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Research report

Striatal 6-OHDA lesion in mice: Investigating early neurochemical changes underlying Parkinson's disease

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ABSTRACT

Early phases of Parkinson's disease (PD) are characterized by a mild reduction of dopamine (DA) in striatum and by emergence of psychiatric disturbances that precede overt motor symptoms. In order to characterize the neurochemical re-arrangements induced by such striatal impairment, we used a mouse model in which a low dose of 6-hydroxydopamine (6-OHDA) was bilaterally injected into the dorsal striatum. These mice showed a DA reduction of about 40% that remained stable up to 12 weeks after injection. This reduction was accompanied by changes in DA metabolite levels, such as HVA, transiently reduced at 4 weeks, and DOPAC, decreased at 12 weeks. No change in the 5-hydroxytryptamine (5-HT) levels was found but the 5-hydroxyindoleacetic acid (5-HIAA)/5-HT ratio was increased at 4 weeks. In addition, at the same time-point, the levels of 15-F_{2t}-IsoP, an index of oxidative stress, and of PGE_2, a major product of cyclooxygenase-2, were decreased in different brain areas while BDNF levels were increased. These neurochemical changes were accompanied by altered behavioral responses concerning the emotional reactivity. Overall, the present findings suggest that a change of 5-HT metabolism and a modification of oxidative stress levels may play a role in the early PD degeneration phases.

1. Introduction

Parkinson's disease (PD) is a progressive brain illness characterized by a degeneration of the dopaminergic nigrostriatal pathway [29,30,46]. Though PD is classically diagnosed according to motor symptoms, an increasing body of evidence suggests that motor symptoms are only one aspect of a multifaceted and complex disorder. Indeed, around 60% of people with PD show non-motor symptoms, such as psychiatry disturbances [18,25,38,39] and approximately 40% of PD patients present anxiety disorders and depression [1,15,40,66].

The non-motor symptoms of PD are a major cause of disability and their recognition is of crucial importance for a comprehensive and effective healthcare of PD patients [39,54]. Furthermore, their appropriate consideration allows to refine both research strategies aimed at investigating the cause of the disease and therapeutic approaches aimed at slowing or halting its progression [34]. The relevance of non-motor symptoms in PD should be taken into account also in animal models of PD.

Among the animal models of PD, the one generated through an unilateral 6-hydroxydopamine (6-OHDA) injection into the medial forebrain bundle is one of the most common [4,6,9,16,43,55]. Such a treatment produces an almost complete dopaminergic loss, leading to motor impairments and neurochemical alterations resembling those of the advanced phases of PD [44,52].

More recently, models mimicking the early phases of PD have been generated by bilateral partial lesion of the dopaminergic system. The study of these models, mostly developed in the rat species, provided information on the effects of a mild dopamine (DA) depletion on behavior [3,12,50].

Further exploitation of animal models of the early PD is envisaged to provide additional results relevant for preclinical testing and to identify the earliest pathogenic events, suitable for therapeutic intervention. The development of these models in the mouse species will be relevant for future studies aimed at combining chemical lesions and genetic manipulations.

In the present paper, we have studied a mouse model generated by a bilateral striatal injection of a moderate 6-OHDA dose (8 μg)

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to mimic the mild DA reduction accompanying early PD phases and elucidate the associated molecular alterations, which may underlie the behavioral symptoms preceding overt motor impairment. Given the major involvement of serotonin (5-hydroxytryptamine, 5-HT) in PD reviewed in [22], and considering the role of such neurotransmitter in depression, we examined the long-term lesion effects on striatal levels of both DA, 5-HT and their metabolites. Furthermore, we investigated oxidative stress and cyclooxygenase (COX) activity, as putative pathogenic mechanisms, and brain derived neurotrophic factor (BDNF) expression, which is reportedly affected by dopaminergic lesions [68]. The latter analyses were performed in the striatum and also in the hippocampus and cortex to evaluate the impact of 6-OHDA injection on brain areas involved in controlling behaviors having face validity with PD non-motor symptoms including anxiety-like and cognitive alterations.

2. Materials and methods

2.1. Animals

Male CB57Bl/6 mice (25–30 g of weight; Charles River, Germany) were used. Upon arrival at the laboratory, the animals were housed in an air-conditioned room (temperature 21 ± 1 °C, relative humidity 60 ± 10%) with lights on from 08:00 to 20:00 h. Males were housed in same-sex groups of 5 individuals in 32 cm × 14 cm × 12 cm Plexiglas boxes with a metal top and sawdust as bedding, and with pellet food (Enriched standard diet purchased from Mucedola, Settimo Milanese, Italy) and tap water *ad libitum*. Experimental subjects were weighed at 4 and 12 weeks after the lesion. Animal care and use followed the directives of the Council of the European Communities (86/609/EEC).

2.2. 6-OHDA injection

Under Equithesin (3 ml/kg i.p.) anesthesia, the animals were placed on a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Bilateral injections of 6-OHDA (4 μl of a 2 μg/μl in 0.2% ascorbic acid saline solution; each animal received a total of 16 μg of 6-OHDA, 8 μg at each side) was performed in the dorsal striatum [coordinates with respect to bregma: $A = +1$; $L = \pm 1.7$; $V = -2.9$ mm, according to the stereotaxic atlas of [48] using a Hamilton syringe (Reno, NV, model 701). 6-OHDA was injected manually at a rate of 1 μl/min. After the injection the needle was left in place for 3 min before slowly retracting it to prevent reflux. Control (SHAM) animals received the same volume of vehicle according to the same procedure. After surgery, the animals were allowed to recover for 2 weeks before starting the behavioral assessment.

2.3. Monoamine and monoamine metabolites determinations

Mice were sacrificed at 4 and 12 weeks after 6-OHDA injection (SHAM and 6-OHDA mice were respectively, 4 and 6 at 4 week and 6 and 6 at 12 weeks) for the determination of DA, 5-hydroxytryptamine (5-HT) and their metabolites. For monoamine and monoamine metabolites assay, the brain was rapidly removed and the striata were dissected out on ice, weighed in polypropylene vials and homogenated in percloric acid (PCA) 0.1 M. Dihdyroxybenzylamine (DHBA) was added as internal standard (100 mg/ml in PCA 0.1 M). Samples were centrifuged for 20 min at 15,000 × *g* (4 °C), then the supernatant was filtered in micropure 0.22 μ for 30 s and used for monoamine and monoamine metabolites assays. The endogenous levels of DA, DA metabolites (homovanillic acid, HVA and 3, 4-dihydroxyphenylacetic acid, DOPAC), 5-HT and 5-HT metabolite (5-hydroxyindolacetic acid, 5-HIAA) were assayed by microbore HPLC, according to a previous published protocol [17].

2.4. BDNF assay

Mice ($n = 5$ per group) were sacrificed 4 weeks after 6-OHDA injection for BDNF assays according to a previously described procedure [13]. Briefly, brain areas (cortex, hippocampus and striatum) from lesioned and SHAM mice were rapidly dissected on ice and homogenized in lysis buffer (137 mM NaCl, 20 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml leupeptin, 0.5 mM sodium vanadate, 10 μg/ml aprotinin). Homogenized samples were centrifuged at 2000 × *g*. for 20 min at 4 °C and supernatants were collected and frozen at −80° until the time of the assay.

Enzyme-linked immunosorbent assays (ELISA) were performed according to manufacturer's instructions using the BDNF Emax kits (Promega, WI, USA). The *R* value for the standard curve sensitivity limits was consistently ≥0.99. All sample values were in the linear region of the standard. Based on standard curve of known BDNF concentration, levels of mature BDNF were calculated as pg/mg of total proteins.

2.5. PGE_2 and 15-F_{2t}-IsoP assays

Mice ($n = 5$ per group) were sacrificed 4 weeks after 6-OHDA injection for PGE_2 and 15-F_{2t}-IsoP assays. After sacrifice, brain areas (cortex, hippocampus and striatum), were immediately dissected out, placed in plastic tubes, weighted, frozen on dry ice and stored at −80 °C until metabolite extraction. A detailed procedure for prostaglandin E_2 (PGE_2) and F_{2t}-isoprostane (15-F_{2t}-IsoP) extraction has been described elsewhere [42]. In brief, 200 μl of ice-cold Tris–HCl buffer pH 7.5 containing 10 μg/ml of the COX inhibitor indomethacin (stock solution 100× in ethanol) to avoid ex vivo PGE_2 synthesis, and 10 μM of the radical scavenger butylated hydroxytoluene (stock solution 100× in ethanol) to avoid auto-oxidation, were added to each frozen sample, which was quickly thawed, homogenized with a Teflon pestle (Sigma) – 20 cycles in an ice bath – vigorously vortexed and centrifuged at 14,000 rpm for 45 min at +4 °C. Supernatants were collected and stored at −80 °C until analysis. Pellets were used for protein analysis (BCA assay; Pierce). PGE_2 and 15-F_{2t}-IsoP were measured in tissue extracts by high sensitivity colorimetric enzyme immunoassays (EIA kits, detection limit for PGE_2: 7.8 pg/ml, Assay Designs, Inc. Ann Arbor, MI; detection limit for 15-F_{2t}-IsoP: 2 pg/ml; Cayman Chemical, Ann Arbor, MI). According to the manufacturers, the cross-reactivity of the anti-PGE_2 antibody with 8-15-F_{2t}-IsoP was less than 0.25% and that of anti-15-F_{2t}-IsoP antibody for other prostaglandins was less than 1% (0.02% for PGE_2).

2.6. Behavioral tests

Mice were tested at 4 weeks after surgery. All tests were carried out between 09:30 and 15:30 h. Animals were transferred to the experimental room at least 40 min before the test in order to let them acclimatize to the test environment. Animals were weighed before starting the behavioral test. Each single experimental procedure was performed in a time window not longer of 2 h. Behavior was videorecorded using a digital videocamera (TR 7000E, Sony, Tokyo, Japan) and a digital video recorder (RDR GX 350, Sony, Tokyo, Japan). All behavioral analyses was performed using the commercial software Observer 3.0 (Noldus, Wageningen, The Netherlands) with the exception of the Morris water maze test (see below for details). All scores were assigned from the same observer who was unaware of animal treatment. The behavioral tests were performed every other day in the following order: open-field, elevated plus-maze, Morris water maze and forced swim test.

2.6.1. Open field

Mice were tested in the open -field according to a previously described procedure [14]. Mice ($n = 7$ SHAM; $n = 8$, 6-OHDA) were individually transferred from the home cage to an open-field arena (40 cm × 40 cm) made of gray Plexiglas. The test started by placing the animal in the center of the arena that was illuminated at 5 Lux light intensity. During the test, divided in two sessions, the animal was videotaped. During the first session, lasting 15 min divided in three 5-min blocks, the following behavioral parameters were scored: distance moved (self explained) and thigmotaxis (time spending near the walls). In the second 5-min session, a stimulus object (a glass sphere, 1.2 cm diameter) was placed in the center of the arena. During this period, the latency to the first contact and the frequency and duration of contacts with the object were scored. Mice not sniffing the object within 5 min were arbitrarily assigned a latency time of 5 min. After testing each animal, the apparatus was thoroughly cleaned with cotton pads wetted with 40% ethanol.

2.6.2. Elevated plus-maze

Mice were tested in the elevated plus-maze according to a previously described procedure [11]. The elevated plus-maze apparatus comprised two open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15 cm) that extended from a common central platform (5 cm × 5 cm), as described in previous works. The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level and was illuminated with dim light (0, 6 Lux). Each mouse ($n = 7$ SHAM; $n = 9$, 6-OHDA); was individually placed on the central platform facing an closed arm and allowed to freely explore the maze for 5 min. Immediately after each session, the apparatus was thoroughly cleaned with cotton pads wetted with 40% ethanol. Behavioral parameters observed were: frequencies of total, open and closed entries (arm entry = all four paws into an arm), % open entries [(open/total) × 100], and time spent in open and closed arms and in the central part of the platform. Furthermore, frequency, duration and latency of rearing (standing with the body inclined vertically, forequarters raised), wall-rearing (standing on the hindlimbs and touching the walls of the apparatus with the forelimbs), immobility (total absence of movement), grooming (rubbing the body with paws or mouth and rubbing the head with paws), head dipping (exploratory movement of head and shoulders over the edge of the maze) and stretched-attend postures (SAP; exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion) were scored.

2.6.3. Morris water maze

Mice were tested in the Morris water maze according to a modified Morris' procedure [26]. The water maze apparatus consisted of a white Plexiglas circular pool 88 cm in diameter and 33 cm in height, and was placed in the middle of an experimental room (dimension 5 m × 4 m × 3 m). The pool was filled with water kept at a temperature of 26 ± 1 °C. A transparent plastic platform (8 cm in diameter)