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Full Length Research Paper

Convenience of the traversal beam test modified to evaluate the model of Parkinson's disease in Rat lesioned in SNPC

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The nigrostriatal degeneration underlying Parkinson's disease is commonly studied in experimental animals by injection of the neurotoxin 6-hydroxydopamine (6-OHDA). The present study describes a modified version of a beam traversal test which allows the quantification of the motor deficit through the time spent to arrive to the platform once all four paws of the animals are in contact with the beam (escape latency), the time spent before falling (tumbled down latency) and the number of errors committed for the animals in each beam. The shape and the diameter of the cross section of the beams were modified from rectangular and circular cross section with 2.5 cm of diameter to the same shape with 1 cm of diameter, which induced a high difficulty to the execution of the test. Three groups of Wistar rats were examined: untreated (n = 15), lesioned with 6-hydroxydopamine (n = 14), and sham-operated (n = 14). All variables studies showed significant differences between control and hemiparkinsonian rats. The escape latency and the error were increased and the tumbled down latency was decreased in hemiparkinsonian rats for all beams in comparison with control rats. In tumbled down latency, the significant differences between groups were more evident ($p < 0.001$) for the beams with high cross section irrespective of the shape of the cross section (rectangular or circular). The beam traversal test is a convenient sensorimotor test that does not need to be trained extensively, and require averse motivation or food deprivation and appears to be very useful in evaluating the motor deficits in established unilateral model of Parkinson's disease and also other experimental models.

Key words: Beam traversal test, 6-OHDA, dopamine.

INTRODUCTION

In the most common rat analogue for Parkinson's disease (PD), dopaminergic fibers are destroyed by unilateral injections of the neurotoxin 6-hydroxydopamine (6-OHDA) into striatum, *substantia nigra pars compacta* (SNPC) or its major efferent projection, the medial forebrain bundle (MFB) (Schober, 2004). The lesion can result in almost complete unilateral dopamine (DA) depletion, causing moderate to severe functional asymmetries (Zigmond et al., 2002). Animals with dopaminergic depletions display pronounced sensory and motor deficits, especially those in walking, which are very similar to those displayed by humans with PD (Doan et al., 2008). The difficulty in understanding the rather complex behavioural changes resulting from unilateral DA depletion is illustrated in the study of the asymmetry in turning. End point measures of rotational behaviour show that spontaneously and in response to treatment with amphetamine rats usually turns

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ABBREVIATIONS: PD; Parkinson's disease, **6-OHDA**; 6-hydroxydopamine, **SNpc**; substantia nigra pars compacta, **MFB**; Medial forebrain bundle, **DA**; Dopamine, **i.p.**; Intraperitoneal, **NaCl** ; Physiological saline solution, **RCS**; Rectangular cross section, **CCS**; Circular cross sections, **TH**; Tyrosine hydroxylase, **SNK**; Student-Newman-Keuls test, **PPN**; pedunculopontine nucleus.

ipsilateral to their lesion while after injection of apomor-0phine they turn contralateral to their lesion (Fornaguera et al., 1994; Metz and Whishaw, 2002). Turning has been variously explained as a result of a postural or stepping asymmetry, asymmetrical levels of excitation in central DA circuits and asymmetry in responsiveness to sensory stimuli (Miklyaeva et al., 1995; Metz and Whishaw, 2002). Despite the range of views concerning the causes and direction of rotation none of these explanations is viewed as being exclusive.

Although the test with amphetamine or apomorphine addresses the imbalance in DA available for release and DA receptor function, respectively, the functional integrity of nigrostriatal DA neurons in PD is more reliably assessed without drugs. This is also true in the rat model, provided that the behavioural tests are appropriate (Cenci et al., 2002).

The hemiparkinsonian model is characterized by many of the motor abnormalities of PD (Cenci et al., 2002). For example, in Rats with severe DA depletion, akinesia is found in the forelimb contralateral to the lesion in a stepping test (Johnston et al., 1999; Stein, 2009) and on the paw-reaching and staircase test (Whishaw et al., 1997). In addition, Rats cannot effectively use the limbs contralateral to the lesion to initiate movements that shift body weight and control posture (Schallert et al., 2000; Woodle et al., 2008) or to regain stable equilibrium during experiment imposed weight shifting challenges in a bracing test (Metz et al., 2004).

A different approach to the effects of DA depletions is to examine how its affects the skill of animals to maintain the balance and the walk pattern in a narrow surface (Allbutt and Henderson, 2007). Here, we describe a modified version of a square beam test (Thullier et al., 1996) in which Rats are placed at one end of an elevated narrow beam and their ability to cross the beam is scored. The main modification is that the diameter and the shape of the beams are changing beginning with a beam with 2.5 cm of diameter and rectangular cross section (RCS) followed by a beam with the same diameter and circular cross section (CCS). The next evaluation was carried out with beams showing RCS and CCS but it had 1 cm of diameter. The advantages of this test are that 1. Parkinsonian movements can be monitored specifically and separately, 2. The behaviours being monitored are spontaneous, in the sense that there is no drug or externally manipulated eliciting stimulus, 3. Specific training procedures are not strictly required.

MATERIALS AND METHODS

Subjects

The study used male adult Wistar rats weighing from 200 to 250 g, provided by the Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Three individuals were housed per cage throughout the experiment. They were kept in a 12 h light-dark cycle and had unrestricted access to food and water. During the investigation, every effort was made to minimize animal suffering. The experimental work complied with the Guidelines for the care, use and reproduction of laboratory animals (Guidelines, CENPALAB, Cuba, 2000). The experiments were approved by the Ethic Committee of CIREN.

Surgery procedures

The surgical table and the stereotaxic frame were cleaned with alcoholic chlorhexidene and aqueous solution of Betadine. Rats were injected with desmethylimipramine (15 mg/kg, i.p. route) 30 min prior to the intracerebral infusion to protect noradrenergic neurons. Ocular lubricant Lacrilube was applied to prevent corneal drying. Furthermore, on the day of surgery they were treated with buprenorphine (0.05 mg/kg, s.c. route) for trans-operative analgesia. Body temperature was measured rectally throughout the surgery and maintained between 36.5 and 38.5°C by use of a heating pad. The animals were anesthetized by intraperitoneal (i.p.) administration of a chloral hydrate solution (420 mg/kg of body weight). Following anesthesia the rats were placed in a stereotaxic apparatus for rodents (Stoelting, U.S.A) and their skull was exposed. A small burr hole was drilled through the skull at the following coordinates (cm) AP = -0.49; ML = 0.17 relative to bregma; DV = 0.81 from the dura, as described in the Atlas by Paxinos and Watson, 1998. Afterwards, the rats were injected with 3 µl of 6-OHDA solution (St. Louis, USA, 8 µg per 3 µl of 0.9% physiological saline solution (NaCl) and 0.5 mg/ml ascorbic acid) at a rate of 1 µl per min in the right SNpc. The syringe needle was left in place for 3 min after the injection to prevent reflux of the solution. Finally, the burr hole was sealed with bone wax and the scalp incision sutured, and the animals were then allowed to recover in a humidified incubator before being returned to their home cages.

Drug-induced rotation

One month after the SNpc lesion, the rotational activity induced by D-amphetamine (5 mg/kg body weigh, i.p. route) was studied according to the procedures described (Blanco et al., 2008a). The study only included animals with at least 7 turns per minute, corresponding to a degree of dopaminergic denervation of 90% or higher (data not shown). The Sham-operated group was formed by animals receiving a solution of vehicle (3 µl of 0.9% NaCl and 0.5 mg/ml ascorbic acid) with the same volume and stereotaxic coordinates. A total of three experimental groups were organized: untreated rats (n = 15), rats with SNpc injury (n = 14) and sham-operated rats (n = 14).

Behavioural assessment, apparatus and procedure

The beam traversal test was carried out under proper conditions of silence and illumination, evaluating their results by direct observation. The rats were placed at the middle point of a beam at a height of 60 cm from a supporting surface, equipped with escape platforms at each end (Figure 1A). A little square air bed was collocated immediately under the beam and platform in order to protect the animals from the bang into the floor. Sixty seconds were allotted for each assay, which quantified the time it took for each animal to reach any of the escape platforms (escape latency). Failure to reach either platform or falling from the beam (tumbled down latency) was scored as a latency of 60 s. The number of errors was also simultaneously counted in each assay. An error was defined as any failed attempt to hold on to the beam with either the extremities or the tail, any loss of balance, or any fall from the beam before the 60 s assigned to each assay. The beams were 60 cm long with RCS or CCS used in order of increasing test

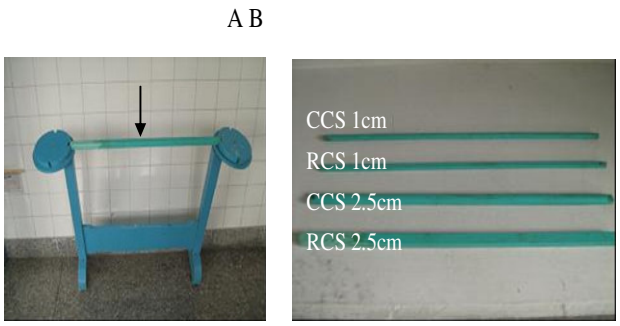

Figure1. Experimental set prepared for the study of motor coordination through the beam traversal test. A. Two escape platforms are united by means of a beam. The arrow indicates the middle point of the beam where the rat is positioned at the beginning of each assay. B. Beam employed in the test: RCS 2.5cm = transversal beam with rectangular cross section of 2.5cm of diameter, CCS 2.5cm = transversal beam with circular cross section of 2.5cm of diameter, RCS 1cm = transversal beam with rectangular cross section of 1cm of diameter, CCS 1cm = transversal beam with circular cross section of 1cm of diameter.

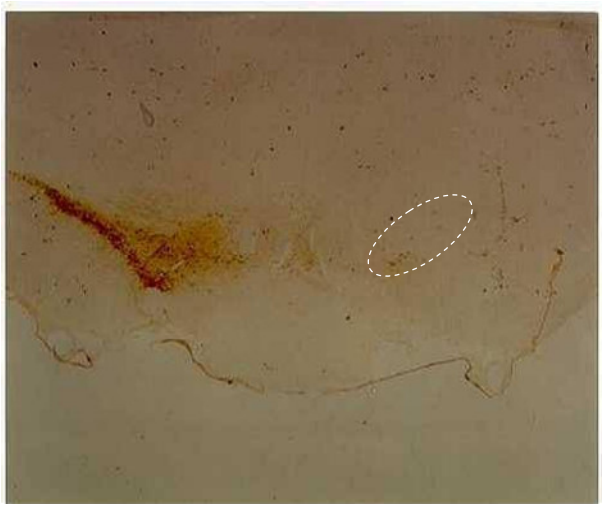

Figure 2. Photomicrograph of representative TH-IR cells in the *substantia nigra pars compacta* (SNpc). Injection of 6-hydroxydopamine induced cell loss in the right SNpc (indicated area) while the left SNpc remained intact. (X10).

complexity (rectangular 2.5 cm diameter, circular 2.5 cm diameter, rectangular 1 cm diameter, circular 1 cm diameter) (Figure 1B). The complete experiment was performed over 2 consecutive days, carrying out 3 assays per day. The final escape and tumbled down latency figures were computed as the mean of the 6 values obtained from all assays.

Tyrosine hydroxylase immunocytochemistry

The animals were anaesthetized with chloral hydrate (480 mg/Kg of body weight, i.p route), and perfused through the ascending aorta. Animal brains were extracted and fixed with 10% formalin for 24 h. Coronal sections (20 µm thick) were obtained (Leitz 1720 cryostat,

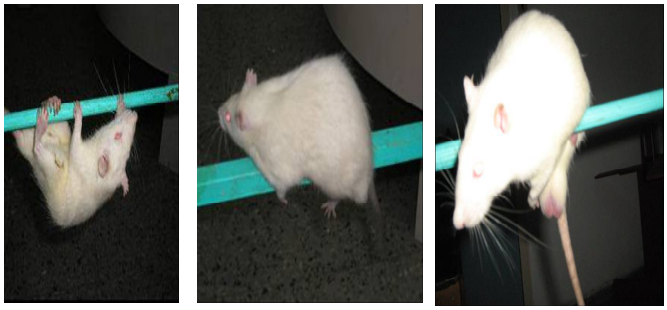

Figure 3. Performing the beam traversal test. A. Untreated rat showed a support strategy which involves the four extremities and also the tail in order to obtain an adequate support on the beams. B. Hemiparkinsonian rat showed a support strategy which did not involve the impaired limbs. C. This support strategy was reinforced when the beams had circular cross section without edges.

Germany) from the areas corresponding to the SNpc. The determination of the extent of nigral dopaminergic degeneration was carried out by the examination of the coronal sections of the SNpc immunohistochemically stained for the tyrosine hydroxylase (TH) enzyme according to the procedure described (Blanco et al., 2008b). The samples from animals where the 6-OHDA injection was in anatomically incorrect location were excluded from the analysis.

Data analysis

The data were tested for normal distribution and homogeneity of variances using the Kolmogorov-Smirnov and the Bartlett tests. The escape and tumbled down latency were compared using one-way analysis of variance, followed by the Tukey's test. The number of errors committed for the animals was compared using a Kruskal-Wallis non parametric analysis of variance, followed by Student-Newman-Keuls (SNK) test. Significance level for statistical analyses was set to 0.05. The data were processed and analyzed using the statistical software application package STATISTICA (StatSoft Inc., 2003).

RESULTS

Inmunohistochemistry study

The 6-OHDA injection caused an almost complete loss of TH-IR cells bodies around the injection site in the SNpc of all animals (Figure 2). The extent of the lesion was variable, with some specimens displaying an affected zone corresponding also with the ventral tegmental area.

Study of motor coordination

All animals were able to execute the beam traversal test. However, they displayed different support and subjection

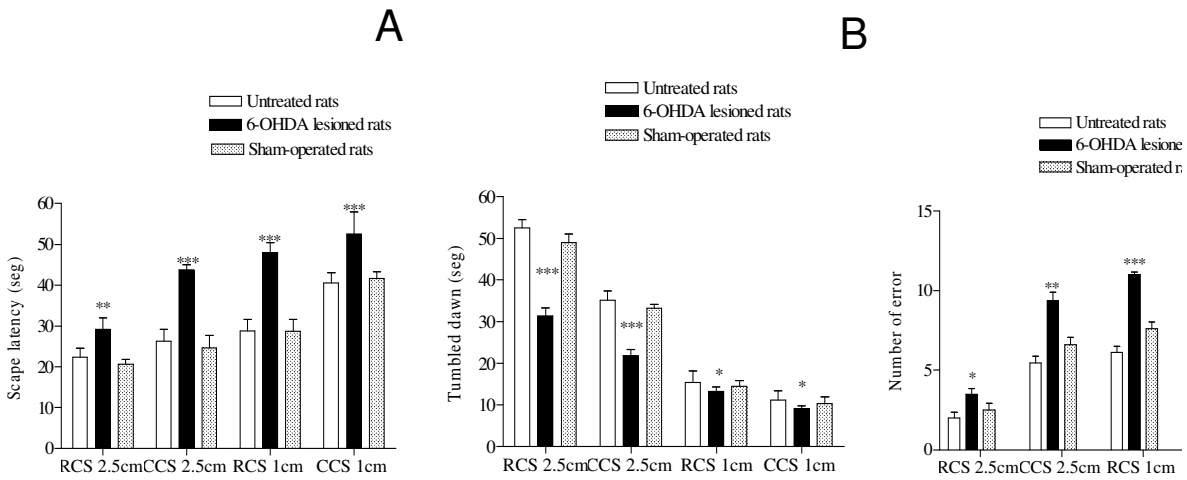

Figure 4. 6-Hydroxydopamine injection effects on motor coordination evaluated by means of beam traversal test. A. Inter-group comparison of escape latency for each beam. B. Inter-group comparison of tumbled down latency for each beam. C. Inter-group comparison of number of errors committed for the animals. The escape and tumbled down latency were compared using one-way ANOVA, followed by Tukey's test. The number of errors committed for the animals was compared using a Kruskal-Wallis non-parametric ANOVA, followed by SNK test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). RCS 2.5 cm: rectangular cross section with 2.5 cm of diameter, CCS 2.5 cm: circular cross section with 2.5 cm of diameter; RCS 1 cm: rectangular cross section with 1 cm of diameter; CCS 1 cm: circular cross section with 1 cm of diameter.

strategies independence of its health condition. Untreated rats showed a good walking strategy and it was performed employing theirs four limbs and also the tail (Figure 3A).

On the other hand, hemiparkinsonian rats adopted an asymmetric posture and they also frequently limped or dragged the impaired hindlimb and often also forelimb during the execution of the test (Figure 3B). This behaviour was emphasized when the beams had circular cross section without edges (Figure 3C).

All variables studied showed significant differences between sham-operated, control and hemiparkinsonian rats. This issue points to the efficacy of the test to evaluate the motor dysfunction in 6-OHDA model.

The escape latency was significantly longer in hemiparkinsonian rats for all beams in comparison with control rats. The difference between groups was increasing with the difficulty of the test. Like that, for the beam with RCS 2.5 cm, the escape latency showed differences slightly significant between groups ($F_{(2, 35)} = 19.69$ $p < 0.01$), meanwhile for the rest of the beams the variable was highly significantly different between groups [CCS 2.5cm: $F_{(2, 41)} = 36.83$ $p < 0.001$; RCS 1cm: $F_{(2, 34)} = 9.91$ $p < 0.001$; CCS 1cm: $F_{(2, 32)} = 4.41$ $p < 0.001$] (Figure 4A).

The tumbled down latency was significantly shorter in hemiparkinsonian rats for all beams in comparison with control rats. Nevertheless, the significant differences between groups were more evident ($p < 0.001$) for the beams with high cross section without taking into account the shape of the cross section (rectangular or circular) [RCS 2.5cm: $F_{(2, 40)} = 15.06$ $p < 0.001$; CCS 2.5 cm: $F_{(2, 35)} = 4.10$ $p < 0.001$; RCS 1cm: $F_{(2, 34)} = 4.49$ $p < 0.05$; CCS 1cm: $F_{(2, 39)} = 7.95$ $p < 0.05$]. This significant differences although were maintained when the rats were evaluated with the beams more narrow, it showed less significant level ($p < 0.05$) (Figure 4B).

The numbers of errors committed for the animals was increased also in the same sense of the difficulty of the test. The hemiparkinsonian rats exhibited a significant increase in this variable for all kind of beams, in comparison with control rats. For the beam with RCS 2.5 cm the difference between hemiparkinsonian and control rats was significant ($H_{(2, 34)} = 6.92$ $p < 0.05$), for the beam with CCS 2.5 cm the difference was slightly significant ($H_{(2, 45)} = 5.48$ $p < 0.01$) and for the rest of the beams (1 cm of diameter) the differences were highly significant (RCS 1cm: $H_{(2, 43)} = 7.31$ $p < 0.001$; CCS 1cm: $H_{(2, 45)} = 9.16$ $p < 0.001$) (Figure 4C).

DISCUSSION

The results indicate that the variables studied allow the evaluation of the motor compromise in the hemiparkinsonian rats. The escape latency reflects the real possibilities of the animals to carry out some compensatory movements whose result is to reach the platform. Meanwhile the tumbled down latency is a direct indication of the sensorimotor deterioration of the hemiparkinsonian rat. Therefore, the increase of the escape latency represents a combination between a higher motor dysfunction of the animal and a higher difficulty of the test. In connection with this idea the hemiparkinsonian rat delays more time to arrive correctly to the platform for all beams but it

is most accentuate in the beam with 1 cm of diameter cross section. In contrast, the decrease of the tumbled down latency points out a higher sensorimotor compromise in the motor execution of the hemiparkinsonian rats. No difference was detected in either the escape latency or the tumbled down latency on the beam when the sham rats were compared to the normal group of rats suggesting that the sham surgery did not affect performance on the beam. On the other hand the quantification of the errors committed for the animals offers an idea of the quality of the motor execution.

In general the score of each variable is worsening at the same time that is increased in the complexity of the test, such as: when the diameter of the cross section of the beam decrease or when the shape of the cross section changes from square to circular. Particularity when motor deficit is evaluated in the beam CCS 1cm, the complexity of the test is maximum in this experimental condition. In this case, the lesser diameter with the circular shape that does not have edge is combined. The edge facilitates the subjection and support in a narrow surface (Schallert and Woodle, 2005).

In the present study we suggest the modification of the test by means of the change in the shape and diameter of the beam and graded the complexity of the test. The change in the diameter of the beam had already been taking into account for other authors (Strome et al., 2006). However, although the diameter was changed during the experiment, the shape of the cross section of the beam was always the same. This study had a training session which involves 10 trials: three trials with the rats starting at the beginning of the narrow end, three starting at the beginning of the medium end and four starting at the wide end (Strome et al., 2006). We are suggesting changing simultaneously the diameter and the shape of the beam in the same assay. It could be interesting to evaluate the effects of some treatment on the motor function recovery. The treatment studied may induce a recovery in the first step of the assay and also it may not induce a recovery in the same measure in the final step of the assay taking into consideration the maximum complexity of the test.

On the other hand other authors have mentioned that the traversal beam test appears to require minimal training for rats to reach maximal performance, reducing the need for extensive training and increasing the reliability of the data (Allbutt and Henderson, 2007).

During our study of motor activity by the beam traversal test all the animals displayed the typically diagonal coupled alternating sequence that characterizes quadruped locomotion (Schallert, 2006). However, and in agreement with previous report in the literature, the hemiparkinsonian rats were handicapped for the use of the limbs contralateral to the lesion side during both postural adjustments and ambulation (Woodle and Schallert, 2004). This dysfunction is the main cause of the errors committed for the animals and it may be related to difficulties for adequately transferring the weight of the body and achieving an optimal postural adjustment in small supporting surfaces (Miklyaeva et al., 1995). In addition it is possible that the unlesioned side is not able to compensate adequately for locomotors' deficits when the lesion is large according to previous report (Warraich et al., 2009).

It has been pointed out that striatal dopaminergic deficiencies represent an obstacle to the controlled application of the force demanded by the coordinated movement of all body parts (Woodle et al., 2008). This obstacle may be manifested as a deficiency in the control of the affected limbs, as limb rigidity, as diminished motor reflexes, or as a number of other sensory and motor deficiencies (Miklyaeva et al., 1995; Johnston et al., 1999).

On the other hand, the hemiparkinsonian rats show a tendency to spontaneously take tight turns in narrow circles. This behaviour further pushes the centre of mass off-balance, adding up to the difficulties already imposed by the diameter and the cross sectional shape of the bridge (Metz and Whishaw, 2002; Schallert et al., 2000). The rotational behaviour deteriorates the locomotor abilities of the animals and results in more frequent failures when attempting to hold onto the beam or just staying on its surface (Metz and Whishaw, 2002).

During the execution in the beam traversal test, the DA depleted rats show a small oscillation that swayed the body of the animal without achieving forward movement, and thus was not involved in forward locomotion (Woodle and Schallert, 2004; 2006). This group of rats never move in a straight line along the beams, instead alternating straight movements with tight turns that forced them to adopt increasingly unstable positions as the diameter of the beam is decreased.

A beam traversal test is a reliable test to study the motor coordination in 6-OHDA model when the toxin is administered in SNpc. This test reflects dysfunction at multiple anatomical areas of the Central Nervous System and peripheral nervous system or muscle (Urakawa et al., 2007). The literature refers some papers where this test has been employed to evaluated rats lesioned in the MFB (Strome et al., 2006; 2007). After 6-OHDA injections into SNpc, dopaminergic neurons start degenerating within 24 h. When injected into the striatum or MFB, however, 6-OHDA produces a more protracted retrograde degeneration of nigrostriatal neurons, which lasts for 1 - 3 weeks (Dauer and Przedborsky, 2003). Taking into account that the exact moment that appears the motor impairment and also the possibilities of spontaneous recovery are different among them, it is very important to consider the best time interval after 6-OHDA lesion to apply the test.

We found that sometimes our hemiparkinsonian rats tumbled from the beams. However this fall has not been reported by other author whose rats were injected in MFB (Allbutt and Henderson, 2007). They have suggested that

balance did not appear to be dramatically affected by the lesion performed in MFB (Allbutt and Henderson, 2007). We can consider that the course of degeneration induced by the injection of 6-OHDA in SNpc is associated also with degeneration of others structures such as the pedunculopontine nucleus (PPN) (Blanco et al., 2007, 2008a). In this hemiparkinsonian condition, the pontine neuron presents a cells death process (Blanco et al., 2007). Meanwhile when the toxin is injected in MFB these neurons are preserved (Heise et al., 2005). The PPN is a very important relay center in the balance and motor coordination control in rats and its dysfunction could be a substrate for the tumbled down behaviour observed for us (Jenkinson et al., 2009). In the future will be very interesting to study the influence of PPN dysfunction in the execution of traversal beam test taking into account the recently interest for this nucleus by deep brain stimulation therapy for parkinsonian patients (Jenkinson et al., 2009).

The present results confirm that the traversal beam test can be applied to evaluate the motor dysfunction in rats injected in SNpc taking into account that the motor disabilities developed for these animals are sensitive to be recording with the test. The behavioural consequences of dopaminergic lesions and dopamine-replacement therapies are commonly assessed in the laboratory using simple screens of motor asymmetry such as drug-induced rotation (Kirik et al., 1998; Dowd and Dunnett, 2005). However, the parkinsonian deficits are significantly more complex than this. Then alternative traverse beam test changing the diameter and the shape of the beam that permits a detailed analysis of the motor dysfunction and the effects of possible treatment is more informative.

Conclusion

The impairments that are displayed by the rats with unilateral DA depletions can be studied by beam traversal test through the direct observation and also through automatic-digital systems. The last one is very expensive, but it has some advantages such as the possibility to evaluate the error separately considering forelimb or hindlimb ipsi- or contralateral to the lesion. The beam traversal test is a convenient sensorimotor test that does not need to be trained extensively and require adverse motivation or food deprivation, for to collect the data. It appears to be very useful in established unilateral model of Parkinson's disease and also it would be useful in evaluating the effect of some pharmacological or surgical (lesion, stimulation or cell transplants) treatments on motor dysfunction of hemiparkinsonian rats.

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