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Review

"In vivo" intraneuronal trafficking of G protein coupled receptors in the striatum: regulation by dopaminergic and cholinergic environment

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Abstract

We have studied "in vivo" neurochemically identified striatal neurons to analyze the localisation and the trafficking of dopamine and acetylcholine G protein coupled receptors (GPCR) (D1R, D2R, m2R and m4R) under the influence of neurotransmitter environment. We have identified receptors in tissue sections through immunohistochemical detection at the light and electron microscopic level. We have identified receptors in normal animals and after acute and chronic stimulations. We have quantified receptors through image analysis at the electron microscopic level in relation to various subcellular compartments. Our results demonstrate that, in normal conditions, GPCRs are mostly associated with plasma membrane of the striatal neurons, mostly at extra-synaptic sites. In certain instances (m4R; D2R), receptors have prominent localisation inside the rough endoplasmic reticulum. Our results also show that two distinct receptors for a same neurotransmitter may have distinct subcellular localisation in a same neuronal population (m2R versus m4R) and that the same neurotransmitter receptor (m4R) can have distinct localisation in distinct neuronal populations (cytoplasm versus cell surface). After acute stimulation, cell surface receptors undergo dramatic subcellular changes that involve plasma membrane depletion, internalisation in endosomes and in multivesicular bodies. Such changes are reversible after the end of the stimulation and are blocked by antagonist action. Chronic stimulation also provokes changes in subcellular localisation with specific pattern: plasma membrane depletion, and exaggerated storage of receptors in rough endoplasmic reticulum and eventually Golgi complex (D1R; m2R and m4R). Decreasing chronic receptor stimulation reverses such changes. These results demonstrate that, "in vivo", in the striatum, GPCRs undergo complex intraneuronal trafficking under the influence of neurochemical environment in conditions that dramatically modulate the number of cell surface receptors available for interaction with neurotransmitters or drugs. This confirms that "in vivo", the trafficking and the subcellular compartmentalization of GPCRs may contribute to regulate neuronal sensitivity and neuronal interactions in physiological, experimental and pathological conditions, including in therapeutic conditions.

Keywords: Neurotransmitter receptors; GPCR; Intraneuronal trafficking; Basal ganglia

1. Introduction

G protein coupled receptors (GPCRs) mediate neurotransmitter actions throughout the nervous system.They are known as transmembrane proteins expressed at the surface of the neurons where they are available for endogenous ligands and related molecules. A large amount of information has been collected regarding the biosynthesis and the intracellular trafficking of such receptors through various approaches including the use of transfected cells and tagged receptors, (Keith et al. 1996, Keith et al. 1998, Koenig & Edwardson 1997, Trimmer 1999, Tsao 2001), and the immunohistochemical detection of these receptors (Beaudet et al. 1998, Bernard et al. 1998, Bernard et al. 1999, Bloch et al. 1999, Csaba et al. 2001, Csaba et al. 2002, Dournaud et al. 1998, Dournaud et al. 1996, Dumartin et al. 1998, Dumartin et al. 2000, Faure et al. 1995, Mantyh et al. 1995a, Mantyh et al. 1995b, Sternini et al. 2000). It is known that these transmembrane proteins are synthesized in the rough endoplasmic reticulum, modified in the Golgi complex and transported through vesicular systems at the surface of the neurons where they are expressed, most frequently at extra-synaptic or perisynaptic locations. It is also known that the acute stimulation of these receptors promotes complex and reversible biochemical and intracytoplasmic events that start with endocytosis and receptor phosphorylation-dephosphorylation and that ultimately lead to receptor desensitisation and recycling

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(Koenig and Edwardson 1997, Tsao and Von Zastrow 2001); such receptor biosynthesis and intracellular trafficking contribute to regulate the number of receptors available for neurotransmitters or related drugs at the surface of the neurons and consequently contribute to modulate neuronal reactivity and drug effects. Little is known of the conditions of expression of these receptors by chemically identified neurons "in vivo" in normal brain; little is also known of how these receptors react "in vivo" to their neurochemical environment; several studies have focused on the analysis of the trafficking and the subcellular localisation of neuropeptide GPCRs (SP, neurotensin, opioids) in brain and have shown that in most instances, acute stimulation of these receptors provokes internalisation and recycling (Csaba et al. 2001, Csaba et al. 2002, Dournaud et al. 1996, Dournaud et al. 1998, Haberstock-Debic et al. 2003, Mantyh et al. 1995a, Mantyh et al. 1995b, Marvizon et al. 1997, Marvizon et al. 1999, Marvizon et al. 2003, Trafton et al. 2000, Wang & Marvizon 2002). In order to better understand the conditions of expression of GPCRs in the brain and to expand these results to classical neutransmitters, we have analysed the expression of acetylcholine (muscarinic) and dopamine

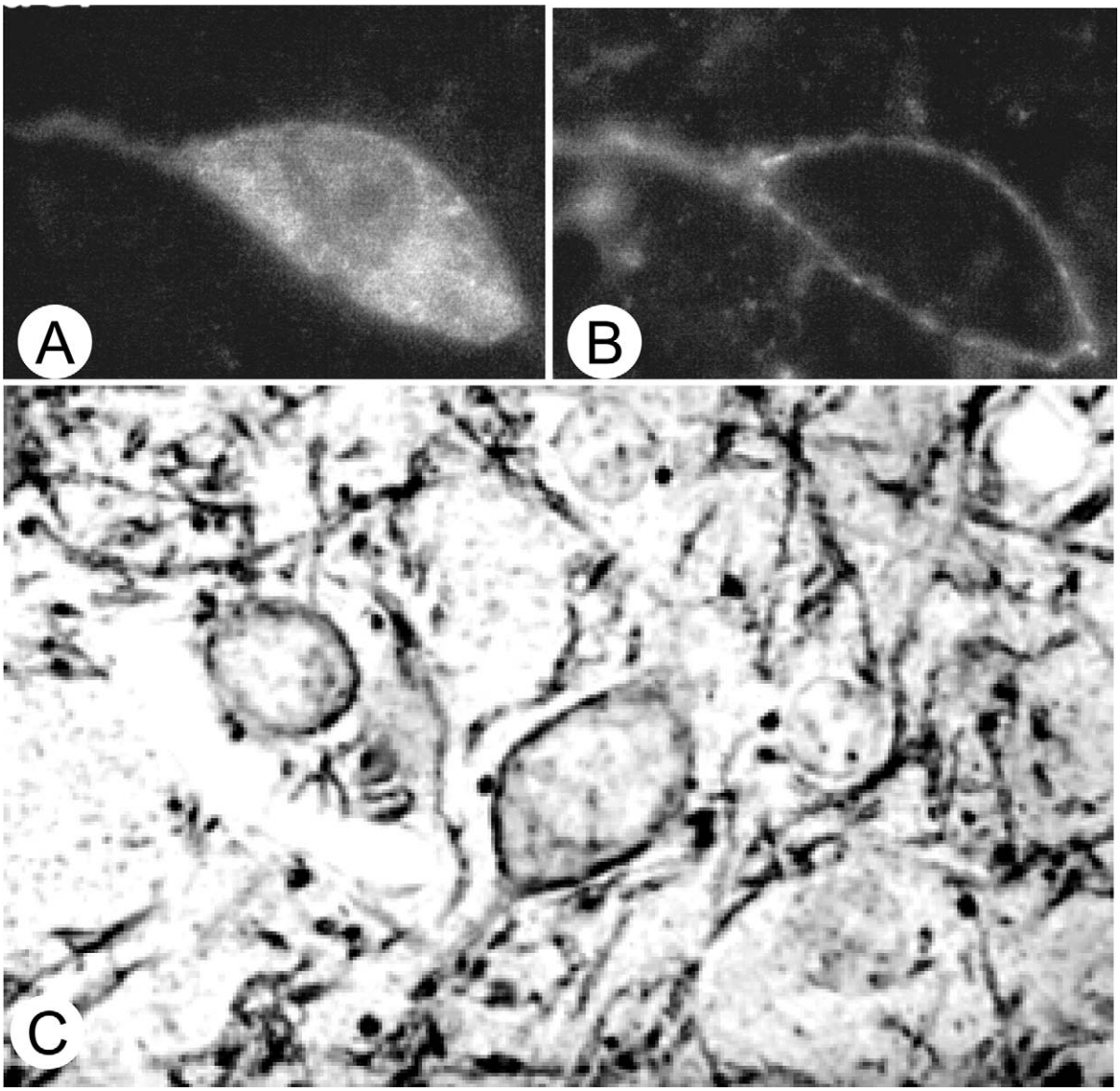

Fig. 1. Localisation of GPCRs in striatal neurons in normal adult rat. Light microscopy.

A and B: Simultaneous detection of choline acetyltransferase (A) and m2R (B). The m2R immunoreactivity is located at the periphery of a cholinergic cell body identified by choline acetyltransferase immunoreactivity in the cytoplasm. Double immunofluorescence.

C: Immunohistochemical detection of D1R (semi-thin section) with immunoperoxidase. D1R immunoreactivity is located at the periphery of neurons. (From Caille et al. 1996).

Methods: D1R was previously prepared as previously described (Caille et al. 1996); m2R was prepared as described in Hersch et al. 1995, Levey et al. 1995 and Levey et al. 1993). Brains were fixed by perfusion of animals with aldehyde (formaldehyde +/– glutaraldehyde), cut with vibratome and processed for light microscopic studies according to various procedures described in details in (Bernard et al. 1998, Bernard et al. 1999, Dumartin et al. 1998, Dumartin et al. 2000 and Liste et al. 2002): primary antibodies were recognized by secondary antibodies coupled to peroxidase or various fluorochromes.

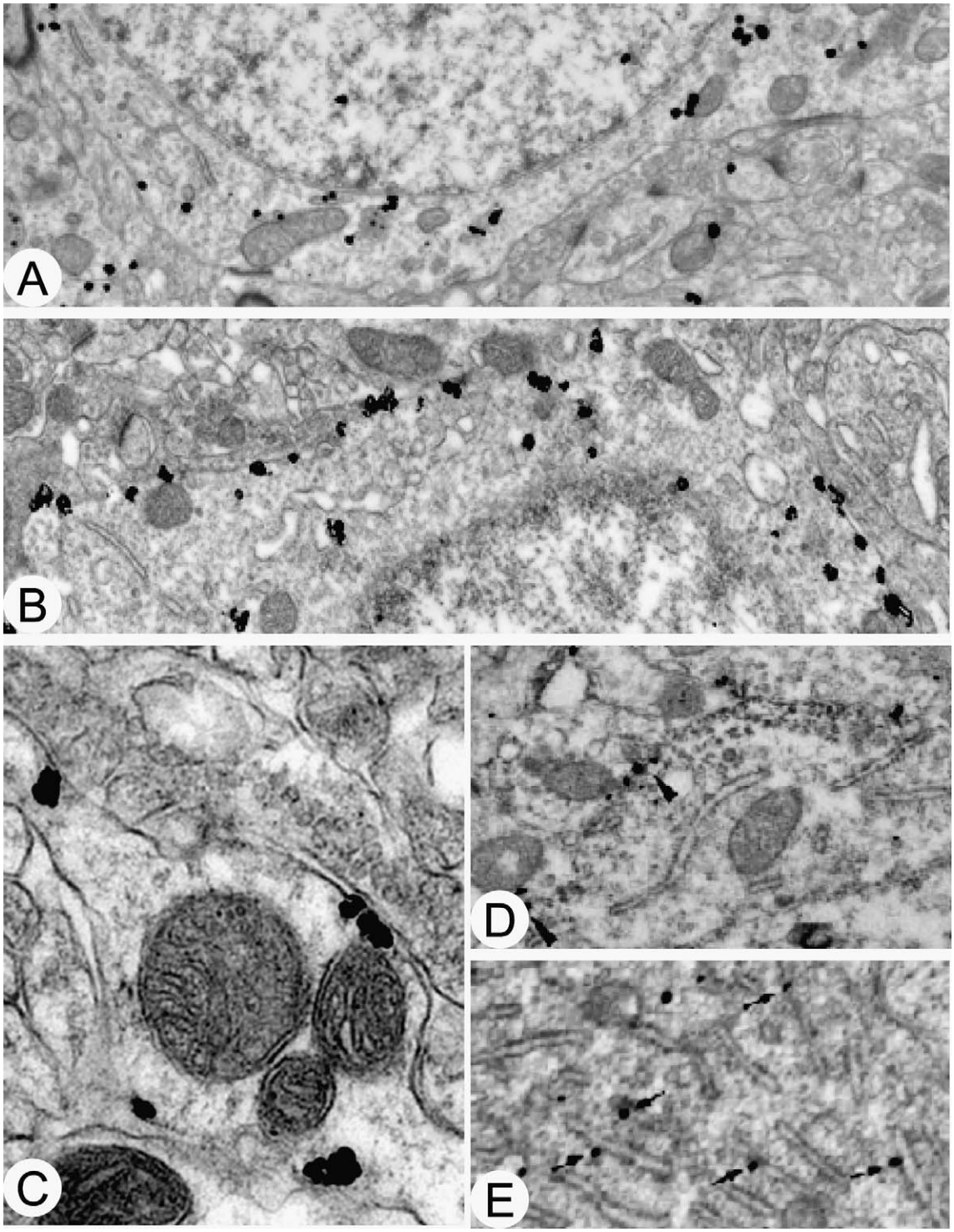

Fig. 2. Localisation of GPCRs in striatal neurons in normal conditions in adult rat. Electron microscopy with immunogold particles.
A to C: Detection of D2R (A) and D1R (B and C) immunoreactivity in striatal neurons. D1R is mostly associated with plasma membrane in cell body (B) and dendrite (C), while D2R is mostly associated with cytoplasm including the endoplasmic reticulum (A) in a cell body and is not present at the cell surface.
D and E: Detail of the detection of m4R immunoreactivity in a cholinergic neuron (D) (identified by double immunohistochemistry with choline acetyltransferase) and in a medium sized neuron (E) (from (Bernard et al. 1999)). The m4R is associated with the plasma membrane in D (arrowheads) while it is associated with the rough endoplasmic reticulum in E (arrows).
Methods: D1R was previously prepared as previously described (Caille et al. 1996); D2R, m2R and m4R were prepared as described in Hersch et al. 1995, Levey et al. 1995 and Levey et al. 1993). Brains were fixed by perfusion of animals with aldehyde (formaldehyde +/– glutaraldehyde), cut with vibratome and processed for electron microscopic studies according to various procedures described in details in (Bernard et al. 1998, Bernard et al. 1999, Dumartin et al. 1998, Dumartin et al. 2000 and Liste et al. 2002): primary antibodies were recognized with secondary antibodies coupled with gold particles that allow exquisite subcellular recognition and quantitative analysis.

receptors in several neurochemically identified neuronal populations of the striatum. We have analysed the subcellular compartmentalization of these receptors after modification of their environment in two ways: acute stimulation and chronic stimulation, including by using drugs that may be of importance in human pathology, such as amphetamine or acetylcholinesterase inhibitors; we have analysed receptor expression through immunohistochemical analysis at the light and electron microscopic level. We present here a synthesis of our research that demonstrates that each receptor has a specific pattern of expression in the normal striatum, depending of its nature and of the nature of the neurons that express it. It also shows that activation provokes dramatic changes in trafficking and compartmentalization of these receptors with distinct pattern depending on conditions of acute or chronic stimulation.

2. Subcellular localisation of dopamine and muscarinic receptors in neurochemically identified striatal neurons in normal conditions

Immunohistochemistry at the light and electron microscopic level demonstrates m2R, m4R, D1R and D2R at the surface and inside neurons in the striatum: D1R, that is expressed in GABA/SP/ dynorphin neurons is prominently associated with the plasma membrane of cell bodies and dendrites (Caille et al. 1996, Dumartin et al. 1998, Hersch et al. 1995, Levey et al. 1993), at the surface of the neurons (Figs. 1 and 2), in most instances at extra-synaptic locations as shown by double immunohistochemistry with tyrosine hydroxylase and D1R antibody (Caille et al. 1996). In the same way, the m2R, that is produced as an autoreceptor in cholinergic neurons is also prominently located at the plasma membrane, at extra-synaptic locations (Bernard et al. 1998, Levey et al. 1995) (Figs. 1 and 2). In contrast, D2R is prominently located in the rough endoplasmic reticulum (Fig. 2A) (Dumartin et al., unpublished results and Levey et al., 1993, Hersch et al., 1995). The m4R displays dramatic differences in subcellular localisation, depending on the neurons in which it is expressed (Bernard et al. 1999): in GABA neurons, m4R displays same expected membrane localisation as D1R and m2R. In contrast, in cholinergic neurons where m4R is co-expressed with m2R, m4R displays prominent intracytoplasmic localisation, inside the rough endoplasmic reticulum and Golgi complex with very little receptor present at the cell surface (Figs. 2D, E and Fig. 3). Our results in normal rats and mice confirm that, in most instances, GPCRs are located at the surface of neurons, at the plasma membrane, mostly in extra-synaptic locations, at cell bodies and dendrites. While we cannot exclude that technical conditions may preclude the detection of receptors located at synaptic clefts, the large abundance of receptors outside synapses, not in direct contact with synaptic buttons bring additional evidence for volume transmission in the striatum for dopamine and acetylcholine action (Agnati et al. 1995), in accordance with similar results regarding neuropeptide

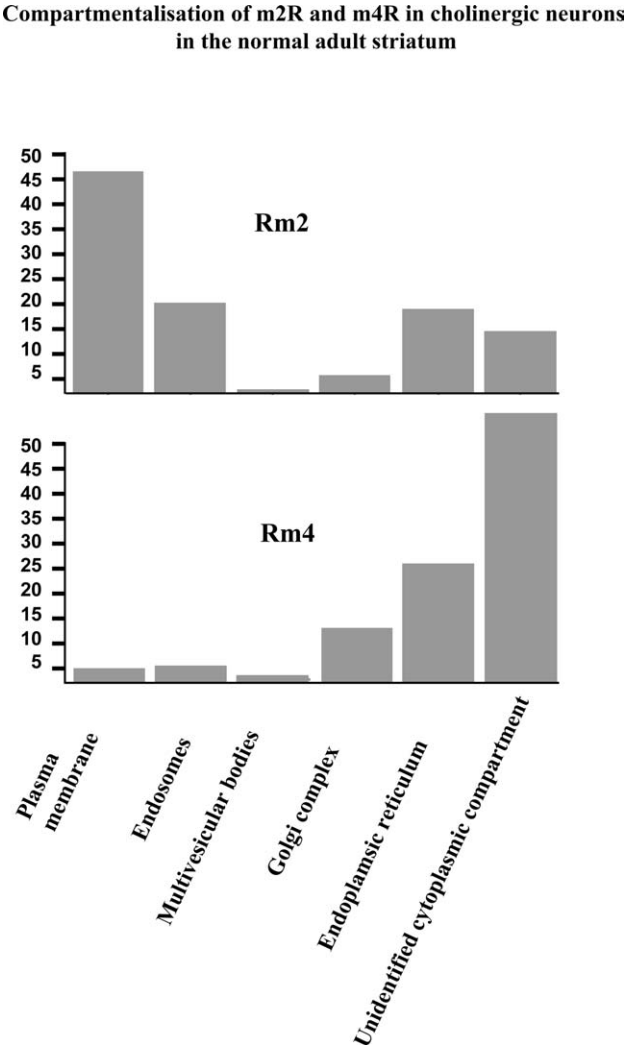

Fig. 3. Comparative quantitative analysis of the subcellular localisation of the m2R and m4R in normal cholinergic neurons: image analysis after ultrastructural immunohistochemistry.

Immunogold particles have been counted in neurons by using Metamorph software according to procedures described in Bernard et al. 1998 and Bernard et al. 1999).Values are expressed as the percentage of total immunoparticles present on a cell body. The m2R is largely associated with the plasma membrane (45%), while the m4R is not (only 3%), and is mostly associated with several intracytoplasmic compartments, including Golgi complex and rough endoplasmic reticulum. (Bernard et al. 1998, Bernard et al. 1999).

Methods: Quantitative analysis was performed after ultrastructural immunohistochemistry in order to establish the abundance of receptor immunoreactivity associated with each subcellular compartment. Metamorph image analysis software was used in order to associate immunoreactive gold particles with plasma membrane or with various cytoplasmic compartments in cell bodies or dendrites as described in Bernard et al. 1998, Bernard et al. 1999, Liste et al. 2002).

receptors (Beaudet et al. 1998, Csaba et al. 2001, Csaba et al. 2002, Dournaud et al. 1996, Dournaud et al. 1998, Faure et al. 1995, Mantyh et al. 1995a, Mantyh et al. 1995b, Marvizon et al. 1999, Marvizon et al. 1997, Marvizon et al. 2003, Svingos et al. 1997). The subcellular localisation of m4R also illustrates that the compartmentalization of a given receptor

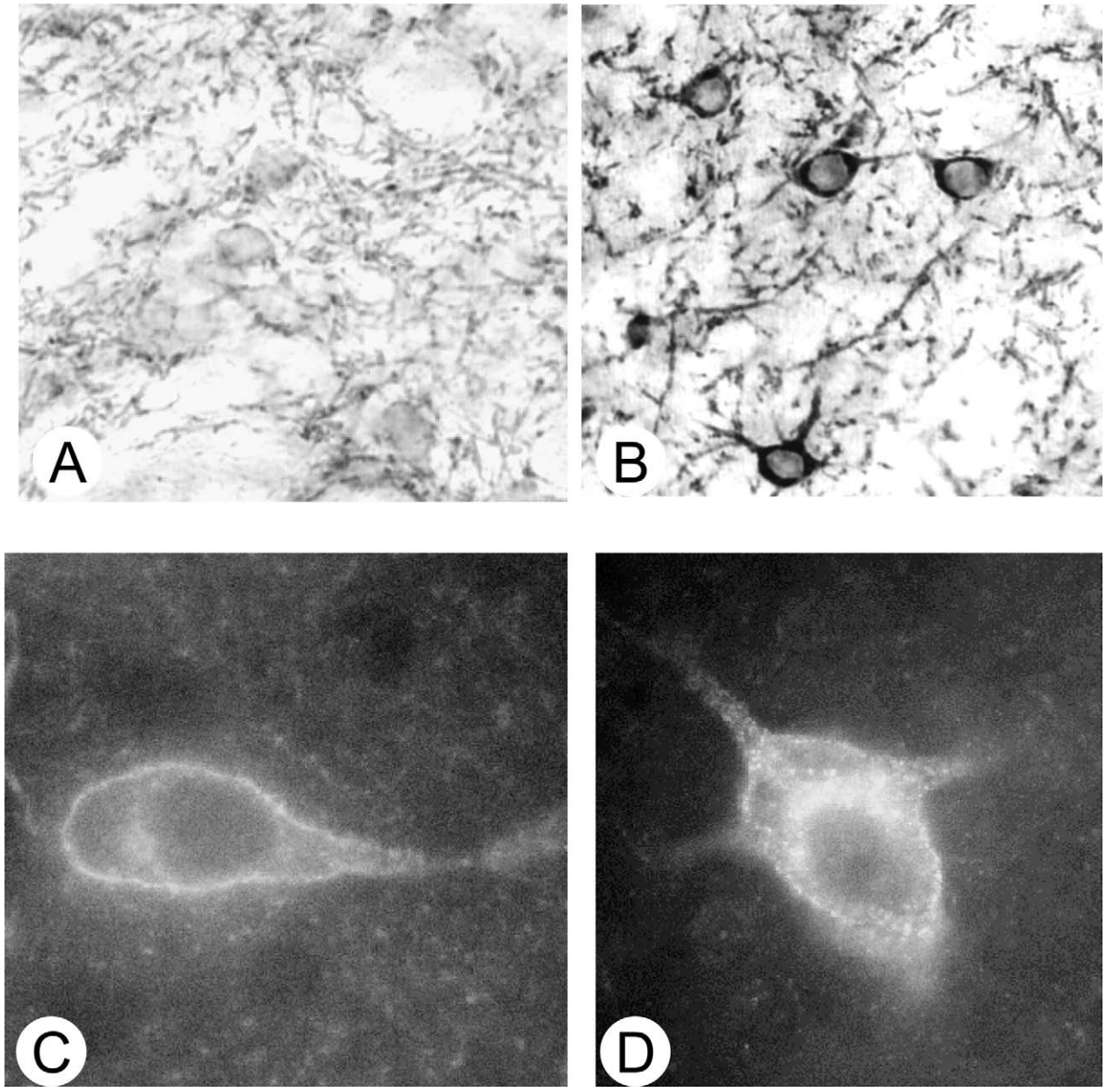

Fig. 4. Localisation of GPCRs in striatal neurons after acute stimulation. Light microscopy.
A and B: Localisation of D1R in normal striatum (A) and in striatum after treatment with the D1R agonist SKF 82958 during 40 min (B). Receptor stimulation provokes a dramatic translocation of D1R from the periphery of the neurons (A) to the cytoplasm (B). (Dumartin et al. 1998).
C and D: Localisation of m2R in normal striatum (C) and in striatum after treatment with oxotremorine (45 min) (D). Muscarinic receptor stimulation provokes intracytoplasmic localisation of m2R in striatal neurons. (Bernard et al., 1998).

depends of the neurochemical characteristics of the neurons that express it: indeed, in GABA neurons, m4R is expressed at the surface of the neurons, as expected for GPCRs. In acetylcholine neurons located beside GABA neurons, m4R is mostly stored in the rough endoplasmic reticulum, while in such neurons, m2R is also present at the plasma membrane. Such results also suggest that two GPCRs for a same neurotransmitter, namely m4R and m2R may have distinct localisations in a same neuronal population ; subcellular localisation of D2R also demonstrates the presence of this receptor in the rough endoplasmic reticulum of GABA neurons while D1R is located at the cell surface (Hersch et al. 1995, Levey et al. 1993). In the same way, it appears that the subcellular localisation of opioid receptors in brain also varies with receptor subtype (Marvizon et al. 1999, McConalogue et al. 1999). Such results demonstrate that each receptor has specific features for subcellular compartmentalization depending on its nature and of the neurochemical nature of the neurons that express it. The functional significance of such differences is still unclear but may be related to the structure of each receptor, to their neurochemical environment or to specific functions in neurochemically defined neurons.

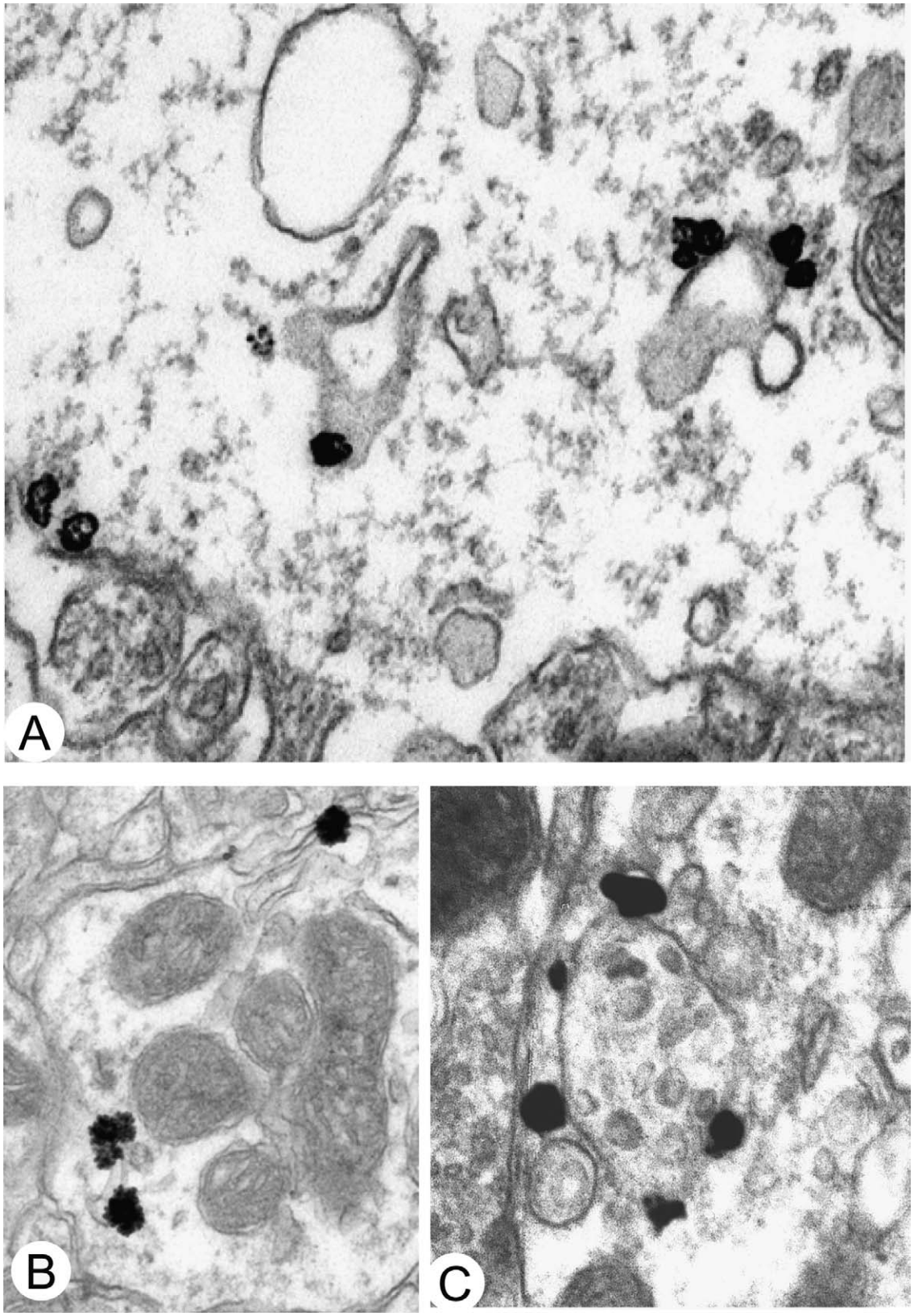

Fig. 5. Localisation of GPCRs in striatal neurons after acute stimulation. Electron microscopy with immunogold particles.
A and B: D1R immunoreactivity after amphetamine treatment during 40 min (A) in the cytoplasm of a striatal neuron and after SKF 82958 during 30 min in a dendrite (B). Immunoreactivity is associated with vesicles that have ultrastructural features of endosomes.(A from (Dumartin et al. 1998)
C: m2R immunoreactivity after stimulation with oxotremorine, a muscarinic agonist during 40 min: m2R immunoreactivity is associated with a typical multivesicular body.

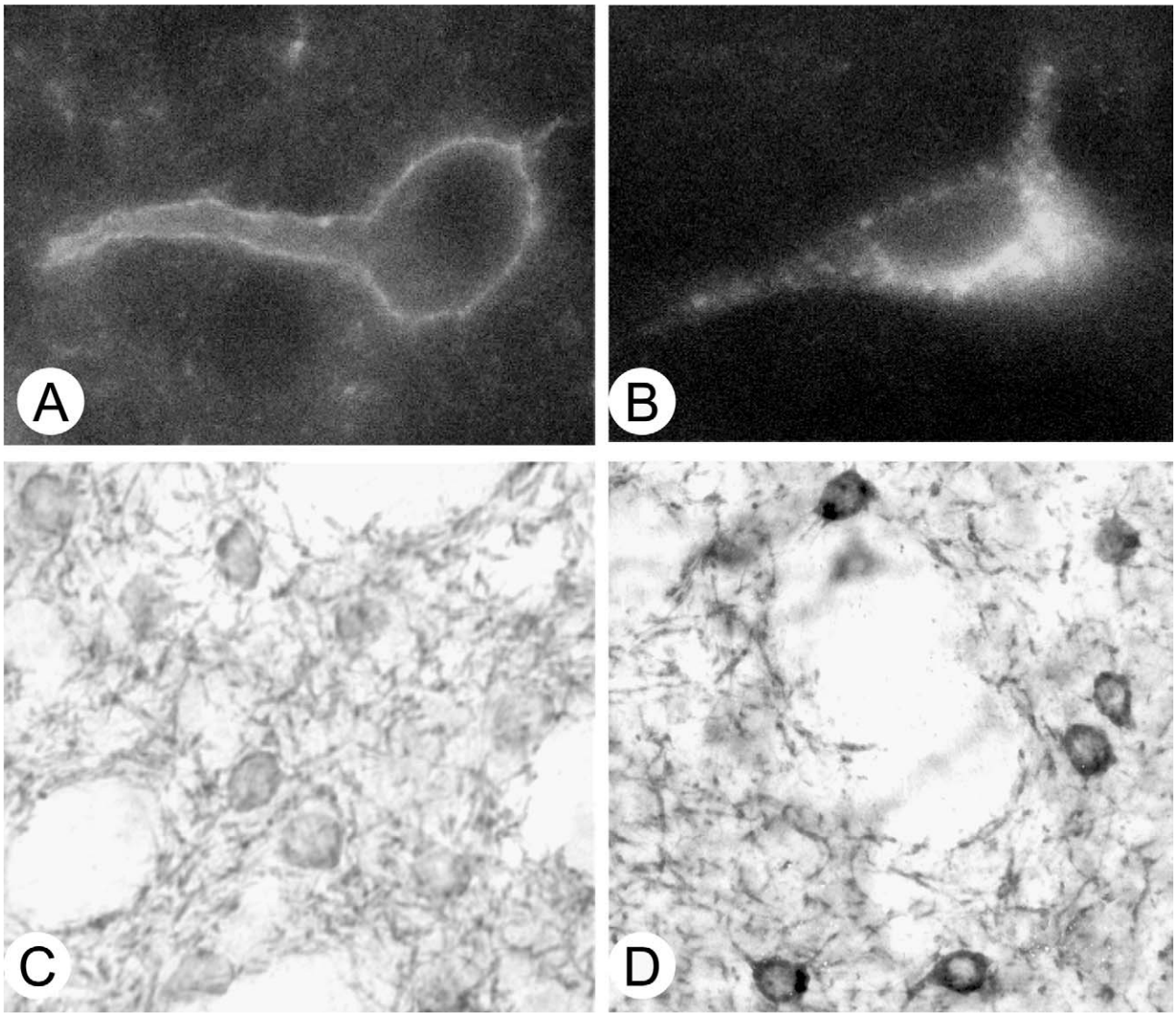

Fig. 6. Immunohistological detection of GPCRs in striatal neurons in mice genetically deficient for acetylcholinesterase (A, B) or for dopamine transporter (C, D). Light microscopy.
A and B: m2R detection in wild type mouse (A) and in homozygous K–O mouse for acetylcholinesterase gene (B). In normal mouse, the m2R is located at the plasma membrane. In acetylcholinesterase deficient mouse, there is a nearly total depletion of m2R at the membrane and a massive accumulation in the neuronal cytoplasm (B). (From Bernard et al. 2003).
C and D: D1R detection in wild type mouse (C) and in mouse having knockout for dopamine transporter (D) and consequently chronic elevated extracellular dopamine concentration. In normal mouse (C), the D1R is located at the plasma membrane. In transporter deficient mouse, there is a massive accumulation of D1R in the neuronal cytoplasm (B). (From Dumartin et al. 2000).

3. Subcellular localisation of dopamine and acetylcholine receptors after acute stimulation

Acute stimulation provokes a rapid (within minutes), dramatic (from 50 to 80%) and reversible depletion of cell surface receptors in the striatum. This occurs when receptors are stimulated by exogenous agonist: SKF 82958 for D1R (Dumartin et al. 1998); oxotremorine for m2R and m4R (Bernard et al. 1998, Bernard et al. 1999). This also occurs when they are stimulated by important release or prolonged action of endogenous agonist (after amphetamine treatment for D1R (Dumartin et al. 1998) or injection of acetylcholinesterase inhibitor such as metrifonate or other molecules for m2R and m4R (Liste et al. 2002). Such membrane depletion is reversible within hours after the end of the treatment and is blocked by simultaneous treatment with antagonist. Such changes are associated with a dramatic accumulation of the receptors inside the cytoplasm of cell bodies and dendrites, as shown by light microscopy analysis (Fig. 4). Electron microscopic quantitative analysis demonstrates that most internalised receptor is associated with vesicles that display ultrastructural features of endosomes (Figs. 5A and B) (Bernard et al. 1999, Bernard 2003, Dumartin et al. 1998). In some instances, the number of multivesicular bodies containing immunoreactive receptor is dramatically increased after acute stimulation, up to 800% (Fig. 5C) (Bernard et al.

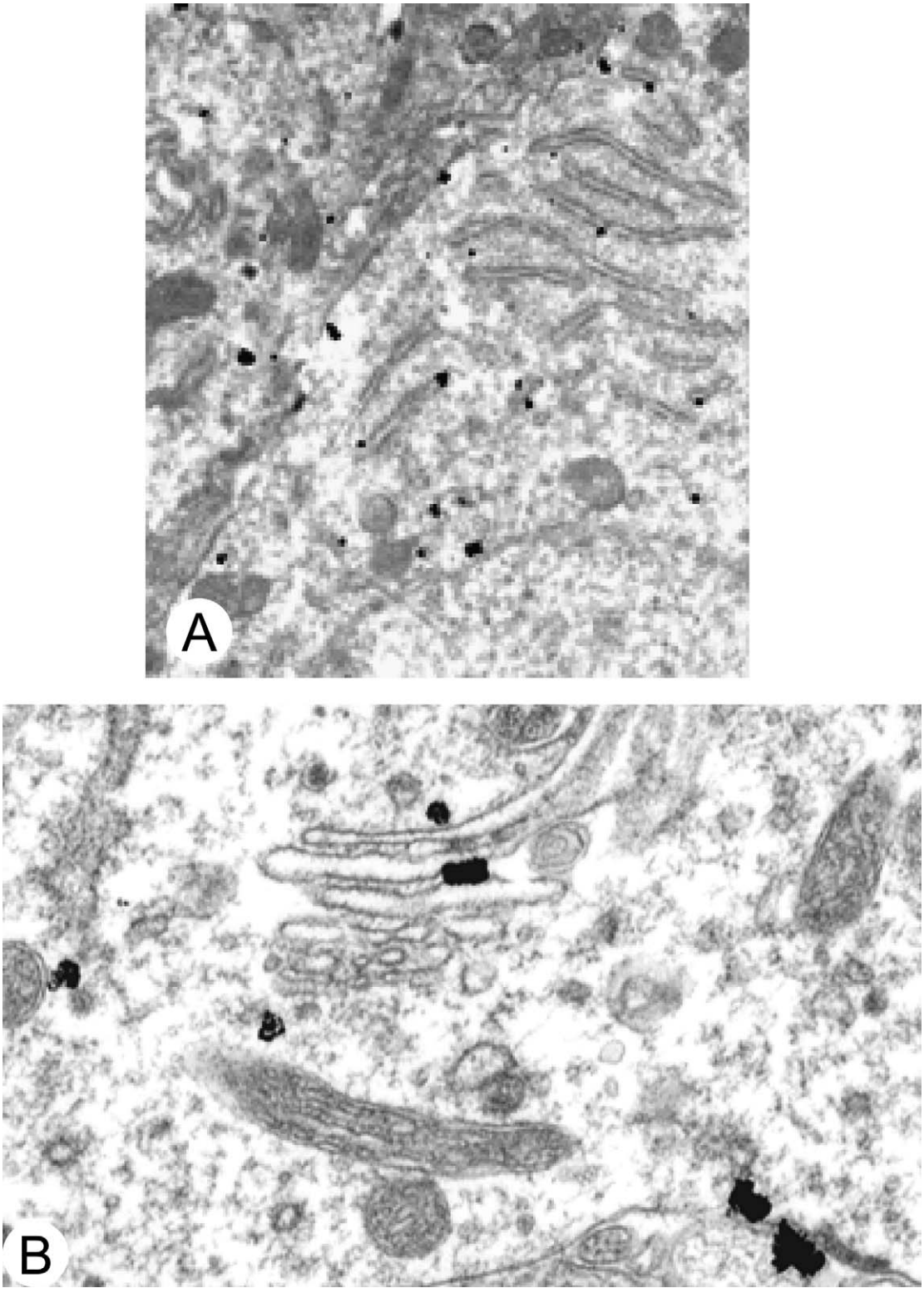

Fig. 7. Immunohistological detection of GPCRs in striatal neurons in mouse genetically deficient for acetylcholinesterase (A) or for dopamine transporter (B). Electron microscopy with immunogold technique.

A: In acetylcholinesterase deficient mouse, m2R is prominently associated with subcellular compartments that are involved in biosynthesis, namely Golgi complex and endoplasmic reticulum. The present document display m2R immunoreactivity associated with rough endoplasmic reticulum. (From Bernard et al. 2003).

B: In dopamine transporter deficient mouse, D1R is prominently associated with subcellular compartments that are involved in biosynthesis, namely Golgi complex and endoplasmic reticulum. The present document display D1R immunoreactivity associated with Golgi saccules. (From (Dumartin et al. 2000).

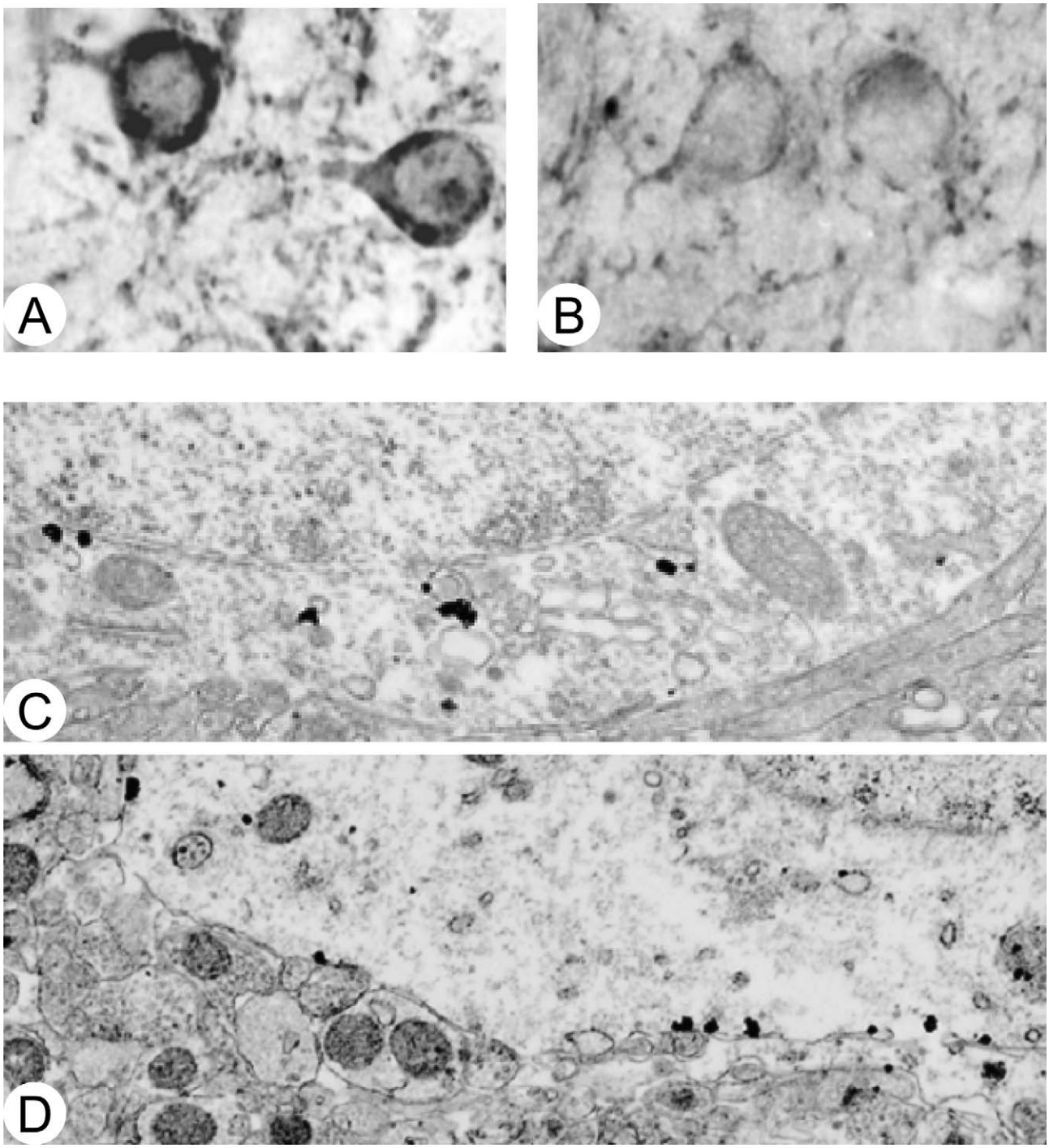

Fig. 8. Immunohistological detection of D1R in striatal neurons in mice genetically deficient for dopamine transporter: effect of the destruction of dopamine neurons.

Documents are from a heterozygous mouse in which the substantia nigra was destroyed on the right side through injection of 6–OH dopamine. A and C show striatal neurons from the intact side, B and D show neurons on the right side in which substantia nigra was destroyed. A and C show D1R immunoreactivity associated with cytoplasmic compartments. B and D show, in the same animal, D1R that is prominently associated with the plasma membrane three days after destruction of the substantia nigra (From Dumartin et al. 2000).

1998, Bernard et al. 1999, Liste et al. 2002). Cessation of drug treatment leads to receptor recovery at the plasma membrane. When the receptor is not present at the plasma membrane in normal condition (m4R in cholinergic neurons or D2R in GABA neurons), acute stimulation does not modify its subcellular localisation (Bernard et al. 1999, Dumartin, Levey and Bloch, unpublished).

4. Subcellular localisation of dopamine and acetylcholine receptors after chronic stimulation

Chronic stimulation also provokes dramatic changes in the localisation of the receptors, but with subcellular features very different from the ones observed after acute stimulation (Bernard 2003, Dumartin et al. 2000, Liste et al. 2002). When the receptor is initially located at the plasma membrane, chronic stimulation provokes a dramatic subcellular redistribution with several features: an important decrease of receptor abundance at the membrane, both in the cell bodies or dendrites (from 60 to 94 %) (Fig. 6); an exaggerated accumulation of receptor associated with rough endoplasmic reticulum and in certain conditions with Golgi complex (Fig. 7). Such changes are observed for D1R in mice knockout for dopamine transporter that are known to have permanently high content of extracellular dopamine and behavioural features of chronic hyperdopaminergy (Dumartin et al. 2000, Giros et al. 1996). In the same way, chronic treatment (twenty days) with metrifonate also provokes such effect for m2R in cholinergic neurons and m4R in GABA neurons (Liste et al. 2002). The most dramatic example is observed in mice knock-out for acetylcholinesterase that display functional symptoms of chronic hypercholinergy and demonstrate nearly total depletion of membrane bound m2R (– 94%) with huge accumulation inside the rough endoplasmic reticulum (Bernard 2003) (Fig. 6A, B and 7A). In most instances, there is no modification of the abundance of receptor associated with endosomes or multivesicular bodies after chronic treatment, with the noticeable exception of the neurons of rats treated with metrifonate (Liste et al. 2002). Dynamic experiments demonstrate that such a situation is reversible when decreasing receptor stimulation: this is observed for m2R within organotypic cultures of acetylcholinesterase K–O mice in which treatment with antagonist (atropine) or cocultured neurons producing acetylcholinesterase provokes m2R receptor delivery to the membrane (Bernard 2003). In the same way, the destruction of dopamine neurons in dopamine transporter knockout mice provokes a return of the D1R to the plasma membrane of the neurons within a few days (Dumartin et al. 2000); (Fig. 8).

5. Rationale and functional significance for intraneuronal trafficking

Our experiments clearly demonstrate that, “in vivo”, dopamine and muscarinic receptors react after acute stimulation in conditions that were previously described “in vitro” for these receptors in non neuronal cells, or “in vivo” for peptide GPCRs: membrane depletion and internalisation in endosomes occur within minutes after stimulation, suggesting that activation dramatically modifies the number of receptors available for neurotransmitter or related drugs. The functional significance of such events is still unclear: it does not seem to be directly related to the potency of activation by agonists, since certain very active agonists such as morphine do not provoke “in vitro” internalisation of opioid receptors (McConalogue et al. 1999, Sternini et al. 2000, Whistler et al. 1999). In any case, endogenous agonists, namely acetylcholine and dopamine have potent internalising effects. Similarly, it appears that neurokinin receptor internalisation is directly related to substance P release by afferent neurons in the spinal cord (Marvizon et al. 1997, Marvizon et al. 2003). The presence of certain receptors in multivesicular bodies after acute or chronic activation demonstrates that internalisation promotes in certain instances, a degradation of the receptor since multivesicular bodies are known to be a pathway to the lysosomal system. Nevertheless, numerous events involved in the endocytic and degradative process of the GPCRs escape investigation at the moment. It is important for example to known whether restoration of normal stock of receptors after internalisation is due to new receptor synthesis or to the recycling of endocytosized receptors inside the neurons. “In vitro” studies suggest that, indeed, receptor recycling is a major component of these events (Koenig & Edwardson 1997, Martin-Negrier in press, Tsao and Von Zastrow 2001). In the same way, little is known about the subcellular modifications induced by activation at axonal varicosities or endings; studies throughout basalocortical neurons demonstrate that localisation and intraneuronal trafficking of m2R in axonal varicosities in the cortex is different after activation to that observed in the somatodendritic field of the same neurons (Decossas et al. 2003). This demonstrates that in a same neuronal system, the fate of a receptor may depend on the part of the neuron in which it is expressed. Other recent data demonstrate complexity of intraneuronal trafficking of GPCRs: it appears that SP internalisation or conversely D1R plasma membrane delivery can be induced by NMDA activation (Marvizon et al. 1997, Scott et al. 2002). Neuropeptide exocytosis may be a way by which GPCRs are delivered to the plasma membrane of neurons after receptor activation (Bao et al. 2003). It also appears that certain proteins interact specifically with GPCRs to regulate their subcellular compartmentalization (Bermak et al. 2001).

In our experiments, chronic stimulation also provokes a membrane depletion of GPCRs in the striatum whatever model studied; this demonstrates that the redistribution of receptors under chronic stimulation may contribute to down-regulation of the receptors; analysis of m2R depletion in AchE knock-out mice demonstrates that such depletion can be nearly complete and then can profoundly alter the reactivity of neurons to neurotransmitter or drugs. Such membrane depletion is associated with exaggerated storage of receptors

in the rough endoplasmic reticulum and sometimes in Golgi complex. The most conservative hypothesis to explain this redistribution is that regulation of the GPCR storage in rough endoplasmic reticulum and most probably in Golgi complex contribute to regulate membrane delivery and consequently cell surface receptor abundance under chronic stimulation. Such hypothesis is substantiated by the existence of molecules that specifically retain such receptor in the endoplasmic reticulum (Bermak et al. 2001) and by the fact that such storage is reversible and that normal membrane delivery of receptors can be restored by decreasing chronic stimulation of the receptors as we demonstrated "in vivo" (Dumartin et al. 2000) or in organotypic cultures (Bernard 2003). Nevertheless, chronic stimulation of GPCRs can have opposite effects in brain: chronic stimulation of opioid receptors provoke their membrane delivery from cytoplasmic stores in the spinal cord (Cahill et al. 2001, Cahill et al. 2003).

6. Perspective

The results obtained "in vivo" from the seminal work of Mantyh et al. in 1995 demonstrate that the analysis of trafficking of GPCRs in neurons appears now as an important field of investigation for the understanding of neuronal interactions and neurotransmitter effects. It is very useful to test or validate models of GPCR trafficking established on the basis of "in vitro" experiments at the single cell level. It allows to analyse neuronal interactions in conditions than cannot be developed "in vitro": this includes the possibility to observe the localisation of GPCRs in conditions in which neurochemically identified neurons are submitted to the various brain inputs. This also includes the possibility to analyse the influence of neuronal interactions on receptor trafficking under the influence of molecules or experimental conditions of importance for human pathology, in conditions that cannot be obtained "in vitro". This is true for pain, for understanting of drug addiction, or also for the analysis of acetylcholinesterase inhibitors effects. As an example, recent data (Haberstock-Debic et al. 2003) demonstrate that, "in vivo", morphine has internalising effects on opioid receptors in the accumbens nucleus. Indeed, numerous "in vitro" studies had previously failed to demonstrate such internalising effects despite potent activation of the receptors by morphine (Keith et al. 1996, 1998, Whistler et al. 1999). "In vivo" studies also allow exquisite subcellular resolution including for the analysis of receptor trafficking in endings. At the moment, one of the major goal of such field is to give general understanding of GPCR trafficking "in vivo", to establish links between trafficking and physiology of the neurons that express them, and to establish how the regulation of such trafficking may contribute to effects of neurotransmitters or drugs in physiological, experimental and pathological conditions, including in neurological and psychiatric diseases. Refinements in temporal and spatial resolution of receptor imaging "in vivo" in living animals or humans may also contribute in the future to better approach such questions.

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