Subcellular localization of m2 muscarinic receptors in GABAergic interneurons of the olfactory bulb

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Abstract

We analysed the ultrastructural distribution of the m2 muscarinic receptor (m2R) in the rat olfactory bulb (OB) using immunohistochemical techniques and light and electron microscopy. m2R was differentially distributed within the cellular compartments of γ-aminobutyric acid (GABA)ergic bulb interneurons. It is located in the gemmules of granule cells and in the synaptic loci of the interneurons of the external plexiform layer, suggesting that m2R activation could modulate the release of GABA from these interneurons onto principal cells by a presynaptic mechanism. By contrast, the receptor appears in the somata and dendritic trunks of second-order short-axon interneurons located in the inframitral layers, suggesting that postsynaptic muscarinic activation in these cells could elicit the inhibition of granule cells, leading to a disinhibition of principal cells. We also detail the anatomical substrate for a new putative muscarinic modulation that has not been previously described, and that could influence the reception of sensory information within the olfactory glomeruli. m2R appears in a subset of GABAergic/dopaminergic juxtaglomerular cells innervated by olfactory axons but is absent in juxtaglomerular cells that do not receive sensory inputs. This finding suggests that m2R activation could modify, through dopaminergic local circuits, the strength of olfactory nerve inputs onto principal cells. Activation of the muscarinic receptor may modulate the olfactory information encoding within olfactory glomeruli and may facilitate the bulb transmission to superior centres influencing the GABA release by presynaptic and postsynaptic mechanisms. Taken together, our data provide the neuroanatomical basis for a complex action of m2R at different levels in the mammalian OB.

Introduction

The synaptic organization of the olfactory bulb (OB) is well established. Olfactory receptors transduce olfactory signals and project their axons to the olfactory glomeruli, where they synapse with principal neurons, i.e. mitral and tufted cells. The bulb responsiveness to olfactory stimuli is modulated by inhibitory interneurons: (i) periglomerular cells, granule cells, and γ-aminobutyric acid (GABA)ergic cells of the external plexiform layer (EPL) synapse onto principal neurons and are called first-order interneurons; and (ii), deep, short-axon cells innervate the first-order interneurons, and are therefore termed second-order interneurons. Moreover, strong centrifugal inputs from the nucleus of the diagonal band, the dorsal and median raphe nuclei and the locus coeruleus modulate bulbar output (Shipley et al., 1995; see Shepherd & Greer, 1998 for a review).

The centrifugal projection from the nucleus of the diagonal band includes an important cholinergic component implicated in olfactory memory (Macrides et al., 1982; Ravel et al., 1994; Lévy et al., 1995). It is widely accepted that the bulbar targets of cholinergic fibres are a population of second-order short-axon cells containing acetylcholine (ACh) (Nickell & Shipley, 1988; Le Jeune & Jourdan, 1991, 1994). However, Kasa et al. (1995) demonstrated synaptic contacts from cholinergic fibres on first-order interneurons and have suggested an additional nonsynaptic effect of ACh on projection neurons. It would thus appear necessary to re-examine the cholinceptive targets of the OB in order to understand the modulatory actions of ACh in the processing of olfactory information.

The cellular actions of ACh in the nervous system are mediated by nicotinic and muscarinic receptors. Autoradiographic and binding studies have revealed the presence of both receptor families in the OB (Le Jeune et al., 1995, 1996) and electrophysiological experiments support their functional involvement in mediating the modulatory role of the cholinergic input (Elaagoubey et al., 1991). Recently, Castillo et al. (1999) reported that the activation of muscarinic receptors influences granule cells in two different ways, i.e. by reducing their excitability and by modulating the release of GABA onto principal cells. To explain this dual effect, both a postsynaptic and a presynaptic location of muscarinic receptors in bulbar interneurons is required. Previous binding autoradiographic studies did not have sufficient resolution to analyse the presumable subcellular compartmentalization of muscarinic receptors (Le Jeune et al., 1995, 1996) but immunohistochemical techniques combined with electron microscopy may resolve this issue.

Here, we investigate the ultrastructural location of m2 muscarinic receptors (m2R) in the OB. Special interest was focused on this muscarinic receptor subtype because the OB is the region of the brain in which this receptor shows one of its highest levels of expression (Cortés & Palacios, 1986). Recently, it has been demonstrated that...
calbindin D-28 k (CB) and calretinin (CR) are present in periglomerular cells, which do not receive sensory input, whereas tyrosine hydroxylase (TH) marks periglomerular cells that are innervated by olfactory axons (Kosaka et al., 1995, 1997, 1998; Toida et al., 1998). Thus, double immunolabelling for m2R and CB and CR and TH was used to investigate the expression of m2R in specific neuronal populations related to the reception of sensory afferences.

Materials and methods

Animals and tissue preparations

Twelve adult male Wistar rats were used in this study. Animals were deeply anaesthetized with 4% chloral hydrate in physiological saline (1 ml/100 g body weight) and intra-aortically perfused first with saline for 1 min followed by 500 ml of fixative according to the experimental procedures explained below. All procedures were in accordance with the guidelines of the European Communities Council Directive (86/609/EEC), and the current Spanish legislation for the use and care of animals, and conformed to NIH guidelines. Five rats were used for the m2R distribution analysis and for the colocalization studies with CR, CB and TH. In these animals, the fixative was 4% (w/v) paraformaldehyde and 2% (w/v) picric acid in 0.1 M phosphate buffer (PB), pH 7.4. To enhance the m2R immunoreactivity, as previously reported (Hajós et al., 1998), two additional animals were first perfused for 2 min with 5% acrolein in PB followed by 500 ml of the fixative described above. For the colocalization analysis of m2R and GABA and for electron microscopy, the five remaining animals were perfused with a fixative composed of 4% (w/v) paraformaldehyde, 1% glutaraldehyde and 2% (w/v) picric acid in 0.1 M PB, pH 7.4.

After perfusion, the brains were removed from the skull, and the olfactory bulbs were dissected and carefully rinsed in cold (4°C) PB overnight. Afterwards, 50-μm-thick transverse sections were cut on a vibratome (VT 1000E, Leica, Nussloch, Germany) and collected in cold (4°C) PB.

Pre-embedding m2R immunohistochemistry

Float-freezing sections from different animals were cryoprotected by overnight immersion in a mixture of 25% sucrose and 10% glycerol in 0.05 M PB at 4°C. The sections were then freeze-thawed three times in aluminium foil boats using liquid nitrogen. Following extensive washes in PB, sections from animals perfused with fixative containing glutaraldehyde or acrolein were treated with 1% NaBH₄ in PB for 30 min and extensively rinsed in PB. For m2R immunocytochemical staining, the avidin–biotin–immunoperoxidase method (Hsu et al., 1981) was used. In this procedure, sections were sequentially incubated in: (i) blocking solution containing 2% bovine serum albumin (Sigma, St. Louis, MO, USA) in PB for 45 min at room temperature; (ii) monoclonal rat anti-m2R (Chemicon International Inc., Temecula, CA, USA; 1:500 in PB) for 48 h at 4°C; (iii) biotinylated rabbit anti-rat immunoglobulin (Vector Labs., Burlingame, CA, USA; 1:250 in PB) for 2 h at room temperature; and (iv) avidin-biotinylated horseradish peroxidase complex (Vector; 1:250 in PB) for 2 h at room temperature. After each step, sections were carefully rinsed in PB (3 x 10 min). The peroxidase reaction was developed using 0.05% 3,3’-diaminobenzidine tetrahydrochloride (Sigma) and 0.003% hydrogen peroxide in PB for 10–20 min until m2R-specific immunostaining could be clearly visualized under the microscope. Sections were then rinsed in PB (3 x 10 min), treated with 1% osmium tetroxide (Sigma) for 1 h and washed repeatedly in PB. The sections were then stained with 1% uranyl acetate for 90 min and dehydrated through a graded ethanol series, cleared with propylene oxide and flat-embedded in Durcupan (ACM, Fluka AG, Switzerland) between slides and coverslips. Durcupan was polymerized overnight at 60°C. Finally, sections were carefully examined microscopically and some were further processed for postembedding CB, CR, or GABA immunohistochemistry as indicated below. After light microscopic analysis, a few sections containing m2R-positive profiles from the animals perfused with 1% glutaraldehyde were re-embedded in Durcupan. The selected sections were cut on an ultramicrotome and serial ultrathin sections were mounted on single-slot formvar-coated copper grids, stained with lead citrate and analysed using electron microscopy.

The primary antibody used in the study has been exhaustively characterized and previously used in the rat nervous system (Levey et al., 1995a, 1995b; Hajós et al., 1998). Additional controls for immunohistochemical specificity were carried out by omitting the first or second antibody in each step, and incubating some sections exclusively in 0.05% 3,3’-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in PB in order to rule out the presence of endogenous peroxidase in the tissue. No residual reactivity was found in the controls.

Colocalization of m2R with the calcium-binding proteins CB and CR for light microscopy

To analyse the colocalization of m2R with CB and CR in the same cells, the m2R-pre-embedding and CB- or CR-postembedding double immunocytochemical method on adjacent semithin sections was performed. After pre-embedding m2R immunohistochemistry (as indicated above), 50-μm-thick flat-embedded sections containing m2R-immunopositive somata were selected and re-embedded in Durcupan. Serial semithin (1 μm-thick) sections were then cut with an ultramicrotome and mounted on gelatin-coated slides. A one-in-two series was kept to identify the m2R-immunopositive cells labelled after the pre-embedding procedures. Alternate sections from adjacent series were employed to detect CB or CR by postembedding immunocytochemistry. In this procedure, the Durcupan was removed by treating the sections for 12 min with a saturated sodium ethoxide solution. The sections were then rinsed and hydrated through a decreasing ethanol series down to distilled water. Because the tissue had been treated with osmium tetroxide in the pre-embedding procedure, antigenic sites were unmasked by incubating the sections with a fresh solution of 2% sodium metaperiodate (Fluka, Switzerland) in distilled water for 20 min; following this the sections were rinsed in distilled water (3 x 5 min) and in PB (3 x 10 min). For the detection of CB and CR, sections were sequentially incubated at room temperature in: (i) blocking solution containing 10% normal goat serum and 0.1% Triton X-100 in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 45 min; (ii) polyclonal rabbit anti-CB or rabbit anti-CR (Swant, Bellinzona, Switzerland; 1:1000 in PBS containing 0.1% Triton X-100) overnight; (iii) biotinylated goat anti-rabbit IgG (Vector; 1:250 in PBS) for 45 min; and (iv), avidin-biotinylated horseradish peroxidase complex (Vector; 1:250 in PBS) for 30 min. After each step, sections were carefully rinsed in PBS (3 x 10 min). The peroxidase reaction was developed using 0.05% 3,3’-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in PBS for 5–10 min, until specific CB- or CR-immunostaining was clearly visualized under the microscope. Sections were then washed in PBS (3 x 10 min) and CB- and CR-immunostaining was enhanced by treating the sections with 1% osmium tetroxide for 2 min. After dehydration through a graded alcohol series, sections were cleared with xylene and mounted with Eukitt (Kindler GmbH, Freiburg, Germany).
m2R-immunopositive neurons and nearby blood vessels were identified in semithin sections and were then drawn with a camera lucida using a 100 × oil-immersion objective. The same profiles were located using capillaries as landmarks on the adjacent semithin sections processed for the demonstration of CB or CR. It was determined whether the m2R-immunopositive cells were or were not immunopositive for CB or CR; only those cells unequivocally present in both adjacent semithin sections were studied.

Omission of the first or second antibodies in each step, and sections incubated exclusively in 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in PBS were used as controls. No residual immunoreactivity was observed. In addition, the patterns of CB- and CR-immunolabeling in sections previously stained for m2R in the pre-embedding procedures were similar to those found for both calcium-binding proteins in standard material, as previously reported (Brignon et al., 1999).

**Colocalization of m2R with TH for light microscopy**

The colocalization of m2R and TH in the same cells was analysed using the double-immunofluorescent technique. Vibratome 50 μm-thick sections were sequentially incubated in: (i) blocking solution containing 2% bovine serum albumin in PB for 45 min at room temperature; (ii) a mixture of 1 : 100 rat anti-m2R and 1 : 1000 mouse anti-TH (Inctar Corporation, Stillwater, MN, USA) in PB for 48 h at 4°C; (iii) biotinylated rabbit anti-rat antibody (Vector; 1 : 250 in PB) for 2 h at room temperature; and (iv), a mixture of avidin fluorescein isothiocyanate-conjugated (Vector; 1 : 75 in PB) and donkey anti-rabbit Texas red-conjugated antibody (Jackson Labs, West Grove, PA, USA; 1 : 400 in PB) for 2 h at room temperature. After washing in PB, sections were mounted on gelatin-coated slides and coverslipped with an antifading-containing medium (0.42 g glycine, 0.021 g sodium hydroxide, 0.51 g sodium chloride, 0.03 g sodium azide, and 5 g N-propyl gallate in 100 ml. of 70% glycerin). Double-labelled neurons were identified under a photomicroscope equipped with epifluorescence and appropriate filter sets.

Controls for the immunohistochemical procedure were prepared by incubating sections without the first or second antibody in each step. No residual reactivity was found. In addition, omission of one of the primary or secondary antibodies resulted in the disappearance of staining for that particular antigen without changing the pattern of immunoreactivity for the other.

**Colocalization of m2R with GABA: post-embedding immunogold staining for GABA**

To test the GABAergic nature of the m2R-containing elements, pre-embedding m2R immunocytochemistry was combined with post-embedding immunogold staining for GABA. After light microscopic analysis, sections including fields containing m2R-immunoreactive somata and neuropil, from the animals perfused with fixative containing 1% glutaraldehyde, were re-embedded in Durcupan. This material was cut with an ultramicrotome and serial ultrathin sections were mounted on single-slot formvar-coated nickel grids. Post-embedding GABA immunostaining was carried out according to Somogyi & Hodgson (1985) with slight variations. All steps were carried out on droplets of solution in humid Petri dishes as follows: treatment with 1% periodic acid for 10 min; washing in distilled water (3 × 3 min); treatment with 2% sodium metaperiodate for 10 min; washing in distilled water (3 × 3 min); washing 0.05 M Tris buffer saline, pH 7.4 (TBS) for 3 min; blocking solution containing 1% chicken egg albumin in TBS for 30 min; washing in TBS (2 × 10 min); rabbit anti-GABA antiseraum (Sigma; 1 : 2500 in 1% normal goat serum in TBS) for 2 h; washing in TBS (2 × 10 min); 1% bovine serum albumin and 0.05% Tween 20 in Tris-HCl buffer (TB) for 10 min; goat anti-rabbit IgG-coated colloidal gold (Sigma, 10 nm, 1 : 15 in TB containing 1% bovine serum albumin and 0.05% Tween 20) for 2 h; washing in distilled water (3 × 5 min) and PBS (4 × 5 min); 2% glutaraldehyde in PBS for 2 min; washing in distilled water (4 × 5 min); silver enhancement for 1 min; washing in distilled water (4 × 5 min); saturated uranyl acetate for 30 min; washing in distilled water (4 × 5 min); staining with lead citrate; and washing in distilled water. Profiles showing a density of colloidal gold particles 10 × background level, in two or three adjacent sections were considered GABA-immunoreactive. Profiles of olfactory nerve terminals or mitral/tufted cells dendrites (presumed glutamatergic elements) were used to establish background density.

Omission or substitution of the GABA antiserum with normal rabbit serum resulted in a lost of specific staining, i.e. no signs of colloidal gold accumulation could be detected.

**Results**

**Light microscopic observations of m2R distribution in the OB**

A laminar distribution pattern was found for m2R in the OB (Fig. 1A). m2R-immunoreactive elements were detected in all of the OB layers with the exception of the olfactory nerve layer (ONL), which did not display either immunoreactive cells or fibres. An intense immunostaining was observed in the glomerular layer (GL), the internal plexiform layer (IPL) and the superficial portion of the granule cell layer (GCL), but the highest density of m2R-containing elements appeared in the external plexiform layer (EPL). The reaction product covered the surface of the soma and/or processes of the immunopositive elements, with faint immunoreactivity deeper in the cytoplasm. The overall m2R distribution pattern was consistent among all animals used in the study. Nevertheless, the staining intensity was stronger in animals perfused with acrolein, allowing optimal visualization of the m2R-positive elements as previously reported (Hájos et al., 1998).

In the GL, an abundant subpopulation of juxtaglomerular m2R-containing small or medium-sized round neurons was located in the periglomerular region of the olfactory glomeruli (Fig. 1B). These cells had one, or rarely two dendritic trunks innervating the glomerular core. They showed m2R-immunoreactivity in their cell body and dendritic shafts but their axons were not stained. According to their size and location the small and the medium-sized juxtaglomerular cells could correspond to periglomerular cells and external tufted cells, respectively. No differences regarding the density of m2R immunoreactive cells were found among any of the olfactory glomeruli. Even for the atypical glomeruli, in which cholinergic innervation is paramount (Zheng et al., 1987; Le Jeune & Jourdan, 1991, 1993; Crespo et al., 1995), the distribution of m2R-containing elements showed no variance.

The strongest m2R immunolabeling in the OB was found in the neuropil of the EPL (Fig. 1A). In this layer, the density of m2R-containing processes was high enough to mask the presence of interneurons and tufted cells in the 50 μm-thick sections. To determine whether these neurons displayed m2R-immunoreactivity, some sections were re-embedded and re-sectioned at a thickness of 1 μm. In these sections, the large somata of tufted cells and their thick dendritic trunks, as well as the cell bodies of the interneurons located in the EPL, such as superficial short-axon cells, Van Gieson cells and multipolar cells, were m2R-immunonegative. Even with the semithin sections, light microscopy did not allow identification of the neuronal types which gave rise to the dense m2R-containing neuropil of this layer.
In the mitral cell layer (MCL), the somata of the mitral cells lacked m2R-immunostaining. In the inframitral regions, occasionally a few granule cell perikarya were labelled (less than 1% of the whole population of granule cell somata) (Fig. 1C). The granule cell somata containing m2R were preferentially located in the IPL and superficial portion of the GCL rather than in the deep portions of this layer. The reaction product in these neurons was also found in the apical dendrites coursing towards the EPL, but not in the basal dendrites.
coursing towards deep portions of the GCL. Despite the presence of a few m2R-positive granule cell somata, the vast majority of these interneurons did not display m2R-immunoreactivity in their cell bodies. Another neuronal population showed m2R immunoreactivity in the inframitral regions. It included large neurons (15–20 μm) with piriform, horizontal or multipolar morphologies. The location and morphological characteristics allowed us to identify these neurons as different morphological types of deep short-axon cells, including vertical cells of Cajal (Fig. 1D) and horizontal cells (Fig. 1E). In the white matter (WM), the m2R-containing deep short-axon cells displayed fusiform morphology with one or two thin dendrites, running parallel to the bulbar lamination (Fig. 1F). Although deep short-axon cells showed the reaction product in their somatodendritic portions, no axons arising from these interneurons could be identified.

Electron microscopic analysis of m2R distribution in the OB. Colocalization with GABA

Glomerular layer

Electron microscopic observations confirmed the light microscopic analysis. m2R was found in the somata and dendrites of interneurons, whereas the receptor was never observed in the profiles of principal cells. In the periglomerular region of the olfactory glomeruli, most m2R-containing elements consisted of small round neurons with their nuclei almost completely filling the cell body, surrounded by a thin rim of cytoplasm. The nuclei often presented nuclear indentations. These ultrastructural features corresponded to those of periglomerular cells (Pinching & Powell, 1971a).

Inside the olfactory glomeruli, the glomerular neuropil is subdivided into two different compartments. The sensory-synaptic
Fig. 3. Subcellular distribution of m2R immunoreactivity in the EPL. (A and B) Synaptic contacts (arrows) between dendritic profiles of mitral/tufted cells (M/T) and m2R-containing gemmules of granule cells. In (B), note the presence of a typical reciprocal synapse (open arrows). (C) Large m2R-immunopositive profile making a symmetrical synaptic contact (arrow) on a proximal dendrite of a mitral/tufted cell (M/T). The m2R-immunolabeled profile exhibits the same ultrastructural features (large size and a high density of mitochondria at the synaptic loci) as those of GABAergic cells of the EPL. (D) Low-magnification electron micrograph depicting an internal tufted cell (T) within the EPL. The squared area is shown at higher magnification in E. (E) Synaptic contact (arrow) between a m2R-containing profile and the soma of the internal tufted cell (T) shown in D. In addition, this profile receives a synaptic contact from the dendritic trunk of an adjacent mitral/tufted cell (M/T) (open arrow). Scale bars, 0.5 µm (A–C and E); 5 µm (D).

compartment includes sensory axons and terminal dendritic branches of periglomerular cells and principal neurons. By contrast, the central-synaptic compartment comprises dendritic branches of principal neurons and periglomerular cells but is devoid of sensory axons (Chao et al., 1997; Kasowski et al., 1999). We found profiles containing m2R homogeneously distributed in both neuropil zones. On the basis of ultrastructural criteria, these profiles were identified as the dendritic shafts and terminal dendritic branches of the m2R-containing juxtaglomerular cells (Fig. 2). The sensory axons and the processes of mitral/tufted cells were easily recognized on the basis of previously reported ultrastructural descriptions (Pinching & Powell, 1971b) and they were always m2R-immunonegative.

The synaptic relationships of the positive elements found within the glomerular neuropil were as follows. In the sensory-synaptic zone, the m2R-immunopositive gemmules of juxtaglomerular cells received asymmetrical synaptic contacts from sensory axons (Fig. 2A). In addition, dendrodendritic synapses between m2R-labelled juxtaglomerular cells and principal neurons were found in both glomerular compartments (Fig. 2B). The synaptic relationships included asymmetrical synaptic contacts from the mitral/tufted...
dendrites to the juxtaglomerular cell profiles and symmetrical contacts from juxtaglomerular cell gemmules to the mitral/tufted dendrites. In the symmetrical contacts from m2R-containing juxtaglomerular cell dendrites to projection cell processes, m2R appeared in a presynaptic location. Both asymmetrical and symmetrical contacts were frequently located side by side forming reciprocal synapses.

Post-embedding immunogold staining for GABA was carried out on ultrathin sections in order to investigate the GABAergic nature of the m2R-immunopositive profiles, demonstrating that all the m2R-containing elements (n=100) examined in the GL were GABA-immunopositive (Fig. 2C–E).

**External plexiform layer**

In the EPL, a dense neuropil was immunostained for m2R, confirming the light microscopic observations. Detailed analysis revealed that most of the m2R-containing elements displayed the ultrastructural features previously reported for the gemmules of granule cells (Fig. 3A and B) and for the synaptic loci of the dendrites of the interneurons present in this layer (Fig. 3C–E). On the contrary, the receptor was never found in processes or somata of the mitral/tufted cells.

The m2R-containing gemmules of granule cells received asymmetrical contacts from mitral/tufted dendrites and made symmetrical contacts onto them (Fig. 3A and B). These synapses were frequently reciprocal (Fig. 3B). As has been described for the periglomerular cell gemmules, the m2R-immunoreactivity displayed a presynaptic location in the symmetrical contacts from granule cells to principal cell processes.

The other m2R-immunoreactive profiles that integrate the stained neuropil of the EPL were larger in size than granule cell gemmules and showed a prominent abundance of mitochondria (Fig. 3C and E). Moreover, they differed from granule cell gemmules in their location within the EPL; whereas granule cell gemmules innervated different portions of the secondary mitral/tufted cell dendrites, the larger m2R-immunostained profiles were preferentially located in the perisomatic region of mitral/tufted cells and close to their proximal dendrites (Fig. 3D and E). These morphological features and distribution
Fig. 5. Colocalization of m2R with CB, CR and TH in juxtaglomerular cells. Panels on the left correspond to m2R-immunoreactivity. Panels on the right correspond to CB, CR and TH. (A and B) Photographic matching par of consecutive semithin sections showing pre-embedding m2R immunohistochemistry and postembedding immunohistochemistry for the detection of CB. The CB-immunoreactive cells depicted in B lack m2R immunoreactivity in A (asterisks). Open arrows indicate two m2R-immunopositive/CB-immunonegative neurons. (C and D) Photographic matching par of consecutive sections showing pre-embedding immunohistochemical detection of m2R and postembedding CR immunoreactivity. Neurons showing CR-immunostaining in D are m2R-immunonegative in C (asterisks). Open arrows indicate m2R-immunopositive neurons that do not express CR. (E and F) Double immunofluorescence method for the detection of m2R (E) and TH (F) in the same sections demonstrates the colocalization of both markers in a large population of juxtaglomerular cells (open arrows). Scale bar, 10 μm.

Correspond to those previously reported for the synaptic loci of the dendrites of those GABAergic interneurons located in the EPL that contain the calcium-binding protein parvalbumin (Toida et al., 1994, 1996). These m2R-containing profiles showed a synaptic connectivity similar to that of granule cells gemmules. They received asymmetrical contacts from the somata or proximal dendrites of mitral/tufted neurons and made symmetrical synapses onto them (Fig. 3C and E).

The receptor displayed a presynaptic location in the symmetrical contacts. Although light microscopic analysis did not detect m2R immunostaining in the cell bodies of the interneurons of the EPL, these somata were also analysed at the electron microscopic level confirming the lack of receptor.

Post-embedding immunogold detection of GABA demonstrated the GABAergic nature of all the m2R-containing elements found in...
the EPL, including the gemmules of granule cells (Fig. 4A) and the synaptic loci of the dendrites of the interneurons of the EPL (Fig. 4B and C).

**Infra- mitral regions**

Electron microscopy confirmed that although a few granule cell perikarya showed m2R-immunoreactivity, the vast majority of granule cell somata did not contain m2R in the inframitral layers. Most of the m2R-positive elements found in these regions corresponded to the somata and dendritic shafts of deep short-axon cells. All the m2R-containing elements found in these regions were GABAAergic as demonstrated the postembedding immunogold staining.

**Colocalization of m2R with CB, CR, and TH in the juxtaglomerular cells at light microscopic level**

Juxtaglomerular cells do not constitute an homogeneous population of interneurons (Kosaka et al., 1995, 1997, 1998; Toida et al., 1998). In this respect, two different subpopulations of periglomerular cells have been described on the basis of their connectivity with olfactory nerve fibres. These two subpopulations can be identified according to their neurochemical features. One subpopulation of periglomerular cells receives synaptic contacts from the olfactory nerve axons, and these neurons are neurochemically characterized by the presence of TH and/or GABA in their somata. The other subpopulation of periglomerular cells does not establish synaptic contacts with the olfactory nerve axons, and these cells may be neurochemically identified by the presence of CB or CR and by the absence of GABA in their cell bodies. Because we had found a subpopulation of m2R-immunopositive juxtaglomerular cells in the GL, it was of particular interest to investigate whether they corresponded to one or both of the two above reported subpopulations of periglomerular cells. Our electron microscopic observations demonstrated that m2R-containing profiles in the GL received asymmetrical synapses from the olfactory nerve fibres and that all of them were GABAAergic (a neurochemical feature of the periglomerular cells innervated by the olfactory axons). Nevertheless, the colocalization of m2R with CB and CR was studied to check whether m2R is also present in juxtaglomerular cells that do not receive synapses from the olfactory fibres.

The m2R-pre-embedding and CB- or CR-postembedding double immunocytochemical staining on adjacent semithin sections allowed us to be conclusive regarding this question. The m2R-immunostaining was clearly identifiable in semithin sections and appeared covering the surface of the soma and/or processes of the positive cells (Fig. 5A and C). By contrast, the immunostaining found against CB or CR filled the whole cell body, including the cytoplasm and the nuclear region of the immunopositive neurons (Fig. 5B and D). A total of 200 m2R-containing juxtaglomerular cells were located on semithin sections and the profiles of the same perikarya were analysed on the adjacent sections incubated to detect one of the two calcium-binding proteins (100 for CB and 100 for CR). No m2R-immunopositive juxtaglomerular cells contained CB (Fig. 5A and B) or CR (Fig. 5C and D). It was therefore concluded that the subpopulation of juxtaglomerular cells that is not innervated by the olfactory axons does not express the muscarinic receptor.

Almost 50% of the GABAAergic periglomerular cells contacting with olfactory axons colocalize TH (Kosaka et al., 1995). Thus, in order to investigate whether the m2R-positive juxtaglomerular cells belong to this GABA/TH-positive subpopulation of periglomerular cells or to the GABA-positive/TH-negative population, the m2R/TH double immunofluorescence procedure was carried out. A total of 100 m2R-containing juxtaglomerular cells were analysed in the double-stained sections. These colocalization experiments demonstrated that the vast majority, if not all, of the neurons containing m2R contained TH as well (Fig. 5E and F).

**Discussion**

The major findings of this study are: (i) m2R showed a subcellular compartmentalization in GABAAergic interneurons of the OB. The receptor appeared in the gemmules and synaptic loci of first-order interneurons and by contrast, it appeared in the somata and dendritic trunks of second-order interneurons. This provides anatomical evidence in favour of a dual action of ACh, via m2R activation in the OB, including presynaptic modulation of neurotransmitter release and postsynaptic modulation of electric activity in cholinceptive interneurons; and (ii), m2R is expressed in juxtaglomerular cells receiving synapses from sensory axons, but it is absent in those juxtaglomerular cells that do not receive sensory inputs. This result strongly supports that muscarinic activation could influence the response of mitral/tufted cells to odourant stimuli through a subset of juxtaglomerular cells.

**Presynaptic location of m2R in the EPL**

The most intense staining for m2R in the OB has been found in the neuropile of the EPL. This layer contains the granule cell gemmules establishing reciprocal synapses with the dendrites of principal neurons (Shipley et al., 1995; Shepherd & Greer, 1998). In addition to granule cell processes, this layer contains a population of GABAAergic interneurons previously described using parvalbumin-immunocytochemistry (Toida et al., 1994, 1996). These neurons also establish reciprocal synapses with the dendrites and somata of principal neurons (Toida et al., 1996) and should thus be considered, in addition to granule cells, as first-order interneurons. Electron microscopy demonstrated high levels of m2R-immunoreactivity in the transmitter release sites of granule cells, whereas granule cell somata or proximal dendrites lack the receptor in the GCL. Moreover, we found a high expression of m2R in the synaptic sites of the population of GABAAergic interneurons of the EPL, but the receptor was absent from the somata and proximal dendrites. Our results clearly demonstrate a specific presynaptic localization of m2R in the EPL, and provide neuroanatomical support for a presynaptic modulation of the GABA release from these interneurons onto principal cell dendrites in this layer.

A presynaptic localization of m2R has been largely reported in different brain regions (Hersch et al., 1994; Rouse et al., 1997; Bernard et al., 1998; Hájos et al., 1998; Rouse et al., 1998; Nikbakht & Stone, 1999; Plummer et al., 1999). To date, only inhibitory actions of m2R located presynaptically have been shown. In this sense, it has been reported that activation of m2R reduces the release of glutamate from corticostratial fibres (Calabresi et al., 1998) or inhibits the release of ACh from individual release sites on basal forebrain neurons (Allen, 1999). In the hippocampus, m2R appears located on the axon terminals of parvalbumin-containing basket and axo-axonic cells innervating the perisomatic region of pyramidal neurons (Hájos et al., 1998). In these loci, the activation of m2R reduce the synaptic release of GABA by a presynaptic mechanism (Pitler & Alger, 1992; Behrens & Bruggencate, 1993; Hájos et al., 1998). The presynaptic localization of m2R in granule cells and GABAAergic interneurons of the EPL argues for a similar cholinergic modulation of GABAAergic neurotransmission in the rat OB. Presynaptic m2R would decrease GABA release from these first-order interneurons onto principal cell dendrites in the EPL. This effect could account for the reduction in inhibitory action upon principal neurons reported by Elagrouby et al. (1991) after local application of ACh and carbachol near the MCL of
the rat OB. However, Castillo et al. (1999) recently reported that activation of muscarinic receptors would facilitate GABA release from granule cells onto principal cells. This excitatory effect of muscarinic receptors might originate from a nonselective action of muscarinic drugs and from the effect of other muscarinic receptor subtypes in the rat OB. In this sense, autoradiographic and binding studies have revealed the presence of m1 muscarinic receptors in the rat OB (Le Jeune et al., 1995, 1996), and this muscarinic receptor subtype has been involved in the increase of the GABA release from GABAergic neurons in the neostriatum (Harsing & Zigmond, 1998).

The m2R has been postulated to be a presynaptic cholinergic autoreceptor in many brain regions (Vilaro et al., 1992; Hersch et al., 1994; Rouse et al., 1997; Bernard et al., 1998). The presynaptic localization of this receptor in cholinergic terminals suggests a direct involvement of m2R in the modulation of ACh release. Our results do not support a similar location of this muscarinic receptor in the cholinergic centrifugal axons coursing to the OB because m2R was exclusively located in GABAergic local elements.

**Postsynaptic location of m2R in the inframitral layers**

In addition to the presynaptic distribution of m2R, found in the synaptic loci of first-order interneurons, the muscarinic receptor appeared in somatodendritic portions of GABAergic second-order interneurons of the inframitral layers. It has been demonstrated that the effect of m2R activation on postsynaptic domains is likely to increase the excitability of GABAergic neurons (Müller & Misgeld, 1986; Baba et al., 1998). Thus, we propose that postsynaptic m2R activation of second-order interneurons of the inframitral regions of the rat OB could elicit the inhibition of granule cells, decreasing their firing rate and thus leading to a disinhibition of mitral/tufted cells. This model is in agreement with previous electrophysiological data from Elagoughy et al. (1991) that found a phasic muscarinic modulation of bulbar interneuronal activity that could finally reduce the inhibitory action of granule cells and thus facilitate the transmission of bulbar output neurons to central structures. These authors proposed that muscarinic activation of GABAergic interneurons presynaptic to granule cells could account for this effect. Now, we provide neuroanatomical basis for this presumably cholinergic modulation of bulbar circuits.

Moreover, it has been recently reported that activation of muscarinic receptors in inframitral layers decreases granule cell firing frequency by an activation of GABAergic input onto granule cells (Castillo et al., 1999). These authors proposed that, rather than short-axon cells or centrifugal fibres, the source of the GABAergic innervation of granule cells is probably neighbouring granule cells somatically activated by cholinergic axons through muscarinic receptors. Previous data do not support this hypothesis as synaptic contacts from granule cells onto neighbouring granule cells have not been demonstrated by neuroanatomical approaches (Price & Powell, 1970; Shepherd & Greer, 1998). Nevertheless, the GABAergic second-order short-axon cells of the inframitral layers directly innervate granule cells and interestingly, we demonstrate that a large subset of these second-order interneurons contain m2R in their somatodendritic portions.
Muscarinic modulation at the glomerular level

Electrophysiological data suggest a dual organization of the OB cholinergic system, matched with a segregation of muscarinic and nicotinic receptors. Whereas the EPL, IPL, and GCL seem to be more susceptible to modulation by muscarinic receptors, the GL has been considered more susceptible to nicotinic receptors (Elagoubi et al., 1991; Castillo et al., 1999). Previous reports showed that ACh increases the firing of interneurons from the GL (Pager, 1983). Nickell & Shipley (1988) identified the cholinceptive targets in the periglomerular region as short-axon cells and Elagoubi et al. (1991) proposed that this cholinergic action would be mediated via nicotinic receptors. More recently, it has been reported that activation of nicotinic receptors excites bipolar periglomerular cells (Castillo et al., 1999). Nevertheless, a muscarinic modulation of the bulbar circuits has not been considered previously in the GL. Here, for the first time, the neuroanatomical basis of a muscarinic action at the level of the olfactory glomeruli is established. A large subset of juxtakolomerular cells expressed m2R in their somatodendritic domain (postsynaptic loci). The lack of electrophysiological data prevents us from considering an effect for muscarinic activation on these interneurons. Despite this, the postsynaptic location of m2R strongly supports that ACh could increase the firing of juxtakolomerular cells via muscarinic activation, as has been demonstrated for nicotinic receptors (Castillo et al., 1999). In addition to their postsynaptic location, the receptors appeared on the gemmules of juxtakolomerular cells. This suggests a presynaptic modulation of the GABA release from these gemmules onto mitral/tufted dendrites, as has been discussed for granule cells. However, this presynaptic role remains to be demonstrated using electrophysiology.

The absence of colocalization m2R/CB and m2R/CR and the colocalization of m2R/GABA/TH clearly demonstrate that only the subset of GABAergic/dopaminergic juxtakolomerular cells innervated by the olfactory axons express m2R. Interestingly, it has been suggested that in the olfactory glomeruli, ACh could inhibit sensory inputs onto mitral cell dendrites through the activation of dopaminergic periglomerular cells (Castillo et al., 1999), because dopamine receptor activation reduces the strength of olfactory inputs (Hsia et al., 1999). Although it has been proposed that this effect could be mediated through nicotinic receptors, our data support that it may be mediated via m2R present in the subset of GABAergic/dopaminergic juxtakolomerular cells. On the other hand, we cannot confirm the possibility that ACh might exert a presynaptic decrease of dopamine release through m2R in juxtakolomerular cell gemmules, but it has been demonstrated that muscarinic receptor activation inhibits the release of catecholamines in rat hippocampal synaptosomes (Birch & Fillen, 1986).

Functional implications

We provide anatomical findings that suggest that muscarinic modulation of the OB is more complex than previously reported (Elagoubi et al., 1991; Nickell & Shipley, 1993; Castillo et al., 1999). The spatial segregation of m2R-containing cells and the subcellular compartmentalization of the muscarinic receptor prompt us to suggest that muscarinic modulation of the bulbar cortex, via m2R, takes place at three distinct levels (Fig. 6). First, in the GL the presynaptic receptor would modulate GABA and dopamine release from juxtakolomerular cells receiving sensory inputs and thus, influence the entrance of olfactory information. Second, in the EPL, postsynaptic m2R would modulate GABA release from first-order interneurons, leading to a disinhibition of mitral/tufted cells. Third, in the inframitral layers postsynaptic m2R would excite second-order interneurons, thus leading to the same effect: a disinhibition of mitral/tufted cells through the inhibition of granule cells. The convergence of presynaptic and postsynaptic m2R actions in the EPL and inframitral layers, respectively, would finally reduce the inhibitory action of granule cells and GABAergic interneurons of the EPL, thus, facilitating transmission of bulbular output neurons to central structures. This is in close agreement with a previous report demonstrating that stimulation of the horizontal limb of the diagonal band of Broca, the source of cholinergic fibres reaching the OB, activates mitral cell unit activity (Kunze et al., 1991). As a consequence, activation of m2R could facilitate the responsiveness of principal cells in the presence of sensory stimulation and thus increase the signal-to-noise ratio as in other sensory cortices.

Despite the high levels of m2R found in the OB, previous autoradiographic and binding studies or mRNA in situ hybridization experiments demonstrate the presence of all four (m1, m2, m3 and m4) muscarinic receptor subtypes in this brain centre (Buckley et al., 1988; Le Jeune et al., 1995, 1996). Similarly to m2R, m1 and m3 subtypes display their highest densities in the EPL (Araujo et al., 1991; Le Jeune et al., 1995). Nevertheless, the lack of detailed neuroanatomical descriptions and electrophysiological data on these muscarinic receptors make it difficult to establish their functional implications in the olfactory circuits. Further studies are needed in order to investigate a possible convergence of muscarinic actions in the cholinergic bulbar transmission, though different muscarinic receptor subtypes.

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Abbreviations

ACh, acetylcholine; CB, calbindin D-28k; CR, calretinin; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; m2R, m2 muscarinic receptor; MCL, mitral cell layer; OB, olfactory bulb; OLN, olfactory nerve layer; TH, tyrosine hydroxylase; WM, white matter.

References
