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Original paper

6-HYDROXYDOPAMINE LESIONS OF THE STRIATUM LEAD TO THE ALTERATIONS OF DOPAMINE RECEPTOR mRNA IN PARKINSONIAN RATS

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Summary: The effects of four-site intrastriatal 6-hydroxydopamine (6-OHDA) lesions were examined in adult male rats. Five days after the lesions the animals were checked for specific rotational behavior induced by middle dose of amphetamine and the results confirmed the effectiveness of the lesions. The RNAs from the striatum were isolated at different time points after the lesion, and the RT-PCR analyse were performed for the D1 and D2 receptor mRNA. The results show a decline in the D2 receptor mRNA level (40%) at 6 h and 24 h points while this change was not observed seven days after the lesion. In contrast, no statistically significant changes in the level of the D1 receptor mRNA after the lesion at any time point were found.

Key words: Parkinsonian rats, 6-OHDA lesions, behavior, D1 and D2 mRNA, RT-PCR.

Introduction

Parkinson's disease (PD) is a common neurological disorder with progressive degeneration of dopaminergic (DA) neurons in substantia nigra pars compacta that is a cause of the main neurological signs associated with PD. Among many animal models of PD, the experimental lesion of nigrostriatal system by 6-hydroxydopamine (6-OHDA) is an excellent model for the study of the adaptive mechanism in response to the loss of DA neurons and for testing pharmacologically active compounds and agents (1). 6-OHDA is a neurotoxin relatively specific for the catecholaminergic neurons and its functional effects depend on both total dose and the site of the injection (1 3). Behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system in the rat, indicate that the four-site intrastriatal 6-OHDA lesion (with 70 80% destruction of DA neurons) may be a relevant model of the neuropathology seen in parkinsonian patients (2, 4 5).

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Unilateral 6-OHDA lesion induces behavioral stereotypies, as well as cellular and molecular modifications in the striatal target tissue, on the dopamine (DA) and dopamine receptor (DA R) levels (6 7). The actions of dopamine are mediated via five different receptor subtypes which are the members of two dopamine receptor classes, D1-like and D2-like. The D1-like receptors include the D1 and the D5 receptor and D2-like receptors include the D2 (with D2long and D2short isoforms), D3 and D4 receptor. The D1 and D2 receptors are the most abundant subtypes in the central nervous system (CNS) and display specific anatomical distribution (8). Although, there are still controversies about co-localization of D1 and D2 receptors, they are mostly present in nonoverlapping, medium-sized, spiny projection neurons throughout the dorsal and ventral striatum. These neurons form the two major output pathways of the striatum and the balance between these pathways is crucial for the normal functioning of the basal ganglia (9 10). Lesions of the dopamine system, such as those occurring in PD, disrupt this balance and lead to the changes in the DA receptor number and sensitivity.

In this paper, we investigated the effects of four site intrastriatal 6-OHDA lesions in adult male rats on behavioral stereotypies and changes in the D1 and D2 receptor mRNA levels at early time points after the lesions, since these changes haven't been extensively studied so far.

Material and methods

Animals and surgical procedures

Adult Mill-Hill hooded male rats, three months old (200 300 g), were housed 2 4 per cage with a 12:12 hour light-dark cycle, at 22±2 °C. Food and water were provided ad libitum. The animals anesthetized with thiopental (50 mg per kg i.p.) received four unilateral stereotaxic injections of 6-OHDA, 4 ×7 μg in a volume of 8 μL, into the right striatum, using a 10-μL Hamilton micro syringe fitted with a 26-gauge still cannula. Coordinates are: AP: +1.3 +0.4 0.4 1.3, ML: 2.6 3.0 4.2 4.5, DV 5.0 mm according to the Paxinos and Watson atlas (5, 11). The 6-hydroxydopamine-hydrobromide (ICN Pharmaceuticals, CA, U.S.A.) was dissolved in ice-chilled 154 mmol/L NaCl with 10 g/L ascorbic acid added to retard the oxidation and the solution was kept cold and protected from exposure to light, during the experiment. The injection rate was 1 μL/min. After each 2 μL of 6-OHDA solution, the cannula was allowed to remain in the brain for 2 min. The incision was closed and treated with antibiotic. Control rats were injected with 10 g/L ascorbic acid in 154 mmol/L NaCl solution.

Behavioral analysis

One week before the lesion, all animals were tested for rotational behavior induced by middle dose of amphetamine (3 mg/kg). Fifteen min after *i.p* injection, rotations were monitored in animal cage for 10 min, and right (CR) and left (CCR) full body turns were observed. Five days after the lesion, the same behavioral experiment was repeated in 6-OHDA-lesioned and control animals, to confirm the effectiveness of the lesion.

RNA isolation

Animals were divided into three experimental groups and sacrificed at different time points after the lesion and the brains were quickly removed. RNA was isolated by the guanidine isothiocyanate (GTC)/cesium chloride (CsCl) centrifugation method (12 14). Seventen striata, left and right separately, were homogenized in 7 mL of GTC puffer containing 4 mol/L GTC, 40 g/L N-lauryl sarcosine, 50 mmol/L Na-acetate, pH 5.5 and 10 g/L β-mercaptoethanol. Dry CsCl, 0.5 g/mL, was added to the homogenate and then layered on 3 mL of CsCl, density of 1.7 g/cm^3, for ultracentrifugation (24 h, 38 000 rpm in a Ti-50 rotor). The obtaining pellets were suspended in precipitation buffer containing 10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA and 5 g/L SDS. After butanol/chlorophorm extraction, the pellets were suspended in one volume of 3 mol/L Na-acetate, pH 5.0 and two volumes of absolute cold ethanol and kept overnight at 20 °C. The solutions were centrifuged (20 min, 10 000 rpm) and the pellets were suspended in DEPC-treated water and kept at 70 °C .

Reverse transcription (RT)

For the synthesis of cDNAs, 5 μg of total RNA was reverse transcribed in RT buffer (containing 50 mmol/L KCl, 10 mmol/L TRIS-HCl, pH 8.3, 5 mmol/L $MgCl_2$) 100 mmol/L DTT, 2.5 μmol/L Oligo (dT)16, dNTPs 0.5 mmol/L each, 1 U RNase inhibitor and 2.5 U of murine leukemia virus reverse transcriptase (MuLV RT) in a final volume of 10 μL. The reaction cycle consisted of 1 h at 42 °C, 10 min at 95 °C, then cooling to 5 °C. The cDNAs were kept at 20 °C. To minimize errors in pipetting among the samples, master mixes of cDNA synthesis buffer containing the dNTPs, RT buffer, oligo (dT), DTT and the enzymes were prepared and used for RT of all RNA samples in experiment.

PCR assay

For PCR amplification, appropriate dilutions of cDNA samples representing 100 200 ng of total RNA were mixed with PCR buffer containing 100 μmol/L dNTPs, 1.5 mmol/L $MgCl_2$, Stoffel Buffer (containing 50 mmol/L KCl and 10 mmol/L Tris-HCl), 0.8 1 μmol/L each of the primers and 1.25 U Stoffel Taq polymerase in a total volume of 25 μL.

A PCR primer pair for D1, D2, β-actin, and cyclophilin (p1B15) sequences, obtained from Gene Bank, were used to design the primer pairs. All oligonucleotide primer pairs spanned the intron/exon splice site, ensuring that PCR products did not arise from DNA contamination present in the RNA preparations. The amplification products were normalized against the »housekeeping« gene β-actin, and cyclophilin (p1B15). The primer sequences and PCR product size were as follows:

D1, amplifying sequences corresponding to amino acid residues 235 310 of the D1 cDNA, with PCR product 225 bp long (15):

5'-CAGTCCATGCCAAGAATTGCC-3' and

5'-AATCGATGCAGAATGGCTGGG,

D2, amplifying sequences corresponding to amino acid residues 228 362 of the D2 long cDNA, which will amplify both D2 long- and D2 short-specific fragments. Specifically, the primers anneal on either side of the alternative splice site and therefore generate two different fragments following PCR of 404 bp and 317 bp, respectively (15):

5'-GCAGTCGAGCTTTCAGAGCC-3' and

5'-TCTGCGGCTCATCGTCTTAAG-3'

Cyclophilin -p1B15 (16)

5'-AGAAGCGCATGAGCATTGTGGAAG-3' and

5'-TGCTCTCCTGAGCTACACAGAAGGAA-3' (195 bp)

β-actin (17)

5'-CAGCCATGTACGTTGCTATCCAG-3' and

5'-GTTTCGTGGATGCCACAGGAC-3'(415 bp)

The samples were denatured initially at 94 °C for 2 min and amplification was performed. Cycle parameters in all cases were: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min (30 cycles) followed by 72 °C for 8 min. PCR amplified products were analyzed by electrophoresis in a 18 g/L agarose gel. All PCRs were performed at least five times. The levels of D1 mRNA were compared and a correction was made for differences in β-actin mRNA. The levels of D2 mRNA were compared and corrected with p1B15 mRNA levels.

Statistical analyses

For the PCRs, relative signal intensities were calculated and the data presented as the mean ± SEM. A one-way analysis of variance (ANOVA), followed by the Tukey test was used for statistical comparisons between the groups. A probability value equal or less then 0.05 (5%) was considered statistically significant.

Results

In order to determine whether the observed rotations of the rats were due to 6-OHDA lesions, turning behavior was measured in presence of amphetamine. The data on full body turns per 10 min, before the lesion and five days after the lesion are presented in *Figure 1*. Before the lesion, there was no statistically significant difference between ipsilateral and contralateral rotation among the animals (*Fig.1a, d*). Still, five days after the lesion, control animals did not express any changes comparing to rotation before the lesion (*Fig. 1b, e*). However, the rats bearing unilateral 6-OHDA lesions demonstrated a statistically significant increase (4 times) in ipsilateral rotation (*Fig.1c*). In contrast, a significant decrease in contralateral rotation was observed five days after the lesion (*Fig.1f*).

To see whether the 6-OHDA lesions cause the changes on the level of DA receptors, the expression of the D1 and D2 mRNA was examined. We performed semi-quantitative RT-PCR assay using primers designed on the basis of the sequences of the D1 and D2 gene.

To assess the changes in D1 mRNA expression, cDNA from different time points (6 h, 14 h, and 7 days) and treatment, were used in the assay to co-amplify either D1 or β-actin mRNAs. As shown in *Figure 2A*, the specific amplified product for D1 receptor mRNA is 225 bp. Densitometric analysis of the agarose gel with PCR products revealed no differences in the level of D1 mRNA following different time points and treatment (*Fig. 2 B,C,D*). Statistical analysis using ANOVA followed by the Tukey test confirmed these results.

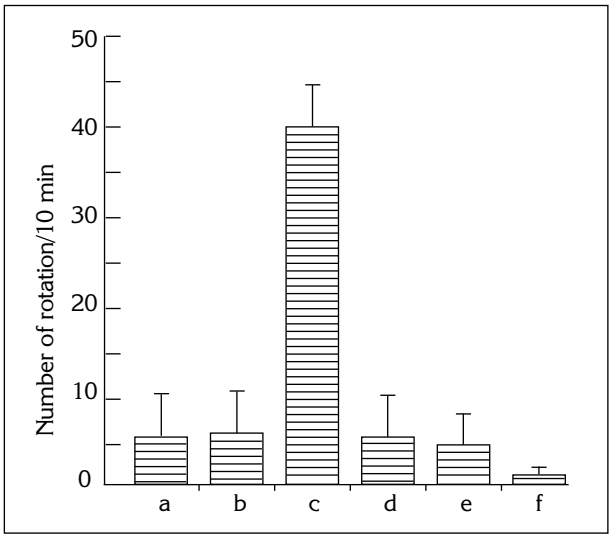

Figure 1 The effect of unilateral, four-site 6-OHDA lesion on rotational behavior of rats. a-clock rotations (CR) before the lesion; b-CR five days after the lesion in control animals; c-CR five days after the lesion in 6-OHDA treated animals; d-contra clock rotations (CCR) before the lesion; e-CCR five days after the lesion in control animals; f-CCR five days after the lesion in 6-OHDA treated animals. Rotational behavior induced by middle dose of amphetamine (3 mg/kg) was monitored during 10 minutes. The data are mean ± S.E.M. obtained from at least three separate experiment repeats under the same conditions. Kruskal-Wallis One Way ANOVA and Dunn's method of multiple comparisons versus control group were used.

In the case of the D2 receptor, two forms generated by alternative splicing amplified different fragments of 404 bp and 317 bp, (long and short form) corresponded to the molecular size of the D2 mRNA. The agarose gel with PCR products for the D2 receptor and cyclophilin mRNAs are presented in *Figure 3A*. There was a significant difference between the control and treated group of animals ($p < 0.05$) in the level of long form D2 receptor mRNA (40%), 6 and 24 h after the lesion on the right side (*Figure 3 B,C*). Seven days after the lesion, no statistically significant differences in D2 long form mRNA on the lesioned side were observed (*Figure 3D*). We also examined the effect of 6-OHDA lesion on the level of the short form D2 receptor mRNA. As shown in *Figure 3 B,C,D,* the short form was also significantly changed for the right and left side of the treatment and between the controls and the treatments, as well. Statistical analysis using ANOVA followed by the Tukey test confirmed these results.

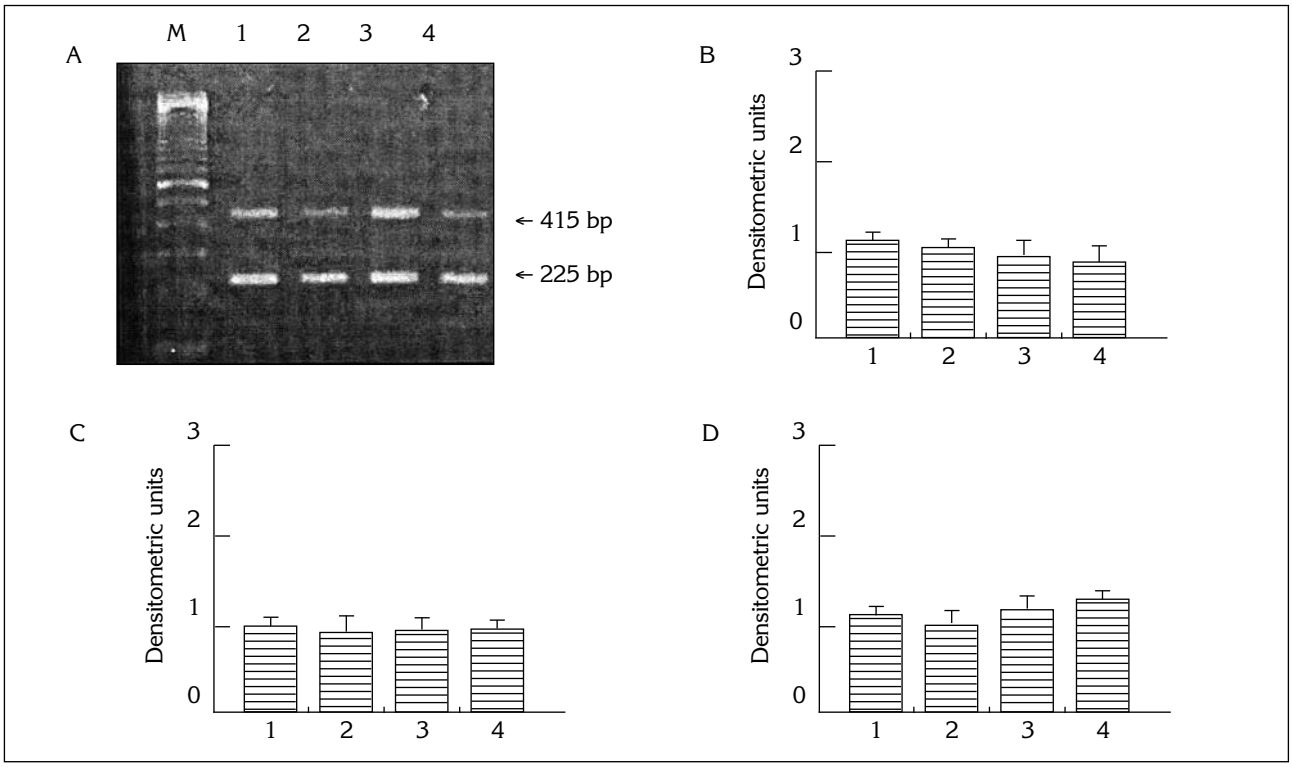

Figure 2 RT-PCR assay of the D1 mRNA in control and 6-OHDA-treated rats at different time points. A- The PCR products for D1R (225 bp) and β-actin (415 bp) were separated on 15 g/L agarose gel stained with ethidium br mide and photographed under UV light. Quantification was carried out using Multi-Analyst/PC Software Image Analysis System (Bio-Rad, Gel Doc 1000). M-Molecular weight marker (100 bp DNA ladders). 1 the PCR products from the left striata of control animals; 2 the PCR products from the left striata of 6-OHDA-treated animals; 3 the PCR products from the right striata of control animals; 4 the PCR products from the right striata of 6-OHDA-treated animals. B-Quantitative analysis of the D1R mRNA expression in control and 6- OHDA-treated animals 6 h after the lesion; C-Quantitative analysis of the D1R mRNA expression in control and 6-OHDA-treated animals 24 h after the lesion; D-Quantitative analysis of D1R mRNA expression in control and 6-OHDA-treated animals 7 days after the lesion. Changes in the D1R mRNA levels were obtained as a ratio of the D1R mRNA and b-actin mRNA levels. 1 relative level of the D1R mRNA in the left striata of control animals; 2 relative level of the D1R mRNA in the left striata of 6-OHDA-treated animals; 3 relative level of the D1R mRNA in the right striata of control animals; 4 relative level of the D1R mRNA in the right striata of 6-OHDA-treated animals. The data represent the means ± S.E.M. for minimum 5 repeats per given time point. One Way ANOVA with Tukey Test were used.

Discussion

6-Hydroxydopamine (6-OHDA) is the most prevalent monoaminergic toxin in degeneration of dopaminergic neurons and has been used extensively in animal models of Parkinson's disease (PD). Degeneration of the nigrostriatal dopamine pathway results in an imbalance in the activity of direct and indirect pathways between the striatum and the output nuclei of the basal ganglia, which is responsible for movement disorder associated with PD. This imbalance is a consequence of the segregation of the D1 and D2 dopamine-receptor subtypes on the direct and indirect striatal projection neurons (8 9).

Though long-term changes in the level of the DA receptors induced by 6-OHDA lesions have been extensively studied, early lesion-induced changes and the time course of these changes have received relatively little attention. These changes are of great interest because of slow and progressive course of Parkinson's disease.

The results presented in this paper demonstrate that four-site intrastriatal injection of 6-OHDA induced early and fast changes in the level of D2R mRNA in the striatum, as a consequence of lesion and postsynaptic adaptations that occur in the striatum, but the changes in D1R mRNA were not statistically significant at these early time points.

Five days after the lesion, middle dose of amphetamine, which increases synaptic levels of DA by inducing its release from, and inhibiting its reuptake into intact terminals, induced ipsiversive rotation (turning is ipsilateral to the lesion), as a result of a greater DA receptor stimulation on the intact side (18 20).

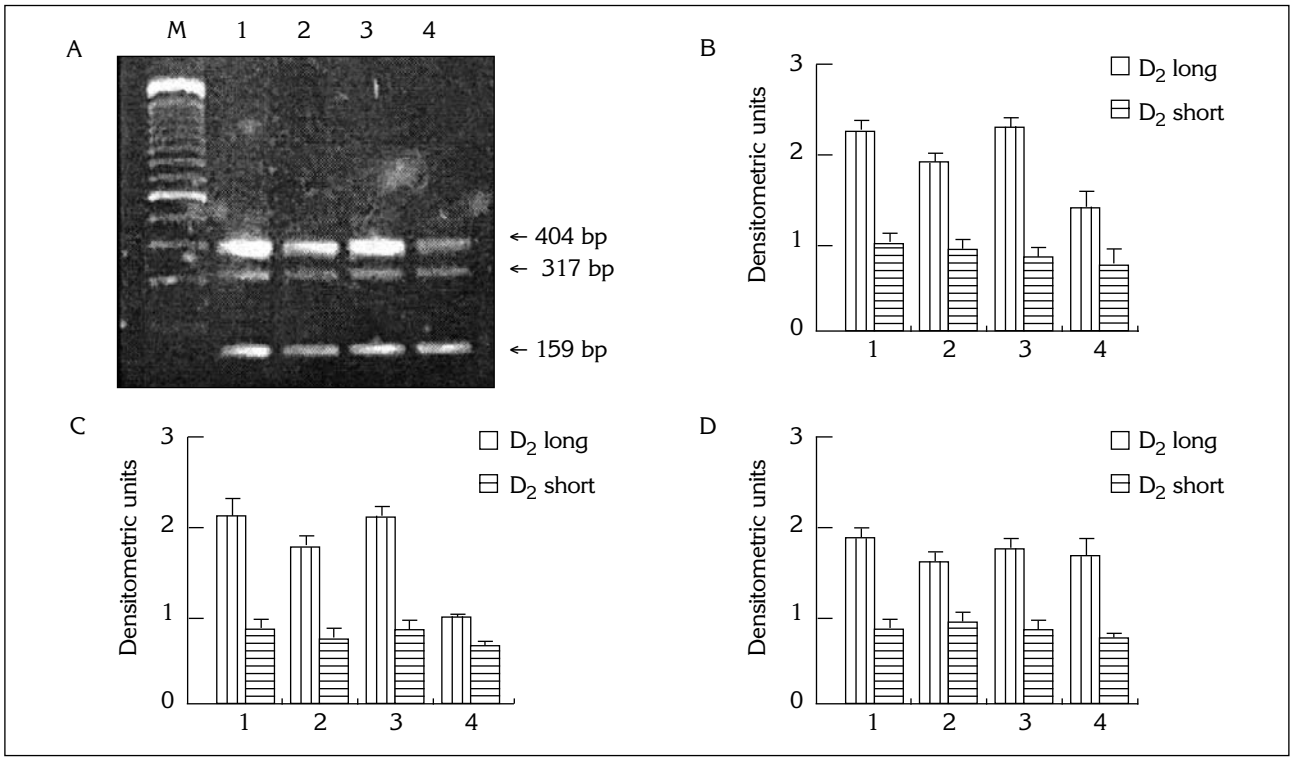

Figure 3 RT-PCR assay of D2 mRNA (both forms) in control and 6-OHDA-treated rats at different time points. A-The PCR products for D2R (404 and 317 bp) and cyclophilin (159 bp) were separated on 15 g/L agarose gel stained with ethidium bromide and photographed under UV light. Quantification was carried out using Multi-Analyst/PC Software Image Analysis System (Bio-Rad Gel Doc 1000). M- Molecular weight marker (100 bp DNA ladders). 1 the PCR products from the left striata of control animals; 2 the PCR products from the left striata of 6-OHDA-treated animals; 3 the PCR products from the right striata of control animals; 4 the PCR products from the right striata of 6-OHDA-treated animals. B-Quantitative analysis of the D2R mRNA expression in control and 6-OHDA-treated animals 6 h after the lesion; C- Quantitative analysis of the D2R mRNA expression in control and 6-OHDA-treated animals 24 h after the lesion; D- Quantitative analysis of the D2R mRNA expression in control and 6-OHDA-treated animals 7 days after the lesion. Changes in the D2R mRNA levels were obtained as a ratio of the D2R mRNA and cyclophilin mRNA levels. 1 relative level of the D2R mRNA in the left striata of control animals; 2 relative level of the D2R mRNA in the left striata of 6-OHDA-treated animals; 3 relative level of the D2R mRNA in the right striata of control animals; 4 relative level of the D2R mRNA in the right striata of 6- OHDA-treated animals. The data represent the means ± S.E.M. for minimum 5 repeats per given time point. One Way ANOVA with Tukey Test were used.

Proliferation of DA receptors (particularly D2 receptors) has been proposed as the cell-level mechanism responsible for this behavioral supersensitivity and recovery of function, but increase in the density of striatal DA receptors has usually been reported to occur two or more weeks post lesion (21 24). Other studies using *in vivo* binding methods have, however, reported proliferation of D2 receptors as early as 4 days after 6-OHDA injection (25 26).

Our results show that 6 h and 24 h after the lesion there is a decrease in D2 receptor mRNA level, probably as a consequence of the mechanical destruction of striatal neurons bearing D2 receptors and as a result of degeneration of dopaminergic fibers that are also bearing these receptors. The differences that exist in time-dependent changes between D2 short and D2 long form may be explained by the differences in the distribution or regulation of the two forms (27 28). Seven days after the lesion, as a result of D2 receptor proliferation, there was no statistically significant difference between the control and treated animals.

However, colocalization of the D1 and D2 receptors on striatal neurons and 6-OHDA-induced changes in D1 receptors remain controversial. Many authors suggest that nearly one-half of all medium spiny neurons bear both types of the receptors (29 33), while the others find that D1 and D2 receptors appear to be expressed largely in distinct neurons (34 36). Savasta (21) and Flores (36) found that as compared to the intact side, the 6-OHDA lesions did not change the D1 receptor. According to Araki et al (23 24), unilateral injection of 6-OHDA in the medial forebrain bundle can cause a significant increase in the D1 receptor

in the striatum from 2 to 4 weeks of post-lesion, but this change is less pronounced than that in dopamine D2 receptor. Gerfen et al (37) even found a 6-OHDA-induced reduction in D1R mRNA. Our result suggests that there are no statistically significant changes in the level of the D1 receptor mRNA during the first postoperative week. One possible explanation is a heterogeneous fashion of dopamine system degeneration following 6-OHDA administration (38) and a probability that neurons that bear only D1 receptors are probably less susceptible to 6-OHDA action or that these neurons require more time for molecular adaptations. A slight increase in the level of the D1R mRNA seven days after the lesion supports such an idea.

A slight increase in the DA-receptor synthesis and sensitivity seems to be insufficient to compensate for a decline in amphetamine-induced DA release five days after the lesion and rotational asymmetry. However, it might be that at this time point an increase in DA receptor synthesis just begain. Fornaguera (20) also observed ipsilateral asymmetry in turning first day after the lesion, but one week later the animals expressed a tendency to recover from deficit as a consequence of compensatory mechanism in the striatum.

Striatal dopamine receptor supersensitivity developed only when 90% or more of striatal DA nerve terminals have been destroyed (39). At this stage, all main signs of parkinsonism become visible. Because of that, the investigation of early changes in the D1 and D2 receptor synthesis may provide a better insight into movement disorder characteristic for PD and may help in developing of novel therapeutic strategies.

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6-HIDROKSIDOPAMINSKA LEZIJA STRIATUMA IZAZIVA PROMENE iRNK ZA DOPAMINSKE RECEPTORE KOD PARKISONIČNOG PACOVA

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Kratak sadržaj: Kratak sadržaj: Ispitivani su efekti četiri ubodne 6-hidroksidopaminske (6-OHDA) lezije striatuma kod odraslih mužjaka pacova. Pet dana nakon lezije, životinje su testirane na specifično rotaciono ponašanje pod uticajem srednje doze amfetamina i rezultati su potvrdili efikasnost lezije. RNK iz striatuma su izolovane u različitim vremenskim tačkama nakon lezije i urađena je RT-PCR analiza iRNK za D1 i D2 dopaminske receptore. Rezultati pokazuju smanjivanje nivoa iRNK za D2 receptor (40%) 6 h i 24 h nakon lezije, dok sedam dana nakon lezije nema promena. Za razliku od ovih rezultata, u nivou iRNK za D1 receptor ne postoje statistički značajne razlike u bilo kojoj vremenskoj tački.

Ključne reči: parkinsoničan pacov, 6-OHDA lezija, ponašanje, dopaminski receptori, iRNK za D1R i D2R, RT-PCR.

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