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Glutamate-like immunoreactivity in axon terminals from the olfactory bulb to the piriform cortex

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Summary. A highly specific anti-glutamate monoclonal antibody, mAb2D7, was used together with light and electron microscopy to elucidate the role played by the amino acid glutamate in the projection from the olfactory bulb to the piriform cortex in the rat. By light microscopy, glutamate-like immunoreactivity was observed in neuronal cell bodies and in the neuropil of the piriform cortex. Double labelling experiments which involved injections of wheat germ agglutinin-horseradish peroxidase into the olfactory bulb and a post-embedding immunogold method for electron microscopy revealed anterogradely labelled terminals making asymmetric synaptic contacts on dendrites in the piriform cortex which contained high levels of glutamate as assessed by quantification. These results further support a role for glutamate as a neurotransmitter in the efferent pathway of the rat olfactory bulb.

Key words: Glutamate, Olfactory cortex, Horseradish peroxidase

Introduction

The olfactory system of the rat is a phylogenetically old part of the nervous system which is proving to be useful for a variety of anatomical and physiological investigations. The morphology and connectivity of the piriform cortex have been studied in some detail by light microscopy (Luskin and Price, 1982, 1983a,b; Haberly, 1983) and electron microscopy (Derer et al., 1977; Heimer and Kalil, 1978).

In the rat, the olfactory cortex is a three-layered structure (Price, 1973; Haberly and Price, 1978a,b) which occupies most of the ventral surface of the forebrain. Layer I, or the plexiform layer, can be divided into a superficial portion, layer Ia, in which synaptic terminals of the olfactory bulb (OB) are concentrated and a deep portion, layer Ib, which contains terminals of association and commissural fibers (Price, 1973). Layer II is characterized by a high concentration of pyramidal and semilunar cell bodies, and their large-caliber proximal dendrites. Intervening islands of neuropil with a composition similar to that of the deep part of layer Ib and the superficial part of layer III are also present.

Several lines of evidence suggest that glutamate acts as a transmitter from the olfactory bulb efferent pathway to the piriform cortex (Johnson and Aprison, 1971; Collins, 1984). The high concentration of glutamate in the piriform cortex is reduced following olfactory bulbectomy or transection of the lateral olfactory tract (Bradford and Richards, 1976).

The present study was undertaken because to date there is no morphological information available regarding the neurotransmitter associated with this efferent pathway.

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Materials and methods

Light microscopy

Untreated adult male Sprague-Dawley albino rats (n=4, 220-250 g) were sedated and deeply anaesthetized with Ketamine hydrochloride (25 mg/kg, intramuscularly injected). Thereafter, the rats were rapidly perfusion-fixed through the heart with 4% formaldehyde for 30 s followed by 1 litre of 5% glutaraldehyde (both fixatives prepared in 0.1M cacodylate buffer, pH 7.4). The brains were removed from the skull and postfixed overnight at 4 °C. Both the lateral olfactory tract and the piriform cortex were dissected out and 350 μm-thick vibratome sections were collected in phosphate-buffered saline (PBS). Subsequently sections were rinsed in the same buffer and then in 0.1M cacodylate buffer (pH 7.4). Finally, sections were treated with 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.4, for 1 h on a shaker at room temperature, dehydrated and embedded flat in Epon 812. Semithin sections (1 μm) were processed for postembedding immunohistochemistry using a peroxidase-antiperoxidase (PAP) procedure and silver intensification of the 3,3'-diaminobenzidine (DAB) end product, as previously described in detail

(Liu et al., 1989). The anti-glutamate monoclonal antibody, mAb2D7, was used at dilutions from 0.2 to 0.1 μg protein/ml of purified ascites fluid.

Electron microscopy

Since olfactory axon terminals are not readily identifiable at the electron microscopic level, we pressure-injected 0.1 μl of a 10% WGA-HRP in 0.9% saline solution into the left olfactory bulb of two deeply anaesthetized rats. Twenty-four hours later, the animals were perfused transcardially with the same perfusion solutions as described above for non-injected animals. Vibratome sections cut at 40 μm from the forebrain were processed for WGA-HRP histochemistry with tetramethylbenzidine (TMB) (Weinberg and Eyck, 1991). Subsequently, tissue sections were osmicated as described above, rinsed in 0.1M cacodylate buffer, dehydrated in graded alcohols with a final rinse in propylene oxide and embedded flat in Epon 812; areas containing anterograde label were then selected by light microscopy. Ultrathin sections were collected on nickel mesh grids and processed using a postembedding immunocytochemical method to localize glutamate-like immunoreactivity (Glu-Li) at the electron microscopic level.

Following a treatment with 1% periodic acid for 7 min, 1% sodium metaperiodate for 7 min and 1% sodium borohydride for 1 min, ultrathin sections were preincubated with a blocking solution of 7.5% bovine serum-Tris phosphate-buffered isotonic saline (TPBS 10 mM; pH 7.4) and incubated overnight at 4 °C with mAb2D7 at 8 μg protein/ml of purified ascites fluid prepared in 1% bovine serum-TPBS. Thereafter, they were incubated with a goat anti-mouse antibody conjugated to colloidal gold (10 nm diameter; Amersham, 1:10) for 90 min, stained with uranyl acetate and lead citrate and examined in a JEOL 100SX electron microscope.

Statistical analysis

To assess the density of gold particles over various profiles in single as well as double-labelled ultrathin sections, randomly selected electron micrographs were digitized by means of an Apple scanner and processed by the image analysis programme "Image" 1.37. Statistical analysis of the data was performed using the "Statworks" programme and the Fisher LSD procedure. By light microscopy, Glu-Li was observed in neuronal cell bodies of the olfactory nuclei, piriform cortex and olfactory tubercle. In addition, numerous heavily glutamate-immunoreactive (Glu-ir) dots were seen between cell bodies and dendritic profiles.

Results

In the piriform cortex Glu-Li was observed throughout the whole extent of the three layers. Layer I was particularly stained with immunoreactive dots (Fig. 1A).

At electron microsocpic level synaptic terminals, with round vesicles and making asymmetric synaptic contacts, were labelled with a heavy accumulation of gold particles in the presynaptic portion. The immunoreactive terminals had two different morphological characteristics. One of them had the typical aspect of a synapse including vesicles, mitochondria and a flat synaptic junction and the other population was characterized by the presence of a scalloped synaptic cleft. Some of these later terminals showed a hole in the scalloped membrane (Fig. 1B) as corroborated by serial sections. The pre- and postsynaptic membranes appeared to be intact and continuous in the periphery of the gap.

After WGA-HRP injections in the olfactory bulb, labelled axons were found in the investigated areas, as previously described. In the piriform cortex the labelled axons were distributed in the three layers, being more prominent in layer I where the olfactory axons ended. Layers II and III were weakly labelled with the presence of anterogradely transported crystals. Retrogradely labelled cell bodies were also found in layers II and III of the piriform cortex.

The ultrastructural study showed axon terminals labelled with electron-dense rod-like crystals. Two types of terminals were present. One of them had asymmetric morphology without any distinctive characteristics and the other had a scalloped synaptic membrane. On occasions, a hole was present in this scalloped synaptic cleft.

Double-labelled sections showed distributed label of gold particles in the neuropil and cellular profiles. Both of the terminal types described before and labelled with TMB crystals, seemed to contain more gold particles (Fig. 1C,D) than surrounding structures. The postsynaptic profiles were generally dendritic spines, although dendritic shafts were also found.

Electron micrographs from material processed for double staining (WGA-HRP and immunohistochemical methods) were digitized and processed by an image analysis programme. Digitized image analysis allowed us to subtract the very low (1.6 particles/μm^2) mean background level, assessed as particles over empty resin, from positive samples. The corrected values obtained

Fig. 1.A. Low-power photomicrograph of the piriform cortex showing immunoreactive dots distributed abundantly in layer I (I) and sparsely in layer II (II). Bar: 100 μm. **B.** Electron micrograph of glutamate-like immunoreactivity in the rat piriform cortex showing a synaptic contact whith a hole in the scalloped membrane. The pre- and postsynaptic membranes appear to be intact and continuous in the periphery of the gap. Bar: 0.5 μm. **C and D.** Electron micrographs of glutamate-like immunoreactivity and WGA-HRP labelling in the rat piriform cortex. The immunoreactive olfactory terminals labelled with TMB crystals (arrows) contain round synaptic vesicles, dark mitochondria and make asymmetric synaptic contacts with dendritic profiles (C). Some of the terminals (D) show a synaptic specialization (arrowhead) known as a "perforated synapse". Bars: 0.5 μm.

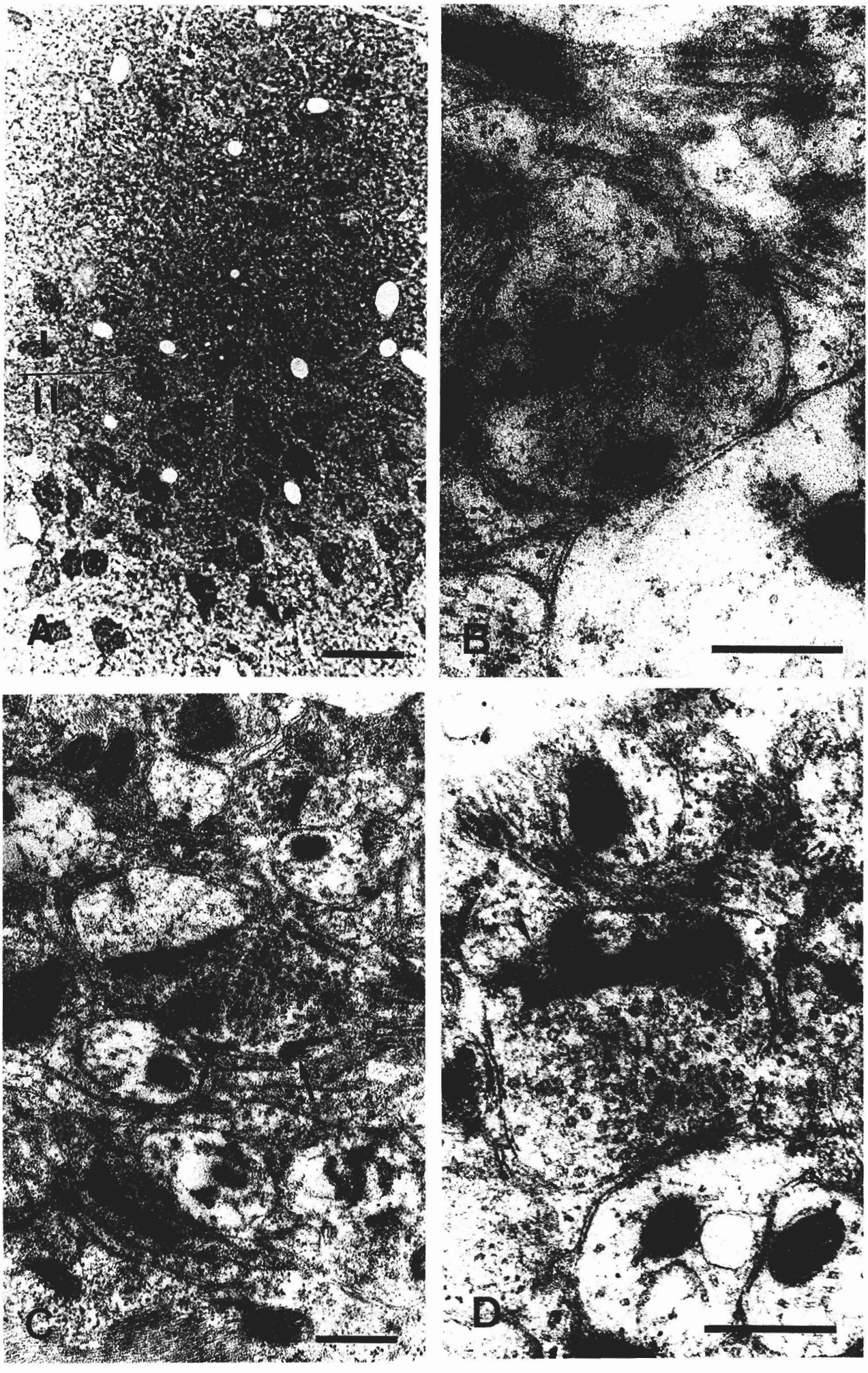
I
II
A
B
C
D

Table 1. Statistical comparison of glutamate immunoreactivity in analysed profiles from the rat olfactory cortex.

PROFILES	MEAN GOLD PARTICLES PER μm^2±SEM	PROFILE NO.	STATISTICAL COMPARISON			
OT	26.04 ±1.26	35	OT			
PE (OT)	11.20 ±1.02	34	<0,01	PE (OT)		
SC	11.39 ±1.10	7	<0,01	>0.05*	SC	
PE (SC)	9.31 ±1.18	8	<0,01	>0.05*	>0.05*	PE (SC)
Glia	7.76 ±1.18	6	<0,01	>0.05*	>0.05*	>0.05*

Differences in gold particle densities between the various profiles were assessed statistically by means of the Fisher LSD procedure after significant ($p<0.01$) Anova test. *: not statistically different. Gold particle density values over tissue profiles corrected for density of gold particles over empty resin (1.6 particles/μm^2). All the OT terminals used in the quantitative analysis were identified by the presence of WGA-HRP crystals. OT: olfactory terminals; PE (OT): elements postsynaptic to olfactory terminals; SC: symmetric synaptic contacts; PE (SC): elements postsynaptic to symmetric contacts.

from the olfactory cortex showed that olfactory-labelled terminals were twice more heavily gold labelled than their postsynaptic profiles and symmetric terminals as well as three times the corresponding glial values. These differences in the levels of Glu-Li were statistically significant (Table 1).

Discussion

The specificity of mAb2D7 has been demostrated in previous studies (Liu et al., 1989; Grandes et al., 1994; Ortega et al., 1995) and a very important finding was that mAb2D7 discriminated well between glutamate and aspartate, two chemically-related amino acids (Palaiologos et al., 1989). The pattern of Glu-Li seen in the olfactory cortex was comparable to that observed using tracing techniques. Layer I of the olfactory cortex showed abundant labelled fibers and dots, irrespective of the method used.

The ultrastructural study showed two types of terminals anterogradely labelled with crystal of WGA-HRP, flat and scalloped, both of them with similar mean values of gold particle density over their profiles.

In the neuropil of the olfactory cortex we have found scalloped synapses that have been related to terminals of olfactory bulb origin (Haberly and Feig, 1983). Moreover, in the HRP-WGA material rod-like deposits were common in synaptic terminals with scalloped profiles, providing additional evidence of their olfactory bulb origin. The present study indicates that scalloped terminals may use glutamate as a neurotransmitter because of the high levels of glutamate found by immunohistochemical methods. In addition, several of these scalloped terminals presented holes in the synaptic specialization. The presence of perforated synapses has previously been described in other areas (Calverley and Jones, 1990) and associated with several neuronal functions such as learning and growth. However, despite the large number of morphological studies devoted to the olfactory cortex, this type of synaptic contact has not been described to date in this structure, although it is possible to observe some of them in previous works (Haberly and Behan, 1983; Haberly and Feig, 1983).

The results from the present investigation indicate that olfactory terminals in the piriform cortex are glutamate-immunoreactive, which provides additional support for the role of glutamate as a neurotransmitter in the primary olfactory projection.

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References

Bradford H.F. and Richards C.D. (1976). Specific release of endogenous glutamate from piriform cortex stimulated in vitro. Brain Res. 105, 168-172.

Calverley R.K.S. and Jones D.G. (1990). Contribution of dendritic spines and perforated synapses to synaptic plasticity. Brain Res. Rev. 15, 215-249.

Collins G.G.S. (1984). Amino acid transmitter candidates in various regions of the primary olfactory cortex following bulbectomy. Brain Res. 296, 145-147.

Derer P., Caviness V.S. Jr. and Sidman R.L. (1977). Early cortical histogenesis in the primary olfactory cortex of the mouse. Brain Res. 133, 27-40.

Grandes P., Ortega F. and Streit P. (1994). Glutamate-immunoreactive climbing fibres in the cerebellar cortex of the rat. Histochemistry 101, 427-437.

Haberly L.B. (1983). Structure of the piriform cortex of the opossum. I. Description of neuron types with Golgi methods. J. Comp. Neurol. 213, 163-187.

Haberly L.B. and Behan M. (1983). Structure of the piriform cortex of the opossum. I. Ultrastructural characterization of synaptic terminals of association and olfactory bulb afferent fibers. J. Comp. Neurol. 219, 448-460.

Haberly L.B. and Feig S.L. (1983). Structure of the piriform cortex of the opossum. II. Fine structure of cell bodies and neuropil. J. Comp. Neurol. 216, 69-88.

Haberly L.B. and Price J.L. (1978a). Association and commissural fiber systems of the olfactory cortex of the rat. I. Systems originating in the piriform cortex and adjacent areas. J. Comp. Neurol. 178, 711-740.

Haberly L.B. and Price J.L. (1978b). Association and commissural fiber systems of the olfactory cortex of the rat. II. Systems originating in the olfactory peduncle. J. Comp. Neurol. 181, 781-802.

Heimer L. and Kalil R. (1978). Rapid transneuronal degeneration and death of cortical neurons following removal of the olfactory bulb in

adult rats. J. Comp. Neurol. 178, 559-609.

Johnson J.L. and Aprison M.H. (1971). The distribution of glutamate and total free amino acids in thirteen specific regions of the cat central nervous system. Brain Res. 26, 141-148.

Liu C.J., Grandes P., Matute C., Cuenod M. and Streit P. (1989). Glutamate-like immunoreactivity revealed in rat olfactory bulb, hippocampus and cerebellum by monoclonal antibody and sensitive staining method. Histochemistry 90, 427-445.

Luskin M.B. and Price J.L. (1982). The distribution of axon collaterals from the olfactory bulb and the nucleus of the horizontal limb of the diagonal band to the olfactory cortex, demonstrated by double retrograde labeling techniques. J. Comp. Neurol. 209, 249-263.

Luskin M.B. and Price J.L. (1983a). The topographic organization of associational fibers of the olfactory system in the rat, including centrifugal fibers to the olfactory bulb. J. Comp. Neurol. 216, 264-291.

Luskin M.B. and Price J.L. (1983b). The laminar distribution of intracortical fibers originating in the olfactory cortex of the rat. J. Comp. Neurol. 216, 292-302.

Ortega F., Hennequet L., Sarría R., Streit P. and Grandes P. (1995). Changes in the pattern of glutamate-like immunoreactivity in rat superior colliculus following retinal and visual cortical lesions. Neuroscience 67, 125-134.

Palaiologos G., Hertz L. and Schousboe A. (1989). Role of aspartate aminotransferase and mitochondrial dicarboxylate transport for release of endogenously and exogenously supplied neurotransmitter in glutamatergic neurons. Neurochem. Res. 14, 359-366.

Price J.L. (1973). An autoradiographic study of complementary laminar patterns of termination of afferent fibers to the olfactory cortex. J. Comp. Neurol. 150, 87-108.

Weinberg R.J. and VanEyck S.L. (1991). A tetramethilbenzidine/tungstate reaction for horseradish peroxidase histochemistry. J. Histochem. Cytochem. 39, 1143-1148.

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