1.2 KH₂PO₄, 28 CH₃CH(OH)COONa, 15 glucose, 5 HEPES, and 0.4 thio urea and 10% dextran 40000 MW] saturated with a 95% O₂-5% CO₂ gas mixture at 10°C was perfused at a flow rate of 40–50 ml/min through the ascending aorta for 3 min. Then transcardial perfusion was stopped, the head was severed from the body, and the skin over the skull and the periostium was dissected away. The rostral part of the nose including the vomeronasal organs was dissected at the anterior edge of the olfactory turbinate. The upper jawbone was cut, and the nasal bone, the premaxillar bone, and the olfactory turbinates on the left side were removed. The olfactory turbinate was separate from underlying premaxillar bone by using a fine razor blade. The dorsal surface of the cerebellum and spinal cord was exposed. The spinal cord was transected between C₂ and C₃. The parietal, occipital and a part of frontal bones were removed and the dorsal surface of the brain without OB was exposed. A part of the remaining frontal bone was removed to expose the LOT. The head was turned upside down and arteries and nerves on the ventral surface were cut. Finally, the brain was separated by cutting the skull around OB with meticulous care. After isolation, the brain and the preserved OE was transferred to a recording chamber at 14°C with the ventral side up and loosely fixed with strings at the spinal cord and the olfactory turbinate. Then the brain was perfused through the basilar artery with the same oxygenated artificial plasma solution utilized for the intracardiac perfusion, at 6.0 ml/min rate. The preparation was slowly warmed ≤27°C at the rate of 1°C /6 min. Low temperature was maintained as substitute for anesthesia. Oxygenized Ringer solution was constantly supplied into the nasal cavity from a tube inserted between the septum and the medial turbinate of the nose, to keep the olfactory epithelium vital. The most caudal part of the inferior portion of the nasal epithelium was slightly opened to allow air bubbles come out of the nasal cavity.

**Electrophysiology**

Local field potentials (LFPs) were recorded with glass micropipettes filled with 2 M NaCl (DC resistance was 1–3 MΩ) and a tungsten multi-channel electrode (homemade, impedance of each channel was 300 kΩ at 200 Hz, 20 nA). Each recording channel was arranged on-line at 100-μm intervals. The electrical signals were passed through a preamplifier (JB-101J, Nihon Kohden) and low-pass filtered at 3 kHz (MEZ-8300, Nihon Kohden) and amplified 1,000 times (AVM-11, Nihon Kohden), stored on a personal computer through a 12-bit A/D converter (9111DG, Japan Data System) at a 2- to 10-kHz sampling rate. Extracellular recordings of spike discharges were conducted with electropolished and glass-insulated Eligily (Rocky Mountain Orthodontics) electrodes. Signals were passed through a preamplifier, amplified 10,000 times, band-pass filtered (150 Hz to 1 kHz; MEG-1200, Nihon Kohden), and digitally stored with a DAT recorder (PC204Ax, SONY). Spike isolation was performed offline using Multi-Spike Detector (ALPHA OMEGA Engineering). All recordings were monopolar, referenced to Ag⁺/AgCl ground wire placed in recording chamber. Electrodes were penetrated approximately perpendicular to the brain surface. To test cortical activities and monitor the tip position of the recording electrode, LFPs were recorded in response to electrical stimulation (square pulse: 5–20 μA, 100 μs) to the LOT through a bipolar tungsten electrode. In OB, LFPs were recorded between the glomerular layer (GL) and mitral cell layer (MCL), and spike discharges were recorded from MCL. In APC, LFPs were recorded from layer I–III, and spike discharges were recorded from layer II. In the early stage of this study, LFP recordings over different depths were performed with a four-channel electrode the channel tips of which were positioned at different depths at 100 μm intervals. LFPs were repeatedly recorded at partly overlapping different depths (0–300, 200–500, 400–700, 600–900, 800–1,100 μm) in different sessions. If the shape of such traces overlaid, traces from multiple sessions were combined to form a laminar profile over the depth of 0–1,100 μm in 100-μm intervals. Twelve-channel electrodes (homemade) were also utilized to record LFPs simultaneously at the depth of 0–1,100 μm in 100-μm intervals. To perform the CSD analysis, second derivative was calculated at each recording depth (Ketchum and Haberly 1993b). For cortical micro-stimulation to the rostral part of APC (APCvr) and the anterior olfactory cortex (AOC), bipolar tungsten electrodes were positioned at cell body layer of those cortices. The stimulation was a 100 μs square current pulse of 50–100 μA. To confirm the recording and stimulating positions, each site was marked by passing positive current to the Elgiloy electrode (10 μA, 5 s) and the preparation was fixed with a mixture of 2% potassium ferrocyanide and 10% formalin after experiments. Coronal sections of the brain (100 μm thick) were made on a freezing microtome (SM2000R, LEICA) and stained with thionine.

**Odor stimulation**

To activate various types of olfactory receptor and maximize the response in OB and following olfactory cortices, we used lavender oil (Spectrum Chemical) as odor stimulation. For odor stimulation, odorant solutions in which various volume of lavender oil in the range from 0.01 to 1,000 ppm was mixed were applied. Because lavender oil is hydrophobic, 0.1% (volume/volume) dimethyl sulfoxide was added in Ringer solution. In this solution, upper limit of the volume of lavender oil soluble in Ringer solution was around 100 ppm. When >100 ppm lavender oil was mixed in Ringer solution, oil droplets were observed near the surface of the solution. To the experimenter, odorant solutions including >10 ppm lavender oil smelled very sharp and even noxious, and <0.01 ppm were too weak to be detected.
For application of odor stimulation, a double-barrel Teflon tubing was inserted into the nasal cavity (Fig. 1A). Odorants dissolved in Ringer solution were applied to the OE through one of the paired tubing by gravity-driven flow. The other one of the paired tubing was filled with Ringer solution to wash out the odorants after the odor stimulation. When coming in contact with the olfactory membrane surface, the odorant substance first diffuses into the hydrophilic mucus that covers the cilia and then it binds with receptor proteins in the membrane of each cilium. Therefore it is assumed that applying odorants as aqueous solution can induce the physiological activation of olfactory receptors, followed by the neural response in OB and in cortical olfactory areas. Flow rate of odorants solution was 0.1 mm/ms at the point where 1 mm distant from the tip of the inserted tube, and flow volume was 60 mg/s. The duration of odor stimulation was 1–2 s, and interstimulus interval (ISI) was >3 min to minimize the desensitization and habituation of olfactory receptor cells. As odorant solutions flowing out from the nose may possibly have toxicity to the brain, bath solution was aspirated ahead of the nose for 1 min just after odor stimulations.

RESULTS

Responsiveness of the isolated whole brain with OE to odor stimulation

The first objective of this study was to develop a new technique to prepare an isolated whole brain preparation preserving OE, to enable recording of natural odor-induced responses in all olfactory areas. The ventral view of the preparation is shown in Fig. 1A. Electrical stimulation to LOT (not shown) evoked an antidromic response in OB, a typical triphasic LFP in PC, and a polysynaptic LFP in the lateral entorhinal cortex (LERC). These results indicate that the preparation maintains a responsiveness for synaptic inputs.

To examine the responsiveness of this preparation for odor stimulation, we applied odorants dissolved in ringer solution into the nasal cavity and recorded electrical responses simultaneously with glass microelectrodes placed on the various sites of the ventral surface of the brain (Fig. 1A). Odor-induced receptor potential from OE, and LFPs from OB, dorsal part of the anterior PC (APCd), posterior PC (PPC) and LERC were recorded as shown in Fig. 1B. To maximize the response, mixture odorant (lavender oil, 100 ppm) was used as odor stimulation for 1 s. To minimize the habituation or sensitization, ISI was set to >3 min. As there was no cyclic motion noise derived from aspiration and heart-beat in this preparation, stable recording was possible for ~5 h. When odor stimulation was applied, a receptor potential was recorded at the surface of OE. This result suggested the olfactory sensory neurons maintained their sensitivity for odorants. In OB and olfactory cortices, characteristic LFPs lasting >1 s were observed. In the OB and APCd, a transient beta oscillation at 20 Hz was observed (n = 12/20). The amplitude spectra of the oscillation in OB and APCd are shown in Fig. 1C. Both oscillations had peak amplitude at 20 Hz; only in APCd, 10-Hz oscillation had relatively large amplitude. The beta oscillation was also observed in PPC and LERC (n = 2/12, data not shown). These oscillatory fluctuations built up gradually after 200–300 ms from the onset of field potential changes in each region. As a result, it is assumed that the isolated whole brain preparation with OE maintains responsiveness to odor stimulations.

To examine the concentration dependency of odor-induced response, we made simultaneous recording in OE, OB, and
APCD with different concentration of lavender oil \((n = 8, \text{Fig. 2})\). In all cases, the odor-induced responses increased in a concentration-dependent manner. The peak amplitude of EOG was approximately proportional to the logarithm of odor concentration within the range from 0.01 to 100 ppm. In OB, LFPs grew larger and the oscillatory fluctuation became clearer with the increase of odor concentration. In APCD, the odor stimulation of relatively low odorant concentration induced only a transient potential changes, whereas that of relatively high concentration induced the characteristic LFPs including the oscillatory potential changes at beta range as well as in Fig. 1. The duration of the oscillatory fluctuations gradually increased \(\approx 100 \text{ ppm}\). Odor stimulation of hydrophilic odorants at far higher concentration also induced the same typical responses in each region (data not shown). Regardless of the odor concentration, the waveforms of odor-induced LFPs in APCD were quite different from those of LFPs evoked by electrical stimulation to OB or LOT. Control stimuli (Ringer solution used as the solvent of odorants) induced very weak EOG in OE and LFPs in OB, but no clear LFPs were observed in APCD.

**Odor-induced local field potential in APC**

The second objective of this study was to describe the network activity involved in the odor-induced response by taking advantage of in vitro preparation, which enabled stable recording of neural activity from deep structures of the brain. As shown in Figs. 1B (trace 3) and 2, in the superficial layer of APCD, an odor stimulation generally induced a small transient surface-negative potential change, which was followed by a transient large surface-positive potential change. Although small negative potential change is not very clear in trace 3 of Fig. 1B, it is obvious in the top four traces of a laminar profile in Fig. 3B and the traces of Fig. 6A. The transient, large surface-positive potential change was followed by oscillatory potential changes. The initial component of the odor-induced LFP change was quite different from that induced by an electrical stimulation to LOT, which was composed of two surface-negative potentials, as shown in Fig. 3A (top). The laminar profile of LFPs obtained by LOT stimulation in this preparation (Fig. 3A) was very similar to those described in the previous studies (Biella et al. 1996a,b; Ketchum and Haberly 1993b). Then, we compared the initial component of the odor-induced response with LOT-evoked response sequentially recorded at identical cortical depths. To figure out the synaptic events underlying these responses, we made contour plots of CSD laminar profiles for both of responses (Fig. 4, A and B, bottom). For odor-induced response, the CSD profile (Fig. 4B, top) and the CSD contour plot of the period included between the filled and open triangles in Fig. 3B traces are shown. Triangles above the CSD profile in Fig. 4B indicate the same time points.

The laminar profile of LFPs for LOT stimulation (Fig. 3A) and its CSD contour plot (Fig. 4A) had the same characteristics as described in the previous studies (Biella et al. 1996a,b; Ketchum and Haberly 1993a,b), which explained their underlying neural mechanisms as follows. In response to LOT stimulation, strong afferent input evoked a monosynaptic excitatory postsynaptic current (EPSC) in layer Ia, and then followed by a strong disynaptic EPSC in layer Ib (association input), resulting in the generation of current sinks in layer Ia.
and Ib, as shown in Fig. 4A. Sinks and sources and their intensities are represented by contour plot. These active inward currents are accompanied by a passive outward current at deep layer, which is observed as a current source in Fig. 4A (dashed lines). The EPSCs are followed by an inward membrane current sink in layer II–III, presumably due to the inhibitory postsynaptic current (IPSC) generated by the depolarizing Cl\(^-\)-mediated inhibitory postsynaptic potential (IPSP) at layer II–III (GABAergic input) induced by inhibitory interneurons.

The CSD contour plot for odor-induced response showed somewhat different sink/source pattern (Fig. 4B, bottom). A continuous sink with amplitude peaks coincident with large potential deflections was observed at layer Ib but not in layer Ia. The lack of sink in layer Ia may be due to relatively weak afferent inputs evoked by natural odor stimulation compared with those evoked by electrical stimulation (see DISCUSSION). As PC neurons project associative fibers to a large number of pyramidal cell dendrites in layer Ib in PC itself (and other olfactory areas), an amplified input to layer Ib through this reverberating associative circuit could be responsible for the sink.

To further analyze the characteristics of this CSD, we divided the initial component of the odor-induced response into three periods (Fig. 4B, broken vertical lines). During period 1, a sink was observed in layer Ib, and sources were observed at layer Ia and II. This pattern may reflect the association input to layer Ib. The sink/source pattern changed clearly during period 2. The reinforcement of the Ib sink was coupled to additional sinks in layer II and III. As a sink in layer II is formed by depolarizing Cl\(^-\)-mediated IPSP in response to LOT stimulation (Fig. 4A), the layer II CSD pattern may reflect inhibitory process in this area (see DISCUSSION). In period 3, a source was observed at layer Ia, and sinks were observed in layers Ib, II, and III. As this pattern is similar to that in the former half of period 2, it can be assumed that the similar synaptic events are occurring in this period. Shunting inhibition may have been caused by feedback inhibition by GABAergic interneurons, as the strong inward current reduced very rapidly just before the beginning of period 3. To understand the accurate neural activity generating sink/source pattern as shown in CSD analysis, additional experiments are needed.

An irregular waveform was observed following the initial component of odor-induced response. This was thought to be a reflection of transitional state before the formation of oscillatory rhythm of synaptic events. After that period, a typical beta oscillation was observed, which lasted \( \leq 3\ s \) after the offset of odor stimulation. In correlation to the beta oscillation, alternating sink/source pattern was observed in layer I and II, with half-shifted cycles between the layers (Fig. 5).

**Multiunit activity evoked by odor stimulation**

To examine the relation between the time course of LFP and the timing of the spike discharge in APCD pyramidal cells, we recorded multiunit activity of layer II neurons in the middle APCD (Fig. 6, B–D) simultaneously with LFPs at layer Ia (Fig. 6A). In response to odor stimulations, a phasic unit discharge was observed after \( \sim 50\ ms \) from the onset of the surface-negative potential change, which was followed by a silent period that lasted \( \sim 100\ ms \). A phasic-tonic discharge followed the tonic element of which lasted until the offset of the odor stimulation. We found a good correspondence in timing and pattern between these multiunit discharges and LFPs recorded in layer Ia. The initial phasic multiunit discharge corresponded to period 1 of the LFPs, and the inhibitory period of the multiunit activity corresponded to the former half of period 2 of the LFPs. Then the following phasic-tonic multiunit discharge started during the latter half of period 2 of the LFPs. From these results, it appears that the initial negative potential change in period 1 may reflect an excitatory process induced by either afferent or association input to layer I. LFPs in the early part of period 2 appears to be a reflection of an inhibitory process, as a strong inhibition was seen in the multiunit discharge at that time. The positive slope of LFPs in the late part of the period 2 may reflect an excitatory input to pyramidal cells, as spike discharges were regenerated with this potential change.
Electrical stimulation to olfactory areas

To specify the fiber projection to APCD that generates the surface-positive deep-negative potential change and inhibits spike discharges, we applied electrical stimulations to rostrally positioned olfactory areas that are suggested to send projections to APCD, such as AOC and APCvr (Ekstrand 2001b). In AOC and APCvr, stimulation electrodes were positioned at the depth of cell body layer, as illustrated in Fig. 7A. We also stimulated the caudal segment of severed LOT to evoke association inputs without stimulating the direct afferent input carried by LOT fibers. Recordings were made in the middle portion of APCD. Figure 7B shows superimposed LFPs recorded simultaneously at different depths following stimulation at different sites. Electrical stimulation to AOC evoked surface-negative deep-positive response [onset latency: 4.5 ± 0.29 (SE) ms; peak latency: 16.3 ± 1.18 ms, n = 4]. On the contrary, electrical stimulation to APCvr evoked the surface-positive deep-negative potential change (onset latency: 7.2 ± 0.56 ms; peak latency: 20.5 ± 0.97 ms, n = 15). These results suggest that the surface-positive deep-negative potential change is evoked by synaptic inputs from APCvr neurons to APCD neurons. In addition, the association input evoked by electrical stimulation to the caudal segment of severed LOT showed surface-negative deep-positive LFPs. These findings indicate that the synaptic inputs from APCvr neurons may not be mediated by the associative excitatory input to layer Ib.

CSD analysis of APCD laminar profiles during APCvr stimulation (Fig. 7C) showed that a major sink was observed in layer II and III, without any active sink in superficial layers.

Figure 4. CSD analysis of laminar profiles in APCD during LOT stimulation evoked response (A) and odor-induced response (B). A: CSD contour plot for LOT-evoked response. CSD profile of this contour plot was calculated from the laminar profile shown in Fig. 3A. Sink/source and their current intensities are represented by solid and dashed lines. B: CSD laminar profile (top) and contour plot (bottom) for the odor-induced response shown in Fig. 3B. In the CSD profile, positive and negative deflections correspond to the sink and source respectively. From the strength of inward currents in layer Ib, initial component of odor-induced response was divided into three periods (periods 1–3). Intensity of inward and outward current is represented with solid and dashed lines, respectively. Filled and open triangles above CSD profile indicate the same timing as those in Fig. 3B.

**DISCUSSION**

We describe a novel technique to prepare an isolated whole brain of the guinea pig with OE. Due to the technical difficulties, we used young (2–4 postnatal weeks) male guinea pigs for experiments. We consider that the neural circuit of the olfactory system at this age is basically mature and comparable to that of a mature adult. As the function of OE is preserved, this in vitro preparation is suitable for investigating the neural activity induced by natural odor stimulation in the brain. The present report represents the first attempt to perform a natural stimulation in an in vitro condition. Previous in vitro studies performed on the isolated brain preparation utilized LOT stimulation to mimic olfactory input to the piriform and entorhinal region (Biella and de Curtis 1995; Biella et al. 2003; Gnatkowsky et al. 2004; Uva et al. 2003, 2005). Even though electrical tract stimulation is widely accepted to perform system physiology studies, LOT stimulation does not reproduce all the features of natural stimulation and exclude the contribution of the olfactory areas rostral to the site of stimulation. Natural odor stimulation to OE induced a receptor potential and a typical LFP response in OB and APCD that may be due to the afferent and the association input, followed by a population oscillation at beta frequency. Ringer control also induced a small EOG in OE and LFPs in OB as the cleaned air evoked small EOG in previous studies (Brunet et al. 1996; Ottoson 1956). Although our preparation was in the hypothermic state to reduce the background activities, EOG and odor-induced LFPs in OB were similar to those described in previous studies (Brunet et al. 1996; Kashiwadani et al. 1999; Neville and Haberly 2003). Therefore the responsiveness to odorants in the isolated preparation seems to be preserved as in anesthetized animals. We also observed typical multi-unit responses of APCD layer II neurons, characterized by a phasic discharge at the onset of stimulations accompanied by a strong inhibition.
followed by a tonic discharge lasting until the end of stimulations.

The main finding of this study is that a natural odor stimulation to the OE induces a characteristic response in APCD that is quite different from that evoked by an electrical stimulation to LOT (Fig. 3, A and B). Whereas LOT stimulation evoked a large monosynaptic response in superficial layer Ia, followed by a disynaptic potential in layer Ib mediated by intrinsic associative connections (Neville and Haberly 2004), natural odor stimulation in our experiments induced a prevalent potential generated by a sink in layer Ib, presumably carried by intra-PC cortical association fibers. This difference was maintained regardless of the intensity of the electrical stimulation to LOT or the odor concentration. Odor stimulations of any intensity, whether it was low around the threshold or high, induced characteristic LFPs in APCD that were clearly different from those evoked by electrical stimulation. Two possible reasons for this difference can be assumed. First, as natural odor stimulations might activate a part of olfactory sensory neurons, only a part of principal cells in OB that send afferent input to APC might be excited. Thus the relatively weak afferent input would activate a small part of APC pyramidal cells not numerous enough to produce a field response in which subtending current generators could be detected. A relatively large number of pyramidal cells is activated by association inputs that generate the sink located in layer Ib. Intra-PC...
associative fibers are indeed widely distributed and could amplify the propagation of activity generated by a weak afferent input. On the other hand, electrical stimulation of the LOT activates almost all afferent fibers and therefore generates a large Ia sink (Ketchum and Haberly 1993a,b). As a result, the amplitude of the surface-negative potential change in the initial component of natural odor-induced response should be expected to be much smaller than that of the LOT-evoked response. Second, the main source of inhibitory inputs in the initial component of natural odor-induced response might not be interneurons within APC_D, but basket cells in APCvr, which were suggested to make synapses with pyramidal cells in APC_D layers II and III (Ekstrand et al. 2001b). An alternative explanation is that the inhibition may be mediated by excitatory pyramidal cells in APCvr synapsing on basket cells that are intrinsic to APC_D (Ekstrand et al. 2001a). As shown in Fig. 7B, electrical stimulations to APCvr evoked the surface-positive deep-negative potential associated to a layer II sink similar to that observed in the initial component of natural odor-induced response. As a result, in case of natural odor stimulation, relatively strong afferent input may activate either inhibitory basket cells or excitatory pyramidal cells in APCvr. Then in turn, the former can directly inhibit cells in layer II and III in APC_D, or the latter can indirectly inhibit them through intrinsic inhibitory basket cells in APC_D, which may result in the surface-positive potential change and the associated layer II–III sink in the initial component of the response observed in our experiments. On the contrary, in case of electrical stimulation of LOT, strong afferent and association inputs activate a large number of pyramidal cells in APC_D all together that will result in the strong Ia and Ib sinks and in feedback inhibition caused by interneurons within APC_D itself. By taking into account such a difference in the extent and the temporal pattern of the possible underlying neural activities, it is understandable why odor-induced LFP is quite different from that evoked by LOT stimulation.

Our findings suggest that a phasic inhibition follows the onset of the associative excitation at the onset of odor stimulation. CSD analysis demonstrated that the onset of the odor-induced Ib sink (period 1) was followed by a small sink in layer II–III coupled to a reinforcement of the Ib sink. In line with the previous studies by the Haberly group, the layer II–III sink could be attributed to an inhibitory, possibly feedback, potential (Ketchum and Haberly 1993b; Neville and Haberly 2004). Even though the definitive confirmation of the existence of an inhibitory component in the response could be attained by intracellular recordings, extracellular unit recordings performed in our study strongly support this conclusion. A clear inhibition of neuronal firing was, indeed, observed in layer II units in correspondence to period 2 of the odor-induced potential. Because the nature of the afferent input induced by natural odor stimulation may be quite different from that evoked by LOT stimulation, an alternative hypothesis could be proposed. Although LOT stimulation activates afferent fibers instantaneously, natural odor stimulation induces constant afferent inputs. Mitral/tufted cells in OB are known to generate phasic spike discharges after the onset of natural odor stimulation with some latency and, after a silent period of ≈100 ms, they generate continuous spike discharges for several seconds (Kashiwadani et al. 1999; Wilson 1998). The same firing pattern was observed in APC_D layer II neurons during our experiments (Fig. 6). Moreover, CSD analysis of natural odor-induced response showed that the time interval between the onset (period 1) and the peak (period 2) of the inward current in layer Ia was ~100 ms (Fig. 4B). Thus the characteristic

FIG. 7. Electrical stimulation to the rostral APCv induced surface-positive potential change in APC_D. A: arrangement of stimulation electrodes. In AOC and the rostral part of APCv (APCvr), the tip of stimulation electrodes were positioned at cell body layer. LOT was cut at a caudal site, and stimulation electrode was positioned at the caudal segment of severed LOT (cLOT). Open circle in the middle portion of APC_D indicate the recording site. B: superimposed LFPs evoked by electrical stimulation to AOC, APCvr and the cLOT from the surface to the depth of 1,100 μm (100-μm intervals). C: CSD contour plot for the APCvr stimulation evoked response. Sink/source and their current intensities are represented by solid or dashed lines.

large Ia sink (Ketchum and Haberly 1993a,b). As a result, the amplitude of the surface-negative potential change in the initial component of natural odor-induced response should be expected to be much smaller than that of the LOT-evoked response. Second, the main source of inhibitory inputs in the initial component of natural odor-induced response might not be interneurons within APC_D, but basket cells in APCvr, which were suggested to make synapses with pyramidal cells in APC_D layers II and III (Ekstrand et al. 2001b). An alternative explanation is that the inhibition may be mediated by excitatory pyramidal cells in APCvr synapsing on basket cells that are intrinsic to APC_D (Ekstrand et al. 2001a). As shown in Fig. 7B, electrical stimulations to APCvr activated the surface-positive deep-negative potential associated to a layer II sink similar to that observed in the initial component of natural odor-induced response. As a result, in case of natural odor stimulation, relatively strong afferent input may activate either inhibitory basket cells or excitatory pyramidal cells in APCvr. Then in turn, the former can directly inhibit cells in layer II and III in APC_D, or the latter can indirectly inhibit them through intrinsic inhibitory basket cells in APC_D, which may result in the surface-positive potential change and the associated layer II–III sink in the initial component of the response observed in our experiments. On the contrary, in case of electrical stimulation of LOT, strong afferent and association inputs activate a large number of pyramidal cells in APC_D all together that will result in the strong Ia and Ib sinks and in feedback inhibition caused by interneurons within APC_D itself. By taking into account such a difference in the extent and the temporal pattern of the possible underlying neural activities, it is understandable why odor-induced LFP is quite different from that activated by LOT stimulation.

Our findings suggest that a phasic inhibition follows the onset of the associative excitation at the onset of odor stimulation. CSD analysis demonstrated that the onset of the odor-induced Ib sink (period 1) was followed by a small sink in layer II–III coupled to a reinforcement of the Ib sink. In line with the previous studies by the Haberly group, the layer II–III sink could be attributed to an inhibitory, possibly feedback, potential (Ketchum and Haberly 1993b; Neville and Haberly 2004). Even though the definitive confirmation of the existence of an inhibitory component in the response could be attained by intracellular recordings, extracellular unit recordings performed in our study strongly support this conclusion. A clear inhibition of neuronal firing was, indeed, observed in layer II units in correspondence to period 2 of the odor-induced potential. Because the nature of the afferent input induced by natural odor stimulation may be quite different from that evoked by LOT stimulation, an alternative hypothesis could be proposed. Although LOT stimulation activates afferent fibers instantaneously, natural odor stimulation induces constant afferent inputs. Mitral/tufted cells in OB are known to generate phasic spike discharges after the onset of natural odor stimulation with some latency and, after a silent period of ≈100 ms, they generate continuous spike discharges for several seconds (Kashiwadani et al. 1999; Wilson 1998). The same firing pattern was observed in APC_D layer II neurons during our experiments (Fig. 6). Moreover, CSD analysis of natural odor-induced response showed that the time interval between the onset (period 1) and the peak (period 2) of the inward current in layer Ia was ~100 ms (Fig. 4B). Thus the characteristic
temporal pattern of natural odor-induced response in APC_D could be generated by the time course of neural activity in the OB.

In the isolated brain with OE, beta oscillations were observed in OB, APC, PPC, and LERC, as described in many previous in vivo studies (Becher and Freeman 1968; Bressler 1984; Chapman et al. 1998; Freeman 1959; Martin et al. 2006; Neville and Haberly 2003; Vanderwolf 1992; Zibrowski and Vanderwolf 1997). In APC_D, alternating sink/source pattern was observed during the beta oscillation (Fig. 5). Ketchum and Haberly (1993a) reported a similar sink/source pattern in APC induced by a weak electrical stimulation to LOT. As the lesion of either LOT or centrifugal pathway abolishes beta oscillations in OB and APC, Neville and Haberly (2003) proposed that the beta oscillation might be generated by the loop formed by mitral cells of OB, pyramidal cells in PC, and granule cells in the OB. As the beta oscillation was observed in various olfactory areas, it can be considered that the macro-circuit connecting OB and olfactory areas is intact in our new whole brain preparation with preserved OE. The viability of the OB in the isolated brain preparation has been recently demonstrated by the analysis of LOT-evoked responses in OB (Uva et al. 2005). Gamma oscillations in the olfactory system were also described in the previous studies (Adrian 1950; Boeijinga and Lopes da Silva 1988; Bressler 1984; Freeman 1959, 1978; Kashiwadani et al. 1999; Kay and Freeman 1998; Mori et al. 1992), but they were not observed in this isolated preparation. Fletcher et al. reported that the odor-induced LFP oscillations showed a strong age-dependent change in the dominant frequencies between infant and mature rats (Fletcher et al. 2005). As postnatal week 2–4 guinea pigs were used in this study, the lack of gamma oscillation might be attributed to the immaturity of the neural circuit. The functional role of oscillations in vivo has yet to be examined in the future studies. By preserving OE in this isolated whole brain preparation, we could add the possibility of using natural odor stimulation to the existing advantage of accessing deep brain structures without any artifact derived from heart beat or respiration. We believe that this preparation can serve as a technical tool for further investigating the neural mechanisms of the olfactory system.

References

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