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D4 Dopamine and Metabotropic Glutamate Receptors in Cerebral Cortex and Striatum in Rat Brain

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The characterization of the functional interactions between the metabotropic glutamate receptors (mGluR) and the dopaminergic (DR) receptors in the corticostriatal projections may provide a possible interpretation of synaptic events in the basal ganglia. It has been suggested that presynaptic D2-type receptor located on glutamatergic corticostriatal neurons regulates the release of glutamate. In a first approach we have studied the cellular distribution of the D4R and the mGluRs in cerebral cortex and striatum employing immunocytochemistry. D4R positive neurons were particularly numerous in medial prefrontal cortex mainly occupying layers II and III. An even distribution was found on small round-shaped neurons in the striatum. Group I mGluR1α-like immunoreactivity (mGluR1α-LI) was found in medial and deep layers of the cerebral cortex while group III mGluR4a labeled more superficial layers; group II mGluR2/3 signal was intense on fine fibers with a punctate appearance. In the striatum, mGluR1α and mGluR2/3 stained mainly fibers while mGluR4a labeled round shaped cell bodies. After lateral ventricular injection of colchicine, an axonal transport and firing activity blocker, D4R labeling significantly increased in cerebral cortex and decreased in the striatum. mGluR1α and mGluR4a signal decreased in cerebral cortex and only mGluR4a signal decreased in the striatum. These results support previous reports indicating a presynaptic localization of D4R in the striatum. In contrast, striatal mGluR1α appears to be a postsynaptic receptor probably synthesized in situ. Our results do not support the hypothesis of a colocalization of D4 receptor and one or more of the metabotropic glutamatergic receptors studied here.

KEY WORDS: Corticostriatal projections; immunocytochemical localization; colchicine injections; axonal transport impairment.

INTRODUCTION

Corticostriatal transmission is a key pathway in the regulation of the neuronal activity of the basal ganglia. Almost all regions of the cerebral cortex provide a projection to the striatum and this cortical information is processed by the most abundant input and output neurons in the striatum, the medium spiny neurons. These neurons receive cortical excitatory synapses on the head of the dendritic spines and dopaminergic inputs, from the substantia nigra pars compacta, which end on the neck of the same spines (1–3). The medium spiny neurons are basically GABAergic whereas the corticostriatal neurons are excitatory utilizing glutamate as a neurotransmitter, in such a way that activation of the corticostriatal pathway leads to an increased firing of the striatal GABAergic neurons that

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in turn inhibit the neurons of the output nuclei. In this stimulation-inhibition interplay, the class of receptor present in the synapse greatly influences the type of response. It has been demostrated that D2 type dopaminergic receptors located on corticostriatal terminals regulate the release of excitatory transmitters (4). At this presynaptic terminal the metabotropic glutamate receptors, some located at the perysinaptic area, also regulate their release (5). In this way, there might be an important dopaminergic-glutamatergic interaction involved in the tight regulation of this complex system (cortex-striatum-substantia nigra). For this reason, there is an increasing interest in dilucidating the combination of dopaminergic and metabotropic receptors in corticostriatal projections and there are some clues that relate D2 type and metabotropic receptors in the modulation of striatal neurotransmission (1,6).

The D2 type family receptors include D2, D3 and D4 receptors. Among these, the D4 subtype drove much attention due to its high affinity for the atypical neuroleptic clozapine and to the fact that numerous neuropathological and clinical genetic studies implicated D4 receptors in several neuropsychiatric disorders including schizophrenia; attention deficit hyperactivity disorder and mood disorders, suggesting that D4 may represent a common site of action of antipsychotic drugs that may contribute selectively to their antipsychotic effects (7,8). This hypothesis was supported by the fact that D4R mRNA was described to localize to limbic regions of rat, monkey and human brain such as frontal cortex, midbrain, medulla, amygdala, olfactory bulb, hypothalamus and hippocampus, while relatively low levels were found in the nigrostriatal pathway (9–11). However, the receptor protein was found in caudate putamen among other areas such as the hippocampus, frontal cortex, entorhinal cortex, olfactory tubercle, cerebellum, supraoptic nucleus and substantia nigra pars compacta in different species and with different methodologies (12–16). Thus, these results suggested that D4R could be synthesized in cerebral cortex and targeted to presynaptic terminals of cortical projections to the basal ganglia structures, allowing for dopaminergic regulation of glutamatergic neurotransmission (16). As a matter of fact, Tarazi et al. (17) showed significant D4R losses in both corpus striatum and nucleus accumbens after unilateral surgical ablation of the frontal cerebral cortex to remove descending glutamatergic projections. These results suggest that some D4R are found in excitatory presynaptic corticostriatal afferents.

Glutamate is a major excitatory neurotransmitter in the CNS and is probably the transmitter used in the corticostriatal pathway which has an important function in the regulation of basal ganglia activity. Glutamate receptors are classified into two groups: ionotropic and metabotropic receptors, the latter have been involved in synaptic transmission, modulation and plasticity. It has been suggested that some of the mGluRs may modulate neurotransmitter release acting as presynaptic receptors (5,18,19). There are at least eight types of mGluRs subdivided in three groups (20,21). Group I: mGluR1 and mGluR5 are linked to stimulation of inositol triphosphate (IP3) formation; group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8), both coupled to the inhibition of cyclic AMP synthesis (19,22). The cellular distribution of the metabotropic receptors has been achieved through immunocytochemical and electron microscopy studies in rat and monkey brain (18, 23–27) and exhibit a complex regional pattern of differential localization. Immunocytochemical studies using specific antibodies against metabotropic glutamate receptors have been reported. mGluR1α showed intense immunoreactivity in cerebellum, hippocampus, globus pallidus and substantia nigra and lower levels of staining in cerebral cortex and striatum (23,24,27–31). mGluR2/3 showed immunollabeling within cerebellum, hippocampus, hypothalamus, cerebral cortex, globus pallidus, nucleus accumbens, ventral pallidum, striatum and substantia nigra (26,27,30). Finally, mGluR4a-LI was localized within cerebellum, hippocampus, cerebral cortex, globus pallidus, striatum and substantia nigra pars reticulata (18,30,32).

Electron microscopy analysis showed that mGluR1α was localized at postsynaptic densities in thalamus, at a perisynaptic position in the postsynaptic densities and at non-synaptic sites within the cerebellum and the hippocampus (24,28,30,33). mGluR2/3 antibody labelled presynaptic, postsynaptic and glial structures within hippocampus, cerebral cortex and striatum according to Petralia et al. (26). On the other hand, Shigemoto et al. (30) described mGluR2-LI in the extracellular space and mGluR2/3-LI along the extrasynaptic membrane, in preterminal rather than terminal portions of axons, only rarely detected in the presynaptic membrane and on glial processes within the hippocampus. mGluR4a-LI is presynaptically localized in globus pallidus, with labelling on axon terminals that form symmetrical synapses (18). The mGluR4a hippocampal localization is postsynaptic at asymmetrical synapses and presynaptic at both asymmetrical and symmetrical synapses according to Bradley et al. (32). Although, Shigemoto et al. (30) only described a presynaptic localization at symmetrical and

asymmetrical synapses. Immunogold studies at the electron microscope level have shown that group I was mainly present at the postsynaptic sites, group II at the pre and postsynapsis and group III at the presynapsis in the monkey striatum (25).

The characterization of the functional interactions between the mGluRs and the DRs in the corticostriatal projections may provide a possible interpretation of the synaptic events that take place in the basal ganglia both in physiological and pathological conditions. It has been suggested that presynaptic dopaminergic D2-type receptors located on glutamatergic corticostriatal neurons regulate the release of glutamate; the same hypothesis has been postulated for some subtypes of mGluRs that may act, in the presynaptic membrane, as autoreceptors activated by the release of glutamate (34). In order to identify the receptors subtypes involved in this regulation, our first approach was to study the cellular distribution of the D4R and the mGluRs (mGluR1α, mGluR2/3 and mGluR4a) in cerebral cortex and striatum employing immunocytochemistry. Intraventricular injections of colchicine were administered in order to block the axonal transport of the receptors from its site of synthesis at the neuronal somata to its possible expression site at the presynaptic membrane, giving a clue to their subsynaptic localization (35–38).

EXPERIMENTAL PROCEDURE

Animals and Surgery. Experiments were performed on male adult Wistar rats weighing 200–250 g. The animals were anesthetized with i.p. chloral hydrate (350 mg/kg) and placed on a David Kopf stereotaxic apparatus. A hole was drilled on the right side of the skull at 1.5 mm lateral and 0.8 mm posterior to the Bregma. A 25 μl Hamilton syringe loaded with 20 μl saline, or with 20 μl colchicine (6 mg/ml Sigma), was positioned above the predilled hole and then advanced into the lateral ventricle 4,5 mm, with reference to the stereotaxic atlas of Paxinos and Watson (39). For colchicine injections, the solution was infused at a constant rate for 5 min and the needle remained in position for another 5 min before it was withdrawn. The skin incision was sutured and cleaned. After recovery, the operated animals were returned to the vivarium, and were allowed to survive for 48 h before perfusion. Care was taken to minimize distress, pain or discomfort in the experimental animals.

Tissue Preparation. Operated animals were deeply reanesthetized with chloral hydrate and perfused through the ascending aorta with 50 ml of Tyrode Buffer followed by 400 ml (4°C) of a 4% paraformaldehyde and 0.4% picric acid in phosphate buffer pH 6.9. The brains were then dissected out, immersed in the same fixative for 2 h and transferred to 15% sucrose. Complete series of coronal sections (14 μm thickness) were obtained in a cryostat (Microm, Zeiss, Germany) and processed with the avidin-biotin (ABC) technique.

Immunocytochemistry. The sections were mounted onto chrome alum-gelatin pre-coated glass slides, allowed to dry for at least 1 h and rinsed twice in PBS. Endogenous peroxidase activity was bleached with a 20 min rinse in 0.5% H_2O_2 in alcohol 96%. After this, they were incubated 24 h in a humid chamber at 4°C with rabbit anti-D4R antibody diluted 1:500, rabbit anti-mGluR1α antibody diluted 1:2000, rabbit anti-mGluR2/3 antibody diluted 1:400 and rabbit anti-mGluR4a antibody diluted 1:800 in PBS containing 0.2% (w/v) bovine serum albumin, 0.03% Triton X-100 and 0.1% (w/v) sodium azide. The slides were rinsed twice in PBS and incubated at room temperature for 60 min in a humid chamber with anti-rabbit secondary antibody diluted 1:200 (Vector Laboratories, Burlingame, CA) in PBS containing 0.2% (w/v) bovine serum albumin and 0.03% Triton X-100. They were rinsed twice in PBS and incubated in an ABC (Elite kit, Vector Laboratories) for 1 h in a humid chamber at room temperature. Peroxidase activity was demonstrated by reaction with 3,3′-diaminobenzidine using glucose oxidase and nickel salts for enhancement of the reaction product (40). After dehydration the sections were mounted with Permount medium. For control purposes, sections were incubated only with primary or secondary antibody and pre-immune serum and processed for ABC. All sections were studied in a Eclipse 800 Nikon microscope. Agfapan APX (Agfa Gevaert AG, Leverkusen, Germany) film was used for photography.

Antibodies. Rabbit anti-D4R was raised against a synthetic peptide which corresponds to amino acids 160–172 from the second extracellular loop of the rat D4 receptor (12). Rabbit anti-metabotropic glutamate receptor 1α (mGluR1α) polyclonal antibody was purchased from Chemicon International Inc., and rabbit anti-metabotropic glutamate receptor 2/3 (mGluR2/3) and rabbit anti-metabotropic glutamate receptor 4a (mGluR 4a) polyclonal antibodies were purchased from Upstate Biotechnology.

RESULTS

D4 receptor like immunoreactivity (D4R-LI) was mainly associated to neuronal cell bodies with a widespread distribution throughout the brain. Only rarely, we could observe inmunostained fibers. D4-positive neurons were observed in most areas of the cerebral neocortex in pyramidal and non-pyramidal cells. They were particularly numerous in the medial frontal cortex, mainly occuring in layers II–IV, although they were also present in the deeper layers. In pyramidal cells, the labeling was associated to the plasmatic membrane (Fig 1A). Numerous small round-shaped neurons were labeled at all levels of the striatum where they were evenly distributed (Fig 1C).

Immunoreactivity for mGluR1α in normal rat brain was widely and selectively distributed in neurons and processes, in all subdivisions of the cerebral cortex. mGluR1α-LI was found throughout layers III–VI. The labeling was associated both to the cytoplasm and the plasmatic membrane of the cells. Immunoreactive dendritic profiles were found in all cortical layers (Fig. 2A). In the striatum, mGluR1α-LI was confined to the neuropil. The labeling consisted of numerous fine stained puncta evenly distributed (Fig. 2C).

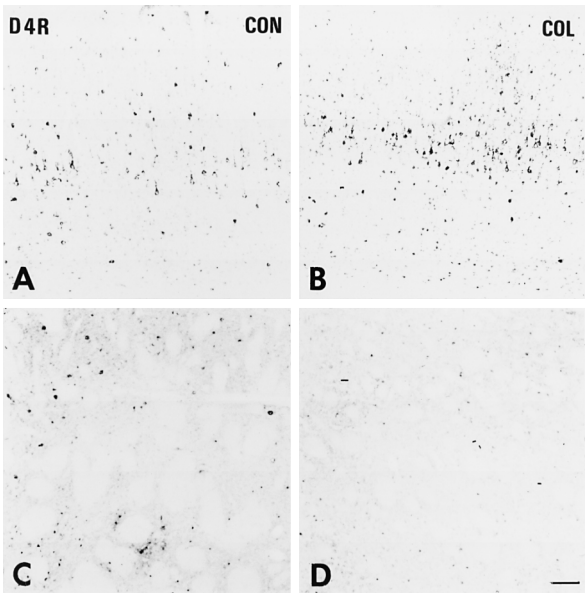

Fig. 1. Photomicrographs showing immunostained sections with D4R-LI in the cerebral cortex (**A, B**) and the striatum (**C, D**) of a control animal (**A, C**) and a colchicine treated rat (**B, D**). Note the higher number of immunostained neurons in the cerebral cortex after colchicine (**B**). Calibration bar: 250 μm.

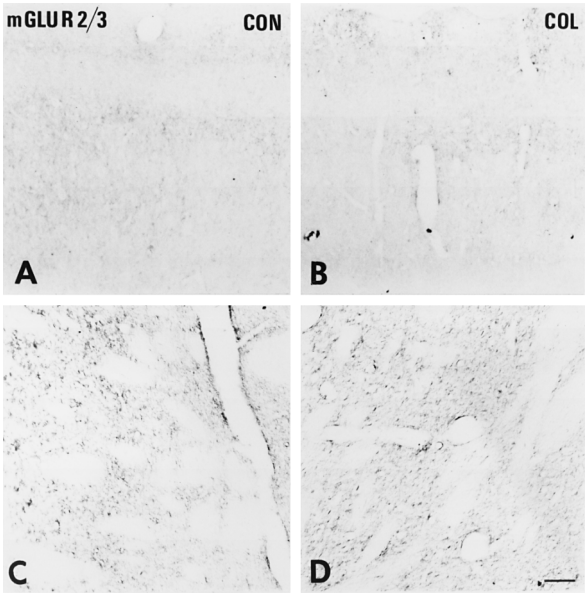

Fig. 3. Photomicrographs showing immunostained sections with mGluR2/3-LI in the cerebral cortex (**A, B**) and the striatum (**C, D**) of a control animal (**A, C**) and a colchicine treated rat (**B, D**). A reduction in the number of immunostained cells is observe in the cerebral cortex of colchicine treated animals. Calibration bar: 125 μm.

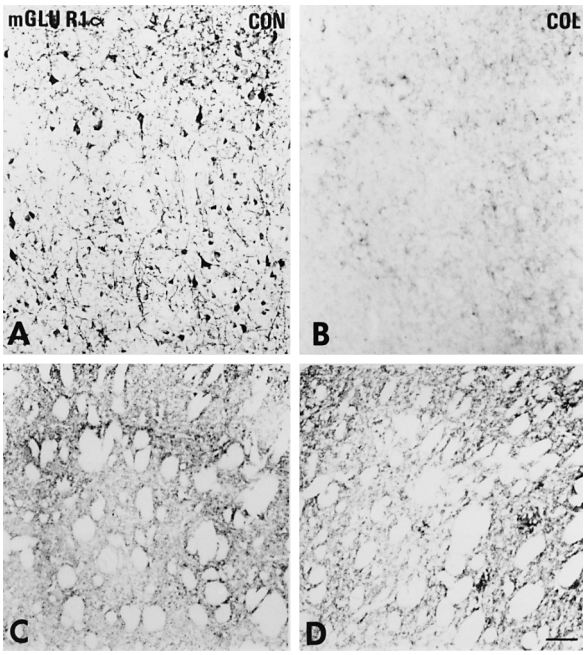

Fig. 2. Photomicrographs showing immunostained sections with mGluR1α-LI in the cerebral cortex (**A, B**) and the striatum (**C, D**) of a control animal (**A, C**) and a colchicine treated rat (**B, D**). The lack of immunostained neurons is notorious in the cerebral cortex after colchicine treatment. Calibration bar: 125 (**A, B**) and 250 (**C, D**) μm.

mGluR2/3-LI displayed a widespread and even light staining within the cerebral cortex which consisted largely of fine immunoreactive punctate within the neuropil. The antibody showed no layer specificity. However, a region with no staining was detected at the border between layers II and III (Fig. 3A). In the striatum, the label was also confined to the neuropil. The staining however, was less intense than that found with the mGluR1α antibody in this region of the basal ganglia. No immunostained neurons were observed in the striatum (Fig. 3C).

Immunohistochemical staining of normal rat brain with the antibody to mGluR4a produced an intense signal in somata within cortical layers II–VI. mGluR4a-LI displayed a patchy like staining of the plasmatic membrane in most labelled cells. Also a light staining of the cytoplasm was frequently observed. mGLuR4a was absent within the neuropil at all levels of the cerebral cortex in all experiments performed (Fig. 4A). Within the striatum, mGluR4a-LI was found in several small multipolar cells bodies showing labelling of the cytoplasm and the plasmatic membrane. Contrary to the cerebral cortex, a fine punctate and intense signal was detected within the neuropil of the striatum (Fig. 4C).

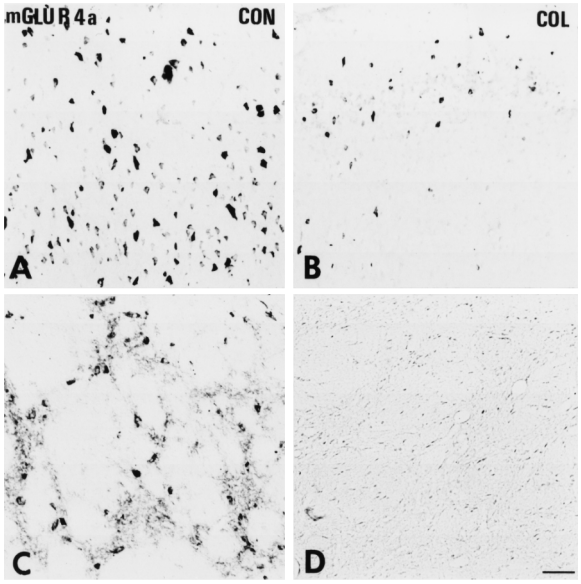

Fig. 4. Photomicrographs showing immunostained sections with mGluR4a-LI in the cerebral cortex (**A, B**) and the striatum (**C, D**) of a control animal (**A, C**) and a colchicine treated rat (**B, D**). Calibration bar: 250 μm.

Control experiments performed with pre-immune serum or without the primary or secondary antibody rendered negative stainings.

Effects of Intraventricular Injection of Colchicine on D4R and mGluRs Expression. After an i.v. colchicine injection, D4R-LI was significantly increased in cerebral cortex and decreased in the striatum. Within the cortex we observed the same distinctive pattern of D4R-positive neurons but with more intense cytoplasmic staining than in control sections. Also a dendritic imunoreactive pattern was detected after the colchicine treatment within layers II–III in the cortex (Fig. 1B). Within the striatum, colchicine induced the disappearance of most of the neuronal cell bodies. Also there was a reduction of the signal in the striatal neuropil (Fig. 1D).

After axonal transport blockade the mGluR1α-LI seen in the cortex of the control animals was largely reduced. There were no mGluR1α-positive cell bodies and only few dendritic profiles remained lightly stained. On the other hand, the striatum mGluR1α-LI remained unchanged (Fig. 2 B,D).

With respect to the mGluR2/3-LI there were no changes neither in the pattern nor in the intensity of the labeling after the i.v. injection of colchicine. The labeling was found in fine fibers of both the cerebral cortex and striatum (Fig. 3 B,D).

Colchicine treatment significantly reduced the number of cell bodies with mGLuR4a-LI immunolabelling in the cerebral cortex. Only neurons within layers II–III persisted with a positive signal. The labeling remained associated to the cytoplasm and to the plasmatic membrane in these cells (Fig. 4B). In the striatum, labelled cells disappeared after colchicine treatment and there was a reduction in the intensity of the immunostaining within the neuropil (Fig. 4D).

DISCUSSION

In the present study, we have employed an affinity purified antibody directed against an extracellular domain of the D4 receptor and commercially available antibodies against the three groups of metabotropic glutamate receptors to study their distribution in the corticostriatal projections in rat brain. In addition we have injected an axonal transport blocker in order to block the receptor transport from the site of synthesis at the neuronal somata to their possible site at the presynaptic membrane, trying to elucidate their subsynaptic localization. The object was to give support to previous suggestions of a presynaptic localization of D4R and mGluRs in corticostriatal projections that gave rise to the hypothesis of a possible presynaptic regulation of glutamate cortical release by D4R and one or more subtypes of mGluR.

The D4 receptor distribution confirms and extends previous work of our laboratory and others showing an intense labeling in pyramidal cells of the cerebral cortex associated to the plasmatic membrane. In the striatum the signal was associated to the plasmatic membrane of small round shaped neurons (12, 41–43). The immunolabeling pattern of D4R after colchicine injection, showing an increase in cortical neuronal signal and decrease in the striatal neuropilar label, is consistent with previous hypothesis that suggests that D4 receptor protein (but not D1-like nor D2-like) is synthesized from genetic material arising primarily from cerebral cortex and then transported to terminal axons projecting to the striatum and nucleus accumbens (17). The axonal transport impairment produced by colchicine could have altered the expression and transport of the D4 receptor producing an accumulation of the protein in the neuronal bodies of the cerebral cortex with the subsequent increase in the immunolabeling of that area. Employing a different approach, Tarazi et al. (17) showed a decrease in D4-like binding detected by quantitative autoradiography in the striatum after cerebral cortex ablation. Our results confirm and extend these findings since we can show the accumulation and further increase in D4R-LI at the

cortical level that these authors could not show since the cortex was removed. It could be argued that D4 signal at the striatal level was also found in neurons suggesting a postsynaptic localization as well. However, an attentive look to our previous study on D4 distribution (12) reveals that the D4 immunolabeling at the striatum shows round shape neurons with the label clearly marginal most probably associated to the plasmatic membrane. With the present results in mind, the label could well be representing the presynaptic membrane of multiple axo-somatic synapses shaping a round neuronal soma.

The immunohistochemical localization of mGluR1α in cerebral cortex is similar to that reported by Martin et al. (24) and Fotuhi et al. (44) in relation to the neuropil staining, whereas in our case more non pyramidal neurons were detected. Employing the same commercial antibody, Testa et al. (27), detected only a low level punctate staining of the neuropil and Baude et al. (28) detected positive immunoreactivity in non principal cells of the cerebral cortex. A similar dendritic profile in striatum was reported by Martin et al. (24) and Testa et al. (27). In cerebral cortex, the neuronal labeling of mGluR1α, was largely reduced after colchicine treatment, whereas no apparent changes were observed in the neuropilar distribution of mGluR1α in the striatum mGluR1α has been described to be mainly localized to the postsynapsis. The protein composition of the postsynaptic densities (PSD) has been the focus of intense investigations lately since it has become evident that the understanding of its precise spatial organization is crucial for regulation of adhesion, control of receptor clustering, regulation of receptor function and morphological adaptations of dendritic spines (45,46). The PSD is specially prominent in excitatory CNS synapses. Among the first proteins that were identified in the PSD fraction is tubulin and although the role of tubulin within the PSD remains unclear it has been related with the initiation of tubulin polimerization and modulation of receptor responses by interacting with specific G proteins (46). More recently, Ciruela et al. (47) reported that mGluR1α shows a direct interaction with tubulin that function to stabilize the receptor at a specific location by interacting with the cytoskeleton. In this context, the presence of colchicine might be disrupting this interaction of tubulin with the receptors unstabilizing the anchorage of the receptor to the plasmatic membrane.

The neuropilar distribution of mGluR2/3 was also reported by Petralia et al. (26) and Testa et al. (27) both in the cerebral cortex and the striatum. Petralia et al. (26) showed that mGluR2/3 can be found in striatal glia as well as neurons whereas Testa et al. (27) could not distinguish this distribution with confocal microscopy. Although the latter reported that mGluR2/3-LI in the striatal neuropil was markedly reduced after decortication, our colchicine treatment showed no changes in the striatal neuropilar signal.

mGluR4a was previously reported to show a low intensity staining in the mouse striatum with intense immunoreactivity in fibers in the globus pallidus (18). Coupling their results with previous in situ hybridization studies showing striatal neurons heterogenously labelled with mGluR4a mRNA (48), Bradley et al. (18) suggest a presynaptic localization on striatopallidal terminals. However, our results show several striatal multipolar cell bodies displaying a peripheral immunolabelling in the cytoplasm. This label does not accumulate in the bodies after axonal transport impairment of the striatopallidal projections as would be expected for the presynaptic striatal localization suggested by Bradley et al. (18).

At present, no reports on mGluR4a localization in cerebral cortex has been published except for the study of Bradley et al. (18) that shows a moderate level of immunoreactivity in the piriform cortex and a low immunoreactivity in neocortex although a systematic study of this brain area was not the object of this report. The interpretation of the reorganization of mGluR4a after colchicine treatment is more complex and distinct from the other groups. The label disappeared from neurons in the striatum and only neurons from layers II–III in the cortex were labeled after colchicine treatment. In this context, there does not seem to be a relationship between the label seen in the soma of these neurons after axonal transport impairment as was seen in D4R neurons. The suggestion of a possible colocalization of D2 type receptors and one of the Group III metabotropic receptors, as suggested by Calabresi et al. (1) in corticostriatal projections, does not seem to be supported by our results. It is interesting to point out that the neurons labelled with mGluR4a in cerebral cortex, that are labelled after colchicine treatment (Fig. 4B) are in the same layer (II–III) as the neurons more densely labelled with D4 antibody. However, preliminary immunocytochemistry studies performed with thin adjacent sections does not favour the suggestion of a colocalization of D4R and mGluR4a in a same single neuron in the cerebral cortex. More specific double labeling protocols need to be performed to confirm the colocalization hypothesis.

The possibility that the decrease in the signal might be reflecting the destruction of the neuron due to a direct neurotoxic effect of colchicine on the cells

was disregarded since the concentration employed is routinely used in immunolabeling studies for neuropeptides where the integrity of the neurons was demonstrated (49). Moreover, it has been reported that the cerebral cortex and the striatum neurons are relatively unaffected after a direct intra-area injection of 0.5–25 μg colchicine (50).

In summary, D4 receptor immunoreactivity present in the striatum seems to belong to nerve terminals whose neuronal somas are located in the cerebral cortex confirming the presynaptic nature of the receptor. None of the metabotropic receptors suspected to be presynaptic (mGluR2/3 and mGluR4a) responds in a similar way to the axonal transport impairment suggesting that each receptor might be expressed in different neurons that show different susceptibility to colchicine. This might be indicating that D4 receptor and mGluR2/3 and/or mGluR4a might not be colocalized as previously suggested.

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