Distribution of the Olfactory Fiber Input Into the Olfactory Tubercle of the In Vitro Isolated Guinea Pig Brain

Giovanni Carriero, Laura Uva, Vadym Gnatkovsky, and Marco de Curtis

Unit of Experimental Neurophysiology and Epileptology, Fondazione Istituto Neurologico Carlo Besta, Milan, Italy

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Carriero G, Uva L, Gnatkovsky V, de Curtis M. Distribution of the olfactory fiber input into the olfactory tubercle of the in vitro isolated guinea pig brain. J Neurophysiol 101: 1613–1619, 2009. First published October 15, 2008; doi:10.1152/jn.90792.2008. The olfactory tubercle (OT) is a cortical component of the olfactory system involved in reward mechanisms of drug abuse. This region covers an extensive part of the rostral ventral cerebrum and is relatively poorly studied. The intrinsic network interactions evoked by olfactory input are analyzed in the OT of the in vitro isolated guinea pig brain by means of field potential analysis and optical imaging of voltage-sensitive signals. Stimulation of the lateral olfactory tract induces a monosynaptic response that progressively decreases in amplitude from lateral to medial. The monosynaptic input induces a disynaptic response that is proportionally larger in the medial portion of the OT. Direct stimulation of the piriform cortex and subsequent lesion of this pathway showed the existence of an associative disynaptic projection from the anterior part of the piriform cortex to the lateral part of the OT that integrates with the component mediated by the local intra-OT collaterals. Optical and electrophysiological recordings of the signals evoked by stimulation of the olfactory tract during arterial perfusion with the voltage-sensitive dye di-2-ANEPQ confirmed the pattern of distribution of the mono and disynaptic responses in the OT. Finally, current source density analysis of laminar profiles recorded with 16-channel silicon probes confirmed that the monosynaptic and disynaptic potentials localize in the most superficial and the deep portions of the plexiform layer I, as suggested by previous reports. This study sets the standard for further analysis of the modulation of network properties in this largely unexplored brain region.

INTRODUCTION

The olfactory tubercle (OT) is a tri-laminar cortical structure that occupies a large portion of the basal and rostral surface of the brain, medially to the lateral olfactory tract (LOT). It has been proposed that the meso-limbic system, OT included, is implicated with the nucleus accumbens in reward behaviors associated with psychomotor stimulants. Results obtained in self-administration and conditioned place-preference tests, and the effect of specific lesions in accumbens, suggest the OT is strongly involved in amphetamine reward behaviors (Lyness et al. 1979; Spyraki et al. 1982). No direct evidence, however, indicates OT involvement in cocaine pharmacological effects (Hoebel et al. 1983; Ikemoto and Sharpe 2001; Lyness et al. 1979; Phillips et al. 1994). Cocaine injection into the OT induces robust locomotion and rearing (Ikemoto 2002; Ikemoto and Witkin 2003). Recently, it was shown that rats self-administer more cocaine in OT than into the nucleus accumbens during cocaine reward tests. Moreover, direct injections of dopamine receptor antagonist into the OT are able to abolish this reward aspect to cocaine (Ikemoto 2003).

Tracing studies suggest that mitral and tufted olfactory bulb cells project their axons to the superficial part of layer I (sublayer Ia) of the OT, mainly in its lateral portion (Heimer 1968; Price 1973; Scott et al. 1980; Shipley 1985). Neurons in layers II and III receive association fibers that originate in different olfactory cortices including the anterior olfactory nucleus, the piriform cortex, the dorsal peduncular cortex, and the ventral tenia tecta (Luskin and Price 1983a,b). These association fibers preferentially terminate in the apical dendrites of layer II–III neurons that are located in the deep part of layer I (sublayer Ib) and to a minor extent in layers II and III (Price 1973). The connectivity pattern and the cytoarchitectonic structure of the OT is quite similar to that described in the piriform cortex positioned lateral and caudal to the LOT. The OT is not implicated in olfaction-related networks only; it receives inputs and projects to structures belonging to limbic system, such as the amygdala formation, the hypothalamus, the mediadorsal thalamus, the anterior entorhinal cortex and the hippocampus (Haberly and Price 1978; Heimer et al. 1987; Kunzle 2005; Luskin and Price 1983b; Millhouse and Heimer 1984; Price et al. 1991; Ray et al. 1992), and the basal ganglia, specifically the nucleus accumbens (Zahm 1987). These studies suggest that OT is involved in different associative networks with the limbic system and the centers related to the control of motor functions.

A small number of studies considered the connectivity of the OT with anatomical methods; even less work is currently published on the electroresponsiveness of this quite extensive and peculiar region. The studies by Owen and Halliwell (2001) characterized the pharmacology of excitatory synaptic potentials evoked in principal OT cells by stimulation of either the LOT or the local cortico-cortical associative fibers, by using both glutamatergic and cholinergic drugs in OT slice preparation. This study documented an important modulator role of acetylcholine (ACh) in regulating synaptic OT responses. LOT-driven field potential was lightly depressed by bath application of the muscarinic receptor agonist, carbachol, whereas associative responses were deeply depressed. Current-source density-like analysis indicates the presence of a monosynaptic sink in the superficial Ia layer after LOT stimulation and a deeper sink evoked by associative fiber activation (Owen and Halliwell 2001). More recently, a study performed on rat OT slices showed...
the existence of three subtypes of firing patterns in different populations of neurons located in the dense cell and multiform layers of the OT (Chiang and Strowbridge 2007) otherwise, respectively, identified as layers II and III.

In this study we used a combined imaging and electrophysiological approach to further investigate the intrinsic associative networks elicited in different areas of the OT and in the piriform cortex by LOT afferent stimulation. The analysis of the functional interactions between interconnected olfactory structures was ensured by the use of an intact preparation of the whole brain of the guinea pig maintained in vitro by arterial perfusion (de Curtis et al. 1991, 1998; Llinas et al. 1981; Muhlethaler et al. 1993).

METHODS

Nineteen guinea pig brains were isolated and maintained in vitro according to the previously described technique (de Curtis et al. 1991, 1998; Llinas et al. 1981; Muhlethaler et al. 1993). Hartley guinea-pigs (150–200 g, Charles River, Calco, Italy) were anesthetized with sodium thiopental (125 mg/kg, ip, Farmotal, Pharmac, Italy) and were transcardially perfused with cold (10°C) and carboxygenated (95% O₂-5% CO₂) saline solution (pH 7.1) composed of (in mM) 126 NaCl, 3 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, 26 NaHCO₃, 15 glucose, and 2.1 HEPES. Dextran (3%: molecular weight = 70,000) was added to the solution to balance osmolarity. The brain was rapidly removed and transferred into the recording chamber, where artificial perfusion was re-established by inserting a polyethylene cannula into the basilar artery. Isolated brains were perfused with the above solution (pH 7.3, at 15°C) at a flow rate of 7 ml/min. The temperature of the perfusate was slowly increased (0.2°C/min) to a final temperature of 32°C. The experimental protocol was reviewed and approved by the Committee on Animal Care and Use and by the Ethics Committee of the Fondazione Istituto Neurologo. Experiments conformed to International Guidelines on the Ethical Use of Animals. Efforts were made to minimize the number of animals used and their suffering.

A custom-made bipolar twisted silver wire, connected to an isolation unit driven by a Grass-Telefactor S88 pulse generator (Warwick, RI), was positioned on the LOT for stimulation. A second stimulus was delivered by a tungsten bipolar electrode (FHC, Bowdoinham, ME) inserted in superficial layers of the anterior piriform cortex (APC). Evoked extracellular potentials were recorded with glass micropipettes (filled with 1 M NaCl) from nine reproducible sites in the OT, identified with a stereoscopic microscope using surface structures (optic chiasm and midline) as reference points (see Fig. 3). Electrophysiological signals were recorded with a multichannel amplifier (Biomedical Engineering, Thornwood, NY) and were digitized via an AT-MIO-63E3 National Board (National Instruments, Milano, Italy) at a sampling rate of 3 kHz. Custom software (ELPHO) developed in Labview (National Instruments) by Vadym Gnatkovsky was used to acquire and analyze the electrophysiological data. The functional preservation of the olfactory-limbic region was shown in previous studies (de Curtis et al. 1991) (Biella and de Curtis 1995; Biella et al. 2003; Uva et al. 2006).

For mono-dimensional current source density (CSD) analysis, OT laminar profiles were recorded with 16-channel linear probes (50-μm contact separation, NeuroNexus Technologies) inserted perpendicular to the cortical lamination. Laminar field potential profiles evoked by either LOT or APC stimulation were performed by averaging 8–10 responses. To show local OT activities, CSD analysis of laminar field potential profiles was performed using a 200-μm separation grid (Biella and de Curtis 1995; Ketchum and Haberly 1993; Mitzdorf 1985). To disconnect the OT from the piriform cortex input, a cut along the edge between the LOT and the APC was performed with a surgical blade, and a thin plastic foil was inserted into the incision to avoid passive diffusion of electrical fields.

In four experiments, 0.36 M solution of the voltage-sensitive dye di-2-ANEPEQ (Invitrogen, Eugene, OR) was perfused according to the previously described technique (Tominao et al. 2000) to record optical fluorescent signals in olfactory cortices. A single staining of the brain allowed recording evoked fluorescent signals for 3–4 h (de Curtis and Pare 2004; Gnatkovsky and de Curtis 2006). The changes in fluorescence generated by neuronal depolarizations of membrane potential were recorded with MiCAM-02 charge-coupled device-based digital high-speed camera system (SciMedia, Irvine, CA; developed by BrainVision, Tsukuba, Japan).

RESULTS

Electrical stimulation of the LOT induces in the OT an evoked potential that shows strong similarities to the response observed in the APC, characterized by two voltage peaks with a delay of 5–6 (Fig. 1, triangle) and 12–15 ms (Fig. 1, circle) from the LOT stimulus artifact. Low LOT stimulus intensity evoked exclusively a single positive field potential response in OT (Fig. 1B, 25 μAmp). A second positive component emerged for higher LOT stimuli intensities (Fig. 1B). To better characterize the biphasic positive responses, we performed the paired pulse test. By subtracting the potential evoked by single LOT stimulation from the paired response evoked during a pairing test with 20-ms interstimulus interval, a monosynaptic response (triangle) was shown (Fig. 1A, traces b-a). The monosynaptic potential was followed by a disynaptic component (circle) that recovered at interstimulus intervals >30 ms during the pairing test. To rule out the possibility that such potentials were mediated by direct activation of the OT because of the diffusion of the stimulus, we stimulated LOT after cutting the LOT fibers entering the OT: in these experimental conditions, no response was recorded in the OT (data not shown). To evaluate the pattern of distribution and the characteristics of LOT-evoked neuronal activity in the OT, we performed a combined study using both electrophysiological and imaging techniques. Detailed high-resolution maps of

![FIG. 1. Typical potentials evoked in the olfactory tubercle (OT) of the isolated guinea pig brain preparation by lateral olfactory tract (LOT) stimulation. Left: the anterior piriform cortex (APC), LOT, and OT are shown in a scheme of the ventral view of a guinea pig brain. A: responses recorded in the OT following paired LOT stimuli at different interstimulus intervals (20, 30, 40, and 60 ms) are shown in b. Bottom: the traces obtained by subtracting the response to a single LOT stimulus (a) from each paired response in b, is shown (b-a). B: field potentials evoked by LOT stimuli at different intensities (25, 50, 75, 100, and 150 μAmp) The triangle and the circle mark the monosynaptic and the disynaptic responses, respectively.](http://jn.physiology.org/10.1152/jn.011005.2009)
activity propagation were generated during perfusion of isolated brains with the voltage-sensitive dye, di-2-ANEPEQ (n = 4). LOT stimulation induced a first visible response with a 7- to 10-ms delay. This optical wave propagated progressively from lateral to medial, appearing first in the rostro-medial and then in the caudal-medial OT (see movie1.mov). As shown in the OS trace, the signal started to rise in the lateral OT at 7–10 ms, and it showed a peak 14 ms from the stimulation (Fig. 2, a and b). A second increase in the OS was observed in the medial OT with a peak at 30–33 ms after the LOT stimulation (OS; Fig. 2, c and d; see also movie1.mov). This component showed a progressively longer onset from lateral to medial. These data show that OT is diffusely and homogeneously activated in response to LOT stimulation.

To further analyze the distribution of LOT-evoked field potentials in the OT, extracellular recordings were carried out simultaneously in different rostro-caudal and medio-lateral OT positions (n = 7). Nine recording positions were identified and reproduced in different experiments by using surface reference points (Fig. 3): two positions in the most rostral OT (positions 1 and 2), three in the middle OT (positions 3–5), and four in the caudal OT (positions 6–9). An additional recording electrode was placed in the APC. Low-intensity LOT stimulation induced monosynaptic responses that decreased in amplitude with distance in the rostral-to-caudal direction (Fig. 3A, right, dotted lines). Polysynaptic components mainly mediated by disynaptic associative cortico-cortical interactions were evoked in all positions when LOT stimulation intensity was increased. The relative contribution of mono- and disynaptic components to the field responses in different positions was evaluated by analyzing input/output curves in four experiments, as shown in Fig. 3, B and C. The amplitude of the monosynaptic responses decreased from rostral to caudal and from lateral to medial. A linear increase of the disynaptic component with the increase of the monosynaptic response, both measured as peak amplitude values, was observed. The divergence between the disynaptic and the monosynaptic curves was enhanced in the rostral part of the OT (Fig. 3B, see positions 1, 2, and 5). Fig. 3C shows the ratios between the disynaptic and monosynaptic peak. Amplitudes calculated for positions 1 and 2 (rostral OT) were 1.26 ± 0.17 and 1.81 ± 0.45 (t-test, P < 0.05; n = 5), for positions 3, 4, and 5 (medial OT) they were 0.8 ± 0.24, 1.11 ± 0.32, and 1.98 ± 0.6 (n = 5; t-test, P < 0.05 for positions 3 and 4 vs. position 5), and for positions 6, 7, and 8 (caudal OT) they were 1.45 ± 0.06, 1.76 ± 0.69, and 1.48 ± 0.27 (no statistical significance; Fig. 3C). The data suggest that disynaptic responses were larger relatively to the monosynaptic component in the medial and rostral OT.

The latencies of mono- and disynaptic responses were also evaluated in different parts of the OT (Fig. 4). The delay from the LOT stimulus artifact of the peak amplitudes of mono- and disynaptic components increased from lateral to medial OT (Fig. 4), whereas no latency variations were observed in the rostral to caudal dimension.

CSD analysis of field profiles was performed in five experiments. Figure 5 shows field potential laminar profiles recorded in position 3 with a 16-channel probe (top) and the relative CSD contour profiles (bottom), obtained by averaging 10 responses to LOT stimulation (left) and local APC stimulation (right). An early sink corresponding to the monosynaptic LOT-evoked field potential located in layer Ia (100–200 µm; triangle), was followed by a deeper sink in layer Ib and in the superficial part of layer II (200–400 µm) associated with the disynaptic response (circle). Direct local APC stimulation evoked a single sink at the same depth as the disynaptic LOT-evoked sink (n = 4), suggesting the existence of a projection from the APC to the OT mediated by associative

Figure 2. Optical imaging of the LOT-evoked responses in the OT of the in vitro isolated guinea pig brain after arterial perfusion with the voltage-sensitive dye, di-2-ANEPEQ. Top left: a picture of the isolated guinea pig brain. The red rectangle outlines the recording field of the optical imaging, shown in the bottom panel, where the borders between olfactory structures are outlined (LOT, lateral olfactory tract; APC, anterior piriform cortex; OT, olfactory tubercle; PPC, posterior piriform cortex). In the same panel are indicated the positions (a–d) from where the optical signals (OS) shown on the right of the figure were recorded. An electrophysiological field response (FP) was also recorded from position b with a glass microelectrode. Vertical dotted lines indicate the time points that correspond to the images of the optical signal changes shown on the bottom of the figure. Bottom: the optical signals recorded 7, 11, 14, and 33 ms after LOT stimulation. Signal intensity was scaled in pseudo-colors. For details see also movie1.mov.

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cortico-cortical fibers that terminate as the same depth where the sink of the local disynaptic is localized.

To further study the contribution of the APC input to the LOT-evoked response in the OT, we sectioned the LOT fibers that enter the APC (see METHODS), excluding the associative contribute of APC-OT fibers. Figure 6A shows data obtained from an experiment in which recordings in different portions of the OT before (continuous line) and after (dotted line) sectioning the LOT fibers that enter the APC. The results observed in seven experiments are shown in Fig. 6B. The ratio between the amplitudes of the disynaptic and the monosynaptic peak components of the LOT-evoked response were plotted before and after the cut of the LOT fibers directed to the APC. Following exclusion of the APC-derived input, the disynaptic response decreased in the lateral part of the OT, whereas no difference was observed in the medial line. To better evaluate the lesion of the LOT fibers that enter the APC, voltage-sensitive dye experiments were performed before and after severing LOT–APC connections (n = 4; Fig. 6C). The cut abolished the optical signal in the APC and reduced the amplitude of the disynaptic response in the OT (dotted lines) in both the electrophysiological trace (FP) and in the optical signal (OS) recording (Fig. 6, A and C, left; see also movie2.mov). These findings strongly suggest that an associative projection arising from the APC contributes to the disynaptic component of the LOT-evoked OT response and show that this input is stronger in the lateral OT.

DISCUSSION

Extensive electrophysiological studies performed in different areas of the isolated guinea pig brain confirmed that this
preparation retains normal physiological properties close to the in vivo condition for several hours when perfused in vitro with a saline solution supplemented with a plasma expander (de Curtis et al. 1991, 1994; Biella et al. 2002; Muhlethaler et al. 1993). Moreover, the structural and functional preservation of the complex relationship between the vascular and the glial/extracellular compartments that form the blood–brain barrier (BBB) has been shown in this preparation (Librizzi et al. 2001; Mazzetti et al. 2004). In this study, we used a combined electrophysiological and imaging approach to analyze the OT synaptic networks elicited by LOT electrical stimulation in guinea pig isolated whole brain. In addition, we tested the contribution of piriform cortex to the generation of LOT-driven field potential in the OT.

We showed that the field potentials evoked by LOT input are widely and homogeneously distributed in the OT, even though some differences in the ratio of afferent to associative input contribute to differences in the field potential in subregions of the OT. The LOT-evoked responses have similar characteristics to the potential recorded in the APC after LOT stimulation. We discriminated two different voltage peaks. The earliest is associated with the monosynaptic response, as shown by the pairing test. It is localized in the superficial portion of the molecular layer, named layer Ia, that is largely acellular and is composed of afferent fibers (Luskin and Price 1983b; Price 1973). The monosynaptic response shows an amplitude gradient that decrease from lateral (close to the origin of LOT fibers) to medial and from rostral to caudal. These data are in agreement with the decrease pattern of LOT fiber termination to the more medial areas of OT (Meyer and Wahle 1986).

The second wave is mediated by a disynaptic circuit formed by intrinsic intra-OT fibers and by associative fibers originating from neighboring cortical structures, such as the APC. Consistent with the anatomical findings, the disynaptic component is generated by a current sink localized in the deeper part of the molecular layer (layer Ib) and in the superficial part of layer II (Luskin and Price 1983b). CSD analysis confirm dense cell layer I as synaptic target for afferent fibers elicited by direct piriform cortex stimulation. The analysis of the delays and of the mono-disynaptic component ratio showed that the disynaptic potential is proportionally larger in the medial part of the OT in comparison to the lateral OT. Despite the reduction in the monosynaptic field potential responses in the medial OT, the ratio between disynaptic and monosynaptic amplitude was increased in the medial and rostral part of the OT. These findings suggest that the role of the most medial portion of the

FIG. 4. Delay distribution of mono- and disynaptic components of LOT-evoked field responses in the OT. A: traces recorded in different portions of the rostral (positions 1 and 2), intermediate (positions 3–5), and caudal (positions 6–9) OT were superimposed. The dotted trace on the right in A shows the potential evoked in the APC. B: average values of the peak amplitude delays of mono- (gray columns) and disynaptic (white columns) components of the LOT-evoked response in 6 sampled sites in the OT. The data were grouped for medial and lateral OT positions.

FIG. 5. Current source density analysis of laminar profiles recorded in the OT (position 3) with a 16-channel silicon probe. LOT and local APC stimulating electrodes are shown in the scheme on the left. Top right: the laminar field potential profiles recorded in the same position following stimulation of the LOT (left) and the APC (right) (average of 10 responses each). Bottom: the relative CSD bi-dimensional contour profiles are shown. Gray shading shows sources; white contour lines are sinks. Isocurrent contour lines: 10 mV/mm². The triangle and the circle mark the monosynaptic sink and the disynaptic and associative sinks, respectively.
OT is predominantly associative and is not strictly related to the processing of the direct olfactory input. Similar findings about the position of direct LOT and associative synaptic terminals are present in classic axonal label and degenerative anatomic study (Haberly and Price 1978; Heimer 1968; Heimer et al. 1987; Kunze 2005; Luskin and Price 1983b; Millhouse and Heimer 1984; Price 1973; Price et al. 1991; Ray et al. 1992; Scott et al. 1980; Shipley 1985) and in the more recent electrophysiological work carried out on OT rat slices (Owen and Halliwell 2001).

Because the disynaptic component in the medial OT was not significantly reduced by the lesion of the associative fibers that originate from the APC, we conclude that it is mainly caused by the activation of intra-OT associative interaction, even though associative inputs from regions other than the APC cannot be excluded. The electrophysiological map of LOT-evoked responses in 5 different positions of the OT before (gray columns) and after (black columns) the isolation of the APC from the LOT input. C: voltage-sensitive optical signals recorded in the olfactory region before and after the cut performed to isolate the APC from the LOT input. See also movie2.mov.

We showed that this technique is very useful to analyze the pattern of distribution of synaptic excitation over large brain regions (Biella et al. 2003; Gnatkovsky and de Curtis 2004). The optical signals recording are most probably generated from synaptic events occurring mainly in the superficial layers (de Curtis et al. 1999), where most of the LOT- and APC-induced activity was identified also by CSD analysis.

In summary, we used electrophysiological methods and imaging techniques to show that LOT evokes field potentials in the OT with an amplitude gradient of the responses from rostral to caudal and from lateral to medial, with a distribution pattern that is quite similar to that observed in the piriform cortex. Based on these findings, we can definitively conclude that the OT is primarily an olfactory brain area that shows intrinsic organization similar to their cortical regions that receive an input from the olfactory bulbs. This study represents the starting point to further test whether other olfactory or nonolfactory brain regions have specific influences on network plasticity in this peculiar and largely unexplored brain region.

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REFERENCES


