THE INFLUENCE OF INTERMITTENT L-DOPA TREATMENT ON STRIATAL MOLECULAR MARKERS IN HEMIPARKINSONIAN RATS*

VPLIV INTERMITENTNEGA DAJANJA L-DOPA NA STRIATNE MOLEKULSKE OZNAČEVALCE PRI HEMIPARKINSONSKIH PODGANAH

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Abstract

Motor complications after chronic l-DOPA treatment in patients with Parkinson's disease may be caused by the fluctuations of l-DOPA availability in the brain that provokes the sensitization of striatal output neurons of dopamine-depleted striatum. The aim of this study was to analyze the effects of intermittent l-DOPA/carbidopa treatment schedule (injection of l-DOPA/carbidopa every fourth day, 6-treatments) on the development of locomotor sensitization of hemiparkinsonian rats to l-DOPA, and on the development of dopaminergic sensitization of striatal output neurons of the indirect and direct pathways. The development of locomotor sensitization was verified by the increased intensity of contralateral turning behavior after the last l-DOPA injection. It is well known that PPT mRNA is expressed predominantly by the neurons of the direct pathway, PENK mRNA by the neurons of the indirect pathway, while GAD67 mRNA is expressed in the neurons of both pathways. Dopaminergic sensitization of striatal output neurons of dopamine-depleted striatum was thus assessed by the analysis of changes of striatal preprotachykinin (PPT), proenkephalin (PENK) and GAD 67 mRNA levels 4 and 12 hours after the last l-DOPA injection. We found, that chronic dopamine depletion by itself down-regulates the expression of striatal PPT mRNA and up-regulates GAD67 and PENK mRNAs. These changes of basal expression were not reversed by the intermittent l-DOPA/carbidopa treatment. However, in dopamine-depleted striatum, the intermittent treatment with l-DOPA induced increased responsiveness of striatal PPT and GAD67, but not PENK mRNA expression, to l-DOPA. Our results are in agreement with the hypothesis, that intermittent l-DOPA treatment induces locomotor sensitization that may be linked to the increased dopaminergic responsiveness of striatonigral neurons of the direct pathway, within dopamine-depleted striatum.

Key words

6-hydroxydopamine; preprotachykinin; proenkephalin; glutamate decarboxylase 67; dopaminergic sensitization

Izvleček

Zapleti kroničnega zdravljenja Parkinsonove bolezni z l-DOPA v obliki motenj gibanja (diskinezij) so morda povezani s posledicami nihanja koncentracije tega zdravila v možganih, ki ob pomanjkanju dopamina v striatumu, povzroči dopaminergično senziti- zacijo projekcijskih striatalnih nevronov. Namen raziskave je bil preučiti učinek protoko- la intermitentnega dajanja l-DOPA/karbidopa hemiparkinsonskim podganam (injekcija l-DOPA/karbidopa vsak četrti dan, 6-krat), na razvoj lokomotorne senzitizacije na l-DOPA ter na razvoj dopaminergične senzitizacije projekcijskih nevronov posredne in neposredne poti. Lokomotorno senzitizacijo poskusnih podgan, ki smo jim dajali l-DOPA po intermitent- nem protokolu, smo dokazali s povečanjem intenzitete kontralateralnega kroženja po zad- nji injekciji l-DOPA glede na intenziteto kroženja kontrolnih podgan. Znano je, da se PPT

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mRNK v striatumu izraža predvsem v nevronih neposredne poti, GAD67 mRNK pa v nevronih neposredne in posredne poti. Zato smo dopaminergično senzitizacijo striatalnih nevronov ugotavljali z analizo sprememb ravni izražanja mRNK preprotahikinina (PPT), glutamat-dekarboksilaze z molekulsko težo 67 kDa (GAD67) in proenkefalina (PENK) v denerviranem striatumu 4 in 12 ur po zadnji injekciji l-DOPA. Ugotovili smo, da kronično pomanjkanje dopamina v striatumu povzroči zmanjšanje bazalnega izražanja PPT mRNK ter povečanje bazalnega izražanja GAD67 in PENK mRNK v striatumu. Intermitentno dajanje l-DOPA/karbidopa ni zmanjšalo odklonov bazalnega izražanja omenjenih mRNK v primerjavi z intaktnim striatumom, temveč je povzročilo povečano odzivnost izražanja PPT in GAD67 mRNK na l-DOPA. Rezultati naše raziskave so skladni s hipotezo, da lahko z intermitentnim dajanjem l-DOPA hemiparkinsonskim podganam povzročimo lokomotorno senzitizacijo na l-DOPA, ki bi lahko bila povezana s povečano dopaminergično odzivnostjo striatonigralnih nevronov neposredne poti v striatumu, ki mu primanjkuje dopamin.

Ključne besede   *6-hidroksidopamin; preprotahikinin; proenkefalin; glutamat-dekarboksilaza 67; dopaminergična senzitizacija*

Introduction

Parkinson's disease (PD) is characterized by an extensive loss of dopaminergic neurons in the substantia nigra pars compacta.[1] The resulting motor deficits akinesia, rigidity and tremor could be reversed by dopamine precursor l-DOPA that still remains the best option for the symptomatic treatment of Parkinsonism.[2] Unfortunately, its long-term use is commonly associated with severe motor fluctuations and abnormal involuntary movements.[3] The pathogenesis of l-DOPA-induced dyskinesias is not well understood, but includes a variety of factors: the degree of presynaptic striatal dopamine depletion,[4, 5] denervation-induced postsynaptic adaptations, such as dopamine D_2 receptor up-regulation within striatopallidal neurons and increased dopaminergic sensitivity of D_1-linked intracellular signaling pathways within striatonigral neurons.[6-8] These result in the alterations of the dopamine agonist-induced expression of several striatal neuropeptides and enzymes expressed within these neurons.[9-11]

The rodent model that is commonly used for studying the effects of long-term l-DOPA treatment on the development of dopaminergic sensitization is rat 6-hydroxydopamine-lesioned (6-OHDA) model of Parkinsonism.[12] In unilateral variant of this model, the toxin is stereotaxically injected into the medial forebrain bundle of one of the hemispheres, in order to provoke one-sided degeneration of nigrostriatal dopaminergic neurons.[13] One of the adaptations that follow chronic depletion of dopamine is the development of unilateral striatal dopaminergic hypersensitivity that may be detected as contralateral rotational behavior induced by directly acting dopaminergic agonists.[14, 15] Dopaminergic hypersensitivity could play a role in the development of pharmacological complications of the therapy with l-DOPA.[16] The loss of dopamine within the striatum disturbs the functional organization of basal ganglia networks, by introducing an imbalance between striatal output pathways, i.e. the hypoactivity of striatonigral and hyperactivity of striatopallidal projections.[17] The repeated injection of l-DOPA produces sensitization of motor response, such as enhanced contralateral rotations of unilaterally-lesioned 6-OHDA rats.

l-DOPA side-effects are often associated with high doses or long intervals between l-DOPA administration, producing high fluctuations in bioaviability of dopamine in the brain extracellular fluid, that are bound to elicit aberrant postsynaptic responses in dopaminoceptive neurons.[18] Continuous administration is preferred method for l-DOPA treatment in PD. Enteral levodopa/carbidopa gel infusions and oral sustained-release tablets are therefore used for optimizing l-DOPA pharmacokinetics in this regard.[19-21] In animal experiments, this is mimicked by protocols using continuous infusion of l-DOPA. Using similar protocols, continuous l-DOPA treatment has been shown to have different effects on D1 and D2 agonist-stimulated rotation and peptide levels in striatal output nuclei.[22] On the basis of these studies it has been concluded that continuously administered l-DOPA is associated with relatively few if any neurochemical abnormalities in striatal efferent systems.[23]

To characterize striatal abnormalities in gene expression induced by fluctuations of l-DOPA concentration in dopamine-depleted striatum, we used intermittent l-DOPA treatment schedule (6 injections of l-DOPA/carbidopa every fourth day). It was shown that this kind of treatment could induce even stronger behavioral sensitization in 6-OHDA rats.[22] Our aim was to analyze the effect of intermittent l-DOPA/carbidopa treatment schedule on the responsiveness of the molecular striatal markers that are differentially expressed within striatal output neurons. PPT mRNA is expressed preferentially within striatonigral GABAergic neurons (direct pathway), PENK mRNA is expressed within striatopallidal GABAergic neurons (indirect pathway), while GABA synthesizing enzyme glutamate decarboxylase MW = 67,000 (GAD67) mRNA is expressed in the striatal neurons of both pathways.[24] We hypothesized, that in the dopamine-depleted striatum of 6-OHDA rats, the intermittent l-DOPA/carbidopa treatment increases the responsiveness of the expression of PPT, PENK and GAD 67 mRNAs.

Experimental procedures

Animals

We used male Wistar rats maintained on a 12:12 h light:dark cycle (lights on 07.00 to 19.00 h) in a temperature-controlled colony room at 22 °C with free access to rodent pellets and tap water. They were handled according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of experimental animals and their suffering.

Drugs

The following drugs were used: apomorphine hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9 % saline containing 0.02 % ascorbic acid; l-DOPA and carbidopa (gift from LEK Pharmaceutical Company, Ljubljana, Slovenia) were dissolved in 0.3 % ascorbate made in 100 mM Na phosphate buffer (pH = 5), pH was then adjusted with 10 N NaOH to 6.5–7; 6-OHDA hydrobromide (RBI, Natick, MA, USA) was dissolved in 0.9 % saline containing 0.02 % ascorbic acid.

Unilateral 6-OHDA lesions of the nigrostriatal pathway

Stereotaxic lesions were created as described by Glavan and Zivin.[25] Female Wistar rats weighing between 150 and 200 g were anesthetized with the i. p. injection of 2 % xylazine hydrochloride (8 mg/kg; Rompun®; Bayer, Leverkusen, Germany), ketamine hydrochloride (60 mg/kg; Ketanest®; Parke Davis, Wien, Austria), and atropine (0.6 mg/kg; Belupo, Koprivnica, Croatia), and placed in a stereotaxic frame (TrentWells, South Gate, CA, USA). 8 µg of 6-OHDA hydrobromide was infused into the right medial forebrain bundle at the following co-ordinates: anterior 3 mm from lambda, lateral 1.2 mm from the midline and ventral 7.3 mm from the surface of the dura (stereotaxic coordinates[26]).

Apomorphine test

We used apomorphine test to determine the development of nigrostriatal degeneration. 6-OHDA-lesioned animals were treated with directly acting mixed agonist of dopamine receptors apomorphine (0.5 mg/kg, *s. c.*) in the sixth post-operative week. The number of contralateral turning was recorded by placing the rats in plastic cylindrical chambers (40 cm diameter) of the Lablinc automated rotometer system (Colbourn Instruments, Allentown, PA, USA). Only the 6-OHDA rats that responded with peak turning frequency of at least seven contralateral turns per minute were used in subsequent experiments.

Drug treatment and subsequent behavioral testing

One week after the treatment with apomorphine, 6-OHDA rats (n = 36) were divided into five groups of six animals. The animals received six treatments every 4 days. L-DOPA (5 mg/kg, s. c.) was always injected 20 min after the injection of peripheral DOPA-decarboxylase inhibitor carbidopa (2 mg/kg, i. p.). Groups 1 and 2 received six injection treatments of carbidopa and l-DOPA. Groups 3 and 4 received five injections of carbidopa, while the last injection treatment contained both, carbidopa and l-DOPA. Group 5 (control) received six injections of carbidopa. After every treatment the number of contralateral turns was recorded for three hours. The animals from groups 1, 3 and 5 were killed at 4 h while the animals groups 2 and 4 were killed at 12 h after the last injection. The chosen times for killing animals were based on previous studies showing the elevations of GAD67 and PPT mRNAs in 6-OHDA-lesioned striatum 4 h and 12h after the acute treatments of agonists of dopamine receptors.[9]

Brain preparation

The brains were removed and quickly frozen on dry ice. Coronal sections (10 ěm) were cut through the neostriatum (between 2.2 mm and –0.3 mm from bregma) using a cryostat, thaw mounted onto microscope slides. The sections were then fixed in 4 % phosphate-buffered paraformaldehyde, washed in phosphate-buffered saline, dehydrated in 70 % ethanol and stored in 95 % ethanol at +4 °C until used for *in situ* hybridization histochemistry.

In situ hybridization histochemistry

We performed the standard procedure described in detail by Glavan and Zivin.[25] The sections were incubated with 3' end ^{35}S-labelled oligodeoxyribonucleotide antisense probes (45 bases long) complementary to rat preprotachykinin (PPT) mRNA (bases encoding 136–180, sequence 5'-TCG GGC GAT TCT CTG AAG AAG ATG CTC AAA GGG CTC CGG CAT TGC-3'), rat preproenkephaline (PENK) mRNA (bases encoding 153–109, sequence 5'-GTA GCT GCA TTT AGC GCA GTC CTG GCT GCA GTC TGC CTG CAC TGT-3') and rat glutamate decarboxylase MW = 67.000 (GAD67) mRNA (bases encoding 1827–1783, sequence 5'-TGA CTC CAT CAT CAG GGC TTT GAT CTT GGG AGC CAC CCT GTG TAG-3'). GenBank accession numbers used to design the probes were as follows: PPT M14312, PENK M28263 and GAD67 M34445. To summarize, air dried sections were incubated with ^{35}S-labeled probe in hybridization buffer containing 4x SSC (1 × SSC is made out off 150 mM sodium chloride and 15 mM sodium citrate), 50 % deionized formamide, 50 mM sodium phosphate (pH 7.0), 5 × Denhardt's solution, 100 µg/ml polyadenylic acid, 10 % dextran sulfate and 40 mM dithiothreitol. The oligodeoxynucleotide probes were labeled by incubation for one hour at 36 °C with [35S]-deoxyadenosine 5 = –α-(thio) triphosphate ([35S]-dATP; 1000–1500 Ci/mmol; DuPont NEN, Life Science Products Inc., Boston) and terminal deoxynucleotidyl transferase enzyme (Promega, Madison, WI, USA). The labeled probes were purified using spin columns with Sephadex G50. Specific activities of the labeled probes ranged from 55 to 150× 10^3 d. p. m./µl. Hybridization buffer with labeled probe was applied to each slide and in-

cubated for 16 h at 42 °C in a humid chamber. Washing was performed for 30 min at room temperature followed by 1 h wash at 55 °C in 1x SSC. The sections were then quickly dipped in 0.1x SSC and dehydrated through 50 %, 70 % and 98 % ethanol. Air dried hybridized sections were exposed to X-ray film (Scientific Imaging Film X-Omat™ AR, Kodak, Rochester, NY) that were exposed for 2–3 weeks and developed using standard darkroom techniques.

Image analysis

The hybridization signal was analyzed densitometrically with MCID, M4 image analyzer (Imaging Research Inc., Canada) in the region of dorsal striatum using 3mm diameter circle template. Relative optical density (ROD) measurements in the striatum were performed on three sections of each animal. Nonspecific background signal, defined as the ROD of parts of the film without hybridization signal, was subtracted from the ROD measurements.

Statistical analysis

To evaluate the statistical significance of the chronic l-DOPA/carbidopa treatment on turning of animals we performed the one-way analysis of variance (ANOVA) followed by Scheffe's multiple-comparison test by comparing contralateral rotations after different number of injections. The numbers of ipsilateral turns were previously subtracted from the number of contralateral rotations. To evaluate the statistical significance of the effects of dopamine depletion on the intensity of striatal PPT, PENK and GAD67 mRNA signals we performed the paired Student's t-test by comparing ROD measurements of lesioned vs. non-lesioned side. To evaluate the statistical significance of the intermittent l-DOPA/carbidopa treatment on striatal PPT, PENK and GAD67 mRNA signals we used the one-way analysis of variance (ANOVA) followed by Scheffe's multiple-comparison test to compare the l-DOPA acutely, l-DOPA intermittently treated and control group of animals. This statistical analysis was done separately for the striatum of the 6-OHDA-lesioned and the intact side. All data are expressed as means ± S. E. M. Statistical significance was set at $P < 0.05$.

Results

L-DOPA-induced behavioral sensitization

Repeated treatment with l-DOPA produced a progressive increase of the rotation behavior (Figure 1). The numbers of contralateral rotations following the 2nd, 3rd, 4th, 5th and 6th injections were significantly increased when compared to the number of rotations induced by the 1st administration of the same drug (Figure 1, one-way ANOVA followed by Scheffe's multiple-comparison test, $P < 0.05$). The last, 6th injection of l-DOPA produced a significant elevation of the number of rotations as compared to contralateral turning behavior induced by other subsequent injections of l-DOPA (Figure 1, one-way ANOVA followed by Scheffe's multiple-comparison test, $P < 0.05$). The re-

peated injections of carbidopa did not significantly induce turning behavior or behavioral sensitization (data not shown, one-way ANOVA followed by Scheffe's multiple-comparison test, $P > 0.05$).
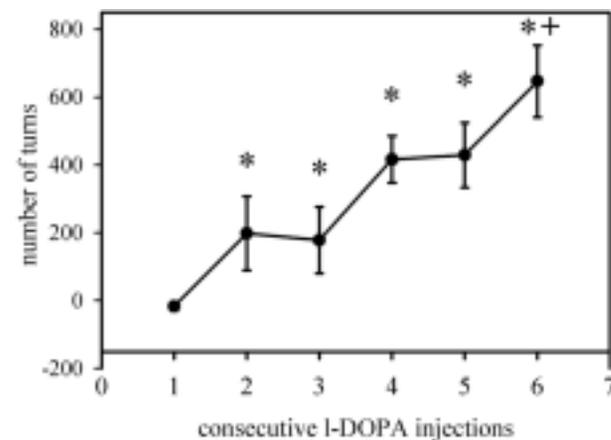

Figure 1. *The sensitization of contralateral turning response following intermittent l-DOPA administration in the 6-OHDA-lesioned rat model. The graph represents the average total number of turns in three hours. Error bars indicate S.E.M. The ipsilateral turns were subtracted from contralateral. The animals received six treatments every 4 days. L-DOPA (5 mg/kg, s.c.) was always injected 20 min after the injection of peripheral DOPA-decarboxylase inhibitor carbidopa (2 mg/kg, i.p.). * – significantly higher than after 1st treatment, + – significantly higher that after 1st, 2nd, 3rd, 4th, and 5th treatments (one-way ANOVA followed by Scheffe's multiple-comparison test, $P < 0.05$).*

Oligonucleotide probe specificity

For each probe, brain sections were hybridized in the presence of 100-fold excess of unlabeled probe to determine the specificity of the probes. The specificity was thus confirmed by the disappearance of the authoradiographic signal for all probes used by our studies (not shown). We also show that the distribution pattern of all probe's hybridization signals on brain section matched to that described by our previous[25, 27] and other reports.[24]

Effects of 6-OHDA lesion on striatal gene expression

The 6-OHDA lesion of dopaminergic nigrostriatal neurons resulted in a significant up-regulation of striatal PENK (Figure 2, control, by +80 %), GAD67 (Figure 2, control, by +79 %) and in the down-regulation of striatal PPT (Figure 2, control, by –45 %) hybridization signals (*all* paired Student's t-test, $P < 0.05$).

Acute effect of l-DOPA on gene expression in the denervated striatum

The acute treatment with l-DOPA significantly elevated the PPT mRNA signal in the denervated striatum 4h (by +64 %) that decreased at 12 h after the injection but was still elevated (by +35 %) as compared to
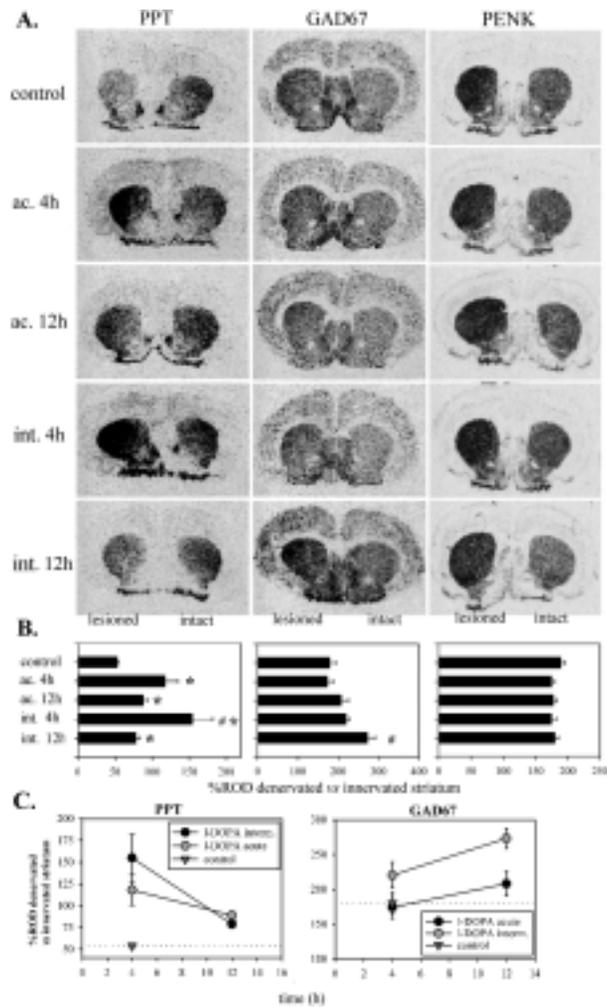
Figure 2. *The effect of acute and intermittent l-DOPA treatment on PPT, GAD67 and PENK mRNA signals in the striatum of 6-OHDA-lesioned rat model for Parkinson's disease. The intermittently treated animals received six treatments of l-DOPA with carbidopa every 4 days. Acutely treated animals received five injections of carbidopa every 4 days while the last injection treatment contained both, carbidopa and l-DOPA. Control received six injections of carbidopa. The animals were killed 4 h or 12 h after the last injection, as indicated. L-DOPA (5 mg/kg, s.c.) was always injected 20 min after the injection of peripheral DOPA-decarboxylase inhibitor carbidopa (2 mg/kg, i.p.). (A) Representative in situ hybridization images for each group of animals. Lesioned side is on the left. (B) Bar charts of average ROD of the denervated dorsal striatum expressed in the % of ROD of innervated striatum of the same animal. (C) Schematic presentation of possible time-courses of PPT and GAD67 mRNA levels for acutely and intermittently l-DOPA treated 6-OHDA-lesioned rats. * – Significantly higher than ROD of the denervated striatum of 6-OHDA control rats (one-way ANOVA followed by Scheffe's multiple-comparison test, P < 0.05, for each group n = 6). # – Significantly higher as compared with ROD of the denervated striatum of group ac. 12 h (one-way ANOVA followed by Scheffe's multiple-comparison test, P < 0.05, for each group n = 6). Error bars indicate S.E.M.*

the denervated striatum of control rats (Figure 2, one-way ANOVA followed by Scheffe's multiple-comparison test, $P < 0.05$). L-DOPA single injection had no significant effect on GAD67 and PENK mRNAs in denervated striatum as compared to the denervated striatum of control rats either at 4 h or 12 h after treatment (Figure 2, one-way ANOVA followed by Scheffe's multiple-comparison test, $P < 0.05$).

In the intact striatum of 6-OHDA rats l-DOPA single injection has no effect on the PPT, PENK or GAD67 mRNA signals as compared to the denervated striatum of control rats (Figure 2, one-way ANOVA followed by Scheffe's multiple-comparison test, $P > 0.05$).

Effect of intermittent l-DOPA on gene expression in the denervated striatum

The repeated injections of l-DOPA resulted in an enhanced responsiveness of PPT and GAD67 mRNA signals to the last l-DOPA injection in the denervated striatum as compared to the acute treatments (Figure 2, one-way ANOVA followed by Scheffe's multiple-comparison test, $P > 0.05$). Significantly elevated GAD67 mRNA in denervated striatum was detected at 12 h, while there was a slight but not significant elevation of this mRNA in denervated striatum after the last treatment. The significant increase of PPT mRNA signal was detected at 4 h, while the intensity of hybridization PPT signal rapidly declined, at 12 h displaying similar striatal mRNA levels in acutely and intermittently treated animals. The intermittent l-DOPA had no effect on PENK mRNA expression in both, denervated and innervated striata.

Discussion

The aim of this study was to analyze the effect of intermittent l-DOPA/carbidopa treatment schedule in groups of hemiparkinsonian rats, on the responsiveness of the expression of PENK, PPT and GAD67 mRNAs, the molecular markers of the direct and indirect striatonigral pathways, to the last l-DOPA injection. We used an intermittent treatment schedule that produces strong behavioral sensitization. We expected increased responsiveness of PPT, PENK and GAD67 mRNA expression in dopamine-depleted striatum of rats that were sensitized by intermittent l-DOPA/carbidopa treatment. We found, that the responsiveness of the striatal expression of PPT and GAD mRNA levels in dopaminergically-sensitized animals was increased, while the responsiveness of PENK mRNA levels remained unaffected.

All 6-OHDA animals developed behavioral hypersensitivity as a consequence of severe striatal dopamine depletion, verified by the intensive contralateral turning induced by apomorphine. It is known that the intermittent treating induces stronger locomotor sensitization as compared to continuously injected l-DOPA.[22] In our experiments intermittent treatment with l-DOPA with a 4 days washout periods produced a progressive increase of the contralateral turning

behavior. These experimental data in regard to the l-DOPA treating protocols confirm the results of previous studies.[12]

We confirmed the severity of striatal dopamine depletion also indirectly, through demonstration of the postsynaptic changes in the expression of PPT, PENK and GAD67 mRNAs. We found up-regulation of PENK and GAD67 mRNAs and down-regulation of PPT mRNA in the denervated striatum of 6-OHDA rats. Similar changes in the basal striatal neuropeptide mRNA levels in dopamine depleted striatum were shown only to occur when striatal dopamine depletion exceeds 90 % of.[28, 29]

The finding that acute administration of l-DOPA resulted in the elevation of PPT and GAD67 mRNAs in the denervated striatum of hemiparkinsonian rats is consistent with other studies.[9-11] While PPT mRNA is elevated only in direct pathway after acute l-DOPA treatment, GAD67 mRNA could be up-regulated in both, direct and indirect pathways.[30] The acute l-DOPA administration did not have any effect on PENK mRNA. This finding is also in agreement with an earlier report[24] that demonstrated that the denervation-induced up-regulation of striatal PENK mRNA could be affected only by chronic treatment with D2 agonists.

Many experiments strongly suggest the connection of behavioral sensitization with the molecular changes of the striatonigral neurons, implicating a D1 receptor-dependent mechanism.[31, 32] Striatal neurons in the direct pathway express D1 receptors use GABA and inhibit the internal globus pallidus (GPi), and substantia nigra pars reticulata (SNr).[1, 3] Striatal neurons in the indirect pathway that express D2 receptors use GABA and connect to the GPi/SNr *via* synaptic connection in the external globus pallidus (GPe) and subthalamic nucleus (STN). GPi/SNr neurons are then connected with cortex through thalamus. In the proposed model of l-DOPA-induced dyskinesias, the decreased firing of GPi/SNr is thought to result in an increase of thalamo-cortical drive leading to dyskinesias.[3] It has also been proposed that the appearance of abnormal pattern of discharges received from GPe-STN-GPi circuit could be responsible for the emergence of dyskinesias.[3]

To characterize the mechanisms that may mediate response of neurons to the stimulation of dopamine receptors after intermittent l-DOPA injections we examined the responsiveness of PPT, PENK and GAD67 mRNA levels. Our results show elevated responsiveness of GAD67 and PPT mRNA levels to the last l-DOPA treatment in dopamine-depleted striatum of rats sensitized by intermittent l-DOPA treatment, while the levels of PENK mRNA remained unaffected. This is in agreement with previous studies that also demonstrated that intermittent l-DOPA administration could lead to further increases in the responsiveness of GAD67 mRNA levels within dopamine-depleted striatum.[9] However, this is the first report demonstrating that the intermittent treatment with l-DOPA elevates also the responsiveness of PPT mRNA levels to the last l-DOPA injection. Knowing that PPT mRNA is expressed predominantly in neurons of the direct stria-

tonigral pathway, while PENK mRNA is expressed in indirect pathway and GAD67 mRNA in both, we conclude that in our experiment only the responsiveness of the direct pathway was elevated due to the intermittent l-DOPA treatment.

Most of the authors that have studied long-term changes of the striatal gene expression in the dopamine-depleted striatum after prolonged, daily treatment with l-DOPA, were studying the so called long duration response, by killing the animals several days after the last injection of l-DOPA. They showed that after chronic treatment with l-DOPA there is a reversal of some of the 6-OHDA-induced changes of striatal gene expression toward the pre-lesioned levels, however, the reversal was only transient.[10, 11] By killing the animals at earlier time-points after the last l-DOPA injection, we showed that the highly intermittent treatment with l-DOPA leads to an increased responsiveness of the expression of striatal GAD67 and PPT mRNAs. To best of our knowledge, there has been only one report revealing that repeated l-DOPA treatment elevates the responsiveness of striatal GAD67 mRNA expression to l-DOPA injections.[9] By analyzing the levels of GAD67 and PPT mRNA at 4 hours and 12 hours after the last l-DOPA injection we also detected the differences in the time-course of l-DOPA-induced changes. While the level of PPT mRNA was significantly elevated after 4 hours and declined already 12 hours after the last l-DOPA injection, striatal GAD67 mRNA levels were higher at 12 hours, as compared to the levels at 4 hours after the last l-DOPA injection.

We conclude that highly intermittent l-DOPA/carbidopa treatment schedule (6 injections of l-DOPA/carbidopa every fourth day) induces a substantial increase of dopaminergic responsiveness of the dopamine-depleted striatonigral neurons, corroborating the hypothesis that these neurons may play a crucial role in the development of l-DOPA-induced dyskinesias.

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Abbreviation list

ANOVA, analysis of variance;
GAD67, GABA synthesizing enzyme glutamate decarboxylase MW = 67.000;
GPe, globus pallidus pars eksterna;
GPi, globus pallidus pars interna;
PD, Parkinson's disease;
PENK, proenkephalin;
PPT, preprotachykinin;
ROD, relative optical density;
SNc, substantia nigra pars compacta;
SNr, substantia nigra pars reticulata;
6-OHDA, 6-hydroxydopamine.