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Quantitative Autoradiographic Study on Receptor Regulation in the Basal Ganglia in Rat Model of Levodopa-induced Motor Complications*

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Summary

In order to study neurotransmitter receptor regulation in the basal ganglia involved in the functional changes underlying levodopa-induced motor complications, quantitative autoradiography was used to observe receptor bindings of dopamine D1 and D2, N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) and amino butyric acid (GABA) in the basal ganglia of rats that had unilateral nigrostriatal lesions and had been chronically treated with levodopa until motor complications developed. The rats were randomly assigned to three groups: normal, denervated and treatment-complicated groups. The results showed that response duration to levodopa became progressively shorter and abnormal involuntary movement (AIM) score was progressively increased during the course of levodopa treatment. Chronic treatment augmented D1 receptors more than denervation, and reduced D2 receptors that were also increased by dopamine denervation. Striatal NMDA receptors were substantially up-regulated in the treatment-complicated group. Levodopa treatment did not change receptors of nigral AMPA, pallidal GABA, and subthalamic GABA, which remained the same as that in denervation group. However, chronic treatment reversed the increase of nigral GABA receptors caused by the lesion. It was concluded that a shortening of response duration and AIM mimicked levodopa-induced motor complications of Parkinson's patients. These data suggested that up-regulation of dopamine D1 and NMDA receptors in the striatum leads to an imbalance of stimulation through the striatal output pathways, which is associated with levodopa-induced motor complications.

Keywords

levodopa-induced motor complications; Parkinson's disease; glutamate receptors; GABA receptors; dopamine receptors; autoradiography

Motor complications associated with dopamine replacement treatment are the common problems that affect long-term efficacy of the therapy of Parkinson's disease (PD). Among these complications, response fluctuations and dyskinesias, appear to be related to drug

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exposure as much as disease progression^[1, 2]. The post-synaptic side plays a major role^[3, 4]. Although dopamine action is mediated mainly through dopamine D1 and D2 receptors located on striatal medium spiny neurons, the striatum and the basal ganglia output are broadly influenced by other neurotransmitter systems^[5, 6]. Glutamate is utilized by corticostriatal terminals as well as subthalamic nucleus (STN) neurons projecting to both segments of the globus pallidus (internal-GPi and external-GPe) and substantia nigra pars reticulata (SNr). Amino butyric acid (GABA) mediates the transmission from striatal output neurons to pallidal segments, and is used by both GPi and GPe neurons. The receptor status of the various neurotransmitter systems associated with those complications remains uncertain. To understand the functional implications of multiple receptor regulation in basal ganglia, binding sites of different receptors have to interact with each other. In this study, we examine the expression of various transmitter receptors in basal ganglia under standardized conditions of dopamine loss and motor complications induced by chronic treatment. We used a PD rat model for quantitative autoradiographic determination of dopamine D1 and D2, GABA, NMDA and AMPA receptors.

1 MATERIALS AND METHODS

1.1 Main Reagents

6-hydroxydopamine (6-OHDA), apomorphine, L-3, 4-dihydroxyphenylalanine methyl ester (Levodopa), and benserazide were obtained from Sigma-RBI (St. Louis, MO, USA). ¹²⁵I-SCH 23982, [³H]-MK-801, [³H]-AMPA were purchased from DuPont/NEN, Boston, USA. [³H] Muscimol and ¹²⁵I-sulpiride were procured from Amersham Inc., Arlington Heights, USA. ³H-ultrafilm were purchased from LKB Instruments Inc. Gaithersburg, USA.

1.2 Preparation of PD Rat Model and Drug Treatment

Male SD rats, provided by the Experimental Animal Center of Tongji Medical College, weighing 240–260 g were allowed to habituate for 1–3 d prior to surgery. Unilateral 6-hydroxydopamine (6-OHDA) lesions of the left nigrostriatal dopaminergic system were induced as previously described^[7]. To determine the result of the lesion, animals were tested for rotation induced by apomorphine (0.05 mg/kg, sc, dissolved in 0.2% ascorbic acid in saline) three weeks after surgery, and the rats that rotated contralateral to the lesion more than 100 turns were considered to be PD rats and included in the study.

Following a period of 3 to 5 d after the apomorphine screening, 13 PD rats were given levodopa methyl ester plus benserazide (25/6.25 mg/kg, ip, dissolved in saline) once a day for 29 days and assigned to the levodopa-treated group. Six PD rats received saline instead of levodopa and served as control.

1.3 Behavioral Assessment

1.3.1 Motor Response Duration—The rotational number during any 5 min interval following levodopa administration was recorded. The duration of the turning response was measured as the efficacy half-time (time from first interval when turning exceeded the half-average rate to first interval when it fell below the half-average rate) of levodopa-induced contraversive turning.

1.3.2 Levodopa-induced Abnormal Involuntary Movement (AIM)—AIM including stereotypy and contraversive rotation, were quantified using the rat AIM rating scale as described by Lee^[8].

1.3.3 Fore-limb Akinesia (Stepping Test)—The rat was held by the experimenter with one hand fixing the hind-limbs and slightly raising the hind part. The other hand fixed the

fore-limb not to be monitored, and then moved slowly sideways (5 s for 0.9 m). The number of adjusting steps was counted for right paw (contralateral to the lesion) in the fore-hand directions of movement. The test was performed 30 and 180 min after levodopa treatment as “on” stage and “off” stage, respectively.

1.4 Tissue Preparation and Autoradiographic Procedure

At the end of the treatment period, animals were sacrificed by decapitation and the brains were removed, immediately frozen in isopentane, and stored at -70°C until sectioning. Coronal sections of 20- μm thick were cut in a cryostat at -20°C and thaw-mounted onto gelatin-coated slides. Sections were taken by duplicate for total and non-specific binding, at four regions of the brain: striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra, pars reticulata (SNr). The slides were dried on a warming plate and stored at -70°C until binding assay.

Quantitative receptor autoradiography for dopamine D1 and D2, NMDA, AMPA and GABA binding sites was performed with ^{125}I -SCH 23982, ^{125}I -Sulpiride, ^3H -MK-801, ^3H -AMPA and ^3H -muscimol, respectively. Autoradiography was performed as previously described by Penney *et al*^[9]. Dopamine D1 and D2 receptor binding sites were assayed in the striatum, and the D1 binding was also assayed in the substantia nigra. NMDA, AMPA and GABA binding sites were studied in all five regions described above. Receptor binding was compared between different states: normal (intact side of control), denervated (lesioned side of control), and treatment-complicated (lesioned side of levodopa treated group). NMDA receptor binding was determined in the presence of glutamate and glycine. All assays were performed at a single concentration of the ligand (K_d). Slides were apposed to LKB ^3H -ultrafilm along with ^3H or ^{125}I micro-scale standards (Amersham, USA) containing known amounts of radioactivity per tissue wet weight (nCi/mg). Exposure time was 6 h (4°C) for dopamine receptor binding, 3 weeks (at room temperature) for excitatory amino acid receptor binding and 10 d (room temperature) for GABA receptor binding. Films were developed in Kodak D-19.

Ligand binding was quantitated with computer-assisted densitometry using a Macintosh-based image analysis system. The optical density of standards was determined and a standard curve was obtained using a third-degree polynomial regression equation. Optical density measured in autoradiograms was converted into nCi/mg wet weight of tissue from the standard curve. For each region, both total and non-specific binding was measured in two sections for each brain. Then values were averaged and specific binding was calculated by subtraction of the non-specific binding from the total binding. Knowing the specific activity of the ligand, values (nCi/mg) were finally converted to fmol/mg wet weight of tissue.

1.5 Statistical Analysis

Differences in specific binding (fmol/mg wet weight tissue) between the lesioned and intact sides in the control and levodopa-treated group were analyzed together by two factor analysis of variance followed by the Duncan's test for areas in which the F value was significant. Values were expressed as $\pm s$, and $P < 0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Behavioral Responses to Levodopa Following Repeated Administration in PD Rats

2.1.1 Response Duration—The response duration to levodopa became progressively shorter during a course of 29 d of treatment with levodopa (149.62 ± 11.55 , 118.46 ± 8.52 ,

98.08±4.58, 93.85±5.13 and 88.08±2.86 min on day 1, 8, 15, 22 and 29, respectively), and reduced by 41% from day 1 to day 29 ($P<0.01$).

2.1.2 Levodopa-induced AIM—Levodopa-induced AIM during the induction period included stereotypy and increased contraversive rotation. Stereotypy, only found on the side contralateral to 6-OHDA lesion, included limb dyskinesia, axial dyskinesia and masticatory dyskinesia, and accompanied by contraversive rotation. The AIM score was progressively increased during levodopa treatment and reached a peak by day 22 and then remained at a plateau until day 29 (0.39±0.27, 4.62±0.96, 5.31±0.73, 10.08±0.91 and 9.69±0.82 on day 1, 8, 15, 22 and 29, respectively). On day 22 and 29, the AIM score was significantly higher than that of control group and on days 1, 8, 15 ($P<0.01$).

2.1.3 Fore-limb Akinesias—At “off” stage, there was a highly significant impairment in right paw performance compared with at “on” stage (1.1434±0.0682 vs. 2.0016±0.1008 on day 1) ($P<0.01$), which resulted in a dragging paw when the rat was moved sideways by the experimenter. However, right paw performance was progressively impaired at “on” stage during levodopa treatment (2.3157±0.1799, 1.8927±0.2031, 1.5338±0.1271 and 1.6625 ± 0.1678 on day 8, 15, 22 and 29, respectively), and reached a plateau at day 22. On day 22 and 29, the steps were significantly less than that on days 1, 8, 15 ($P<0.01$).

2.2 Autoradiographic Study for Ligand Binding

2.2.1 Dopamine Receptor Binding—Striatal dopamine D1 receptor binding increased after dopamine denervation and was further augmented by subsequent levodopa treatment (fig. 1A and 1B). Binding density was 20% higher on the lesioned side than the intact side in the untreated group ($P<0.05$), and increased by an additional 32% on the lesioned side in the levodopa-treated group ($P<0.01$). In the SNr, dopamine D1 receptor binding was not affected by 6-OHDA-induced lesions, but rose by 19% on the lesioned side as a result of levodopa treatment ($P<0.05$) (fig. 1C and 1D, table 1).

Striatal dopamine D2 receptor binding increased by 23% after dopamine denervation ($P<0.01$) (fig. 1E). In contrast to its effects on D1 receptors, chronic levodopa administration partially reversed the lesion-induced rise in striatal D2 receptor binding (from 23% to 11%, $P<0.01$) (fig. 1F, table 1).

2.2.2 Excitatory Amino Acid Receptor Binding—The striatal NMDA receptor binding tended to diminish after nigrostriatal dopaminergic lesions although the decline was not statistically significant (fig. 1G). Chronic levodopa treatment substantially increased striatal NMDA receptor binding. Binding density was 39% higher in the levodopa-treated group than the same side in the untreated group ($P<0.05$) (fig. 1H). Nigral NMDA receptor binding declined by 16% after dopamine denervation. The difference between the lesioned and intact side from denervated and treatment-complicated groups taken together showed statistically significant ($P<0.05$), which reflects a net effect of the lesion and no effect of levodopa treatment. NMDA receptor binding in GP and STN was not altered by either dopamine denervation or chronic levodopa administration (table 2).

Nigral AMPA receptor binding was down-regulated by nigrostriatal dopaminergic lesions. Binding density was 18% lower on the lesioned side than the intact side in the untreated group ($P<0.01$) (fig. 1I). No differences in AMPA receptor binding between the lesioned and intact side were found in other regions, and levodopa treatment did not change AMPA receptor binding in any region (fig. 1J, table 3).

2.2.3 Gamma-amino Butyric Acid Receptor Binding—Pallidal GABA receptor binding decreased by 20% following dopamine denervation ($P<0.01$, comparison between total values of intact and lesioned sides, as described above for nigral NMDA receptor binding) and was unaffected by levodopa treatment (fig. 2A and 2B). In the STN, GABA receptor binding increased by 19% after dopamine denervation ($P<0.05$, comparison between total values of intact and lesioned sides) (table 4), and levodopa administration produced no further change. Nigral GABA receptor binding augmented by 28% after 6-OHDA lesions ($P<0.01$) (fig. 2C). Chronic levodopa treatment reversed the effects of the lesion in the SNr. Binding density was 32% lower ($P<0.01$) on the lesioned side of the treated group than the same side of the control group (fig. 2D). Striatal GABA receptor binding was not changed by effect of dopamine denervation or levodopa treatment.

3 DISCUSSION

3.1 Animal Model

Unilateral 6-OHDA-lesioned rat has been widely used as rodent model of PD, which exhibits some behavioral changes following repetitive levodopa treatment. Previous data showed a shortening of response duration along with failure to respond to levodopa treatment developed progressively, and these features mimic the “wearing-off” phenomenon and “on-off” fluctuations of Parkinson’s patients^[7] and levodopa-induced AIM in PD rats shares the same characteristics as peak-dose dyskinesia in patients with PD, and is considered to be animal model of levodopa-induced dyskinesia (LID)^[8]. In our study, PD rats gradually exhibited AIM of neck, trunk and forelimb on the side contralateral to the dopamine-denervated striatum, which showed an increasing severity upon repeated administration of the same drug dose. The duration of response to levodopa became progressively shorter during the course of levodopa treatment. These behavioral changes are consistent with previous studies.

Fore-limb stepping is a sensitive test to monitor lesioned/and drug-induced changes in fore-limb akinesia in PD rats as suggested by Olsson *et al*^[10]. Therefore, stepping test was performed to evaluate anti-Parkinsonian action of levodopa in this study. The results showed that there was a highly significant impairment in right paw at “off” stage, which reflects loss of anti-Parkinsonian action of levodopa, and right paw performance was progressively impaired at “on” stage during levodopa treatment, which reflects progressively impaired anti-Parkinsonian action of levodopa following chronic levodopa treatment. The phenomenon parallels effect impairment of levodopa in PD patients.

3.2 Dopamine Receptor

In this study, binding changes in our levodopa-treated animals are correlated to motor complications of long-term levodopa therapy. We found that treatment differentially regulated dopamine D1 and D2 receptors. Following denervation-induced up-regulation of both receptors, levodopa treatment further increased striatal D1 receptors while it decreased D2 receptors. Nigral D1 receptors also rose after treatment. Thus, denervation-induced dopamine receptor supersensitivity becomes D1 receptor supersensitivity after chronic treatment. These data are consistent with previous studies on expression of several immediate early genes^[11, 12], and changes of peptides’ levels such as dynorphine and enkephaline^[13]. Noteworthy, D1 receptor supersensitivity appears to derive from intermittent, but not continuous, drug administration. This suggests that normal expression of D1 receptors depends upon the more physiological sustained dopamine stimulation. On the other hand, the disparate sensitivity expressed by D1 and D2 receptors with intermittent treatment represents an imbalance of dopamine stimulation. This imbalance seems to turn the dopamine action on both neuronal populations to excitatory effect, which results in D1

supersensitivity. Whether an imbalance of receptor-mediated mechanisms is the key factor responsible for levodopa-related motor complications remains to be determined. Nevertheless, a prominent role of the D1 receptor in motor complications appears indubitable. This notion is also in line with the recent study by Picconi *et al*^[14] who studied striatal synaptic plasticity in relation to LID and showed that specific changes occurred along the dopamine D1 receptor signaling pathway.

3.3 Excitatory Amino Acid Receptors

Striatal NMDA receptors tended to drop after dopamine denervation, although this decrease was not statistically significant. In the SNr, however, NMDA receptor binding reduced significantly because of the lesion. In rodents, SNr and EPN form the output basal ganglia nuclei, analogues of GPi in primates. Because the glutamatergic input to the SNr derives from the subthalamic projection, down-regulation of nigral NMDA receptors represents overactivity of STN neurons, and this is in keeping with the current knowledge of function and anatomy of basal ganglia^[15, 16]. Moreover, nigral AMPA receptors substantially decreased after development of dopaminergic lesions. In fact, studies of glutamate receptor distribution in basal ganglia demonstrated that the most predominant subtype in SNr is the AMPA receptor^[17]. Thus, our data strongly suggest that dopamine denervation results in increased the effect of glutamatergic STN on the output nuclei of basal ganglia through both NMDA and AMPA receptors. It might work by blocking subthalamic inputs to the GPi and thereby AMPA antagonists potentiate the anti-Parkinsonian effects of levodopa.

Following chronic treatment, striatal NMDA receptors were markedly up-regulated, resulting in a state of striatal NMDA supersensitivity. The concept of striatal NMDA supersensitivity in relation to levodopa-induced motor complications is consistent with findings of behavioral studies. Different from intranigral infusion, intrastratial infusion of the NMDA antagonist MK-801 increased the shortened response duration after chronic administration of levodopa^[18]. NMDA antagonists were also shown to possess anti-dyskinetic effects in MPTP-treated primates. Systemically administered drugs, such as the competitive NMDA antagonist LY235959 or the allosteric NMDA inhibitor Co101244, markedly decrease LID^[19, 20]. The effects on dyskinesias may be mediated through blocking striatal supersensitive receptors. This notion of supersensitivity is further supported by changes in the functional state of receptors. Increase in phosphorylation of protein residues (tyrosine and serine) in striatal NMDA receptor subunits is associated with levodopa-related motor complications^[21, 22].

Our study failed to find any effect of levodopa treatment on nigral AMPA receptors, which was as low as that following dopamine denervation. Persistent down-regulation of AMPA receptors is indicative of a diminished restorative function of dopamine replacement. Dopamine receptors located on STN cells might also be involved in maintaining an increased STN output to the SNr^[23].

3.4 Aminobutyric Acid Receptors

Following dopamine denervation, GABA receptors were reduced in GP and increased in STN. The GP in rodents is the homologue of GPe in primates. Thus, GABA binding in GP and STN was consistent with the expected functional changes after loss of inhibitory dopamine action on indirect striatal output neurons. Overactivity of striatal cells, with increased gabaergic output, down-regulated pallidal GABA receptors and, secondarily, excessively inhibited the GP, with the reduced gabaergic influence on STN, and resulted in up-regulated subthalamic GABA receptors. Moreover, we found a substantially increased nigral GABA receptors after denervation. With the direct pathway, because of suppression

of excitatory dopamine action, the striatum-SNr/EPN connection becomes hypoactive, and GABA receptors in those regions are thus up-regulated.

Chronic treatment of the rats with levodopa completely reversed the increased nigral GABA receptors caused by denervation. Our results were consistent with increased gabaergic influx from direct striatal projections, which is most likely related to up-regulated striatal dopamine D1 and NMDA receptors. In contrast, with the indirect pathway, levodopa treatment did not change the GP and STN GABA binding. These data are in line with the foregoing lack of changes of nigral NMDA/AMPA binding after levodopa treatment. Thus, while functional alterations in the indirect pathway remain unchanged, hypoactivity in the direct pathway is completely reversed after chronic treatment. These results suggest that levodopa-induced motor complications are associated with imbalanced activity of the output pathways, and conceivably with augmented activity through the direct pathway.

3.5 Implications for PD

In conclusion, findings about receptor binding suggest that levodopa-induced motor complications are associated with hyper-activity of striatal dopamine D1 and NMDA receptors, that leads to an imbalanced stimulation through the striatal output pathways. The mechanisms by which increased function of those receptors leads to such imbalance remain elusive. However, up-regulation of D1 and NMDA receptors may be induced by transcription factors that regulate gene expression^[24-26]. These transcription factors correspond to the family of immediate-early genes (IEGs) that rapidly respond to a variety of stimuli, although CREB can also be linked to long-term processes like memory and learning^[27, 28]. Conceivably, specific transcription factors related to chronic treatment might regulate the expression of receptor subunits, like some NMDA subunits^[29, 30]. This regulatory process may play a major role in the pathogenesis of motor complications. This study strongly suggests that the drugs targeting the glutamate system, especially the NMDA division, can be used for the treatment of advanced PD.

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Fig. 1.

Autoradiograms of striatal and nigral dopamine D1 (A-D), striatal dopamine D2 (E, F), striatal NMDA (G, H), and nigral AMPA (I, J) receptors. Pictures on the left of each pair correspond to the control group (A, C, E, G, I), on the right to the levodopa-treated group (B, D, F, H, J). Arrows indicate the striatum or SNr in the lesioned sides. Darker images denote an increase in binding density, as shown by dopamine and NMDA receptors in the lesioned sides. AMPA binding is decreased (lighter) in the lesioned sides. Contrast and brightness of the images have been adjusted digitally for visual clarity.

Fig. 2.

Autoradiograms of GABA receptors Pictures correspond to sections through the GP and SNr from control (A and C) and levodopa-treated (B and D) groups. Arrows indicate the GP (A and B) and SNr (C and D) in the lesioned sides. Pallidal GABA binding is decreased (lighter) in the lesioned side of both groups. Nigral GABA binding is increased in the lesioned side of the control group and is decreased to the same level of the unlesioned side in the levodopa-treated group. Contrast and brightness of the images have been adjusted digitally for visual clarity.

Table 1

Dopamine receptor autoradiographic binding values (fmol/mg tissue, $\bar{x} \pm s$)

Groups	Dopamine D1				Dopamine D2			
	Striatum		SNr		Striatum		Striatum	
	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side
Control	3.8±0.59	4.6±0.59 ^a	3.3±0.38	3.4±0.30	4.4±0.22	5.4±0.26 ^b		
Levodopa	4.6±0.58	6.1±0.69 ^{b, d}	2.7±0.39	4.0±0.49 ^{b, c}	4.2±0.12	4.8±0.15 ^d		

^a $P<0.05$

^b $P<0.01$ vs intact side (same group);

^c $P<0.05$

^d $P<0.01$ vs control group (same side)

Table 2

NMDA receptor autoradiographic binding values (fmol/mg tissue, $\bar{x} \pm s$)

Groups	Striatum		GP		STN		SNr	
	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side
Control	164±15.4	133±12.2	25.6±6.3	34.8±5.9	29.9±5.6	37.1±6.9	45.3±2.7	38.3±4.9 ^e
Levodopa	158±18.7	185±21.4 ^c	38.6±15.2	34.5±8.4	50.7±8.4	42.4±4.2	37.4±4.9	31.1±5.9 ^e

^c $P<0.05$ vs control group (same side);

^e $P<0.05$ lesioned side vs intact side from both groups taken together.

Table 3

AMPA receptor autoradiographic binding values (fmol/mg tissue, $\bar{x} \pm s$)

Groups	Striatum		GP		STN		SNr	
	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side
Control	404±43.3	441±29.2	95.9±5.2	93.6±11.1	111±11.0	113±9.3	113±13.4	92.9±11.8 ^b
Levodopa	481±41.7	435±44.2	89.4±10.8	83.9±10.5	104±11.3	102±10.7	97.4±10.4	88.1±6.9

^b $P<0.01$ vs. intact side (same group)

Table 4

GABA receptor autoradiographic binding values (fmol/mg tissue, $\bar{x} \pm s$)

Groups	Striatum		GP		STN		SNr	
	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side	Intact side	Lesioned side
Control	266±30.4	252±21.7	208±13.7	173±4.7 ^f	146±19.0	167±18.3 ^e	303±35.5	387±48.5 ^b
Levodopa	263±15.4	269±15.2	230±34.2	179±14.7 ^f	126±15.3	158±23.1 ^e	247±16.7	264±25.0 ^d

^b $p < 0.01$ vs intact side (same group);

^c $p < 0.05$

^d $p < 0.01$ vs control group (same side);

^e $p < 0.05$

^f $p < 0.01$ lesioned side vs. intact side from both groups taken together