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COMPARATIVE EFFECTS OF ACUTE OR CHRONIC ADMINISTRATION OF LEVODOPA TO 6-HYDROXYDOPAMINE-LESIONED RATS ON THE EXPRESSION OF GLUTAMIC ACID DECARBOXYLASE IN THE NEOSTRIATUM AND $GABA_A$ RECEPTORS SUBUNITS IN THE SUBSTANTIA NIGRA, PARS RETICULATA

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Abstract—Current evidence suggests that behavioral sensitization to the chronic administration of levodopa (L-DOPA) to dopamine-depleted animals involves a plasticity of GABA-mediated signaling in output regions of the basal ganglia. The purpose of this study was to compare in adult rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion the effects of an acute or chronic (for 3 or 7 days) injection of L-DOPA on mRNA levels encoding for glutamic acid decarboxylase (GAD65 and GAD67) in the striatum and $GABA_A$ receptor α1, β2 and γ2 subunits in the substantia nigra, pars reticulata (SNr), by *in situ* hybridization histochemistry. In addition, immunostaining levels for the α1 subunit were examined in the SNr. In agreement with previous studies, we found that L-DOPA administration increased GAD mRNA levels in the striatum of 6-OHDA-lesioned rats. However, the magnitude of this effect increased with the number of injections of L-DOPA. On the other hand, we found that 6-OHDA lesions resulted in increases in α1, β2 and γ2 mRNA levels in the ipsilateral SNr, which were normalized or decreased compared with the contralateral side by the acute or chronic administration of L-DOPA. In addition, α1 immunostaining in the SNr was significantly decreased in rats injected for 7 days but not for 3 days or acutely with L-DOPA. Our results demonstrate that a chronic administration of L-DOPA results in a progressive increase in GAD and decrease in $GABA_A$ receptor expression in the striatum and SNr, respectively. They provide further evidence that behavioral sensitization and dyskinesia induced by a chronic administration of L-DOPA in an experimental model of Parkinson's disease is paralleled by a plasticity of GABA-mediated signaling in the SNr.

Adult rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of dopamine neurons, a well-characterized model of Parkinson's disease, exhibit widespread alterations in cell activity throughout the basal ganglia. In particular, changes in gene expression have been documented, which show increased preproenkephalin (PPE) and glutamic acid decarboxylase (GAD; synthesizing enzyme of GABA) gene expression in striatopallidal neurons and decreased preprotachykinin and preprodynorphin gene expression in striatonigral/entopeduncular neurons (Lindefors et al., 1989; Gerfen et al., 1990; Soghomonian et al., 1992). In this model, it has also been shown that [^{3}H]-flunitrazepam binding to $GABA_A$ receptors is increased in the substantia nigra, pars reticulata (SNr), and entopeduncular nucleus (EP) and decreased in the globus pallidus on the side of the lesion (Pan et al., 1985; Gnanalingham and Robertson, 1993; Nielsen and Soghomonian, 2004). More recently, an increase in gene expression of the α1 and β2 subunits of the $GABA_A$ receptor has been evidenced in SNr neurons by *in situ* hybridization histochemistry (Chadha et al., 2000). Altogether, these findings are consistent with current models of Parkinson's disease, which propose that loss of dopamine input to the neostriatum results in decreased and increased GABA-mediated signaling in striatonigral/EP and striatopallidal neurons, respectively.

Levodopa (L-DOPA), the metabolic precursor of dopamine, remains the primary drug treatment for patients suffering from Parkinson's disease. Chronic systemic administration of L-DOPA to 6-OHDA-lesioned rats results in increased GAD and prepropeptide gene expression in striato-nigral/EP neurons (Engber et al., 1991; Cenci et al., 1998; Consolo et al., 1999; Henry et al., 1999; Carta et al., 2001, 2002; Bacci et al., 2002; Nielsen and Soghomonian, 2004). In accordance with other findings showing that L-DOPA can increase GABA release in slices of the SNr (Aceves et al., 1991), these gene expression studies suggest that the systemic administration of L-DOPA increases GABAergic input from the neostriatum to the SNr and EP. This hypothesis is corroborated by evidence that chronic systemic administration of L-DOPA to 6-OHDA-lesioned rats decreases levels of [^{3}H]-flunitrazepam binding to GABA receptors in both the SNr and EP (Gnanalingham and Robertson, 1993). In addition to its therapeutic effects, chronic administration of L-DOPA for several months/years can trigger abnormal involuntary movements such as dyskinesia. It has been hypothesized that abnormal involuntary movements may involve excessive GABAergic inhibition of SNr neurons by striatonigral/EP neurons (review in Obeso et al., 2000). Consistent with this hypothesis, it has been reported that the severity of abnormal involuntary movements induced by chronic L-DOPA in rats with a unilateral 6-OHDA lesion correlates with the magnitude of increased GAD67 mRNA levels in the neostriatum (Cenci et al., 1998). Rats with a unilateral 6-OHDA

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Abbreviations: ANOVA, analysis of variance; EP, entopeduncular nucleus; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; L-DOPA, levodopa; OD, optical density; PPE, preproenkephalin; SNr, substantia nigra, pars reticulata; 6-OHDA, 6-hydroxydopamine.

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lesion of dopamine neurons exhibit a rotational behavior following the administration of L-DOPA. The number of rotations is known to increase with the daily repeated administration of the same dose of L-DOPA and is typically observed in rats with an extensive loss (i.e. more than 90%) of dopamine neurons (e.g. Rouillard et al., 1987; Papa et al., 1994; Thomas et al., 1994; Henry et al., 1998). The basis for this model of behavioral sensitization is unclear and could be explained by a progressive increase and/or alteration of GABA-mediated signaling in striatonigral/EP neurons. To further investigate this hypothesis, we examined the effects of an acute or chronic systemic administration of L-DOPA to adult rats with a unilateral 6-OHDA lesion on mRNA levels encoding for the two isoforms of GAD, GAD67 and GAD65, in the neostriatum and for subunits of $GABA_A$ receptors in the SNr using *in situ* hybridization histochemistry. In addition, immunohistochemistry was used to examine the effects of L-DOPA on levels of the α1 subunit of the $GABA_A$ receptor in the SNr.

EXPERIMENTAL PROCEDURES

Animals

A total of 27 adult male Sprague–Dawley rats (Charles River, Wilmington, MA, USA) were used in this study of which 15 were used in a first subgroup (vehicle, acute and 3 days of L-DOPA administration) and 12 were used in a second subgroup (vehicle and 7 days of L-DOPA administration). Rats were divided into two different subgroups because the total number of histological sections used in the entire study was too large to process in a single experiment. All animals were housed in a colony room maintained at 23 °C with a 12-h light/dark cycle. Food and water were available *ad libitum*. Every effort was made to minimize the suffering and the number of animals used in this study. All experimental procedures were performed in full accordance with the Institutional Animal Care and Use Committee guidelines at Boston University School of Medicine and were consistent with international guidelines on the ethical use of animals.

Unilateral 6-OHDA lesions and L-DOPA treatment

Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally and placed into a stereotaxic frame. All rats underwent unilateral dopamine lesion generated by intracerebral injections of 6-OHDA (8.0 μg of free-base in 2 μl of saline with 1.0% ascorbic acid) into the left middle substantia nigra, pars compacta (A/P=3.4 mm, L=2.0 mm, H=2.8 mm) and the left caudal MFB (A/P=4.2 mm, L=1.2 mm, H=1.9 mm) with the incisor bar at 0 mm. Manual injections were administered at a rate of 0.5 μl/min over a 2-min period and the cannula was left in place for an additional 5 min post-injection. Twenty-one days after the 6-OHDA lesion, rats received s.c. injections of vehicle or L-DOPA. In the first subgroup, rats received vehicle, a single injection of L-DOPA (acute) or two daily injections of L-DOPA for 3 days. In the second subgroup, rats received vehicle or two daily injections of L-DOPA for 7 days. L-DOPA (L-DOPA methyl ester; Sigma Chemical Co., St. Louis, MO, USA) was injected at a dose of 100 mg/kg/day along with benserazide at a dose of 25 mg/kg/day (Sigma Chemical Co.) in order to inhibit peripheral decarboxylase activity. All solutions were made fresh each day prior to the injections.

Behavioral testing and tissue preparation

Rotational behavior was evaluated for 180 min immediately following the morning injection of L-DOPA on days 1, 3 and 7. The rats were habituated to rotation bowls prior to the injections and rotational behavior was assessed using an automated rotometer (Columbus Instruments, OH, USA). To obtain the net number of rotations, ipsilateral turns were subtracted from contralateral turns and expressed as mean±S.E.M. Statistical analysis of rotations values between days was carried out with a repeated measures analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons test, significance at $P<0.05$. Only rats that exhibited a number of rotations of more than three per minute following the first injection of L-DOPA were used for this study. Earlier studies have documented that such rotational scores occur in rats with an extensive lesion of dopamine neurons (Thomas et al., 1994; Metz and Whishaw, 2002). In addition, the extent of the lesion was further confirmed by postmortem analysis of [^{3}H]-mazindol binding levels in the striatum as previously described (Nielsen and Soghomonian, 2004). In particular, all 6-OHDA-lesioned rats used in this study including those injected with vehicle had more than 95% loss of mazindol binding in the striatum. Three hours following the last injection of L-DOPA or vehicle, rats were sedated with CO_2 vapors and killed by decapitation. The brains were dissected out and stored at −70 °C until further use. Ten micrometer-thick cryostat-cut sections at the level of the striatum (A3.40 to A3.70 according to Paxinos and Watson's atlas) or substantia nigra (A10.00–A10.20) were collected on gelatin-coated slides. Slides were kept at −70 °C until processing for *in situ* hybridization or immunohistochemistry.

In situ hybridization

For the visualization of GAD mRNA, sections from the neostriatum were processed for *in situ* hybridization histochemistry using [^{35}S]-radiolabeled complementary (cRNA) riboprobes for GAD67 or GAD65 as previously described (Nielsen and Soghomonian, 2004). Briefly, 10 μm-thick coronal sections of the neostriatum were quickly dried under a constant flow of air at room temperature and immediately fixed for 5 min in 3% paraformaldehyde in a phosphate buffer (pH 7.2). Subsequently, sections were rinsed in 2× SSC and 0.1 M phosphate buffer before being washed for 10 min in 0.1 M triethanolamine with 0.25% acetic anhydride, for 30 min in 1 M Tris–glycine and then dehydrated in ethanol. Sections were then hybridized at 52 °C for 4 h with 4 ng in 20 μl of radiolabeled GAD65 or GAD67 cRNA probe diluted in a hybridization solution containing 40% formamide, 10% dextran sulfate, 4× SSC, 10 mM dithiothreitol, 1.0% sheared salmon sperm DNA, 1.0% yeast tRNA and 1× Denhardt's solution and incubated overnight at 42 °C. Post-hybridization washes were done sequentially in 50% formamide at 52 °C for 5 and 20 min, in RNase A (100 μg/ml; Sigma Chemical Co.) and 50% formamide for 5 min at 52 °C. At the end of the experiment, sections were dehydrated and apposed to Kodak Biomax X-ray films for 7 days at room temperature.

For the visualization of $GABA_A$ receptor subunits, [^{35}S]-dATP-radiolabeled oligonucleotide probes specific for the α1, β2 or γ2 subunits were used as previously described (Chadha et al., 2000; Wisden et al., 1992). Brain sections at the level of the SNr were dried under a flow of air, fixed and rinsed in pre-hybridization solutions as described above for riboprobe hybridization. Each section was then hybridized with 3×10^5 cpm in 50 μl of radiolabeled oligonucleotide probe. Post-hybridization washes were carried out at 42 °C in 1× SSC for 1 h and 0.5× SSC for 30 min. Following ethanol dehydration, slides were dipped in Kodak NTB3 nuclear emulsion (diluted 1:1 with water containing ammonium acetate), dried for 3 h and stored in light-tight boxes for 10 days. Slides were developed in Kodak 19 developer, stained with hematoxylin and eosin and mounted with Eukitt mounting medium. The sequences of $GABA_A$ receptor subunit oligonucleotide probes used in this experiment are shown below:

Subunit sequence bp

α1 GGGGTCACCCCTGGCTAAGTTAGGGGTATAGCTGGT TGCTGTAGG 1162–1206