Amphetamine-induced rotation and L-DOPA-induced dyskinesia in the rat 6-OHDA model: A correlation study

Elisabetta Tronci a, Eunju Shin a, Anders Björklund a, Manolo Carta a,b,*

a Wallenberg Neuroscience Center, Division of Neurobiology, Department of Experimental Medical Science, Lund University, Lund 221 84, Sweden
b Department of Biomedical Science, Cagliari University, Cittadella Universitaria SS 554 km 4.5, Monserrato 09042, Italy

1. Introduction

Unilateral 6-OHDA-lesioned rats are widely used as a model for Parkinson’s disease (PD) to study mechanisms involved in neuronal dopaminergic degeneration, and to explore new therapeutic interventions (Ungerstedt, 1968; Björklund, 1991; Cenci et al., 1998). The dopaminergic toxin 6-hydroxypapamide (6-OHDA) can be injected at different levels along the nigro-striatal dopamine pathway, producing various degrees of dopamine cell loss. Nigral or medial forebrain bundle (MFB) delivery is generally meant to produce complete (or near-to-complete) loss of dopaminergic neurons, while targeting of the striatum generally produces a partial and more progressive degeneration, with significant preservation of striatal dopamine fibers (Kirk et al., 1998).

Abbreviations: 6-OHDA, 6-hydroxydopamine; AIMS, abnormal involuntary movements; KPBS, potassium phosphate buffered saline; LID, L-DOPA-induced dyskinesia; MFB, medial forebrain bundle; PBS, phosphate buffered saline; PD, Parkinson’s disease; SERT, serotonin transporter; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

* Corresponding author at: Department of Biomedical Science, Cagliari University, Cittadella Universitaria SS 554 km 4.5, Monserrato 09042, Italy. Tel.: +39 070 675 4142; fax: +39 070 675 4320.
E-mail address: manolocarta@unica.it (M. Carta).

In recent years, 6-OHDA-lesioned rats have been used to study mechanisms underlying L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) (Cenci et al., 1998; Cenci, 2007). Abnormal involuntary movements (AIMs) are induced in dopamine-lesioned rats by sub-chronic administration of L-DOPA, thus recapitulating the appearance of the dyskinesias in PD patients.

Amphetamine is a psychotropic compound able to bind the dopamine transporter, inducing reversal of this protein, and cytosolic dopamine release (Kuhr et al., 1985; Sulzer et al., 1993, 1995). As a consequence, a dose-dependent motor activation can be observed following administration of the drug. Because of these properties, amphetamine is commonly used to investigate the extent of dopamine cell loss induced by 6-OHDA. In fact, when administered to unilateral 6-OHDA-lesioned rats, dopamine is released in greater amounts in the intact striatum than in the lesioned side, thus producing an asymmetric motor activation of the right and left sides of the body. The result is an intense ipsilateral rotational behavior, which has been correlated to the extent of dopamine denervation (Ungerstedt, 1971; Carman et al., 1991; Schwarting and Huston, 1996).

Based on early correlation studies, a cut-off of 6 turns/min over a 90 min period after administration of amphetamine has been used in most of studies from several groups to select 6-OHDA-lesioned rats with a dopamine depletion sufficient to induce deficits in motor tasks (Winkler et al., 2002). Later, when the dyskinesia model was...
introduced, the same cut-off value has been used to recruit animals in these types of investigations. However, only a single study investig- 
tigated correlation between amphetamine-induced rotation rate and development of dyskinesia following chronic administration of different l-DOPA doses (Puttman et al., 2007).

To investigate whether the rotation rate induced by 2.5 mg/kg of amphetamine is predictive of development of dyskinesia induced by sub-chronic l-DOPA treatment (6 mg/kg s.c.), we collected data from 312 female 6-OHDA-MFB-lesioned rats (from different sets of experiments). All rats were subjected to the amphetamine-induced rotation test, and then sub-chronically treated with l-DOPA to establish dyskinesias.

Moreover, we also evaluated whether the magnitude of dopamine depletion is a good predictor of LID susceptibility.

Re-defining the inclusion criteria, and, thus, finding the opti- mal cut-off value for the amphetamine-induced rotation test in our experimental conditions will maximize the number of animals to be included in our investigations. These results have both economical and ethical implications.

2. Materials and methods

2.1. Housing

Data were collected from different sets of experiments over one year period where 6-OHDA-lesioned rats were treated with daily l-DOPA in order to make them dyskinetic. A total of 312 adult female Sprague-Dawley rats weighing 225–250g were used in the present study (Charles River, Germany). The animals were housed under a 12 h light/12 h dark cycle with free access to water and food. All experiments were performed according to the regulations set by the Ethical Committee for use of Laboratory animals at Lund University.

2.2. Experimental parkinsonism

6-OHDA injections were conducted under anesthesia induced by an injectable 20:1 mixture of Fentanyl and Dormitor (Apoteksbolaget, Sweden) using a stereotoxic frame (Stoeling, Wood Dale, Illinois) with an attached Hamilton syringe. The animals received 6-OHDA (Sigma–Aldrich AB, Sweden) injection into the MFB (14 μg free base in 4 μl of saline containing 0.02% ascorbic acid) in order to achieve a complete lesion of the nigrostriatal pathway, at the following coordinates (relative to bregma, see Paxinos and Watson [1998]): AP: −4.4 mm, ML: −1.2 mm, DV: −7.8 mm from the dura). The toothbar was set at −2.4 mm. Injection speed was 1.0 μl/min and the syringe was kept in place for an additional 3 min before it was slowly retracted.

2.3. Amphetamine-induced rotation

Amphetamine-induced rotation was performed at 3 weeks after the 6- 
OHDA injection to evaluate the extent of the dopaminergic lesion. Right and left full body turns were recorded over 90 min, using automated rotometer bowls (Accus- 
cr Implant Inc., Columbus, Ohio), following an i.p. injection of 2.5 mg/kg of d-amphetamine sulfate (Apoteksbolaget, Sweden). The data are expressed as net full body turns per min, where rotation towards the side of the lesion was given a positive value.

2.4. l-DOPA-induced dyskinesia

Starting three days after the amphetamine-induced rotation test, 6-OHDA-lesioned rats were treated with daily l-DOPA (6 mg/kg plus benserazide 10 mg/kg s.c.) for about 4 weeks. AIMS were evaluated during the chronic treat- ment until the average dyskinesia score reached stable values. AIMS were evaluated according to the rat dyskinesia scale described in detail previously (Lee et al., 2000; Lundblad et al., 2002). Briefly, rats were placed individually in transparent plastic cages without bedding material and scored every 20 min following the injection of l-DOPA for the entire time course of dyskinesia (about 120 min). The AIMS were classified into four subtypes according to their topographic distribution as Forelimb, Orolinguial, Axial and Locomotive behaviors. The severity of each AIM subtype was assessed using scores from 0 to 4 (1: occasional, i.e. present less than 50% of the time; 2: frequent, i.e. present more than 50% of the time; 3: continuous, not interrupted by strong sensory stimuli; 4: continuous, not interrupted by strong sensory stimuli). In this study, rats with an AIMS score >28 were considered highly dyskinetic, while rats with an AIMS score <28 either low or non-dyskinetic.

2.5. Tissue processing and immunohistochemistry

Within the 312 female 6-OHDA-lesioned rats employed for collecting the behavioural data, a group of 50 lesioned rats were used for immunohistochemistry analysis after termination of chronic l-DOPA treatment. Three days after the last l-DOPA injection rats were trans-cardially perfused with 100 mL of 0.9% saline, followed by 250 mL of ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were post-fixed for 2 h and then transferred to 25% sucrose in PBS for cryo-protection overnight. Sections of 35 μm, obtained using a freezing microtome (Leica), were stored at −20°C in an anti-freeze solution (PBS containing 30% glycerol and 30% ethylene glycol) until free-floating immunohistochemistry was performed.

Briefly, sections were rinsed in potassium phosphate buffer saline (KPBS) and then endogenous peroxidase activity was quenched in 3% H2O2 and 10% methanol in KPBS for 20 min. After three rinsing steps in KPBS, the sections were incubated in a blocking solution consisting of 5% normal goat serum in KPBS and 0.25% Triton X- 
100, to block non-specific binding sites. Sections were then incubated overnight at room temperature in the same block solution as described above with the primary antibody, rabbit anti-TH (1:1000, AB152, Chemicon) or rabbit anti-SERT (1:1000, MAB1564, Chemicon). On the second day, the sections were rinsed three times in KPBS and then incubated in blocking solution for 20 min before being incubated for one hour in a 1:200 dilution of biotinylated secondary antibody, goat anti-rabbit (Vector Laboratories), in blocking solution. After rinsing, the sections were treated with avidin–biotin complex (ABC Elite kit, Vector Laboratories) in KPBS for one hour before being rinsed again. The colour reaction was developed by incubation in 25 mg/ml 3,3’-diaminobenzidine and 0.005% H2O2. Sections were mounted on gelatin-coated glass slides, dehydrated in an ascending series of alcohols, cleared in xylene and cover-slipped with DePex mounting media (BDH Chemicals).

2.6. Estimation of TH positive cells in SNc

Immunostained sections containing left and right SNc were captured at 20× magnification using an Olympus Optipan microscope. The entire intact and lesioned SNc, corresponding to three frames, was digitized for the analysis. For each animal, three sections corresponding to rostral (−4.8 mm from bregma), medial (−5.3 mm), and caudal (−5.8 mm) SNc levels (accordingly to Rat Brain Atlas, Paxinos and Watson, 1998) were analyzed for TH positive cell number evaluation with Canvas software (Media Cybernetics, 2009). Positive cells were counted in the whole intact and lesioned SNc to give a total number of cells per each level. Data are expressed as percentage of the total TH positive cells in the lesioned SNc compared to the intact side.

2.7. Evaluation of SERT expression in the striatum and globus pallidus

Two groups of rats, either highly dyskinetic (n = 6) or non-dyskinetic (n = 7), were selected from the rats showing a rotation rate ≥6 turns/min for evaluation of the density of the serotonin innervation in the striatum and globus pallidus in SERT stained sections. The density of the SERT-positive innervation was assessed in four measuring fields (0.19 μm²; two in caudal striatum and two in globus pallidus) by estimating the area covered by SERT-positive elements (fibers and terminals; excluding the white matter tracts) as a fraction of the total area of grey matter covered by the measuring frame.

2.8. Statistics

Correlations in Figs. 1–3 were estimated with a nonparametric Spearman’s test. In Table 1, significance between groups was calculated with one-way ANOVA analysis followed by Tukey’s post hoc test. Statistical significance was set at p < 0.05. All statistical analysis was performed using STATISTICA software.
Table 1

<table>
<thead>
<tr>
<th>n. rats (tot. 312)</th>
<th>Amphetamine-induced rotation rate</th>
<th>AIMS average</th>
<th>n. dyskinetic rats (AIMs score &gt;28)</th>
<th>% dyskinetic rats (AIMs score &gt;28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>&lt;0</td>
<td>15.03</td>
<td>6</td>
<td>37.5</td>
</tr>
<tr>
<td>33</td>
<td>0–2</td>
<td>3.34</td>
<td>2</td>
<td>6.1</td>
</tr>
<tr>
<td>22</td>
<td>2–3</td>
<td>17.78</td>
<td>7</td>
<td>31.8</td>
</tr>
<tr>
<td>25</td>
<td>3–4</td>
<td>21.84*</td>
<td>10</td>
<td>40.0</td>
</tr>
<tr>
<td>27</td>
<td>4–5</td>
<td>25.57*</td>
<td>13</td>
<td>48.1</td>
</tr>
<tr>
<td>34</td>
<td>5–6</td>
<td>18.83</td>
<td>11</td>
<td>32.4</td>
</tr>
<tr>
<td>31</td>
<td>6–7</td>
<td>23.17*</td>
<td>11</td>
<td>35.5</td>
</tr>
<tr>
<td>33</td>
<td>7–8</td>
<td>23.01*</td>
<td>15</td>
<td>45.5</td>
</tr>
<tr>
<td>20</td>
<td>8–9</td>
<td>25.41*</td>
<td>9</td>
<td>45.0</td>
</tr>
<tr>
<td>20</td>
<td>9–10</td>
<td>21.18</td>
<td>8</td>
<td>40.0</td>
</tr>
<tr>
<td>17</td>
<td>10–11</td>
<td>27.29*</td>
<td>8</td>
<td>47.1</td>
</tr>
<tr>
<td>34</td>
<td>&gt;11</td>
<td>29.62*</td>
<td>20</td>
<td>58.8</td>
</tr>
</tbody>
</table>

Based on their amphetamine-induced rotation rate, rats were divided in subclasses and in each class AIMS score mean and percentage of dyskinetic rats were calculated. One-way ANOVA analysis showed that a similar percentage of dyskinetic rats and mean AIMS score were obtained among the classes with a rotation rate ≥3 turns/min (*P<0.05 vs 0–2 subgroup).

3. Results

3.1. Correlation between amphetamine-induced rotation rate and LID

Three weeks after the 6-OHDA lesion, rats were treated with amphetamine (2.5 mg/kg, i.p.), and the rotation rate was calculated over a 90 min period by an automated system. Starting three days later, all rats received a sub-chronic treatment with L-DOPA (6 mg/kg plus benserazide 10 mg/kg, s.c.) for about four weeks, until a stable expression of dyskinesia was achieved. A very weak correlation was found between amphetamine-induced rotation rate and AIMS score (n = 312; R² = 0.052; P < 0.0001, Fig. 1). For a detailed overview, rats were then classified based on their rotation rate; mean dyskinesia and percentage of dyskinetic rats in each class were calculated. Results are summarized in Table 1. Interestingly, all categories of rats among those rotating ≥3 turns/min appear to have similar mean AIMS scores and percentage of dyskinetic rats.

3.2. Correlation between LID and TH positive cell number in the lesioned SNc

In order to evaluate the predictive value of TH positive cell loss in SNc on dyskinesia development, we investigated correlation between TH positive cell number and the AIMS score in a subgroup of rats. Fig. 2 shows a very weak correlation between percentage of TH positive cell number in the lesioned SNc and AIMS score (n = 50; R² = 0.12; P = 0.013).

3.3. Correlation between amphetamine-induced rotation test and TH positive cell number in the lesioned SNc

Opposite to previous reports, (Hefti et al., 1980; Hudson et al., 1993; Kirik et al., 1998; Olds et al., 2006; Putterman et al., 2007), no correlation was found between rate of amphetamine-induced rotation and TH positive cell number in the lesioned SNc (n = 50; R² = 0.018; P = 0.35, Fig. 3). However, it is worth noting that about 90% of the animals with cell loss greater than 90% rotated at least 3 turns/min.

3.4. SERT expression in the caudal striatum and globus pallidus of dyskinetic and non-dyskinetic rats

Serotonin neurons are known to play a major role in the induction of L-DOPA-induced dyskinesia and selective serotonin lesions have been shown to suppress LID in 6-OHDA-lesioned rats (Carta et al., 2007). Thus, we have investigated whether loss of serotonin terminals, measured as SERT expression levels, may have contributed to the different dyskinesia levels seen in our amphetamine-responsive rats. Indeed, Rylander and co-workers have recently shown significant difference in SERT expression in striatum and globus pallidus between dyskinetic and non-dyskinetic subjects across different species (Rylander et al., 2010). Two clusters of rats, either highly or non-dyskinetic, were selected for this analysis among those rotating ≥6 turns/min. In fact, a significant difference was found in the mean SERT levels between dyskinetic and non-dyskinetic rats in both caudal striatum and globus pallidus (SERT mean density in the caudal striatum: 26 and 16.1 for dyskinetic and non-dyskinetic rats, respectively, P < 0.05; SERT mean density in the globus pallidus: 24.6 and 16.1 for dyskinetic and non-dyskinetic rats, respectively, P < 0.05, t test, n = 6–7).

Fig. 2. TH positive cell number and LID correlation. The percentage of TH positive cell number in the lesioned SNc was correlated with AIMS score. Nonparametric Spearman’s test revealed a very weak correlation between these two variables.

Fig. 3. TH positive cell number and amphetamine-induced rotation rate correlation. The percentage of TH positive cell number in the lesioned SNc was correlated with amphetamine-induced rotation rate. Nonparametric Spearman’s test revealed no correlation between these two variables.
4. Discussion

Data from different studies performed in our lab where dyskinesia was induced by a sub-chronic treatment with L-DOPA were collected in order to investigate the predictive value of the amphetamine-induced rotation test on development of LID in unilateral 6-OHDA-lesioned rats. Rotational response to amphetamine administration is widely used as behavioral index to evaluate success of the 6-OHDA lesion in the rodent model of PD, and it has been shown to correlate with the extent of the lesion (Ungerstedt, 1968, 1971; Hefti et al., 1980; Hudson et al., 1993; Kirik et al., 1998; Olds et al., 2006; Putterman et al., 2007). A rotation rate ≥ 6 turns/min after administration of amphetamine has been shown to correlate with a level of dopamine depletion sufficient to induce signs of hemi-parkinsonism in 6-OHDA-lesioned rats, although a cut-off of 5 turns/min is also used by other groups (Winkler et al., 2002; Olds et al., 2006; Putterman et al., 2007). To our knowledge, only a single study evaluated the predictive value of the amphetamine-induced rotation test on development of AIMs, where a positive correlation was found (Putterman et al., 2007). However, an amphetamine dose of 5 mg/kg was used in that study, while 2.5 mg/kg was used in our experiments (as for other groups working on dyskinesia) in order to induce fewer side effects. Moreover, female rats are employed in our investigations, while males were used in the Putterman’s study, where the 6-OHDA toxin was also delivered at a different site. Thus, it should not appear surprising that conclusions from the Putterman’s study cannot be applied to such different experimental conditions, as the ones used here; in fact, our results showed that the amphetamine-induced rotation rate was poorly correlated with LID development.

Allocation of rats in different sub-groups based on their rotation rate revealed that propensity to develop LID does not increase with increasing rotational response to amphetamine after 3 turns/min. Only rats with a rotation rate lower than 3 turns/min developed significantly less dyskinesia than those rotating 6 turns/min (the previously employed cut-off value) or more. These results do not only suggest that the amphetamine-induced rotation test has limited ability to predict development of dyskinesia, but they also indicate that using a cut-off of 6 turns/min appears to be excessively strict, and may lead the investigators to exclude too many animals. Rather, our data suggest that all animals rotating ≥ 3 turns/min could be employed in dyskinesia studies.

It is known that partial 6-OHDA-lesioned rats are less prone to develop dyskinesia than complete lesioned rats (Carta et al., 2007). However, our results showed that TH positive cell loss in SNc was poorly correlated with LID, suggesting that the magnitude of dopamine depletion is not a good predictor of LID susceptibility. As shown in Fig. 2, it is worth noting that a TH positive cell loss of at least 90% appears to be necessary but not sufficient for development of AIMs, and therefore, cannot be the only factor involved in LID development. In fact, a significant number of rats with a marked TH positive cell loss showed either none or very mild dyskinesia, despite a dose of L-DOPA able to induce severe abnormal movements in a significant number of rats. Using the same group of animals, we also correlated amphetamine-induced rotation rate with TH positive cell number. Results revealed a lack of positive correlation, in disagreement with previous studies (Olds et al., 2006; Putterman et al., 2007). It should be pointed out, however, that earlier studies have used densitometry as measurement of dopamine depletion, while we performed cell counting in our investigation.

While several reasons may have contributed to the poor correlation between dyskinesia score and amphetamine-induced rotation in our study, the existence of a significant number of well-depleted/amphetamine-responsive rats that are resistant to development of LID suggests that mechanisms other than dopamine depletion must contribute to the induction of LID. In light with previous work, we have speculated that partial loss of serotonin innervation in some critical area may contribute to this resistance. In support of this hypothesis, we formerly showed that 5,7-di-hydroxy-tryptamine (5,7-DHT, a specific toxin for serotonin neurons) lesions suppressed LID in dyskinetic rats, and prevented its development (Carta et al., 2007). In fact, we found here significant lower SERT levels in both caudal striatum and globus pallidus in non-dyskinetic rats as compared to dyskinetic ones. Interestingly, Rylander et al. (2010) have recently demonstrated that L-DOPA treatment induced sprouting of serotonin terminals in dyskinetic subjects, both rats and monkeys. Our data are in line with this report and suggest that reduced striatal and pallidal serotonin innervation may have contributed to the low expression of dyskinesia observed in some of the amphetamine-responsive animals. However, reduction in striatal/pallidal serotonin innervation cannot be the only factor accounting for the full protection observed in some rat; in fact, Eskow et al. (2009) have shown that a striatal denervation > 50% is required to significantly affect LID upon 5,7-DHT lesions. Thus, resistance to the induction of maladaptive alterations at the level of post-synaptic striatal neurons, which are ultimately responsible for dyskinesia, is also likely to play a role.

5. Conclusions

In summary, our data showed that the amphetamine-induced rotation test is a poor predictor of development of dyskinesia, at least at the dose of amphetamine used here in female rats. Based on early reports investigating motor deficits in 6-OHDA-lesioned rats, animals with a rotation rate lower than 6 turns/min (or 5 in some study) have usually been excluded from dyskinesia studies before treatment with L-DOPA was even initiated. Results from the present investigation suggest that all rats with an amphetamine-induced rotation rate ≥ 3 turns/min should actually be recruited for the L-DOPA treatment when using an amphetamine dose of 2.5 mg/kg. This finding has both financial and ethical implications that should be taken into consideration when designing studies focusing on dyskinesia in the rat 6-OHDA model.

Acknowledgments

We thank Bengt Mattsson, Ulla Jarl, Anneli Josefsson, for expert technical assistance. This study was supported by grants from the Swedish Research Council (AB and MC).

References


