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Subcellular Redistribution of m2 Muscarinic Acetylcholine Receptors in Striatal Interneurons *In Vivo* after Acute Cholinergic Stimulation

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The purpose of our work was to investigate how the cholinergic environment influences the targeting and the intracellular trafficking of the muscarinic receptor m2 (m2R) *in vivo*. To address this question, we have used immunohistochemical approaches at light and electron microscopic levels to detect the m2R in control rats and rats treated with muscarinic receptor agonists.

In control animals, m2Rs were located mostly at postsynaptic sites at the plasma membrane of perikarya and dendrites of cholinergic and NPY–somatostatin interneurons as autoreceptors and heteroreceptors, respectively. Presynaptic receptors were also detected in boutons. The m2Rs were usually detected at extrasynaptic sites, but they could be found rarely in association with symmetrical synapses, suggesting that the cholinergic transmission mediated by m2R occurs via synaptic and nonsynaptic mechanisms. The stimulation of muscarinic receptors with oxotremorine provoked a dramatic alteration of m2R compartmentalization, including endocytosis with a decrease of the density of m2R at the membrane (−63%) and an increase of those associated with endosomes (+86%) in perikarya. The very strong increase of m2R associated with multivesicular bodies (+732%) suggests that oxotremorine activated degradation. The slight increase in the Golgi apparatus (+26%) suggests that the m2R stimulation had an effect on the maturation of m2R. The substance P receptor located at the membrane of the same neurons was unaffected by oxotremorine.

Our data demonstrate that cholinergic stimulation dramatically influences the subcellular distribution of m2R in striatal interneurons *in vivo*. These events may have key roles in controlling abundance and availability of muscarinic receptors via regulation of receptor endocytosis, degradation, and/or neosynthesis. Further, the control of muscarinic receptor trafficking may influence the activity of striatal interneurons, including neurotransmitter release and/or electric activity.

Key words: endocytosis; G-protein-coupled receptors; substance P receptor; basal ganglia; immunohistochemistry; multivesicular bodies

Receptors coupled to G-proteins belong to a large family of receptors mediating the functions of most classical neurotransmitters, such as norepinephrine, dopamine, acetylcholine (ACh), or neuropeptides, including substance P or neurotensin. *In vitro* experiments using cells transfected with G-protein-coupled receptors demonstrated that their agonist stimulation induces different events, including phosphorylation, endocytosis of the receptor, dissociation of the ligand from the receptor, dephosphorylation, and either degradation of the receptor or recycling to the plasma membrane (Koenig and Edwardson, 1997). These events are triggered via mechanisms involving changes in the subcellular compartmentalization of receptors, including their translocation from the plasma membrane into endosomes (Koenig and Edwardson, 1997). However, the mechanisms regulating *in vivo* the compartmentalization and recycling of receptors in neurons and their control by neurotransmitters in physiological, experimental, and pathological circumstances are still poorly understood. Recent studies have shown that *in vivo* the acute activation of G-protein-coupled receptors for neuropeptides (substance P, neurotensine, and opiates) or monoamines, such as dopamine, provokes dramatic changes in their subcellular compartmentalization directly linked to the type of stimulation and the cellular response (Faure et al., 1995; Sternini et al., 1996; Marvizon et al., 1997; Dumartin et al., 1998). Dumartin et al. (1998) have recently demonstrated that the acute activation of dopamine receptors induced internalization of D1 receptor in endosomes and recycling at the membrane in striatal dopaminoceptive neurons.

To better understand the subcellular trafficking of classical neurotransmitter receptors after their activation *in vivo*, we have investigated whether the cholinergic environment may influence the compartmentalization and metabolism of ACh receptors in striatal neurons. ACh plays a key role in striatal function, including regulation of motor behavior (Hornykiewicz, 1981; Jabbari et al., 1989; Nieoullon and Kerkérian-Le Goff, 1992). ACh regulates striatal neuronal activity, as well as neurotransmitter release or neuropeptide gene and proto-oncogene expression (Kemel et al., 1992; Stoof et al., 1992; Bernard et al., 1993; Nisenbaum et al., 1994; Wang and McGinty, 1996a,b, 1997; Wang et al., 1997). The cDNAs coding for five muscarinic receptors have been cloned (m1R–m5R) (Bonner et al., 1987, 1988; Bonner, 1989). *In situ* hybridization and immunohistochemical studies have shown that

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m1R and m4R are present mostly in efferent neurons as heteroreceptors but also in cholinergic neurons as autoreceptors (Levey et al., 1991; Bernard et al., 1992; Hersch et al., 1994; Rouse et al., 1997; Ince et al., 1997). In contrast, m2R is expressed exclusively in striatal interneurons as a presynaptic autoreceptor in cholinergic neurons (James and Cubeddu, 1987; Weiler et al., 1984; Bernard et al., 1992) and as a heteroreceptor in somatostatinergic interneurons. The m2R is thus of particular interest, because it is involved directly in autoregulation of ACh release in striatum (Weiler et al., 1984; James and Cubeddu, 1987; Murakami et al., 1989; Billard et al., 1995; Rouse et al., 1997). The subcellular events after the stimulation of m2R may play a key role in the function of cholinergic interneurons, especially in the regulation of their neuronal activity and/or of the inhibition of ACh release.

In this context, the purpose of the present study was to determine whether the cholinergic environment may control and modify the subcellular localization of m2R in striatal interneurons *in vivo* by using immunohistochemical approaches at light and electron microscopic levels. First, we have examined the cellular and subcellular distribution of m2R in striatal neurons of control animals. Second, we have studied the effect of the stimulation of muscarinic receptors with agonists on the localization of m2R at cellular and subcellular levels, and we have determined the time course of this effect. To better understand the fate of the receptor after activation, we have quantified the modification of the distribution of m2R gold immunolabeling in the different subcellular organelles by using image analysis. Third, to investigate the specificity of the mechanisms involved in the regulation of the distribution of receptors, we have determined whether cholinergic agonists may regulate also the subcellular localization of receptors for another neurotransmitter receptor coexpressed in the same neurons, the substance P receptor (SPR) (Gerfen, 1991; Kaneko et al., 1993). For this purpose, we have compared the distribution of the SPR and m2R after stimulation of muscarinic receptors.

MATERIALS AND METHODS

Animals and tissue preparation. Sprague Dawley male adult rats (200–300 gm; Centre d'élevage Janvier, Le Genest St. Isle, France) were used in this study. Environmental conditions for housing the rats and all procedures that were performed on them were in accordance with the guidelines of the French Agriculture and Forestry Ministry (decree 87849, license 01499), with the approval of the Center National de la Recherche Scientifique, and in accordance with the policy on the use of animals in neuroscience research issued by the Society for Neuroscience.

The rats received the following treatments: (1) several groups of rats were treated with a single injection of a muscarinic receptor agonist (oxotremorine or pilocarpine) (Table 1); (2) one group of rats was pretreated with atropine, a muscarinic receptor antagonist, 15 min before oxotremorine to block the effect of the agonist; and (3) control animals were treated with saline as a single injection or in association with oxotremorine or atropine. All drugs were injected intraperitoneally (0.1 ml/100 gm). The animals were usually euthanized 45 min after the last injection of each drug. To examine the time course of the effect of oxotremorine, the animals were allowed to survive from 90 sec to 24 hr (Table 1). All drugs were diluted in 0.9% NaCl. Oxotremorine free base, pilocarpine hydrochloride, and atropine sulfate salt were obtained from Sigma (St Louis, MO).

The rats were deeply anesthetized with sodium chloral hydrate and then perfused transcardially with 50–100 ml of 0.9% NaCl, followed by 250 ml of fixative consisting of 4% paraformaldehyde (PFA) with 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C at a rate of ~15 ml/min. The brain was quickly removed and left overnight in 4% PFA at 4°C. Sections from neostriatum were cut on a vibrating microtome at ~70 μm and collected in PBS (0.01 M phosphate, pH 7.4). To enhance the penetration of the immunoreagents in the preembedding procedures, the sections were equilibrated in a cryoprotectant solution (0.05 M PB, pH 7.4, containing 25% sucrose and 10% glycerol) and freeze-thawed by freezing in isopentane cooled in liquid nitrogen and thawed in PBS (von Krosigk and Smith, 1991). The sections were then preincubated in 4% normal goat serum (NGS) in PBS for 30 min at room temperature.

Table 1. Treatments and number of animals used

Treatment		Dose (mg/kg)	Number of animals	Survival time
Muscarinic agonists	oxotremorine	0.5	2	90 sec
		0.5	2	3 min
		0.5	2	10 min
		0.5	2	20 min
		0.5	17	45 min
		0.5	2	90 min
		0.5	2	3 hr
		0.5	1	7 hr
		0.5	1	24 hr
	pilocarpine	1	2	45 min
		10	3	45 min
		50	2	45 min
Blockade experiments	atropine + oxotremorine	5 + 0.5	2	45 min
Controls	saline	9	15	45 min
	saline + oxotremorine	9 + 0.5	2	45 min
	atropine + saline	5 + 9	2	45 min

Immunohistochemistry. The m2R was detected by immunohistochemistry using a monoclonal antibody raised in rat against a fusion protein derived from a sequence of the receptor corresponding to the third intracytoplasmic loop (Levey et al., 1995). The specificity of the antibody has been described in detail previously (Levey et al., 1995). The SPR was detected using a polyclonal antibody raised in rabbit against a fusion protein containing a C-terminal intracellular portion of the receptor (Shigemoto et al., 1993). The cholinergic and somatostatin–neuropeptide Y (NPY) neurons containing m2R immunoreactivity were identified by their expression of choline acetyltransferase (ChAT) or NPY immunoreactivity, respectively. ChAT and NPY were detected using polyclonal antibodies raised in goat (Chemicon, Temecula, CA) or rabbit (Tabarin et al., 1992), respectively.

Immunofluorescence. Sections of striatum were treated for the detection of m2R by single immunofluorescence or for the simultaneous detection of m2R and SPR, m2R and ChAT, or m2R and NPY by double immunofluorescence. After perfusion–fixation as described above, 70-μm-thick sections were cut on a vibratome and incubated in 4% NGS or normal donkey serum (NDS) for 30 min and then in either the antibody against m2R (1:500) or in a mixture of m2R (1:500) and another antibody: SPR (1:4000), ChAT (1:400), or NPY (1:8000) antibodies, supplemented with 1% NGS or NDS for 15 hr at room temperature (RT). The sections were then washed in PBS and incubated in goat anti-rat IgG coupled to the fluorochrome cyanine 3 (CY3) (Jackson ImmunoResearch, West Grove, PA) for the single detection of m2R. For the double detection of m2R and SPR or m2R and NPY, the sections were incubated in a mixture of CY3-conjugated goat anti-rat IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) at a dilution of 1:400 in PBS for 45 min at RT. For the double detection of m2R and ChAT, the sections were first incubated in biotinylated donkey anti-goat (1:200) and then in a mixture of CY3-conjugated goat anti-rat IgG (1:400) and FITC-conjugated streptavidin (1:1000). After washing, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy with filters selective for FITC and CY3. The specificity of the labeling techniques was proven by the absence of m2R labeling when the primary antibody (single detection) or one or both secondary antibodies (double detection) were omitted.

Preembedding immunogold method. The preembedding immunogold method was performed as described previously (Bernard et al., 1997).

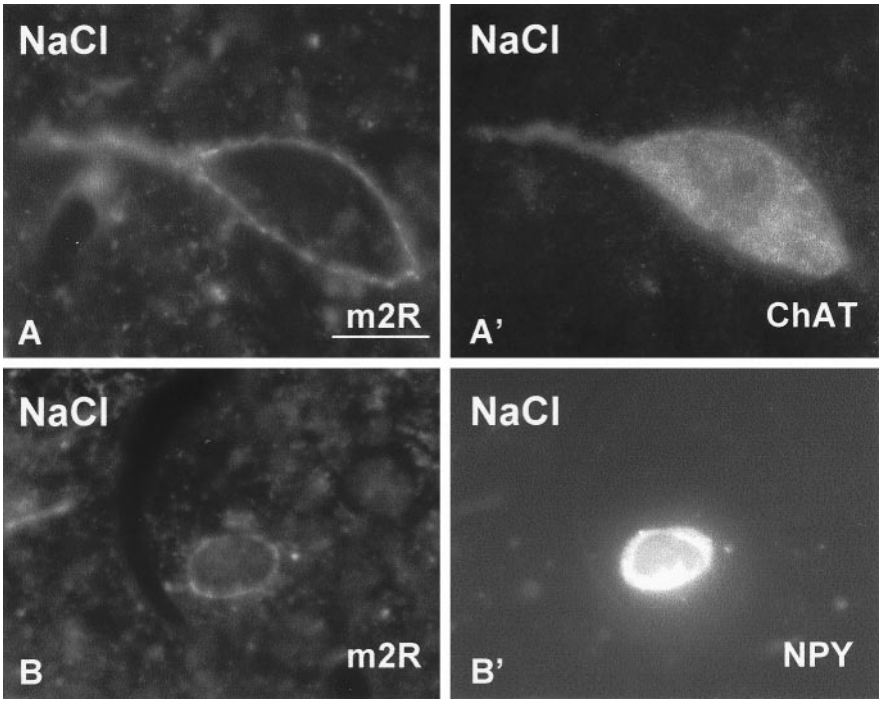

Figure 1. Phenotypical identification of the striatal interneurons expressing m2R immunoreactivity in normal animals using a double-immunofluorescence method. *A*, *A′*, A large-sized neuron expressing m2R immunoreactivity located at the membrane (*A*) is also immunoreactive for ChAT (*A′*). *B*, *B′*, A medium-sized neuron expressing m2R immunoreactivity (*B*) is also immunoreactive for NPY (*B′*). Scale bar (in *A*), 10 μm.

Some spare sections from the same animals were treated also for the immunofluorescence localization of m2R. Briefly, the sections were incubated for 15 hr at RT with constant gentle shaking in primary antibody solutions (m2R and SPR at a dilution of 1:500 and 1:4000, respectively) diluted in PBS that was supplemented with 1% NGS. After washing [two times in PBS and two times in PBS supplemented with 2% bovine serum albumin-c (BSAc) and 0.5% cold fish gelatin (PBS-BSAc) (Sigma)], they were incubated in goat anti-rat or goat anti-rabbit IgGs conjugated to gold particles (1.4 nm in diameter; 1:100 in PBS-BSAc; Nanoprobes, Stony Brook, NY) for 2 hr at RT. The sections were then washed (three times in PBS) and post-fixed in 1% glutaraldehyde in PBS for 10 min. After washing (two times in PBS and two times in sodium acetate buffer and 0.1 M, pH 7.0), the gold labeling was intensified using a silver enhancement kit (HQ silver; Nanoprobes) for 5–10 min at RT in the dark. The sections were finally washed in acetate buffer and then in PB.

Preparation for electron microscopy. Immunogold-treated sections were post-fixed in osmium tetroxide (1% in PB 0.1 M, pH 7.4) 10 min at RT. After washing (three times in PB), they were dehydrated in an ascending series of dilutions of ethanol, which included 1% uranyl acetate in 70% ethanol. They were then treated with propylene oxide (two times in 10 min), equilibrated in resin overnight (Durcupan ACM; Fluka, Buchs, Switzerland), mounted on glass slides, and cured at 60°C for 48 hr. Immunopositive neurons were first visualized in the light microscope. Areas of interest were cut out from the slide and glued to blank cylinders of resin. The selection was made to have several labeled neurons on the same block (usually four to five). All of the immunoreactive neurons identified on thick sections were cut in semithin sections (1-μm-thick) and then in ultrathin sections on a Reichert Ultracut S. Ultrathin sections were collected on pioloform-coated single-slot copper grids. The sections were stained with lead citrate and examined in a Philips CM10 electron microscope.

Quantitative analysis of the distribution of m2R in striatal neuronal compartments. The distribution of m2R in different compartments of striatal perikarya in NaCl- and oxotremorine-treated animals was analyzed from immunogold-treated sections at the electron microscopic level. The analysis was performed on negatives of micrographs at a final magnification of 3900×, using the Metamorph software on a personal computer (Universal Imaging Corporation, Paris, France). After scanning the negative (Magic scan, version 3.1; Umax), the image was converted into a positive picture and magnified to allow the identification of the subcellular element showing immunoparticles. The measures were performed on three NaCl-treated and three oxotremorine-treated rats. A mean of 15.7 ± 1.7 neurons per animal was analyzed. The immunoparticles were identified and counted in association with six subcellular compartments. The five compartments are the plasma membrane, endosome-like vesicles, multivesicular bodies, the Golgi apparatus, and the endoplasmic reticulum. Some immunoparticles were classified as associated with a sixth unidentified compartment, because they were associated with either no detectable organelles or an organelle that could not be identified as one of the five previous ones. The results were expressed as (1) the percentage of immunoparticles associated with the different subcellular compartments in normal animals, and (2) the number of immunoparticles per membrane length (micrometers), cytoplasmic surface (square micrometers), multivesicular body, or Golgi apparatus in normal and treated rats (see Fig. 7). We assume here that the number of immunoparticles is proportional to the absolute number of m2R. The values from NaCl- and oxotremorine-treated rats were compared using the nonparametric Mann–Whitney *U* test.

RESULTS

Cellular and subcellular distribution of m2R immunoreactivity in the striatum in control rats

Light microscopic observations

Immunoreactivity for m2R was detected in occasional neurons in the normal striatum, as evidenced by observations of immunofluorescence-treated sections at the light microscopic level. These neurons were either medium- or large-sized and had an indented nucleus and thus were characterized as aspiny interneurons (Figs. 1, 2, 3). Double-immunofluorescence experiments demonstrated that the large-sized m2R immunoreactive neurons also express immunoreactivity for ChAT and that the medium-sized ones express NPY immunoreactivity (Fig. 1). No labeling was detected in neurons with characteristics of medium-sized spiny neurons. The m2R labeling was intense and clearly associated with the neuronal membrane (Figs. 1*A*,*B*, 2*A*,*B*, 3*A*). Minimal weak staining was detected in the cytoplasm. Immunoreactive neurons frequently displayed labeling in proximal dendrites. Occasional dendritic shafts were also strongly immunoreactive for m2R throughout the striatum. No obvious difference was observed in the labeling between neostriatum and the nucleus accumbens and along the rostrocaudal and dorsoventral axes. The immunogold labeling, as observed in the light microscope, showed

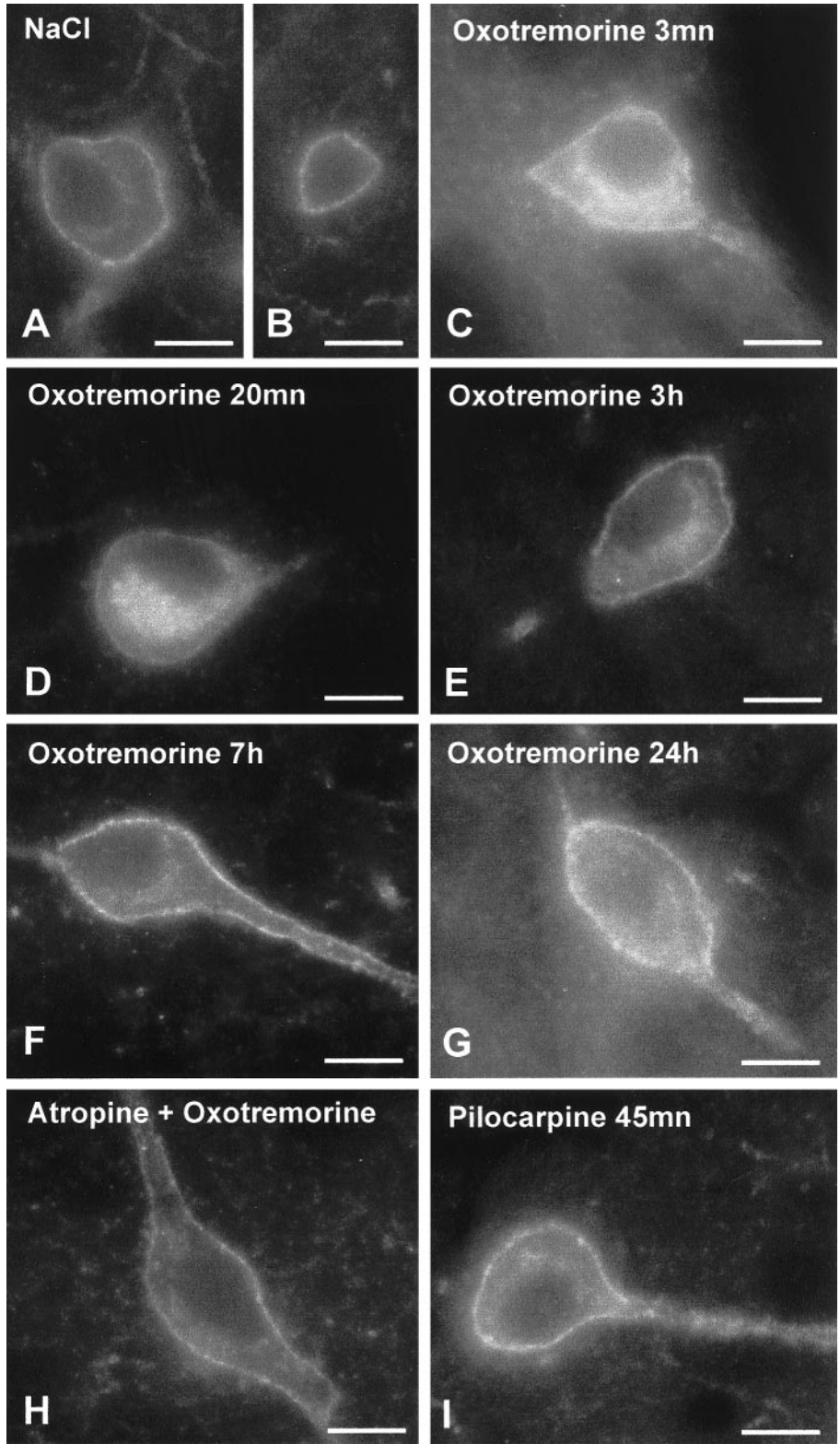

Figure 2. Detection of m2R in striatal interneurons after treatment with muscarinic agonists using an immunofluorescence method. *A*, *B*, In a control animal, m2R immunoreactivity is detected at the membrane of large-sized (*A*) and medium-sized neurons (*B*). A very faint labeling is seen in the cytoplasm (*A*). *C–G*, Evolution of the m2R labeling as a function of the survival time. Three (*C*) and 20 min (*D*) after treatment, m2R immunoreactivity is present in the cytoplasm in a perinuclear area. A labeling is detectable at the membrane. Three hours after treatment (*E*), the m2R immunolabeling is weak in the cytoplasm and strong at the membrane. Seven (*F*) and 24 hr (*G*) after treatment, an intense labeling is detected at the membrane. *H*, m2R immunoreactivity is localized at the membrane when the rat is treated with atropine 15 min before oxotremorine. *I*, After treatment with pilocarpine, the m2R labeling is restricted to the plasma membrane. Scale bars (in *A–I*), 10 μm.

a similar pattern of staining to that produced by the immunofluorescence method. No glial cell labeling was observed in the striatum.

Electron microscopic observations

The subcellular localization of m2R was performed by analysis of immunogold-treated sections (Figs. 4–6). The observation at the electron microscopic level confirmed the light microscopic analysis that m2R was detected only in cell bodies with characteristics of aspiny interneurons, i.e., an indented nucleus and a large volume of cytoplasm (Fig. 4*A*). The m2R immunoreactivity was localized at postsynaptic sites in cell bodies and dendritic shafts and at presynaptic sites in boutons (Fig. 4*A*,*C–E*). The immuno-

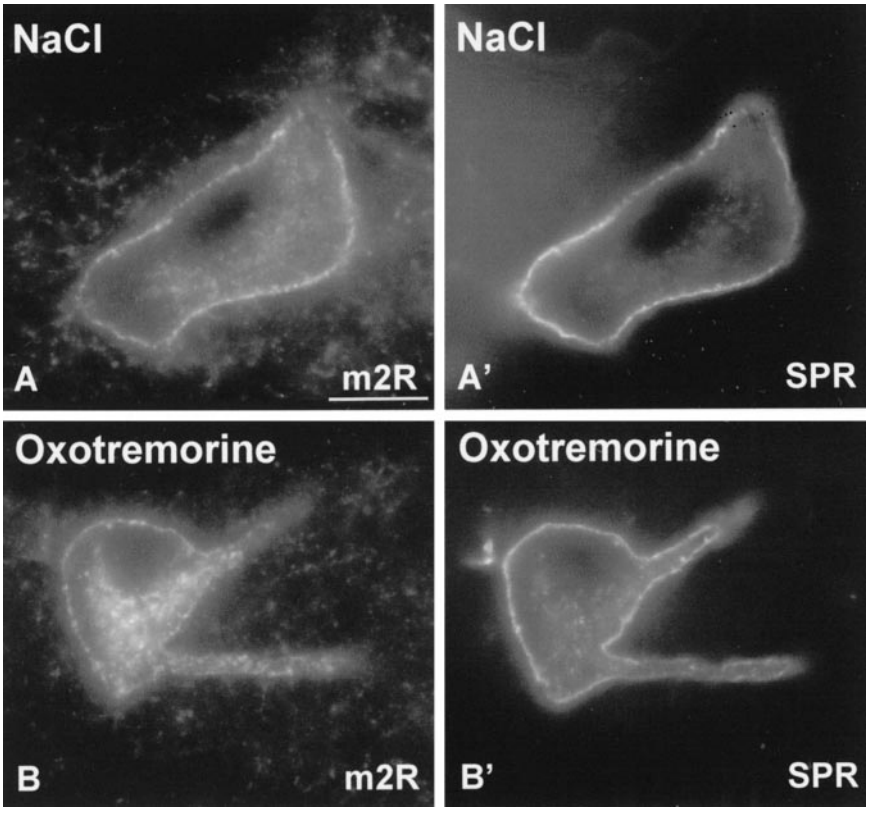

Figure 3. Comparative localization of m2R and SPR immunoreactivity in striatal interneurons in control animals and in animals treated by oxotremorine (45 min) using a double-immunofluorescence method. *A*, *A′*, In a control animal, m2R and SPR immunoreactivities are colocalized in a same neuron at the plasma membrane. *B*, *B′*, After treatment with oxotremorine, m2R labeling is detected in the cytoplasm (*B*), whereas the signal for SPR is still at the membrane (*B′*). Scale bar (in *A*), 10 μm.

particles were mostly associated with the internal cytoplasmic side of plasma membranes. No labeling was detected in dendritic spines. In cell bodies and dendrites, the immunoparticles were usually detected at extrasynaptic sites, although they could be localized rarely in association with postsynaptic specializations of symmetrical synapses (Fig. 4*A*,*C–E*). In perikarya, the immunoparticles were identified and counted in association with six subcellular compartments: plasma membrane, endosome-like vesicles, multivesicular bodies, Golgi apparatus, endoplasmic reticulum, and unidentified compartments. The endosome-like vesicles were small (100–200 nm in diameter) round or irregular-shaped vesicles. The multivesicular bodies were large round vesicles (500–600 nm in diameter) containing small round-shaped vesicles with a clear content. The immunoparticles were mostly associated with the internal side of the plasma membrane (47% of the total number of immunoparticles) (Figs. 4*A*, 7*A*). Immunoparticles were also detected in the cytoplasm in association with the cytoplasmic side of the endoplasmic reticulum (18%), endosomes (17%), Golgi apparatus (3%), and multivesicular bodies (0.5%) (Fig. 4*A*,*B*, 7*A*).

Control for specificity of the immunohistochemical labeling

The specificity of the labeling techniques was proven by the following data: (1) the cellular localizations were in agreement with the results described previously using the same antibodies or by *in situ* hybridization (Bernard et al., 1992; Hersch et al., 1994; Levey et al., 1995; Rouse et al., 1997); (2) the localization of immunoparticles for m2R on the internal side of the plasma membrane was in agreement with the localization of the epitope included in the fusion protein [third intracytoplasmic loop (Levey et al., 1995)]; and (3) the absence of m2R labeling at the light microscopic level when the primary antibody (single detection) or one or both secondary antibodies (double detection) were omitted.

Cellular and subcellular distribution of m2R immunoreactivity in the striatum after treatment with muscarinic agonists

After treatment with oxotremorine

The observations of the labeling immunofluorescence- and immunogold-reacted sections at the light microscopic level showed dramatic modifications of the distribution of m2R immunoreactivity in striatal interneurons (Figs. 2*C*,*D*, 3*B*); an intense labeling appeared in the cytoplasm and was particularly strong in the perinuclear area. These modifications of the labeling were seen in all immunoreactive large- and medium-sized interneurons. An intracytoplasmic labeling was detectable as early as 3 min after injection of oxotremorine with a faint intensity and was strong at 20, 45, and 90 min (Figs. 2*C*,*D*, 3*B*) and was very weak again after 3 hr (Fig. 2*E*). Seven and 24 hr after injection, m2R immunoreactivity was similar to the labeling observed in control animals (Fig. 2*F*,*G*). Pretreatment of rats with atropine, a muscarinic receptor antagonist, completely abolished the effect of oxotremorine on m2R immunoreactivity (Fig. 2*H*).

The analysis at the electron microscopic level confirmed the modifications of the compartmentalization and demonstrated a decrease of the density of immunoparticles located at the plasma membrane and an increase of the density of immunoreactivity in the cytoplasm of striatal interneurons in oxotremorine-treated rats compared with control animals (Figs. 5, 7*B*). The quantitative analysis demonstrated indeed a decrease of the relative abundance of immunoparticles at the plasma membrane (−63%) (Fig. 7*B*). In contrast, the percentage of particles significantly increased in cytoplasmic organelles (Figs. 6*A*,*B*, 7*B*). A very strong increase

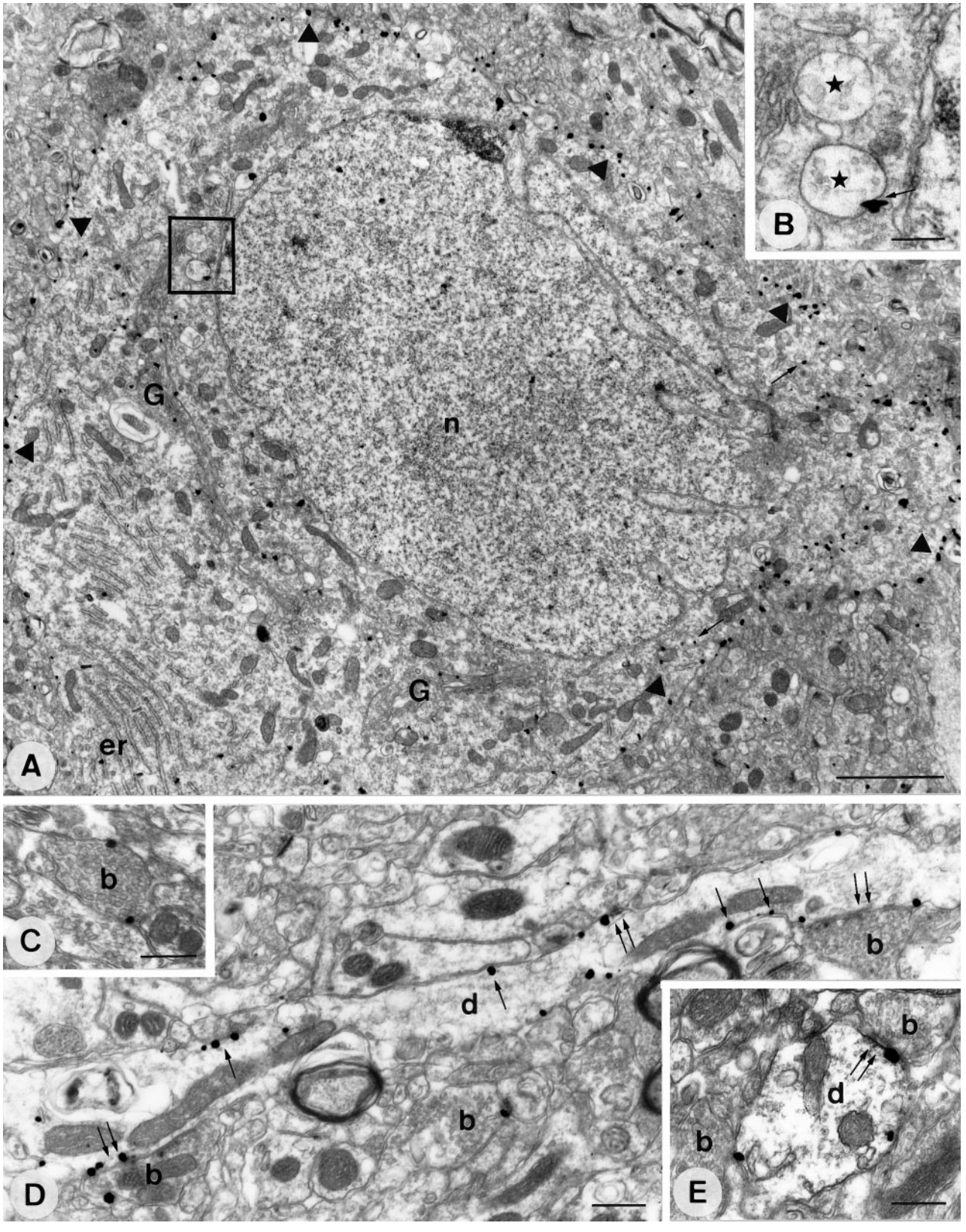

Figure 4. Subcellular distribution of m2R immunoreactivity in the striatum of control rats using preembedding immunogold method with silver intensification. *A*, Immunopositive cell body with an indented nucleus (*n*) and large volume of cytoplasm, characteristic of striatal interneurons. The immunoparticles are associated primarily with the internal side of the plasma membrane (*triangles*). Some immunoparticles are associated with the endoplasmic reticulum (*er*), the Golgi apparatus (*G*), small vesicles (*arrows*), and multivesicular bodies (*frame*). *B*, Detail of the *frame* in *A*, showing two multivesicular bodies (*stars*), one having an immunoparticles associated with it (*arrow*). *C–E*, Some immunoparticles are associated with the internal membrane of dendrites (*d*) (*D*, *E*) and a bouton (*b*) (*C*). Part of the immunoparticles are located at extrasynaptic sites (*single arrows*). Some immunoparticles are located on the main body of postsynaptic membrane of symmetrical synapses (*double arrows*). Scale bars: *A*, 5 μm; *B*, *C*, *E*, 0.5 μm; *D*, 0.2 μm.

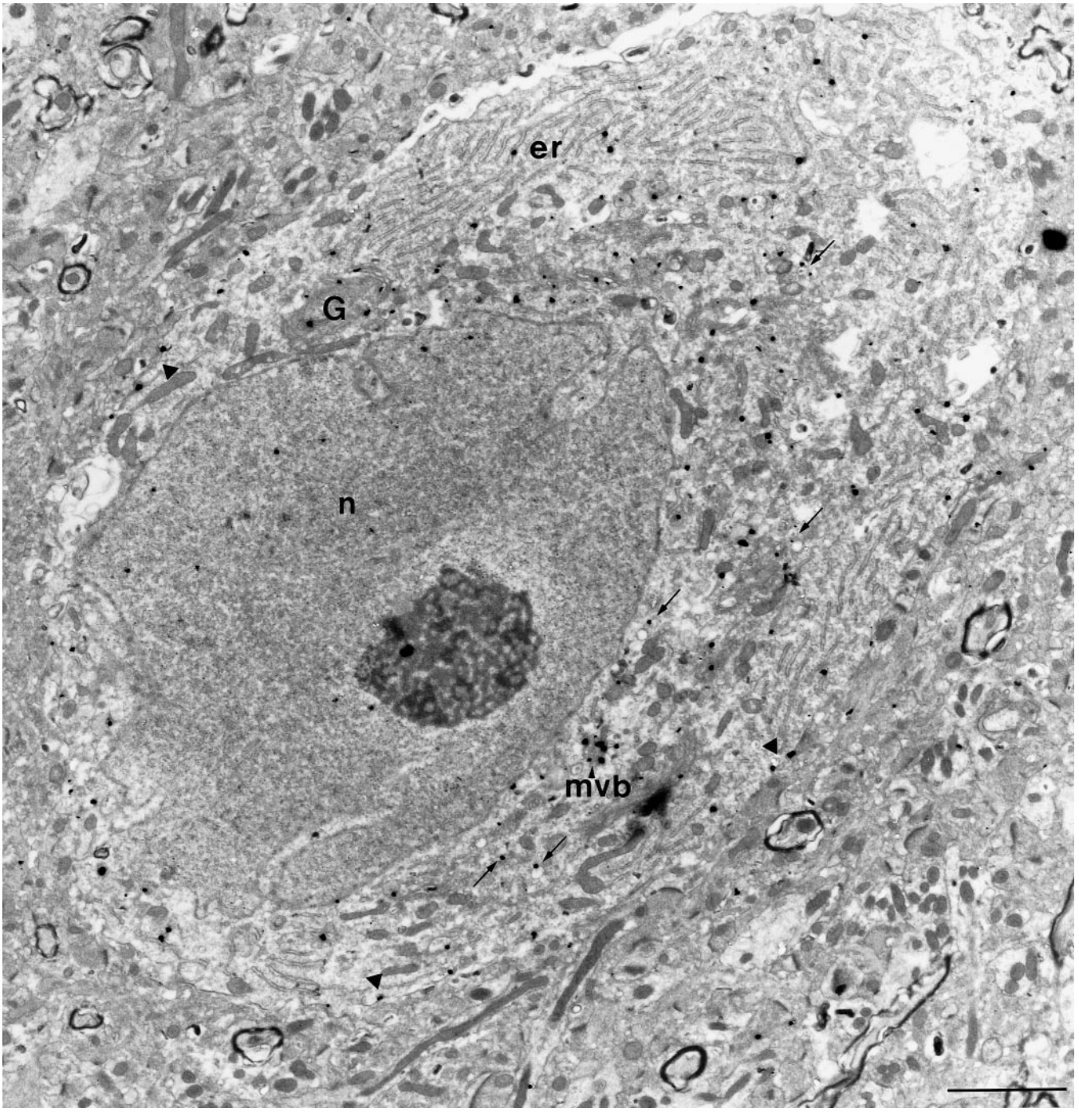

Figure 5. Subcellular distribution of m2R immunoreactivity in the striatum of rats treated with oxotremorine using preembedding immunogold method with silver intensification. The immunopositive neuron has the characteristic features of a striatal interneuron [indented nucleus (*n*) and large volume of cytoplasm]. Numerous immunoparticles are detected in the cytoplasm with a preferential perinuclear localization. They are associated with small vesicles (*arrows*), multivesicular bodies (*mvb*), the endoplasmic reticulum (*er*), and the Golgi apparatus (*G*). Some immunoparticles are associated with the plasma membrane (*triangles*). Scale bar, 5 μm.

was detected for the frequency of particles associated with the multivesicular bodies (+732%). There was also increased labeling associated with endosome-like vesicles (+86%) and with the Golgi apparatus (+26%). No significant difference was shown after treatment in the percentage of immunoparticles associated with the endoplasmic reticulum or with unidentified organelles. In dendrites, endosome-like vesicles and multivesicular bodies displayed m2R immunoreactivity similar to cell bodies (Fig. 6*C*, *D*). All of the immunoparticles detected in terminals were associated with the membrane.

After treatment with pilocarpine

The m2R immunoreactivity observed at the light microscopic level after treatment with pilocarpine did not differ from the

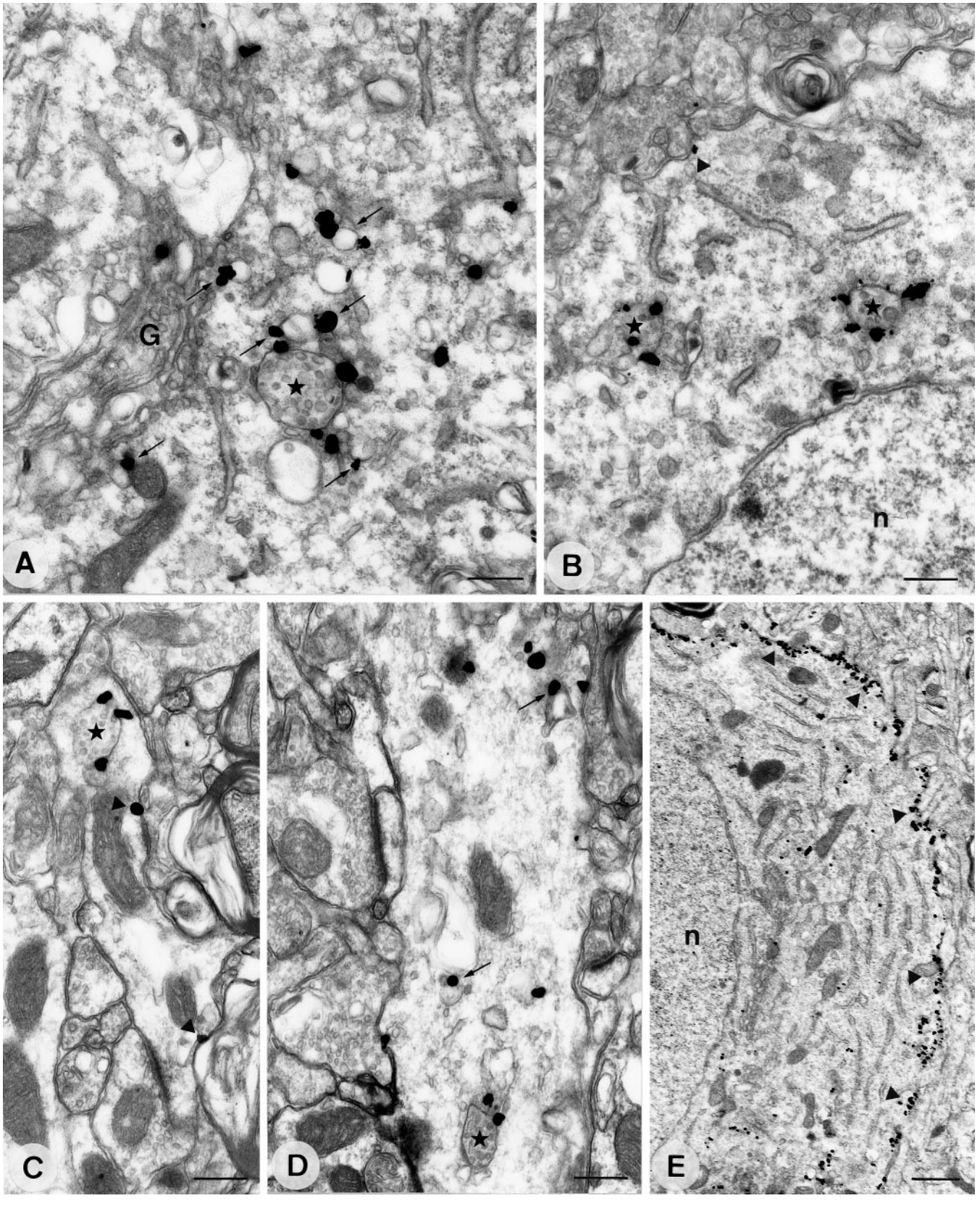

Figure 6. Subcellular distribution of m2R and SPR immunoreactivities in the striatum of rats treated with oxotremorine using preembedding immunogold method with silver intensification. Detail of m2R immunolabeling in the cytoplasm of cell bodies (*A*, *B*) and dendrites (*C*, *D*). Numerous immunoparticles are associated with small vesicles (*arrows*) and multivesicular bodies (*stars*). Some immunoparticles are associated with the plasma membrane (*triangles*). *E* shows a very dense labeling for SPR at the plasma membrane of a cell body (*triangles*). Few immunoparticles are detected in the cytoplasm. *n*, Nucleus; *G*, Golgi apparatus. Scale bars: *A–D*, 0.5 μm; *E*, 1 μm.

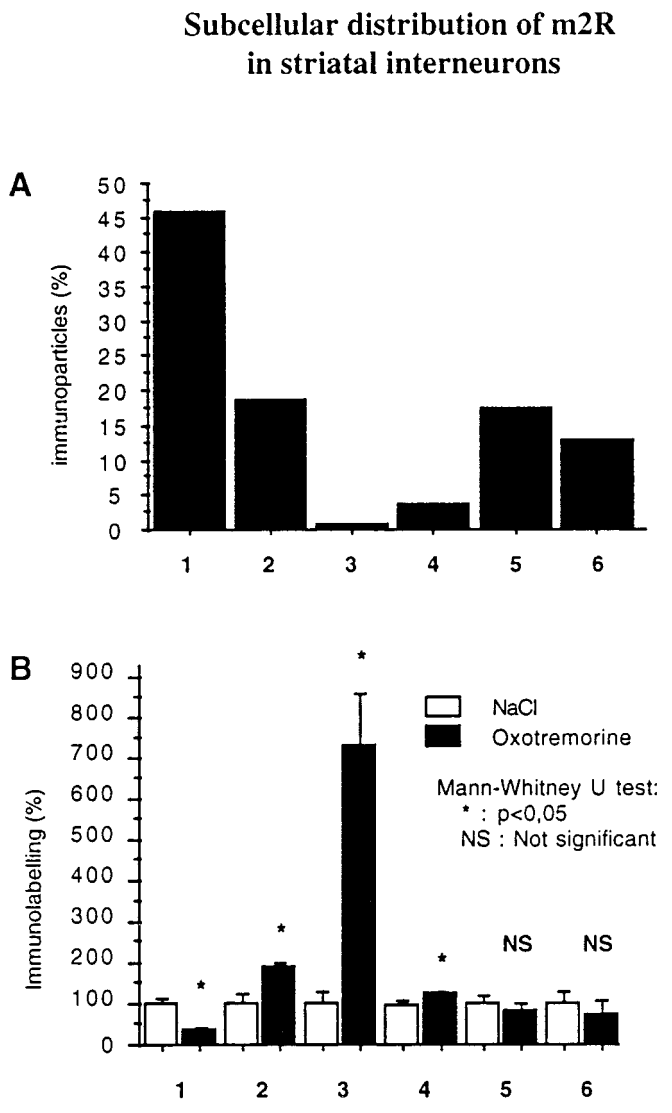

Figure 7. Quantitative analysis of the subcellular distribution of m2R in the striatum of control rats and rats treated with oxotremorine using preembedding immunogold method with silver intensification. *A*, Proportion of immunoparticles associated with different subcellular neuronal compartments in normal animals. For each neuron, the number of immunoparticles associated with each compartment was counted, and the proportion in relation to the total number was calculated. Data are the result of countings in three control rats (16 neurons per animal). The largest portion of immunoparticles are associated with the plasma membrane (*1*). In the cytoplasm, the immunoparticles are detected in association primarily with small vesicles (*2*) and endoplasmic reticulum (*5*). A small proportion of immunoparticles are associated with the Golgi apparatus (*4*) and multivesicular bodies (*3*). Some immunoparticles are not seen in association with any identified compartment (*6*). *B*, Effect of the treatment with oxotremorine on the localization of m2R immunoparticles in cell bodies of striatal interneurons. For each neuron, the number of immunoparticles associated with each compartment was counted in relation to the membrane length (in micrometers) for the plasma membrane (*1*), to the surface of cytoplasm (square micrometers) for small vesicles (*2*), the endoplasmic reticulum (*5*), and the unidentified compartment (*6*). For the multivesicular bodies (*3*) and Golgi apparatus (*4*), the values are expressed as the number of immunoparticles per multivesicular bodies and Golgi apparatus, respectively. Data are the result of countings in three control rats and three treated rats in ~16 neurons per animal. The results are expressed in relation to an arbitrary unit (100) of the control values. The statistical analysis (nonparametric Mann–Whitney *U* test) shows that the labeling strongly decreases at the plasma membrane and increases in small vesicles, very strongly decreases in multivesicular bodies, and more weakly decreases in the Golgi apparatus.

labeling in striatal interneurons of control animals, regardless of the dose. The staining was strong at the membrane with little or no immunoreactivity detected in the cytoplasm (Fig. 2*I*).

Comparative distribution of m2R and SPR in striatal neurons in control and oxotremorine-treated rats

In control and treated rats, the light microscopic observations showed that SPR immunoreactivity was also detected in large- and medium-sized neurons with characteristics of interneurons, i.e., scattered neurons with an indented nucleus as described previously. The double-immunofluorescence experiments indeed demonstrated a colocalization of m2R and SPR immunoreactivity at the plasma membrane of the same interneurons (Fig. 3*A*,*A*′). In control rats, SPR immunolabeling was primarily restricted to the plasma membrane (Fig. 3*A*′). After oxotremorine, the SPR immunoreactivity was identical to the labeling observed in control animals, i.e., remaining at the plasma membrane, whereas m2R immunoreactivity primarily redistributed to the cytoplasm (Fig. 3*B*′). Electron microscopy confirmed these data in control, as well as in oxotremorine-treated, rats (Fig. 6*E*).

DISCUSSION

The present study demonstrates for the first time that acute stimulation of muscarinic receptors dramatically alters the compartmentalization of m2R in striatal interneurons. In control animals, m2R is located primarily at the plasma membrane of cholinergic and somatostatin–NPY interneurons. Treatment with oxotremorine induces internalization of m2R and modification of intracytoplasmic trafficking. The quantitative analysis at the electron microscopic level revealed a dramatic decrease of the receptor at the plasma membrane in oxotremorine-treated rats. Concurrently, the m2R immunolabeling increased in the cytoplasm, strongly in endosome-like vesicles and in multivesicular bodies and weakly in Golgi apparatus. In contrast, oxtremorine had no effect on the localization of SPR in striatal interneurons.

Cellular and subcellular distribution of m2R in striatal interneurons in control animals

Our results confirm and expand previous data demonstrating the expression of m2R in subsets of striatal neurons with hallmarks of interneurons (Levey et al., 1991; Bernard et al., 1992; Hersch et al., 1994; Levey et al., 1995). Light and electron microscopic observations revealed that two types of interneurons displayed m2R immunoreactivity. The first type was large-sized neurons, which we identified as cholinergic aspiny interneurons in agreement with previous data (Bernard et al., 1992; Rouse et al., 1997). The second type was medium-sized aspiny interneurons, which we have identified as neurons producing NPY and somatostatin. These results suggest that m2R may play the double function of autoreceptors in cholinergic neurons and heteroreceptors in somatostatin–NPY. This is in agreement with biochemical data showing the involvement of m2R in the regulation of ACh release (James and Cubeddu, 1987; Dolezal and Wecker, 1990; Billard et al., 1995). Our data suggest also that m2R may also regulate the activity of somatostatin–NPY neurons.

Our electron microscopic study demonstrated that m2R is primarily located at the plasma membrane at presynaptic and postsynaptic sites. Indeed, m2R has a postsynaptic localization at the membrane of cell bodies and dendrites. Immunoreactivity for m2R was also detected at the membrane of some boutons. Some of them have been identified by Rouse et al. (1997) as cholinergic boutons. The wide distribution of m2R along the dendritic and axonal tree suggests that m2R could be directly involved in the

regulation of different functions of cholinergic and somatostatin–NPY neurons, including the modulation of ionic movement through the membrane or the regulation of the release of neurotransmittors. The localization of m2R at the level of terminals provides anatomical evidence in favor of a direct role of presynaptic m2R in the ACh release.

Most m2Rs were detected at nonsynaptic sites in cell bodies, as well as in dendrites. This suggests that the cholinergic transmission in the striatum is primarily a nonsynaptic transmission. This hypothesis is supported by several lines of evidence: (1) most m2Rs are located at the cell surface, whereas cholinergic perikarya receive very little afferents, including cholinergic ones, making synaptic contacts with them (Bolam et al., 1984; Wainer et al., 1984; Phelps et al., 1985); (2) very few symmetrical synapses with characteristics of cholinergic synapses were positive for m2R; and (3) a nonsynaptic component of the neurotransmission has been described in striatum for acetylcholine, as well as for other neurotransmitters, such as dopamine (Descarries et al., 1997). The m2R are likely functional, because they are responsive to their stimulation by modifying their compartmentalization. Alternatively, some extrasynaptic receptors may not be functional and represent a recruitable pool of receptors that would diffuse to the synaptic sites in case of modification of the neuronal environment, as it was shown for AMPA receptors in hippocampus (Rao and Craig, 1997). Nevertheless, we cannot exclude that the preembedding method did not allow us to detect receptors located in the main body of the synapse because of the restricted access of the reagent to the active zone, as suggested previously-(Baude et al., 1995; Nusser et al., 1995; Bernard et al., 1997).

In normal rats, the m2R was detected in the cytoplasm in association with subcellular organelles: primarily with endoplasmic reticulum and with small vesicles but also with the Golgi apparatus. The m2Rs associated with the endoplasmic reticulum and the Golgi apparatus are probably receptors in the process of synthesis before being targeted to the plasma membrane and are thus unlikely functional. The m2Rs associated with endosome-like vesicles may be receptors undergoing normal turnover either before degradation in lysosomes or recycling to the plasma membrane.

Effect of muscarinic agonists on the subcellular distribution of m2R in striatal interneurons

We demonstrate here the translocation of m2R from the plasma membrane into the cytoplasm. The changes in the distribution of m2R are visible in large- and medium-sized cells, i.e., in cholinergic and somatostatin–NPY neurons, suggesting that the modification of the cholinergic environment influences the compartmentalization of autoreceptors, as well as heteroreceptors. The intracellular mechanisms involved after activation of receptors are not completely clear. However, endocytosis seems to be the classical fate for a receptor after its activation, but it has been rarely visualized *in vivo* and *in vitro* (Koenig and Edwardson, 1997; Dumartin et al., 1998). Our detailed electron microscopic analysis supports this hypothesis, because we have demonstrated a decrease (−63%) of the frequency of immunoparticles associated with the plasma membrane after stimulation and, at the same time, an increase of 86% of m2R associated with endosome-like vesicles with characteristic ultrastructural features of endosomes. Our studies are the first data strongly suggesting endocytosis of an acetylcholine muscarinic receptor *in vivo* in the CNS in response to the stimulation by a muscarinic agonist. Our data are in agreement with *in vitro* studies in transfected cells concerning internalization of muscarinic receptors after their pharmacological activation (Koenig and Edwardson, 1996; Barnes et al., 1997) or neuropeptide receptors (Roettger et al., 1995; Koenig and Edwardson, 1996, 1997; Koenig et al., 1997; Marvizon et al., 1997) but also *in vivo* for dopamine or neuropeptide receptors (Faure et al., 1995; Mantyh et al., 1995a,b; Dumartin et al., 1998).

The internalization of the receptor is the first step in the cascade of events occurring after stimulation. The fate of G-protein-coupled receptors after endocytosis is not well understood. They could be either recycled to the plasma membrane and/or degradated in lysosomes. In the present study, we bring the first anatomical evidence suggesting degradation and maturation of a receptor, the m2R, after its activation. Indeed, the number of m2R immunoparticles associated with multivesicular bodies increased more than seven times. These organelles are thought to be the result of the fusion of endosomes and have the function of lysosomes (van Deurs et al., 1993). It may suggest that a process of degradation of the receptor is set up after stimulation. At the same time, we have demonstrated that the relative quantity of m2R immunoparticles is the same in the endoplasmic reticulum but slightly increases in the Golgi apparatus after stimulation. This suggests that there is no neosynthesis of m2R, but there may be activation of the maturation, including phenomena-like glycosylation, sulfatation, or proteolysis. Receptors stored in the endoplasmic reticulum may transfer to the Golgi apparatus to mature and then be recycled to the membrane to compensate for the loss of receptors at this plasma membrane.

We have shown that the m2R internalization is triggered very quickly, because it starts up as soon as 3 min after injection of oxotremorine. This phenomenon is a transient event, because the labeling is back to normal after ~3 hr. In the present study, we have described the distribution of m2R 45 min after stimulation. The sequence of events may vary at different times, and we cannot exclude that there is a dissociation of the effects on the endocytosis, the recycling, the neosynthesis, or the degradation as a function of the time.

We have not detected any modification of the distribution of m2R in striatal interneurons after treatment with pilocarpine. We cannot exclude that the absence of effect was attributable to the fact that the schedules of treatment (doses and time) were not able to induce internalization. However, this could be because of the difference of specificity of both drugs for muscarinic receptors. Although all muscarinic receptors are responsive to the stimulation with both drugs, oxotremorine has a higher affinity for m2R than pilocarpine (McKinney et al., 1991). This suggests that there could be a relationship between the affinity of an agonist for a receptor and the ability of the drug to induce internalization of this receptor, as it has been suggested for opioid receptors (Sternini et al., 1996). An alternative reason for the lack of effect of pilocarpine may be that its structure prevents this ligand from interacting with the part of the m2R that signals internalization in the same way that oxotremorine does, as it has been demonstrated with opiate receptor ligands and opioids (Keith et al., 1998).

Specificity of the mechanism of internalization

We have demonstrated here that m2R and SPR were colocalized in the same striatal interneurons, and SPR and m2R seem to colocalize at the plasma membrane in control animals. The SPR is able to be internalized when stimulating with substance P (Mantyh et al., 1995a). We demonstrate in the present study that the stimulation of muscarinic receptors specifically alters the compartmentalization of m2R, because SPR remains at the mem-

brane. Our data suggest that there is no heteroregulation by the cholinergic environment of the subcellular distribution of this receptor and that two G-protein-coupled receptors located in the same neurons may have independent trafficking and fate under stimulation. Our data suggest that SPR and m2R are localized on different domains of the membrane, because SPR does not seem to be internalized in endocytotic vesicles internalizing m2R.

Functional implications

The functional signification of internalization and trafficking of m2R has to be considered in light of the hypotheses on the localization and functions of G-protein-coupled receptors in striatal interneurons. One of the main findings of the present work is that the stimulation of muscarinic receptors acutely and dramatically modifies m2R localization in neurons, including the m2R pool extrasynaptically located at the surface of striatal cholinergic and NPY–somatostatin interneurons. Because m2R is strongly involved in regulating acetylcholine release and electrical properties of cholinergic neurons (James and Cubeddu, 1987; Dolezal and Wecker, 1990; Billard et al., 1995; Rouse et al., 1997), it can be hypothesized that internalization and modifications of abundance of available m2R at the plasma membrane of neurons may be a means to modulate *in vivo* the response to stimulation of muscarinic receptors after activation of cholinergic transmission in physiological or pathological conditions, such Parkinson's disease. It is known that the motor disorders observed in this disease are attributable, at least in part, to cholinergic overactivity (Nieoullon and Kerkérian, 1992; Calne, 1993). The overstimulation of muscarinic receptors may induce a decrease of the availability of m2R in striatum and thus may be involved in the changes in the neuronal activity and in the clinical symptoms. Further studies using animal models of human diseases may help to elucidate whether modifications of the compartmentalization and traffic of muscarinic receptors may have functional consequences and contribute to regulation of the response of cholinoceptive neurons.

REFERENCES

Barnes PJ, Haddad EB, Rousell J (1997) Regulation of muscarinic M2 receptors. Life Sci 60:1015–1021.

Baude A, Nusser Z, Molnar E, McIlhinney RAJ, Somogyi P (1995) High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. Neuroscience 69:1031–1055.

Bernard V, Normand E, Bloch B (1992) Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 12:3591–3600.

Bernard V, Dumartin B, Lamy E, Bloch B (1993) Fos immunoreactivity after stimulation or inhibition of muscarinic receptors indicates anatomical specificity for cholinergic control of striatal efferent neurons and cortical neurons in rat. Eur J Neurosci 5:1218–1225.

Bernard V, Somogyi P, Bolam JP (1997) Cellular, subcellular, and subsynaptic distribution of AMPA-type glutamate receptor subunits in the neostriatum of the rat. J Neurosci 17:819–833.

Billard W, Binch HR, Crosby G, McQuade RD (1995) Identification of the primary muscarinic autoreceptor subtype in rat striatum as m2 through a correlation of *in vivo* microdialysis and *in vitro* receptor binding data. J Pharmacol Exp Ther 273:273–279.

Bolam JP, Wainer BH, Smith AD (1984) Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi impregnation and electron microscopy. Neuroscience 12:711–718.

Bonner TI (1989) The molecular basis of muscarinic receptor diversity. Trends Neurosci 12:148–151.

Bonner TI, Buckley NJ, Young AC, Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. Science 237:527–532.

Bonner TI, Young AC, Brann MR, Buckley NJ (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine genes. Neuron 1:403–410.

Calne DB (1993) Treatment of Parkinson's disease. N Engl J Med 329:1021–1027.

Descarries L, Gisiger V, Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 53:603–625.

Dolezal V, Wecker L (1990) Muscarinic receptor blockade increases basal acetylcholine release from striatal slices. J Pharmacol Exp Ther 252:739–743.

Dumartin B, Caille I, Gonon F, Bloch B (1998) Internalization of D1 dopamine receptor in striatal neurons *in vivo* as evidence of activation by dopamine agonists. J Neurosci 18:1650–1661.

Faure MP, Alonso A, Nouel D, Gaudriault G, Dennis M, Vincent JP, Beaudet A (1995) Somatodendritic internalization and perinuclear targeting of neurotensin in the mammalian brain. J Neurosci 15:4140–4147.

Gerfen CR (1991) Substance P (neurokinin-1) receptor mRNA is selectively expressed in cholinergic neurons in the striatum and basal forebrain. Brain Res 556:165–170.

Hersch SM, Gutekunst CA, Rees HD, Heilman CJ, Levey AI (1994) Distribution of m1–m4 muscarinic receptor proteins in the rat striatum: light and electron microscopic immunocytochemistry using subtype-specific antibodies. J Neurosci 14:3351–3363.

Hornykiewicz O (1981) Brain neurotransmitter changes in Parkinson's disease. In: Movement disorders (Marsden DC, Fahn S, eds), pp 41–58. Boston: Butterworth.

Ince E, Ciliax BJ, Levey AI (1997) Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons. Synapse 27:357–366.

Jabbari B, Scherokman B, Gunderson CH, Rosenberg ML, Mille J (1989) Treatment of movement disorders with trihexyphenidyl. Mov Disord 4:202–212.

James MK, Cubeddu LX (1987) Pharmacologic characterization and functional role of muscarinic autoreceptors in the rabbit striatum. J Pharmacol Exp Ther 240:203–215.

Kaneko T, Shigemoto R, Nakanishi S, Mizuno N (1993) Substance P receptor-immunoreactive neurons in the rat neostriatum are segregated into somatostatinergic and cholinergic aspiny neurons. Brain Res 631:297–303.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. Mol Pharmacol 53:377–384.

Kemel ML, Desban M, Glowinski J, Gauchy C (1992) Functional heterogeneity of the matrix compartment in the caudate nucleus as demonstrated by the cholinergic presynaptic regulation of dopamine release. Neuroscience 50:597–610.

Koenig JA, Edwardson JM (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. Mol Pharmacol 49:351–359.

Koenig JA, Edwardson JM (1997) Endocytosis and recycling of G protein-coupled receptors. Trends Pharmacol Sci 18:276–287.

Koenig JA, Edwardson JM, Humphrey PP (1997) Somatostatin receptors in Neuro2A neuroblastoma cells: ligand internalization. Br J Pharmacol 120:52–59.

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218–3226.

Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ (1995) Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. J Comp Neurol 351:339–356.

Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu HT, Basbaum AI, Vigna SR, Maggio JE (1995a) Rapid endocytosis of a G protein-coupled receptor: substance P-evoked internalization of its receptor in the rat striatum *in vivo*. Proc Natl Acad Sci USA 92:2622–2626.

Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE (1995b) Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268:1629–1632.

Marvizon JC, Martinez V, Grady EF, Bunnett NW, Mayer EA (1997) Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. J Neurosci 17:8129–8136.

McKinney M, Miller JH, Gibson VA, Nickelson L, Aksoy S (1991) Interactions of agonists with M2 and M4 muscarinic receptor subtypes mediating cyclic AMP inhibition. Mol Pharmacol 40:1014–1022.

Murakami S, Kubota Y, Kito S, Shimada S, Takagi H, Wu JY, Inagaki S (1989) The coexistence of substance P- and glutamic acid decarboxylase-like immunoreactivity in entopeduncular neurons of the rat. Brain Res 485:403–406.

Nieoullon A, Kerkérian-Le Goff L (1992) Cellular interactions in the striatum involving neuronal systems using "classical" neurotransmitters: possible functional implications. Mov Disord 7:311–325.

Nisenbaum LK, Kitai ST, Gerfen CR (1994) Dopaminergic and muscarinic regulation of striatal enkephalin and substance P messenger RNAs following striatal dopamine denervation: effects of systemic and central administration of quinpirole and scopolamine. Neuroscience 63:435–449.

Nusser Z, Roberts JDB, Baude A, Richards JG, Sieghart W, Somogyi P (1995) Immunocytochemical localization of the alpha 1 and beta 2/3 subunits of the GABA(A) receptor in relation to specific GABAergic synapses in the dentate gyrus. Eur J Neurosci 7:630–646.

Phelps PE, Houser CR, Vaughn JE (1985) Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: a correlated light and electron microscopic study of cholinergic neurons and synapses. J Comp Neurol 238:286–307.

Rao A, Craig AM (1997) Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. Neuron 19:801–812.

Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM, Miller LJ (1995) Dual pathways of internalization of the cholecystokinin receptor. J Cell Biol 128:1029–1041.

Rouse ST, Thomas TM, Levey AI (1997) Muscarinic acetylcholine receptor subtype m2: diverse functional implications of differential synaptic localization. Life Sci 60:1031–1038.

Shigemoto R, Nakaya Y, Nomura S, Ogawameguro R, Ohishi H, Kaneko T, Nakanishi S, Mizuno N (1993) Immunocytochemical localization of rat substance-P receptor in the striatum. Neurosci Lett 153:157–160.

Sternini C, Spann M, Anton B, Keith Jr DE, Bunnett NW, von Zastrow M, Evans C, Brecha NC (1996) Agonist-selective endocytosis of mu-opioid receptor by neurons *in vivo*. Proc Natl Acad Sci USA 93:9241–9246.

Stoof JC, Drukarch B, Deboer P, Westerink BHC, Groenewegen HJ (1992) Regulation of the activity of striatal cholinergic neurons by dopamine. Neuroscience 47:755–770.

Tabarin A, Minot AP, Dallochio M, Roger P, Ducassou D (1992) Plasma concentration of neuropeptide Y in patients with adrenal hypertension. Regul Pept 42:51–61.

van Deurs B, Holm PK, Kayser L, Sandvig K, Hansen SH (1993) Multivesicular bodies in HEp-2 cells are maturing endosomes. Eur J Cell Biol 61:208–224.

von Krosigk M, Smith AD (1991) Descending projections from the substantia nigra and retrorubral field to the medullary and pontomedullary reticular formation. Eur J Neurosci 3:260–273.

Wainer BH, Bolam JP, Freund TF, Henderson Z, Totterdell S, Smith AD (1984) Cholinergic synapses in the rat brain: a correlated light and electron microscopic study employing a monoclonal antibody against choline acetyltransferase. Brain Res 308:69–76.

Wang JQ, McGinty JF (1996a) Muscarinic receptors regulate striatal neuropeptide gene expression in normal and amphetamine-treated rats. Neuroscience 75:43–56.

Wang JQ, McGinty JF (1996b) Scopolamine augments c-Fos and zif/268 messenger RNA expression induced by the full D1 dopamine receptor agonist SKF-82958 in the intact rat striatum. Neuroscience 72:601–616.

Wang JQ, McGinty JF (1997) Intrastriatal injection of a muscarinic receptor agonist and antagonist regulates striatal neuropeptide mRNA expression in normal and amphetamine-treated rats. Brain Res 748:62–70.

Wang JQ, Jolkkonen M, McGinty JF (1997) The muscarinic toxin 3 augments neuropeptide mRNA in rat striatum *in vivo*. Eur J Pharmacol 334:43–47.

Weiler MH, Misgeld U, Gheong DK (1984) Presynaptic muscarinic modulation of nicotinic excitation in the rat neostriatum. Brain Res 296:111–120.