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Induction of dopamine D_3 receptor expression as a mechanism of behavioral sensitization to levodopa

(Parkinson disease/6-hydroxydopamine/rotation behavior/prodynorphin/preprotachykinin)

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ABSTRACT **In rats with unilateral lesions of the nigrostriatal dopamine pathway with 6-hydroxydopamine, the motor stimulating effects of levodopa, an indirect dopamine receptor agonist, evidenced by contraversive rotations, become enhanced upon repeated intermittent administration. However, the mechanisms of this behavioral sensitization are essentially unknown. We show that development of sensitization is accompanied by a progressive appearance of D_3 receptor mRNA and binding sites, visualized by *in situ* hybridization and 7-[^{3}H]hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin autoradiography, respectively, occurring in the denervated caudate putamen, a brain area from which this receptor subtype is normally absent. Development and decay of these two processes occur with closely parallel time courses, whereas there were no marked changes in D_1 or D_2 receptor mRNAs. D_3 receptor induction by levodopa is mediated by repeated D_1 receptor stimulation, since it is prevented by the antagonist SCH 33390 and mimicked by the agonist SKF 38393, but not by two D_2 receptor agonists. The enhanced behavioral response to levodopa is mediated by the newly synthesized D_3 receptor, since it is antagonized by nafadotride, a preferential D_3 receptor antagonist, in low dosage, which has no such effect before D_3 receptor induction. D_3 receptor induction and behavioral sensitization are also accompanied by a sustained enhancement of prodynorphin mRNA level and a progressively decreasing expression of the preprotachykinin gene. We propose that imbalance between dynorphin and substance P release from the same striatonigral motor efferent pathway, related to D_3 receptor induction, is responsible for behavioral sensitization.**

Behavioral sensitization, also termed reverse tolerance, is a process by which repeated stimulation of neurotransmitter receptors in brain results in a progressive enhancement of responsiveness (1, 2). This process, particularly well illustrated in the case of direct or indirect dopamine agonists, is involved in the long-term effects of several drugs of abuse as well as of agents used to treat patients with Parkinson disease (PD). In these patients, the reduction in striatal dopamine levels, which results in reduced movement, can be compensated by administration of levodopa, the amino acid precursor of dopamine (3).

Long-term administration of levodopa results in both beneficial and unwanted changes in responsiveness to this drug. Thus, although acute administration of levodopa initially reduces rigidity, repeated administration appears necessary for the resumption of complex motor behavior (4, 5). On the other hand, long-term use of levodopa is associated with development of abnormal involuntary movements as well as psychological disturbances such as hallucinations, both suggestive of excessive response to dopamine.

Behavioral sensitization is also observed in animal models of PD. In agreement, in rats with unilateral lesions of the nigrostriatal dopamine pathway with 6-hydroxydopamine (6-OHDA), the motor-stimulatory effects of levodopa, evidenced by contraversive rotations, become enhanced upon repeated intermittent administration (6, 7). In monkeys with parkinsonism induced by the neurotoxin MPTP, repeated treatment with levodopa elicits the development of a variety of abnormal movements (8).

Despite numerous experimental efforts, the mechanisms responsible for these changes in responsiveness to levodopa have remained unclear. Neither metabolic changes (7) nor dopamine D_1 or D_2 receptor up-regulation (9, 10) seem involved. The observation that the expression of the recently identified dopamine D_3 receptor (11) is highly dependent upon the afferent dopaminergic innervation (12) prompted us to evaluate this receptor participation in the process of behavioral sensitization to levodopa in 6-OHDA-lesioned rats. Normally, the D_3 receptor is absent or almost absent from the dorsal striatum, i.e., caudate putamen (CdPu), the brain area where dopamine deficiency is responsible for the motor disturbances in PD and where this receptor binding-site density represents $<1\%$ that of D_1 and D_2 receptors (11, 13). The D_3 receptor is expressed mainly in the ventral part of the striatal complex, particularly in the shell of nucleus accumbens (AcSh), thought to be involved in reward, emotional, and cognitive processes, and only faintly in the accumbal core (AcCo), an area with an organization and a role in motor function similar to those of CdPu (14, 15).

We show here, in 6-OHDA-lesioned rats, that repeated levodopa administrations result in an ectopic induction of the dopamine D_3 receptor expression in the CdPu, a process that appears responsible for the development of behavioral sensitization.

MATERIALS AND METHODS

Animal Surgery and Treatments. Wistar male rats (180–200 g, Iffa Credo) were anesthetized with pentobarbital (50 mg/kg, i.p.) and infused over 8 min with 6-OHDA (8 μg in 4 μl of 0.05% ascorbic acid in saline) at coordinates A = −3.8 mm, L = 1.5 mm, H = −8.5 mm (16). Three weeks later, they received twice a day, and for various periods of time, i.p. injections of vehicle, levodopa (in all experiments as L-DOPA methyl ester, 50 mg/kg, in combination with benserazide, a

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Abbreviations: AcCo, core of nucleus accumbens; AcSh, shell of nucleus accumbens; CdPu, caudate-putamen; [^{3}H]7-OH-DPAT, 7-[^{3}H]hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; 6-OHDA, 6-hydroxydopamine; PD, Parkinson disease; StPv, periventricular striatum.

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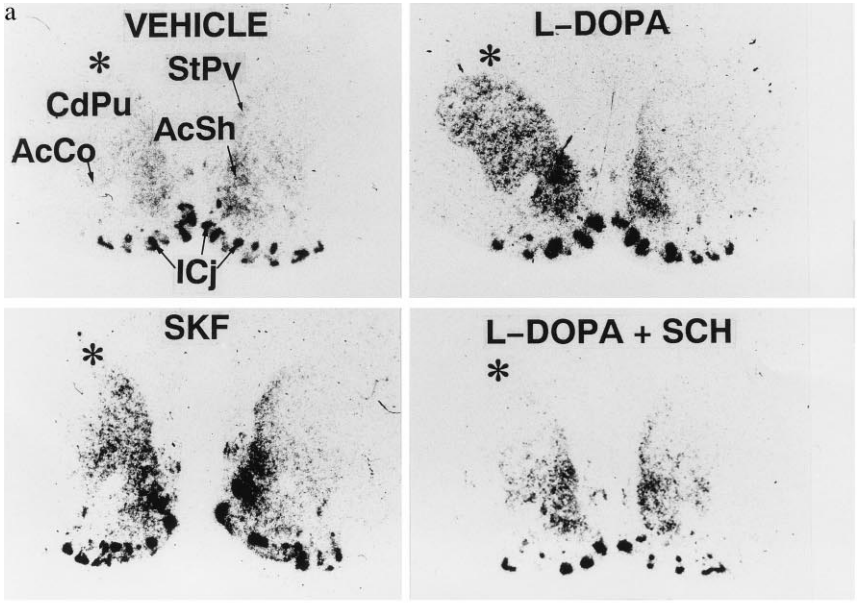
peripheral dopa decarboxylase inhibitor, 12.5 mg/kg) or levodopa plus SCH 23390 (0.5 mg/kg) or plus SKF 38393 (10 mg/kg), bromocriptine (10 mg/kg), quinpirole (0.1 mg/kg).

***In Situ* Hybridization and Receptor Autoradiography.** Rats were sacrificed 4 h after the last drug injection, their brains removed and frozen in liquid monochlorodifluoromethane. Coronal cryostat tissue sections (8–10 μm thick) were thaw-mounted onto slides and used unfixed (for receptor autoradiography), or fixed for 40 min at 4°C in 4% paraformaldehyde (for immunohistochemistry or *in situ* hybridization). The accuracy of the 6-OHDA lesion was checked by immunocytochemistry (17) with an anti-tyrosine hydroxylase antibody (Institut Jacques Roy S.A., Reims, France). Only the rats in which signals in the ventral tegmental area and substantia nigra were undetectable in the lesion as compared with control side were used.

In situ hybridization was performed using ^{33}P-labeled cRNA probes for the D_2 or D_3 receptor (11), D_1 receptor (nucleotides 1382–1708), prodynorphin (nucleotides 391–747), preproenkephalin (nucleotides 335–641), or preprotachykinin (nucle-



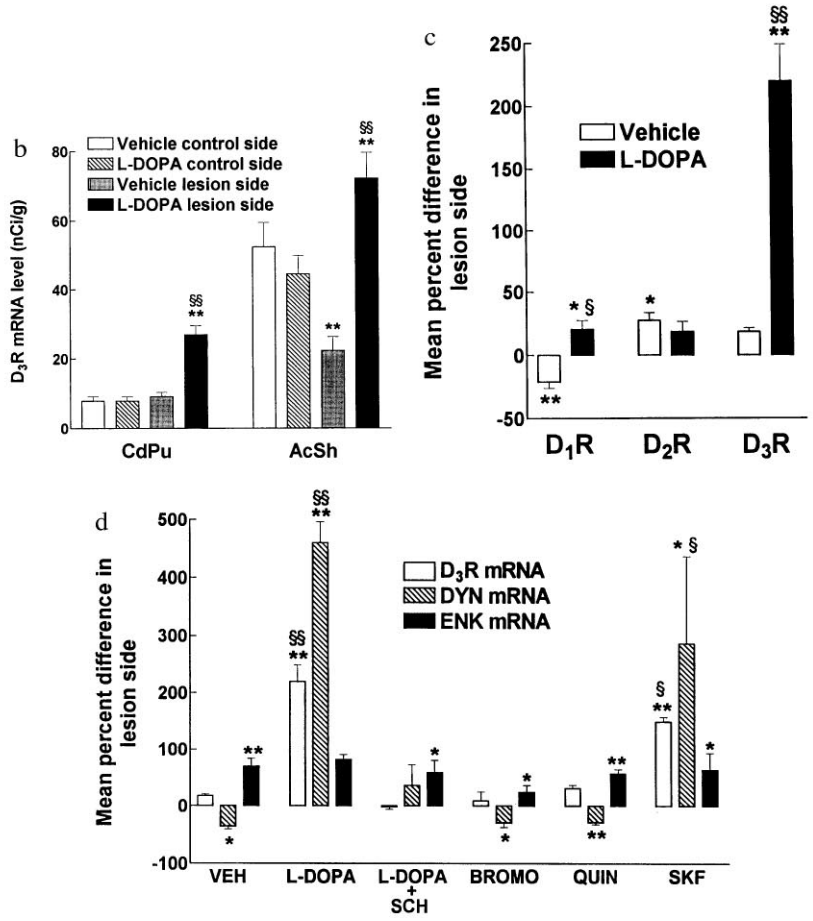


FIG. 1. *In situ* hybridization analysis of the effects of repeated administration of dopamine agonists on dopamine receptor and neuropeptide gene expression in the striatal complex of unilaterally 6-OHDA-lesioned rats. Coronal sections were hybridized with ^{33}P-labeled cRNA probes for D_1, D_2, or D_3 receptors (DRs), prodynorphin (DYN), or preproenkephalin (ENK) mRNAs, apposed to autoradiographic films and resulting pictures analyzed densitometrically. (*a*) Typical pictures showing the increased D_3 receptor mRNA in the lesion side (indicated by asterisk) of the dorsolateral striatum (CdPu), StPv, and shell or core subdivisions of nucleus accumbens (AcSh, AcCo) after a 5-day levodopa (L-DOPA) treatment and the blockade of this effect by SCH 23390 (SCH). The effect of levodopa was partially reproduced by SKF 38393 (SKF). ICj, islands of Calleja. (*b*) Mean D_3 receptor mRNA levels ± SEM (n = 4–6) in the two sides of CdPu (excluding StPv) and AcSh following repeated administration of vehicle or L-DOPA. (*c*) Mean percent difference (±SEM) in D_1, D_2, and D_3 receptor mRNA levels between lesion and control side of CdPu. Same animals as in *b*. The mean (±SEM) signals on the control side of vehicle-treated rats corresponded to 454 ± 54, 597 ± 63, and 8 ± 1 nCi/g (1 Ci = 37 GBq) for D_1R, D_2R, and D_3R, respectively. (*d*) Mean percent difference (± SEM, n = 4–6) in D_3R, DYN, and ENK mRNA levels between lesion and control side of CdPu following 5-day administration of levodopa alone or together with SCH of bromocriptine (BROMO), quinelorane (QUIN), or SKF. The mean (± SEM) *in situ* hybridization signal on the control side of vehicle-treated rats corresponded to 0.22 ± 0.03 and 1.7 ± 0.2 μCi/g for DYN and ENK, respectively. *, $P < 0.05$; **, $P < 0.01$ in lesion vs. control side by the paired Student's t test; §, $P < 0.05$; §§, $P < 0.01$ in lesion side of drug vs. vehicle-treated animals by the Mann–Whitney U test.

otides 80–277) mRNAs. Autoradiograms were generated by apposing the radiolabeled tissue sections to β-max Hyperfilms (Amersham) for 1–4 weeks. Autoradiographic signals were quantified on 2–3 slices per animal using an image analyzer (IMSTAR, Paris). Gray values were converted to microcuries per gram wet weight using ^{14}C standard stripes (Amersham).

Receptor autoradiography was performed as described (13), using 0.5 nM 7-[^{3}H]hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin ([^{3}H]7-OH-DPAT). Nonspecific binding was measured in the presence of 1 μM dopamine. Autoradiographic signals were quantified as above using ^3H standard stripes.

Rotation Behavior. Thirty-five minutes after administration of levodopa alone or in combination with nafadotride or haloperidol in increasing dosages, the rats were placed for a 5-min period of habituation into vertical Plexiglas cylinders (30 cm diameter). Contralateral rotations were counted during the following 5-min period by examiners blind from the treatment.

RESULTS

Induction of the D_3 Receptor Gene Expression. In rats having their left nigrostriatal pathway extensively destroyed by 6-OHDA infusion into the medial forebrain bundle 3 weeks earlier, the D_3 receptor mRNA distribution on the right side of vehicle-treated animals (Figs. 1 *a* and *b*) was similar to that in naive animals (11, 13); it was the highest in the AcSh, lower but still detectable in periventricular striatum (StPv), but hardly detectable over background in the remaining of CdPu and AcCo. On the lesion side of the same animals, D_3 receptor mRNA decreased by 58 ± 4% in AcSh and 32 ± 5% in StPv, but was not significantly modified in CdPu and AcCo.

Levodopa administrations repeated for 5 days resulted in robust induction of D_3 receptor gene expression occurring exclusively on the lesion side of all areas of the striatal complex. The induction was the most dramatic in CdPu or AcCo, where the hybridization signal remained at the limit of detection on the control side (Figs. 1 *a* and *b*). No induction was observed following repeated levodopa administrations to sham-operated animals (not shown). In contrast, there were limited changes in D_1 or D_2 receptor mRNAs (Fig. 1*c*).

We undertook to determine which receptor subtype was involved in D_3 receptor induction in CdPu. Bromocriptine or quinelorane, two preferential D_2 and D_3 receptor agonists, respectively (18), had no effect, whereas SKF 38393, a selective but partial D_1 receptor agonist, produced similar, although less extensive effects than levodopa (Fig. 1*d*). Levodopa effect was completely prevented by SCH 23390, a selective D_1 receptor antagonist (Fig. 1*c*).

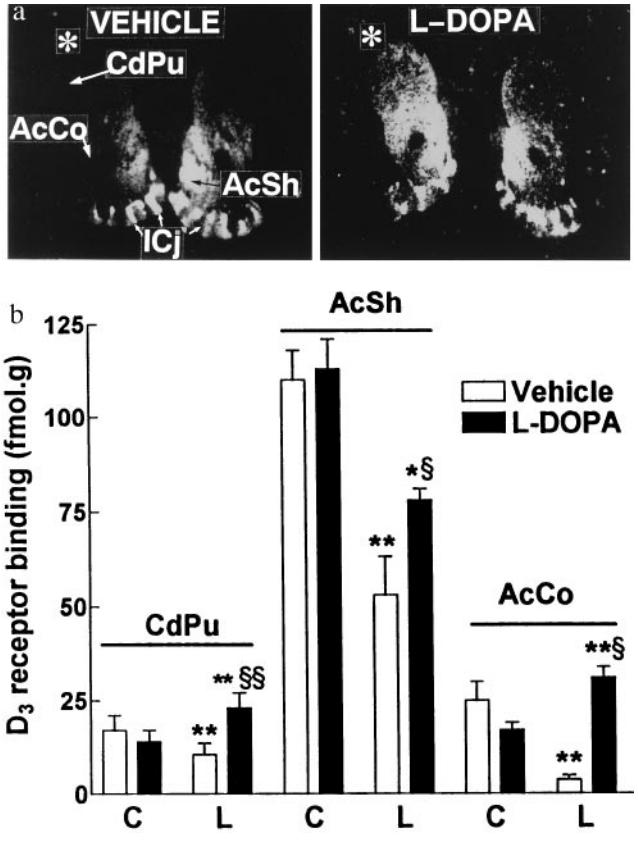


FIG. 2. Changes in D_3 receptor binding elicited by repeated levodopa treatments of 6-OHDA-lesioned rats. (*a*) Digitized autoradiographic D_3 receptor binding picture obtained with [^{3}H]7-OH-DPAT, in animals treated twice a day for 5 days with vehicle (VEH) or levodopa (L-DOPA), in which nonspecific labeling has been subtracted. Asterisk indicates the lesion side. (*b*) Autoradiographic signals generated in *a* were quantified in CdPu, AcSh, and AcCo of control (C) and lesion (L) sides. Mean ± SEM of values from 4–6 animals. Nonspecific binding in CdPu was 50 and 15% of total in vehicle- and levodopa-treated animals, respectively. *, $P < 0.05$; **, $P < 0.01$ as compared with the control side by the paired Student's *t* test; §, $P < 0.05$; §§, $P < 0.01$ in lesion side of drug vs. vehicle-treated animals by the Mann–Whitney *U* test.

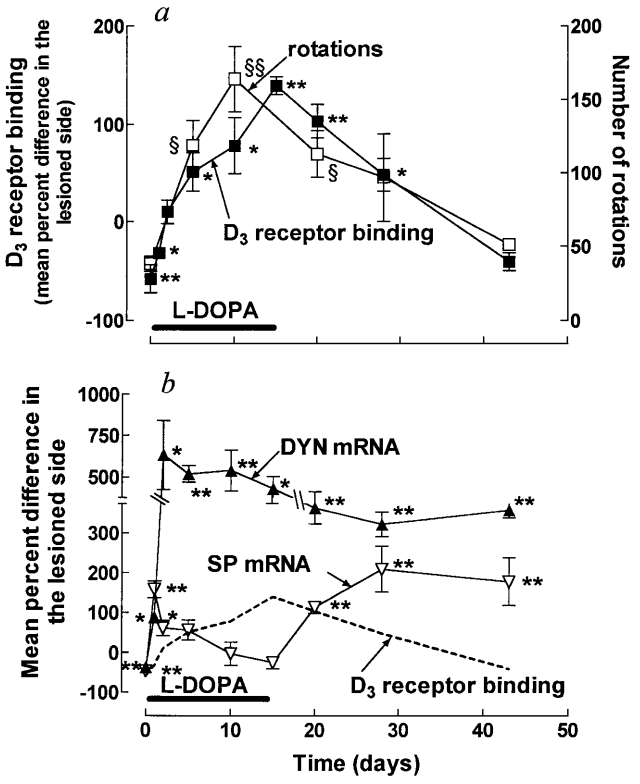


FIG. 3. Progressive changes in D_3-receptor binding and levodopa-induced rotations and neuropeptide mRNAs in CdPu of unilaterally 6-OHDA-lesioned rats following repeated treatment with levodopa and withdrawal. Rats received levodopa (50 mg/kg i.p.) twice daily for up to 15 days (horizontal bar) and were challenged with a single same dose of levodopa at the indicated time during this chronic treatment or after withdrawal. Animals were killed 4 h after the challenge, D_3 receptor autoradiography using [^{3}H]7-OH-DPAT or *in situ* hybridization with probes for prodynorphin (DYN) or preprotachykinin (SP) mRNAs. Note that rotations were not measured in all groups of animals. No significant changes were observed in D_3 receptor binding or *in situ* hybridization signals in the control side. Means ± SEM of values from 4–9 animals. *, $P < 0.05$; **, $P < 0.01$ as compared with the control side by the paired Student's *t* test; §, $P < 0.05$; §§, $P < 0.01$ compared with values obtained following the first levodopa injection by the Mann–Whitney *U* test.

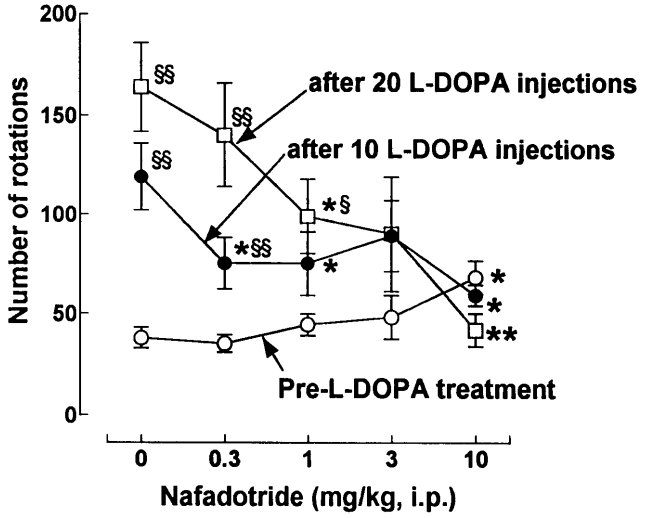


FIG. 4. Effects of nafadotride, a preferential D_3-receptor antagonist on levodopa-induced rotations. Contralateral rotations were measured in animals challenged with levodopa (50 mg/kg i.p.), alone or in combination with nafadotride at indicated doses on three occasions: the first, 4 weeks after the lesion (pre L-DOPA treatment); the second and third, after subsequent treatments with 10 or 20 levodopa (L-DOPA) injections (50 mg/kg i.p., twice daily). Means ± SEM of values from 4–6 animals. *, $P < 0.05$; **, $P < 0.01$ nafadotride vs. saline-treated animals; §, $P < 0.05$; §§, $P < 0.01$ in levodopa-treated vs. untreated animals by the Mann–Whitney U test.

D_3 receptor mRNA induction in the denervated side was accompanied by progressive up-regulation of the D_3 receptor protein itself, as evidenced by [^{3}H]7-OH-DPAT autoradiography (13), occurring in all areas of the striatal complex (Fig. 2). Up-regulation elicited by levodopa was the most dramatic, however, in denervated CdPu and AcCo, reaching +131% and +680%, respectively, as compared with vehicle, after 10 levodopa injections (Fig. 2*b*).

D_3 Receptor Expression and Change in Rotation Behavior. To assess the functional role of these neosynthesized receptors, we measured contralateral rotations produced by a levodopa challenge before, during, or after a period of repeated administration of levodopa. As previously described (6, 7), the intensity of the response to levodopa alone increased 3- to 4-fold upon repeated administration and then progressively declined upon withdrawal of levodopa with a half-life of about 2 weeks (Fig. 3*a*). The time course of the evolution of the rotation behavior paralleled that of D_3 receptor protein appearance and disappearance in CdPu (Fig. 3*a*). In addition, in sensitized animals, the excess of rotations above the pretreatment level was dose-dependently blocked by nafadotride, a preferential dopamine D_3 receptor antagonist (19) with an ID_{50} of 1.1 mg/kg (0.46–2.8, 95% confidence interval) (Fig. 4). Levodopa-induced rotations were also reduced by 41%, 56%, and 83% ($P < 0.02$ by ANOVA) in the same animals after respective administrations of 0.3, 1, and 3 mg/kg i.p. of haloperidol, a preferential dopamine D_2 receptor antagonist (11) (not shown), leading to an ID_{50} for this compound of 0.42 mg/kg i.p. (0.16–1.1, 95% confidence interval).

Changes in Dynorphin and Substance P Gene Expression During Sensitization. Medium-sized spiny neurons make 95% of total neurons from CdPu and are distributed among dynorphin/substance P and enkephalin neurons, harboring the D_1 and D_2 receptors, respectively (20, 21). The variations of D_3 receptor mRNA upon various treatments paralleled those of prodynorphin mRNA rather than those of proenkephalin mRNA (Fig. 1*d*). This suggests that the induction takes place in dynorphin/substance P striatonigral neurons expressing the D_1 receptor, and not enkephalin striatopallidal neurons expressing the D_2 receptor, in agreement with the fact that D_3 receptor induction results from D_1 receptor stimulation.

In addition, there were strikingly reciprocal changes in D_3 receptor binding and substance P mRNA during sensitization and desensitization following levodopa withdrawal, while dynorphin mRNA remained high. Such variations indicate an imbalance between the two neuropeptides at the time when rotation behavior was maximally enhanced (Fig. 3*b*).

DISCUSSION

We show here that repeated intermittent administration of levodopa to hemiparkinsonian rats, under conditions leading to the development of behavioral sensitization to this drug, is accompanied by a dramatic and long-lasting induction of the D_3 receptor gene expression on the denervated side. This induction unexpectedly occurs in the dorsal striatum and core of accumbens, two areas involved in extrapyramidal motor controls and from which the D_3 receptor is normally or nearly absent.

Several observations indicate that the process of D_3 receptor induction is causally related with the development of behavioral sensitization. (*i*) Both processes occur with a similar time course, strikingly parallel changes being observed in the appearance and disappearance of the D_3 receptor protein in the denervated striatum during levodopa treatment and after its interruption, on one hand, and, on the evolution of the rotation behavior during the same periods, on the other hand (Fig. 3*a*). (*ii*) Like the behavioral sensitization process, D_3 receptor induction is shown here to require previous dopaminergic denervation and to depend upon repeated stimulation of the striatal D_1 receptor. (*iii*) Nafadotride, a preferential D_3 receptor antagonist (19), inhibited the levodopa-induced rotation behavior only in sensitized animals expressing the neosynthesized and ectopic D_3 receptor and did so, at a low dosage, compatible with a selective occupancy of this receptor subtype. In agreement, before sensitization levodopa-induced rotations were not impaired by nafadotride, with a slight facilitation even observed, which may reflect the blockade of the homotopic D_3 receptor in nucleus accumbens held responsible for inhibition of locomotor activity (19, 22, 23). It is important to underline that the effect of nafadotride can be safely attributed to D_3 and not D_2 receptor blockade, in spite of the limited (10-fold) preference of this drug for the D_3 over the D_2 receptor. Indeed, it is well known that the rotational behavior in either sensitized or nonsensitized animals can be blocked by preferential D_2 receptor antagonists such as haloperidol (which displays a 5-fold preference for D_2 over D_3 receptors). The inhibition of rotations by nafadotride, occurring only in sensitized animals, was obtained at a low dosage corresponding to a selective D_3 receptor occupancy in rat brain: its ID_{50} (1.1 mg/kg) was only 2–3 times higher than that of haloperidol in this test (see *Results*), whereas in typical behavioral responses mediated by the D_2 receptor, haloperidol is 20–50 times more potent than nafadotride (19).

Our data also allow us to propose possible mechanisms responsible for D_3 receptor induction and those through which the induction and behavioral sensitization may be related. D_3 receptor induction is very likely to take place in D_1 receptor-harboring γ-aminobutyric acid neurons expressing dynorphin and/or substance P. In agreement, D_1 and D_3 receptors normally coexist in other substance P-containing neuronal populations, e.g., granule cells of islands of Calleja (11, 24) or spiny neurons in AcSh (25). Under experimental conditions similar to those used here, transcription factors are markedly activated, i.e., immediate–early gene expression is induced (26–28) and cyclic AMP-responsive element-binding protein is phosphorylated (29) in the same denervated neurons, and such factors may in turn regulate positively D_3 receptor gene expression. When induced in these neurons, the D_3 receptor, which among the various dopamine receptor subtypes displays

the highest sensitivity to dopamine (11), could be responsible for the enhanced responsiveness to dopamine of a motor effector pathway.

Activation of the striatonigral pathway by dopamine, leading to inhibition of γ-aminobutyric acid neurons in substantia nigra pars reticulata (and consequent disinhibition of target motor nuclei) contributes to the generation of movements and rotations in rats with unilateral 6-OHDA lesions (20, 21, 30). To a large extent, changes in neuropeptide gene expression in neurons of this pathway reflect changes in the activity of these neurons as measured with 2-deoxyglucose (31, 32). Although dynorphin and substance P coexist in these neurons (33), they exert opposite actions at the level of substantia nigra: dynorphin inhibits the firing of nigral neurons and potentiates the inhibitory effect of γ-aminobutyric acid (34, 35), whereas tachykinins are excitatory (36, 37). Tachykinins and dynorphin applied to the substantia nigra also exert opposite influences on the activity of dopaminergic neurons (38) and their effects on rotational behavior are differentially affected by unilateral denervation (39). We show here that these two neuropeptide mRNAs change almost reciprocally during the sensitization and desensitization processes, leading to an imbalance in their respective abundance. Similarly dissociated changes in the two neuropeptide mRNAs were already shown to occur in the denervated striatum at the end of chronic "sensitizing" treatments with a D_1 receptor agonist or apomorphine, a mixed agonist (20, 40). The progressive down-regulation of substance P gene expression during sensitization and the reversal of this process during desensitization closely parallels changes in neosynthesized D_3 receptors, indicating that receptor activation may reduce substance P release into the substantia nigra and, thereby, relieve an opposing influence on the nigral γ-aminobutyric acid neurons, thus leading to increased rotation behavior. Differential changes in the expression of several peptides by a single population of neurons as a mechanism of neuronal plasticity are already documented, e.g., cholecystokinin and substance P in the amygdaloid complex during the estrous cycle or vasoactive intestinal peptide and substance P in sensory neurons following axotomy (41).

While the mechanisms through which the D_3 receptor exerts its role remain conjectural at this stage, its involvement in the sensitization process may have wide relevance to PD therapy and prevention of side effects. In agreement, in a preliminary study, D_3 receptor protein and mRNA were found normal or even elevated, however, to a statistically nonsignificant level, in postmortem CdPu from a very limited number of PD patients treated with levodopa (42). Nevertheless, it should be underlined that sensitization mechanisms other than those studied here might also be invoked in the clinical situation, e.g., progressive changes in the activity of residual dopamine neurons (43) or distinct changes mediated by selective D_2 receptor priming (44, 45).

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