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Altered expression of regulators of G-protein signaling (RGS) mRNAs in the striatum of rats undergoing dopamine depletion

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Abstract

Quantitative *in situ* hybridization was used to investigate the effect of prolonged striatal dopamine or monoamine depletion on the mRNA density of regulators of G-protein signaling (RGS) 2–12 proteins. Two types of treatments were studied: a 6-hydroxydopamine-induced unilateral lesion of the nigrostriatal pathway and a 5-day reserpine treatment. The results clearly show a selective increase in the mRNA levels of RGS2, 5 and 8 and a decrease in RGS4 and 9 mRNA levels following nigrostriatal denervation. In this model, we observed no change in the mRNA levels of RGS10 and other RGS proteins that are weakly expressed in the striatum (RGS3, 6, 7, 11 and 12). On the other hand, the mRNA levels RGS2, 4, 5, 8, 9 and 10 were found to be significantly decreased after prolonged reserpine treatment. In contrast, the densities of these transcripts (in particular, RGS2, 4 and 10) tend to increase after an acute administration of reserpine, used as control. These results provide further evidence for the influence of dopamine and/or other monoamines in the regulation of RGS protein expression in the striatum. In connection with the previously documented acute regulation of RGS proteins after modulation of the dopaminergic transmission [Geurts *et al.*, Neurosci Lett 2002;333:146–50], the present study demonstrates that alteration in their genetic expression can be long-lasting and this could reflect the adaptation processes that occur in certain pathological states, such as Parkinson's disease.

Keywords: 6-Hydroxydopamine; Reserpine; *In situ* hybridization; Regulators of G-proteins signaling; Nigrostriatal pathway; Parkinson's disease

1. Introduction

RGS proteins constitute a group of molecules participating in the modulation of G-protein coupled receptor signaling (for reviews see [1–7]). RGS proteins increase the GTPase activity of the alpha subunit of G-proteins and, therefore, have been generally regarded as negative regulators. A positive regulatory function has also been unraveled for RGS4, which increases the G-protein pool available for G-protein gated inward rectifier K^+ channels [8]. Moreover, it becomes obvious that these molecules possess a complex regulatory profile through direct modulation of effectors [9] or even intrinsic effector activity [4]. Finally, by interacting with different partners of the signaling cascades (G_α subunits, receptors, effectors, other scaffolding proteins), RGS proteins have the potential to act as bridges between different transduction pathways [10] or within a particular cascade, assembling molecular complexes that can rapidly turn signaling either on or off [11].

These regulatory proteins have additional properties such as a relative selectivity in function [12,13] and localization [14,15]. Their relevance as potential pharmacological targets would be further reinforced by a selectivity in their regulation (for review see [5]). While some of them—mainly RGS2—are now well characterized for their acute regulation by a variety of drugs (including dopamine receptor antagonists, amphetamine and opioids) [16–23], their modulation after long-term injury or chronic pharmacological treatments remains poorly described. Recent papers, particularly concerning the central dopamine system, suggest a potential role of RGS proteins in long-term adaptation processes observed in response to pharmacological treatment [24,25] or occurring during the development of neurodegenerative diseases [26].

In the present study we have used *in situ* hybridization to measure the mRNA levels of several RGS proteins in the striatum of rats after either 6-hydroxydopamine-mediated

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Abbreviations: RGS, regulators of G-protein signaling.

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denervation of the nigrostriatal pathway or reserpine-induced monoamine depletion. Depending on the RGS protein species examined, both positive and negative modulations of their genetic expression were observed in the 6-hydroxydopamine lesion model, whereas a general decrease was observed after the repeated administration of reserpine.

2. Materials and methods

2.1. Surgery

Unilateral lesion of the dopamine nigrostriatal pathway by stereotaxic microinjection of 6-hydroxydopamine in the substantia nigra was performed as described previously [27]. A pre-treatment with 25 mg/kg desipramine and 5 mg/kg pargyline, 30 min prior to surgery, was demonstrated to enhance both the selectivity and amplitude of the destruction of dopaminergic neurons [28,29]. Three weeks after surgery, rats were killed by decapitation, brains were removed and immediately immersed in isopentane at −40°. Tissues were conserved at −80° for several months. Experimental protocols meet the guidelines of the local and governmental ethical committees.

2.2. Reserpine treatment

Male Wistar rats (250–300 g) were injected i.p. once daily for 5 days or only once with either vehicle (1 mL/kg dimethylsulfoxide) or reserpine (1 mg/kg). Thirty minutes after the last (5-day treatment) or single (acute treatment) injection, rats were sacrificed and brains were conserved as described above.

2.3. In situ hybridization

Twenty micrometer-thick coronal sections were cut on a cryostat at four rostro-caudal levels through the striatum and thaw-mounted onto SuperFrost/plus glass slides. These four levels corresponded to Bregma 2.20, 1.60, 0.20 and −0.40 mm or plates 10, 12, 17 and 20, respectively according to [30]. Dried brain sections were fixed with 4% *para*-formaldehyde, dehydrated and conserved in 70% ethanol for several days. The oligonucleotide probes were complementary to the following published sequences (target, GenBank accession number, nucleotide position): c-*fos*, X06769, 7–36; RGS2, AF279918, 221–249; RGS3, U32434, 19–49; RGS4, NM_017214, 14–43; RGS5, AF241259, 307–337; RGS6, U32436, 20–49; RGS7, NM_019343, 20–49; RGS8, U32432, 16–46; RGS9, AF071475, 91–120; RGS10, U32437, 20–49; RGS11, U32438, 61–91; RGS12, NM_019339, 19–49. After labeling using [^{35}S]ATPαS (>1000 Ci/mmol) and terminal deoxynucleotidyl transferase and dilution in the hybridization mixture (300 mM NaCl, 30 mM sodium citrate, Denhardt's solution, 300 μg/mL herring sperm DNA, 5% dextran sulfate, 1% polyvinylpyrrolidone, 40% formamide, 0.5 mg/mL polyadenylic acid, 100 mM dithiothreitol), these oligonucleotides were applied to each section and incubated for 17 hr at 42°. Following hybridization, sections were washed with solutions containing successively 300 mM NaCl, 30 mM sodium citrate, 150 mM NaCl, 15 mM sodium citrate and 75 mM NaCl, 7.5 mM sodium citrate at 53°. Finally, sections were dehydrated in absolute ethanol, air dried and exposed to Kodak Biomax MR films for 2 weeks to 1 month. Some sections were incubated in the presence of sense oligonucleotides and the labeling was comparable to the background (data not shown). Quantitative densitometry of the autoradiograms was performed in reference to ^{14}C-microscales using a digital image analysis system (MCID 4; Imaging Research).

2.4. [^{3}H]GBR12935 autoradiography

The extent of the unilateral 6-hydroxydopamine lesion was assessed by comparing the specific binding of [^{3}H]GBR12935 to dopamine uptake sites in denervated and non-denervated striata [27]. Protocol was adapted from [31]. Brain sections, adjacent to those used for *in situ* hybridization, corresponding to plates 10–11, 13, 18–19 and 21–22 [30], were conserved at −80° without fixation and served for the assessment of the loss of dopamine transporter. Sections were incubated in the presence of 0.25 nM [^{3}H]GBR12935 in binding buffer containing 50 mM sodium phosphate (pH 7.5), 20 mM NaCl, 0.001% ascorbate, 0.025% fatty acid-free bovine serum albumin and 0.75 μM *trans*-(*E*)-flupentixol. Non-specific binding was measured in the presence of 25 μM nomifensine. Incubation was performed for 30 hr at 0–4° and was terminated by aspiration of the incubation medium. Sections were then washed four times (5 min each) in ice-cold washing buffer (50 mM Tris–HCl, pH 7.5, 450 mM NaCl), briefly rinsed in ice-cold deionized water, air dried and exposed together with ^{3}H-microscales to Hyperfilms-^{3}H for 2 months.

2.5. Materials

Unlabeled oligonucleotides were from Invitrogen. ^{3}H- and ^{14}C-microscales, hyperfilms-^{3}H, Kodak Biomax MR films, [^{35}S]ATPαS (>1000 Ci/mmol), [^{3}H]GBR12935 (47.5 Ci/mmol) and terminal deoxynucleotidyl transferase were purchased from Amersham Pharmacia Biotech. Bovine serum albumin, desipramine hydrochloride, dextran sulfate, formamide deionized (GC), 6-hydroxydopamine hydrobromide, nomifensine maleate, *para*-formaldehyde, pargyline hydrochloride, polyvinylpyrrolidone, reserpine, *trans*-(*E*)-flupenthixol dihydrochloride, herring sperm DNA and Denhardt's solution were from Sigma/RBI. Dithio-1,4-threitol (Molecular Biology Grade) and SuperFrost/plus glass slides were from VWR International.

3. Results

3.1. Lesion of the dopamine nigrostriatal pathway

The extent of the unilateral 6-hydroxydopamine lesion was quantified by comparing the specific binding of [^{3}H]GBR12935 to dopamine uptake sites in denervated and non-denervated striata (Fig. 1). A loss of 80–96% of this presynaptic marker of nigrostriatal neurons was observed 3 weeks after the nigral injection of 6-hydroxydopamine. Such lesion is known to correspond to a dopamine depletion of at least 90% [32–35] and was shown to result in functional hypersensitivity of striatal dopamine receptors [27]. Such amplitude of dopamine depletion or decrease in the number of dopamine uptake sites is observed in the striatum of patients with Parkinson's disease and results in apparent clinical symptoms [36,37].

3.2. Expression of RGS mRNAs in the rat striatum

As reported in our previous work [16], *in situ* hybridization studies revealed the following relative abundance for the RGS mRNA levels in the caudate putamen of control animals: RGS4 > RGS8 > RGS2 = RGS5 = RGS9 = RGS10 (Fig. 2). A weak signal was detected for RGS6 and RGS7, while RGS3, 11 and 12 mRNAs were close to the limit of detection (data not shown). A slight rostro-caudal gradient in the basal expression of RGS mRNAs was observed for RGS2, 4 and 10 (Fig. 2). In addition, the mRNA level of RGS4 was higher in the dorso-lateral quadrant of the striatum (Fig. 4), and there was also a slight gradient for RGS8 mRNAs (not shown). RGS10 mRNAs was more abundant in the ventro-medial quadrant of the striatum (not shown). For the other RGS mRNAs, the distribution in the four quadrants of the striatum was relatively homogeneous.

3.3. Changes in RGS mRNA levels following 6-hydroxydopamine lesion

The mRNA levels of the most abundant RGS detected in the striatum (RGS2, 4, 5, 8, 9 and 10) were measured at the four anatomical levels of the control and denervated striata (Fig. 2). Through the entire caudate putamen, the mRNA level of RGS2 was significantly increased by 60–160% in the denervated striatum as compared to the control side (Fig. 3). Significant increases (from 20 to 50%) were also measured for the RGS8 mRNA with the most pronounced effects corresponding to the most rostral sections. There was also a significant increase (from 10 to 30%) in RGS5 mRNA level. In contrast, RGS4 and RGS9 mRNA levels were significantly decreased in the denervated side (by up to 20 and 10%, respectively). No change was detected for the mRNA levels of RGS10.

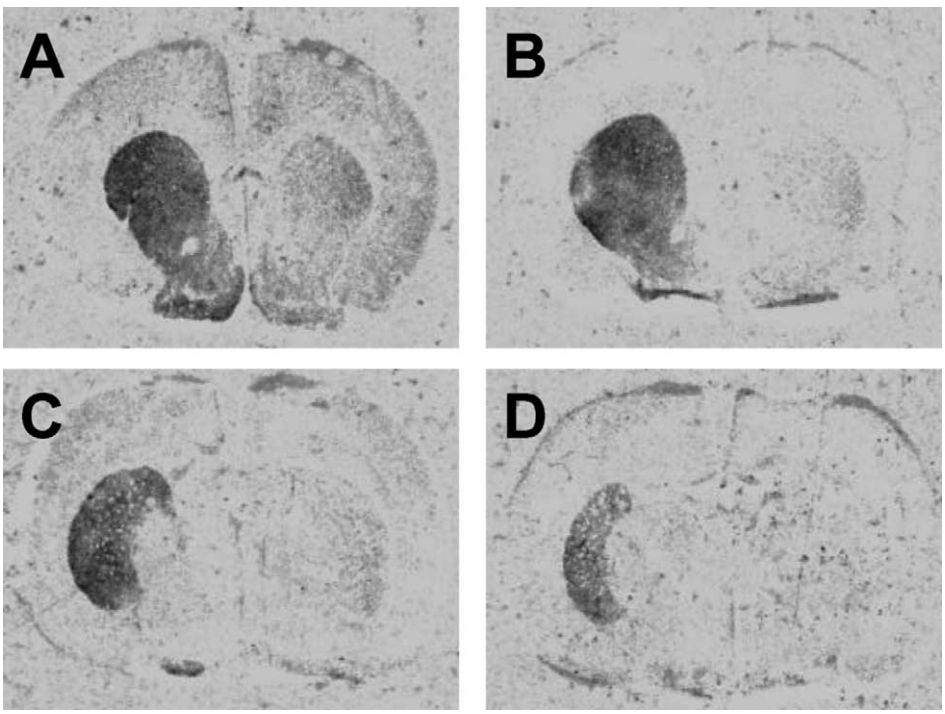

Fig. 1. Decrease in [^{3}H]GBR12935 specific binding after unilateral lesion of the nigrostriatal pathway. Images shown were obtained by subtraction of the digitalized autoradiograms corresponding to the total binding and the non-specific binding. Panels A, B, C, D corresponded to plates 10–11, 13, 18–19 and 21–22 according to [30]. Data were taken in the caudate putamen on the left (control) and right (lesioned) sides of two consecutive slides and quantitative densitometric analysis was performed in reference to ^{3}H-microscales, providing the following values (nCi/mg tissue equivalent) for control and denervated sides, respectively (mean ± SEM from eight independent experiments): A: 4.14 ± 0.85 and 0.70 ± 0.19; B: 3.92 ± 0.58 and 0.71 ± 0.19; C: 3.23 ± 0.65 and 0.63 ± 0.18; D: 3.32 ± 0.53 and 0.15 ± 0.14.

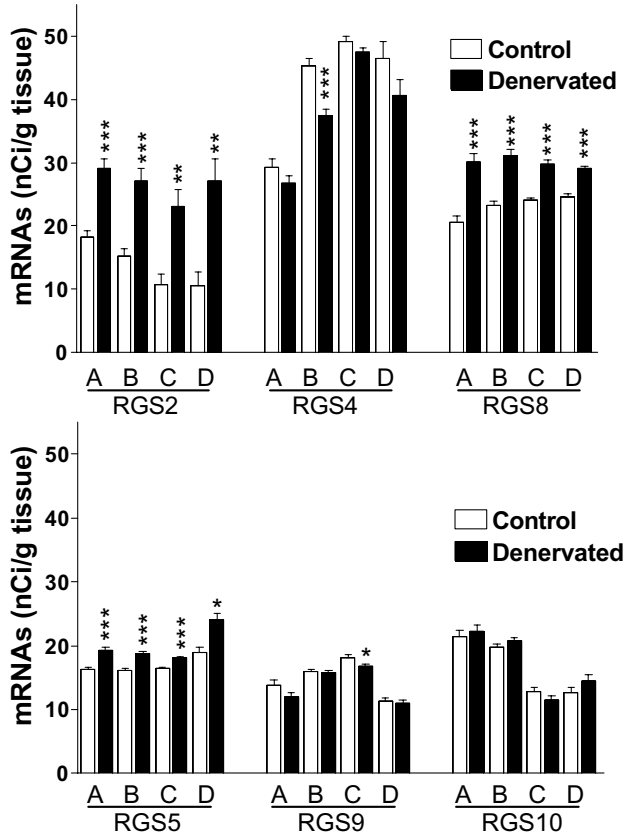

Fig. 2. RGS mRNA levels in the striatum of rats with unilateral denervation of the nigrostriatal pathway. Data were derived from quantitative densitometric analysis of autoradiograms in reference to ^{14}C-microscales and are mean ± SEM of 3–10 independent experiments. Levels A, B, C, D corresponded to plates 10, 12, 17, 20 according to [30]. Statistical analysis between data from control and lesioned sides was performed using Student's unpaired *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Similarities have been observed in the acute regulation of RGS2 and the immediate early gene c-*fos*. Thus, the levels of both mRNAs are rapidly and transiently increased in discrete brain regions upon various acute treatments, including antipsychotic drugs [19,22] and amphetamine [18]. In consequence, the mRNA level of the latter was also examined in the present study. The basal level of c-*fos* mRNA was low (close to the limit of detection) and was not modified after 6-hydroxydopamine lesion. Finally, even if the signals corresponding to RGS3, 6, 7, 11 and 12 were weak, their expression was studied in denervated striata. Although small variations cannot be excluded (due to the sensitivity of the detection), none of these transcripts appeared significantly altered after 6-hydroxydopamine lesion (not shown).

3.4. Changes in RGS mRNA levels following reserpine treatment

The repeated administration of reserpine (for 5 days) was previously found to deplete striatal dopamine by about 80% [38]. After such treatment, a significant decrease in the mRNA levels was observed for the most abundant RGS species detected in the striatum (Fig. 5). Thus, the highest decreases were observed for RGS4 (45–48% decrease, see also Fig. 4) and for RGS2 (25–30%). Modest but significant decreases were also detected for RGS5, 9 and 10 (approximately 20%) and RGS8 (10%). In contrast, the single injection of reserpine was followed by a significant increase in the mRNA density of RGS2, 4 and 10 proteins (Fig. 5). As observed after the 6-hydroxydopamine lesion, the *in situ* hybridization signals detected for the weakly expressed RGS proteins (RGS3, 6, 7, 11 and 12) and c-*fos* were not altered after either single or repeated administrations of reserpine (not shown).

4. Discussion

In the present study, the effect of a lesion of the nigrostriatal pathway on the mRNA levels of several RGS proteins was examined in the striatum of rats. The results were compared to the effect of a non-selective depletion of monoamines. The major findings of the present work are the significant increases in mRNA levels of RGS2, 5 and 8 and the decreases in RGS4 and 9 in the unilateral model of Parkinson's disease. In contrast, the monoamine depletion evoked by the repeated reserpine treatment resulted in significant decreases in the mRNA levels of these RGS proteins.

This is the first report of long-lasting changes in RGS mRNA levels in the rat striatum after alteration of dopaminergic transmission. Previous works tend to suggest that the regulation of striatal RGS proteins observed after diverse pharmacological treatments was transient. Thus, 20 hr following the repeated administration of amphetamine, no change was found in the mRNA levels of RGS2, 3, 4 and 5 [24]. Similarly, 24 hr following a 3-week treatment with haloperidol or risperidone, the RGS2 mRNA level was returned to the basal level [25]. Hence, RGS2 was assimilated to an immediate early gene because it behaves like c-*fos* in acute regulation studies [18,22,25]. In the present study, a noticeable modulation of the mRNA density of diverse RGS proteins was detected 3 weeks after the lesion of the substantia nigra with 6-hydroxydopamine or after non-selective impairment of monoamine transmission resulting from repetitive reserpine administrations. Although marked differences were observed between these experimental models (see below), these results suggest that in addition to a rapid regulation, long-term modulation in the expression of some RGS may occur after sustained alteration in the dopaminergic transmission. At variance with these regulatory proteins, we did not observe long-term regulation of c-*fos* expression as widely reported in previous studies (for review see [39,40]). This result extends previous works clearly showing a differential pattern of mRNA induction for c-*fos* and RGS2 upon long-term treatments with either amphetamine [24] or

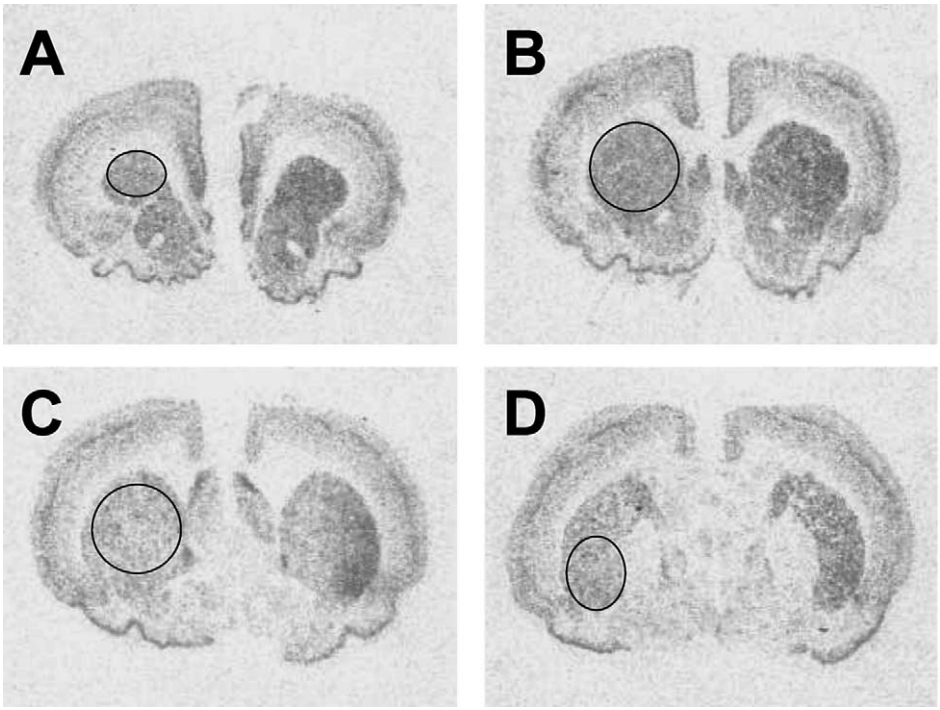

Fig. 3. Increase in striatal RGS2 mRNA levels after unilateral lesion of the nigrostriatal pathway. Autoradiograms of rat coronal brain sections at four levels of the striatum were obtained after *in situ* hybridization using labeled antisens oligonucleotides complementary to RGS2 mRNA. Shown are representative images from 7 to 10 independent experiments. The denervated striatum corresponds to the right side. For quantification (see Fig. 2), data were taken in the caudate putamen on the left (control) and right (lesioned) sides as shown by the circles. Panels A, B, C, D corresponded to plates 10, 12, 17 and 20 according to [30].

antipsychotic drugs [25]. The sustained increase or decrease in RGS mRNA levels could be particularly relevant considering the scaffolding properties of these regulatory proteins towards different molecules of the signaling cascades rather than their GAP (GTPase Activating Protein) activity, which would be more compatible with their rapid and transient acute regulation profile.

As indicated above, the consequences of the nigrostriatal lesion with 6-hydroxydopamine consist of either increases or decreases in the expression of RGS protein subsets. It has been previously suggested that the dopaminergic transmission was participating in the control of the mRNA density of some RGS proteins [16–19,22,24,25]. Through the complex involvement of distinct dopamine receptor subtypes, RGS protein expression may be either increased or decreased in response to alteration in the dopaminergic transmission. Thus, increases in the mRNA density of RGS2 was obtained after pharmacological blockade of D2 dopamine receptors [19,22,24,25], while the selective stimulation of these receptors was found to enhance the

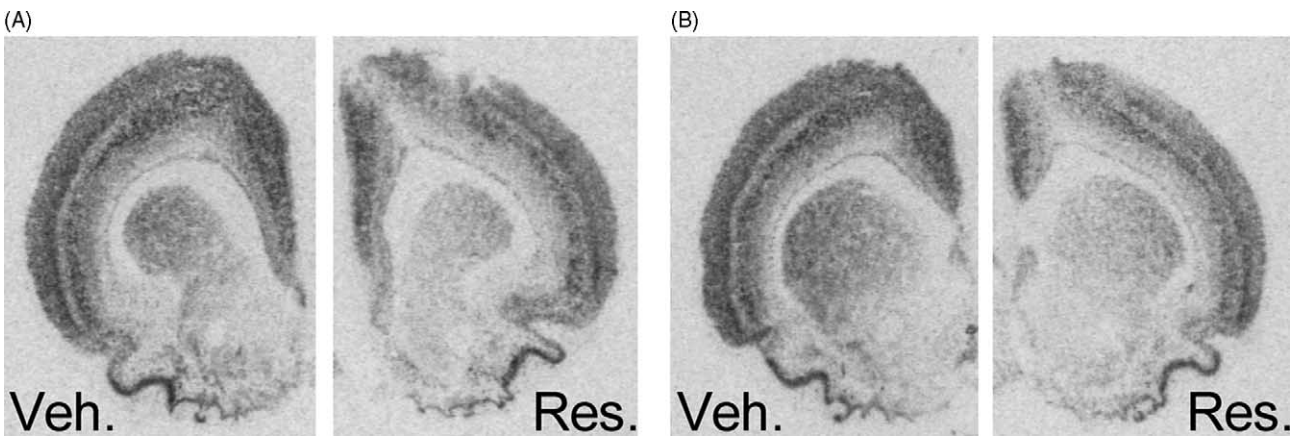

Fig. 4. Decrease in striatal RGS4 mRNA levels after repeated (5 days i.p.) reserpine treatment. Autoradiograms of rat coronal brain sections at two levels of the striatum (panel A: Plate 10 and panel B: Plate 12, according to [30]) were obtained after *in situ* hybridization using labeled antisens oligonucleotides complementary to RGS4 mRNA. Shown are representative images from five independent experiments. For illustration, half-images from vehicle (Veh., left panels) and reserpine (Res., right panels)-treated rats were assembled. However, for quantification (see Fig. 5), data were systematically taken from both hemispheres.

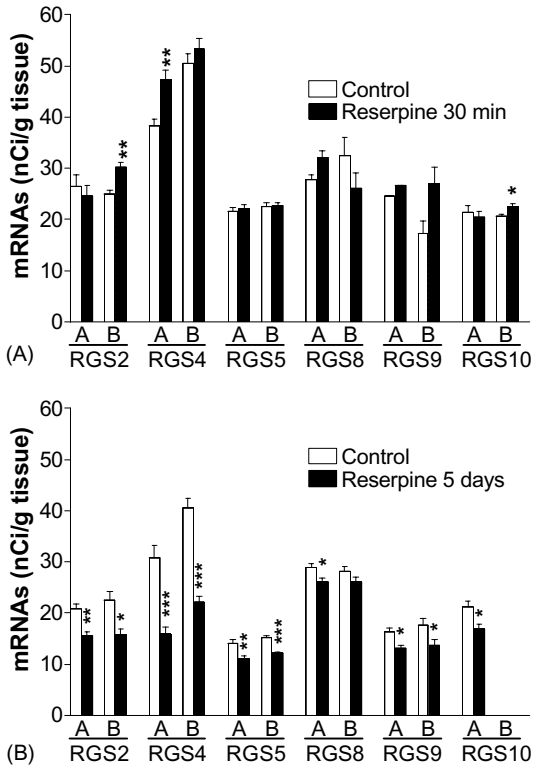

Fig. 5. RGS mRNA levels in the striatum of rats treated acutely (panel A) or for 5 days (panel B) with reserpine. Data were derived from quantitative densitometric analysis of autoradiograms in reference to ^{14}C-microscales and are mean ± SEM of 3–10 independent experiments (except in A: N = 1–2 for RGS9). In each panel, A and B corresponded to plates 10 and 12 according to [30]. Statistical analysis between data from vehicle and reserpine-treated animals was performed using Student's unpaired *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

mRNA density of RGS4 [16,17]. Obviously, regulation of the whole set of striatal RGS proteins is probably not restricted to the influence of the dopaminergic transmission. Accordingly, our data show that the non-selective impairment of monoamine transmission by reserpine results in a decreased expression of all major striatal RGS proteins examined, including those showing enhanced expression after selective dopaminergic lesion. Interestingly, 30 min after a single injection of reserpine, the expression of some of these RGS proteins was found to be increased. This could result from opposite consequences of a single and a repeated administration of reserpine on monoaminergic systems, the former causing a transient release of monoamines, the latter leading to their progressive depletion.

Concerning the molecular mechanisms involved in the modulation of RGS expression, many studies have indicated the influence of cyclic AMP production and protein kinase C activation in their acute regulation [10,21,23,41,42]. In addition, the protein kinase C-induced elevation of RGS2 expression was still evident after stimulation of muscarinic receptors for 24 hr, indicating the possible role of this kinase in long-lasting regulation processes [42]. It is of interest to note that a decreased protein kinase C (α-isoform) immunoreactivity was observed in the striatum after repeated reserpine injection [43] whereas an increase in [^{3}H]phorbol-12,13-dibutyrate binding to protein kinase C was demonstrated in the 6-hydroxydopamine model [44]. These experimental observations could explain the different regulation of RGS2 mRNA levels observed after reserpine treatment (decreased expression) and 6-hydroxydopamine lesion (increased expression). A similar molecular mechanism could also account for the modulation of RGS5 and RGS8 that behave as RGS2 in the two experimental models. In contrast, a hypersensitive G_s signaling pathway takes place in both models [38,45–49] and could explain the changes in the mRNA level of RGS4, and possibly RGS9 which shows a similar regulation profile.

Together, the present results provide further evidence for the influence of dopamine and/or other monoamines in the regulation of RGS proteins expression in the striatum. In connection with the documented regulation of RGS proteins after acute modulation of the dopaminergic transmission [17,18,22], the present study demonstrates that alteration in their genetic expression can be long-lasting and could therefore participate in the adaptation processes that occur in response to various pharmacological treatments or during the development of some neurodegenerative diseases. Although little is known about the physiological influence of RGS proteins on the activity of dopamine receptors, these proteins are generally considered as signal transduction modulators (see introduction). Tekumalla *et al.* [26] recently reported on the elevated level of RGS9 protein immunoreactivity in the striatum of patients with Parkinson's disease and, hence, it was hypothesized that RGS9 inhibition would constitute an attractive therapeutic approach [5]. Paradoxically, it is noteworthy that in the present study, a decreased level of RGS9 mRNA was observed following the lesion of the nigrostriatal pathway in rats. It should be noticed that in the study of Tekumalla *et al.* [26], patients were treated with L-dopa making it difficult to attribute the changes to either the disease or the treatment. Further studies are needed to evaluate the effect of chronic treatment with L-dopa or other dopaminergic agonists on the expression of RGS proteins detected in the striatum.

It is well established that the dopamine transmission is subject to multiple regulation processes that involves alteration in the expression [50] and activity [51] of the different receptor subtypes. Along with our previous work [25] concerning the effect of chronic treatment with dopamine receptor antagonists, the present study reinforces the concept that regulation may occur at additional levels through the signaling cascade. Obviously, additional studies are now required to evaluate the physiological consequences of such alteration in RGS protein expression on the functional response to endogenous dopamine as well as to agonists or antagonists used in therapy.

Acknowledgments

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