Effects of right-unilateral 6-hydroxydopamine infusion-induced memory impairment and oxidative stress: relevance for Parkinson’s disease

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Abstract: Male Wistar rats were subjected to right-unilateral 6-hydroxydopamine (6-OHDA) (2 µg/µl) lesions of the ventral tegmental area (VTA) or the substantia nigra (SN), or were sham-operated, and their ability to acquire the operant task was studied by means of Y-maze and shuttle-box tasks. Lesions of both the VTA and the SN resulted in an impairment of conditioned avoidance response and increase of crossing latency tested by means of shuttle-box task, suggesting significant effects of long-term memory. 6-OHDA significantly decreased spontaneous alternation in Y-maze task, suggesting effects on spatial memory, especially on short-term memory. In addition, 6-OHDA lesions of the VTA and the SN induced reductions in superoxide dismutase (SOD), glutathione peroxidase (GPX) activities and malondialdehyde (MDA) levels in the temporal lobe rather than in the frontal lobe homogenates. Our results provide further support for the toxic effects of 6-OHDA-induced memory impairment and oxidative stress with relevance for Parkinson’s disease.

Keywords: 6-OHDA • Memory • Oxidative stress • Antioxidant enzymes

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1. Introduction

Parkinson’s disease (PD) is a human neurodegenerative disorder which is mainly characterized by a massive and progressive degeneration of the dopaminergic neurons in the substantia nigra (SN). The most widely used animal models of PD involve intracranial infusion of the neurotoxin 6-OHDA directly into the ascending dopaminergic forebrain bundle, thereby inducing severe dopaminergic neuronal degeneration associated with profound deficits in feeding, drinking, sensorimotor and learning functions [1-4]. Alternatively, new Parkinsonian rat models have been developed with 6-OHDA injected directly into the striatum to induce selective and moderate neurodegeneration of dopamine (DA) nerve terminals [5]. Similarly, in PD the progressive degeneration of nigral dopaminergic neurons results in motor deficits only after - 80% of the nigrostriatal system has degenerated [6]. Therefore behavioural studies preferentially involve unilateral destruction of the nigrostriatal pathway with 6-OHDA to avoid the debilitating consequences of a bilateral lesion [7]. Previous results from our group have shown that bilateral 6-OHDA infusions into the ventral tegmental area (VTA) and the SN affecting the retention of both short-term memory, tested in the Y-maze task and long-term memory evaluated with the multi-trial passive avoidance test, do not affect memory acquisition. We found that, as compared to short-term memory, long-term memory is more susceptible to the decreased dopamine levels in nervous structures involved in processing and storage of information [4]. PD has traditionally been considered to be a motor disorder resulting from the bilateral destruction of DA neurons in the SN. One of the most prominent symptoms of PD is the inability to initiate movement [8]. There has been much recent interest, however, in the nature of cognitive impairment in PD ranging from minor disturbances in memory or intellectual function to dementia [9]. In rats, the cognitive function of striatal dopamine has been studied with 6-OHDA lesions to the DA terminals in the striatum [10] and associated with a lesion to the nucleus accumbens [11]. Such lesions produced a disruption of...
active avoidance learning that could result either from a blockade of associative learning (i.e. association between the conditioned stimulus and the avoidance response) or by delaying the initiation of voluntary motor responses.

Although the etiology of PD remains unknown, recent studies have suggested that oxidative stress is an important mediator in its pathogenesis [12]. It is thought that nigral dopaminergic neurons are rich in reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide \((H_2O_2)\), and hydroxyl radicals [13]. Indeed, there are several observations, such as the increased levels of the oxidation products of lipids, proteins, and nuclear acids in nigral cells, that are indicative of the role of oxidative stress in PD [13,14]. There is ample evidence for the involvement of oxidative stress in 6-OHDA-induced degeneration, another animal model of PD. Studies have demonstrated that the neurotoxic effects of 6-OHDA involve generation of hydrogen peroxide and hydroxyl radicals [15], reduction in glutathione (GSH) and SOD activity [16] and an increase in MDA levels in the striatum [17]. In addition, it has been shown that 6-OHDA is toxic to mitochondrial complex I, and leads to production of superoxide free radicals [18,19] and those molecular mechanisms of 6-OHDA-induced cytotoxicity involve hydrogen peroxide-dependent and –independent action [20].

The aim of the present work was to study the effects of right-unilateral 6-OHDA lesions of the VTA or the SN on learning and memory processes evidenced by means of Y-maze task and shuttle-box task, respectively, and on the antioxidant enzymes activities. Our data suggest that a correlation exist between VTA, SN, oxidative stress and expression of cognitive capacities.

2. Experimental Procedures

2.1. Subjects

30 male Wistar rats weighing 200-250 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

2.2. Neurosurgery

All surgical procedures were conducted under aseptic conditions, under sodium pentobarbital (45 mg/kg b.w., i.p., SIGMA) anesthesia. Rats were mounted in the stereotaxic apparatus with the nose oriented 11° below horizontal zero plane. Specific right-unilateral lesions of the dopaminergic neurons located in the SN were produced with 6-OHDA (SIGMA). Eight micrograms (free base) 6-OHDA, dissolved in 4 µl physiological saline containing 0.1% ascorbic acid were administrated through Hamilton syringe over 4.50 min., and the syringe was left in place for 5 min. after injection before being slowly removed. The sham-operated rats were injected with saline. The following coordinates were used: 5.5 mm posterior to bregma; 2.0 mm lateral to the midline; 7.4 mm ventral to the surface of the cortex. For lesioning the VTA, the same quantity of 6-OHDA was injected right-unilateral according to the following coordinates: 5.6 mm posterior to bregma; 0.5 mm lateral to the midline; 7.6 mm ventral to the surface of the cortex [21]. The sham-operated rats were injected with saline. Learning and memory tests began 2 weeks after the operation.

2.3. Y-maze task

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide) and an equilateral triangular central area. The rat was placed at the end of one arm and allowed to move freely through the maze for 8 min. The time limit in Y-maze test was 8 min., and every session was stopped after 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviors was then the total number of arms entered minus 2 and percent spontaneous alternation was calculated as \((actual\ alternations/maximum\ alternations)\times100\ [22,23]\). Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory.

2.4. Shuttle-box task

The shuttle box used in the present study was constructed of grey Plexiglas and measured 66 x 33 cm wide x 39 cm high. The floor made of 2 mm diameter stainless steel rods spaced 0.5 cm apart. The box was divided into two equal compartments by a 5 cm high Plexiglas barrier. Each compartment could be electrified separately. A ringer was mounted in the centre on the top
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Rats were placed in the shuttle box and allowed to freely explore the apparatus for 180 s. Then, they received 10 shuttle trials/day, where they were trained to terminate a shock by jumping over a barrier to the adjoining compartment. Each trial began with 5 s ring tone followed by 5 s 0.3 mA foot shocks (Coulbourn Animal Test Cage Grid Floor Shocker). After 60 s, the next trial was initiated. If the animal crossed the barrier during the ring tone, the stimulus was terminated and no shock was delivered (conditioned avoidance response - CAR). If the animal crossed the barrier during shock delivery, an escape response was measured. After 30 s, the next trial was initiated in this experimental situation. Animals could avoid the shock during sound presentation or interrupt its presentation (escape) by crossing to the opposite side of the chamber. Absence of one of these behaviors was considered an escape failure. The crossing latency (the time elapsed between the delivery of unconditioned stimulation and the start of conditioned avoidance response) was also recorded [24]. During the test session CAR and crossing latency were recorded. Animals that had more than 6 escape failures during the test were considered to be helpless.

2.5. Tissue collection
After the behavioural tests, all rats were anesthetized, rapidly decapitated and whole brain were removed. The frontal and the temporal lobes were collected. Each of the brain tissue samples was weighed and homogenized with a glass homogenizer in double distilled water (1 g tissue/10 ml double distilled water).

Samples were centrifuged 15 min at 3000 x g. Following centrifugation, the supernatant was separated and pipetted into tubes.

2.6. Biochemical estimations
2.6.1. Determination of SOD
Homogenates of the frontal and the temporal lobes were centrifuged at 25,000 x g for 15 min at 4°C and supernatant dialyzed in 50 mM PBS (pH 7.8) containing 1mM EDTA. SOD activity was determined based on inhibition of superoxide-dependent reactions. The reaction mixture contained 70mM potassium phosphate buffer (pH 7.8), 30 µM cytochrome c, 150 µM xanthine, and tissue extract in phosphate buffer diluted 10 times with PBS in a final volume of 3 ml. The reaction was initiated by adding 10 µl of 50 units xanthine oxidase, and the change in absorbance at 550 nm recorded. The results are expressed as unit/mg protein.

2.6.2. Determination of GPX
GPX activities of the frontal and the temporal lobes were analyzed by a spectrophotometric assay, using 2.0 mM reduced glutathione and 0.25 mM H₂O₂ as substrate. One unit of GPX is defined as the quantity that catalyzes the oxidation of 1 nM NADPH/min at 25°C. Protein was measured using the bicinchoninic acid (BCA) protein assay reagent and bovine serum albumin was used as a standard. The results are expressed as unit/mg protein.

2.6.3. Determination of MDA
The amount of MDA in homogenates of the frontal and the temporal lobes was determined. In brief, 0.1 ml of the homogenate diluted 10 times with phosphate buffered saline (PBS) was mixed with 0.75 ml working solution (thiobarbituric acid 0.37% and perchloric acid 6.4%, 2:1, v/v) and heated to 95°C for 1 h. After cooling (10 min in ice water bath), the flocculent precipitate was removed by centrifugation at 3200 x g for 10 min. The supernatant was neutralized and filtered prior to injection on an octadecylsilane 5 µm column. Mobile phase consisted of 50 mM PBS (pH 6.0): methanol (58:42, v/v). Isocratic separation with 1.0 ml/min flow rate and detection at 532 nm using a UV–vis high-performance liquid chromatography detector were performed.

2.7. Histological control
At the end of the experiment, all rats were killed with an overdose of sodium pentobarbital (100 mg/kg b.w., i.p., SIGMA) followed by a transcardial infusion of 0.9% saline and a 10% formalin solution. The brains were removed and placed in a 30% sucrose/formalin solution. The brains were frozen and cut into coronal sections (50 µm) using a freezing microtome and stained with cresyl violet for verification of the point of the syringe needle. Only experimental data from lesions correctly located in the VTA and the SN were used for statistical analysis.

2.8. Statistical analysis
The results of Y-maze and shuttle-box tasks were statistically analyzed using two-way ANOVA (ANOVA: two factor with replication) with lesion as the between groups factor (at two levels —VTA and sham-operated, SN and sham-operated, respectively). The results for antioxidant enzymes activity assays were analyzed using Student’s t-Test. All results are expressed as mean ± S.E.M.; F values for which p<0.05 were regarded as statistically significant.
3. Results

3.1. Histological verification

After 6-OHDA lesions the rats recovered quickly and gained weight by 1 week. In the majority of SN – lesioned rats (8/10) the point of the syringe needle was positioned in the central part of the SN and the lesions extended to a part of adjacent structures including substantia nigra pars reticulata, without any significant damage. In the case of VTA-lesioned rats (7/10) the point of the syringe needle was positioned in the posterior part of the VTA and the lesions extended to adjacent structures without any significant damage.

3.2. Effect of 6-OHDA-induced SN and VTA lesion on learning and memory

Lesioning of the SN evidenced a significant impairment of short-term memory, explored by means of Y-maze task, indicated by a decrease of spontaneous alternation percentage (F(1,2)=3.44, p<0.05) (Figure 1). This effect could not be attributed to decreased motor activity, because the number of arm entries was not significantly changed (F(1,2)=0.23; p>0.05) (Figure 1). In the shuttle-box active avoidance test, the 6-OHDA SN-lesioned rats showed a significant decrease in the number of avoidance responses (F(2,18)=31.48; p<0.0004) (Figure 2), during 10 days training, compared to the sham-operated group, suggesting a significant effect in the long-term memory.

6-OHDA significantly increased crossing latency in SN-lesioned rats compared to the sham-operated group (F(2,18)=94.21, p<0.00001) (Figure 3).

Lesioning of the VTA evidenced a non-significant impairment of short-term memory, explored by means of Y-maze task, indicated by non-significant decrease of spontaneous alternation percentage (F(1,2)=1.64, p>0.05) (Figure 1). This effect could not be attributed to decreased motor activity, because the number of arm entries was not significantly changed (F(1,2)=0.23; p>0.05) (Figure 1). In the shuttle-box active avoidance test, the 6-OHDA VTA-lesioned rats showed a significant decrease in the number of avoidance responses (F(2,18)=31.48; p<0.0004) (Figure 2), during 10 days training, compared to the sham-operated group.
sustaining a significant effect in the long-term memory. 
6-OHDA significantly increases crossing latency in VTA-lesioned rats compared to the sham-operated group (F(2,18)=94.21, p<0.00001) (Figure 3).

3.3. Effect of 6-OHDA-induced SN and VTA lesion on SOD, GPX activities and MDA level

6-OHDA–induced lesion of the SN and the VTA impaired significantly (p<0.05) the SOD activity estimated in the temporal lobe homogenates (Figure 4) and produced non-significant changes in the frontal lobe homogenates on both the SN and the VTA lesioned rats compared with sham-operated groups.

6-OHDA–induced lesion of the SN and the VTA impaired significantly (p<0.05) the GPX activity estimated in the temporal lobe homogenates (Figure 5) and produced non-significant changes in the frontal lobe homogenates on both the SN and the VTA lesioned rats compared with sham-operated groups.

6-OHDA–induced lesion of the SN and the VTA decreased non-significantly the MDA level estimated in the temporal and in the frontal lobe homogenates (Figure 6) on both the SN and the VTA lesioned rats compared with sham-operated groups.

We observed that the effects are pronounced in all the SN-lesioned rats but not in all the VTA-lesioned rats.

4. Discussion

The present study demonstrates that intranigral infusion of 6-OHDA reproduced behavioral, biochemical, and certain neuropathological features of PD.

PD, a result of progressive deterioration of the nigrostriatal dopaminergic tract, is characterized by cognitive deficits in addition to the well-defined motor

deficits. PD is often complicated by a variety of cognitive symptoms that range from isolated memory and thinking problems to severe dementia. While the motor symptoms of PD are well-known (tremor, rigidity, slowness of movement, imbalance), the commonly seen deficits in memory, attention, problem-solving, and language are less understood. Studies have shown that over 50% of people with PD experience some form of cognitive impairment. About 20% have more substantial cognitive impairment. Memory problems in PD are typically milder than in Alzheimer’s disease. In PD, the person may have difficulty concentrating, learning new information and recalling names.

There have been a number of indices of damage that have been studied in animals following unilateral destruction of dopaminergic motor tracts with toxins such as 6-OHDA [5,25].

Our results showed that 6-OHDA-induced the SN and the VTA lesion produced a broad spectrum of behavioral deficits.

6-OHDA-induced unilateral destruction of dopaminergic motor tracts produced a significant decrease in short-term memory (significant decrease in spontaneous alternation percentage in Y-maze task) in the SN-lesioned animals without significantly affecting locomotion (non-significant changes in number of arm
entries in Y-maze test) compared to the sham-operated groups. The VTA has a similar role, but the effect of the SN is more prominent.

Acquisition of active avoidance responding is severely disrupted by dopamine receptor blockade [26] and catecholamine depleting drugs such as reserpine [27]. Disruption of a well-learned active avoidance response in animals occurs only after severe depletion of striatal dopamine induced by 6-OHDA [28]. The dose of 6-OHDA used in the present experiments was high enough to damage dopamine neurons. Previous studies have reported that 6-OHDA injection into SN reduced the dopamine content in the striatum to 30% of the control level 1 day after lesion and that this reduction persisted until 20 days after lesioning [4,29], indicating that dopaminergic neurons were damaged.

In the present study, 6-OHDA significantly decreases the number of avoidance responses tested by means of shuttle-box task, in both SN and VTA lesioned animals during 10 days of training compared to the sham-operated groups, suggesting effects on long-term memory. Motor deficits are well-correlated with 6-OHDA-induced lesion of both the SN and the VTA. Slowed response times of 6-OHDA SN and VTA lesioned animals could be a cause of cognitive impairment evidenced by a significant increase of crossing latencies in both the SN and the VTA lesioned animals compared to the sham-operated groups. Similarly, a decrease of GPX activity may be a cause of a decrease in the levels of GSH, because 6-OHDA induced cytotoxicity via H₂O₂ intracellular production which can be transformed in reactive hydroxyl radicals and produce cell damage.

The present study indicates that 6-OHDA-induced learning and memory deficits on both the SN and the VTA lesioned animals is correlated with the involvement of 6-OHDA in oxidative stress generation.

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