Changes in day/night activity in the 6-OHDA-induced experimental model of Parkinson’s disease: Exploring prodromal biomarkers

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Research

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Abstract

Background:

Parkinson's disease (PD) is a prevalent neurodegenerative disorder for which there is not currently a cure and when it is clinically diagnosed is likely too late. The search for experimental models mimicking an early stage of PD before motor manifestations is fundamental in order to explore early signs and get a better prognosis. Interestingly, our previous studies have indicated that 6-hydroxydopamine (6-OHDA) is a suitable model to induce an early degeneration of the nigrostriatal system without any gross motor impairment. Considering our previous findings, we aim to implement a novel system to monitor rats after intrastriatal injection of 6-OHDA to detect and analyze physiological changes underlying prodromal PD.

Methods:

Rats were unilaterally injected with 6-OHDA or saline solution into the striatum and placed in enriched environment cages where the activity was monitored. After two weeks, the amphetamine test was performed before the sacrifice. Immunohistochemistry was developed for the morphological evaluation and western blot analysis to assess molecular changes.

Results:

Home-cage monitoring revealed behavioral changes in response to 6-OHDA administration including significant hyperactivity and hypoactivity during the light and dark phase respectively turning out in a change of the circadian timing. A preclinical stage of PD was functionally confirmed with the amphetamine test. Moreover, the loss of tyrosine hydroxylase expression was significantly correlated with the motor results, and 6-OHDA induced early proapoptotic events.

Conclusions:

Our findings provide evidence for a novel prodromal 6-OHDA model following a customized monitoring system that could give insights to detect non-motor deficits and molecular targets to test neuroprotective/neurorestorative agents.

Background:

Parkinson's disease (PD) is a complex neurologic disorder in which not only motor impairments occur, but also other non-motor symptoms (NMS) play a relevant role, including hyposmia, sleep disorders, depression, constipation and cognitive deficit [1–3]. Most of them often appear earlier than the motor symptomatology, during the so-called prodromal stage and worsen following the disease’s progression [4]. Remarkably, slight motor symptoms (< 40–60% of dopaminergic neuronal cell loss) have been also
associated to the prodromal stage when they do not meet the criteria for the clinical diagnosis of PD [5]. Thus, it is essential to establish a correlation between the early manifestations of PD (prodromal phase) with the clinical and pathological stage for an early diagnosis of PD.

Thus, studies about early events of the disease are emerging in order to find some biomarker for an early-premotor diagnosis [6]. However, the neuroanatomical and etiological background of NMS in PD remain to be elucidated [4]. One of the first NMS that is suffered by a high percentage of the patients is the sleep disorder, such as sleep fragmentation, excessive daytime sleepiness or “REM behavior disorder” (RBD), which is a pathology based on increasing muscle tone during the REM (rapid eye movement) sleep [7, 8]. Among the mechanisms underlying sleep disturbance, the disruption in the circadian system is attributed as triggering factor [9]. Indeed, growing evidence support an association between circadian disruption and PD, suggesting that the dopamine depletion may lead to circadian rhythm irregularities including the alteration of the circadian control of the rest / activity rhythms [8, 10].

In the search of preclinical models of PD, there is a main challenge for reproducing those functional and pathological changes that could provide the scaffold to find out about the target for the design of neuroprotective therapies before the progression of the disease. In this context, the well-known model of the 6-hydroxydopamine (6-OHDA) which is widely used to study motor deficits, can also be useful for identifying non-motor and early motor impairments specially when combined with advanced technological devices [11]. In this model, the site of administration and time elapsed after the injection are critical for determining the extent and time course of the lesion (Deumens et al., 2002; Kirik et al., 2000). In our hands, this model is also useful for studying the preclinical phase of PD, as intrastratal injection of 6-OHDA in adult male rats induced an early degeneration of the nigrostriatal system without any gross motor impairment as well as upregulation of caspase-3 and downregulation of survival signaling pathways [14, 15].

Therefore, in the present study we aimed to detect prodromal changes in the behavior following an experimental model of PD based on the intrastratal injection of 6-OHDA with a short time of evolution (2 weeks) in adult male rats housed in monitored enriched environment (EE) cages.

Materia And Methods

Experimental design and housing conditions

Twenty adult male Sprague-Dawley rats 3-month-old (280-300 gr) were randomly assigned to the following groups: Saline group (n = 10) as control and 6-OHDA group (n = 10). After stereotaxic injection of toxin or saline solution the animals were housed for two weeks in monitored enriched environment cages (10 animals per cage) consisting of large cage (790 mm × 460 mm × 640 mm) with two floors, which were connected by a plastic ramp and an external running wheel. A 12 h light/12 h dark cycle was established with access to food and water ad libitum as previously described [15]. Current EE cages were supplied with an automatic recording system. This novel system consists, basically, of an infrared camera connected to a raspberry device programmed to collect pictures accurately every 30 seconds. In
order to measure changes in the activity, all images were analyzed by an own designed customized program.

Two weeks after the intrastriatal injection the amphetamine test was performed for evaluating motor deficits and with the purpose of confirming that no rat presented more than 3 turns per minute (tpm). After amphetamine test, rats were sacrificed for histological and biochemical analysis.

All the experimental protocols were approved by the Ethical Committee and Animal Welfare (CEEA) of the University of the Basque Country (CEEA/M20/2016/176), and in accordance with Spanish Royal Decree RD 1201/2005, European Directive 2003/65/EC and the European Recommendation 2007/526/EC on the protection of animals used for scientific purposes. Furthermore, subclinical changes were daily evaluated throughout an observational protocol based in Morton-Griffiths ones [16]. This supervision protocol allowed us to assess the animal welfare by evaluating qualitatively the changes induced by this model regarding a sort of detectable variables.

6-OHDA lesions

Rats were lesioned as we previously described [14,15]. Briefly, rats were unilaterally injected into the striatum with 7.5 micrograms of 6-OHDA, but a short time of evolution (two weeks) was elapsed before sacrifice in order to achieve a preclinical lesion.

Monitoring analysis

An in-house customized automatic system based on image processing with a camera infrared Camera Module v2 (Pi NoIR) was used for the analysis of the rat group behavior. A schematic diagram of the working procedure for image processing is described in Fig. 1.

This process was divided in two phases: image pre-processing and image analysis.

1. In the first phase video sequences were acquired by a high-quality camera and pre-processed by an own toolbox in MATLAB to create series of binary image frames of the rat group activity. Thus, the activity areas (motion detection) were calculated based on a frame difference method. Frame absolute difference was calculated between two consecutive frames with a sampling period of 30s. Images were converted from RGB into grey scale, after that in binary images and then by a threshold filter extracted the main activity area of the system (rats, and objects moved by the rats). Finally, activity areas were defined applying morphological filters to reduce the noise and for smoothing the images.

2. During the second phase the trajectories of the animal group is generated by the system centroid (the average position of all the activity areas in the binary image) evolution. The centroid is estimated by $k$-means algorithm over the binarized frame series [17,18]. Thus, within each frame, the coordinates of the centers of every object were calculated, and K-means were applied to find the center of the entire group, that is the “centroid”. A centroid with two coordinates:
\[ C = (x_c, y_c) \]

3. The group trajectory consists of the evolution of that centroid from the first frame to the last one.

Finally, the rat groups’ behavior was described and modelled by the following parameters:

1) Number of activity areas in the binary images.

2) Shannon entropy of the centroid trajectory. Shannon entropy is a main concept in information theory and is a measure of average uncertainty (information content). Entropy in biosignals gives information about the system evolution and behavior and can be applied to analyze pathological behaviors [17,18].

3) Velocity, speed and acceleration defined as:

\[
\begin{align*}
\{ v_{cx} &= \Delta x_c / \Delta t \\
\{ v_{cy} &= \Delta y_c / \Delta t \\
\end{align*}
\]  

(1)

being speed the absolute value of velocity. An acceleration:

\[
\begin{align*}
\{ a_{cx} &= \Delta v_{cx} / \Delta t \\
\{ a_{cy} &= \Delta v_{cy} / \Delta t \\
\end{align*}
\]  

(2)

Amphetamine-induced rotation test

Two weeks after intrastriatal injection the amphetamine test was developed following the previously described methodology for this behavioral test [19]. Briefly, D-amphetamine (5 mg/kg) in 0.9 % NaCl; Sigma-Aldrich, St. Louis, USA) was intraperitoneally administered and the animals were placed in an individual circular cage (rotameter). After 15 min of latency, the total number of full ipsilateral (IL) rotations was recorded during 90 min (Multicounter LE3806; Harvard Apparatus, Holliston, MA, USA) and data expressed as the number of rotations per minute.

Morphological analysis

Morphological evaluation was performed as we previously described [14,15,20].

Tissue processing for histological evaluation

After behavioral test, five rats from each group were transcardially perfused with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains removed and 50 mm serial coronal sections including striatum and substantia nigra (SN) were collected for further analysis.

TH immunostaining
Tyrosine hydroxylase (TH) immunohistochemical staining was developed on free-floating coronal slices. Briefly, sections were incubated with rabbit polyclonal anti-TH (Ref: AB-152, Millipore; 1:1000). Sections were processed with the avidin-biotin-peroxidase complex using a commercial kit (Ref. PK-6102, Elite ABC kit, Vector Laboratories, Burlingame, CA) and the reaction was shown by using 3,3-diaminobenzidine (DAB).

Images were visualized, captured and analyzed at 4x and 40x magnification by an Olympus BX-50 photomicroscope.

*Double immunofluorescence staining of Caspase-3 and NeuN*

Coronal sections were incubated with rabbit anti-caspase 3 (H-277) (Ref: sc-7148, Santa Cruz Biotechnology Inc; 1:50) and monoclonal mouse anti-NeuN (Ref: MAB377, Chemicon International, Inc.; 1:100). Sections were subsequently incubated with secondary antibodies conjugated to Alexa Fluor-488 (Ref: A11029; Invitrogen; 1:400) and Alexa Fluor-568 (Ref: A11036 Invitrogen; 1:400). Immunostained sections were further reincubated with Hoechst for nuclear counterstaining. Images were finally analyzed at 20x magnification using Olympus Fluoview FV500 confocal microscope.

*Stereological analysis*

The stereological analysis was performed as previously described [14,15,20] by using a computerized image analysis system (Mercator Image Analysis system, Explora Nova, La Rochelle, France) connected to an Olympus BX-50 photomicroscope.

In brief, in order to evaluate the TH-immunoreactivity (ir) in the IL striatum (lesioned striatum) the volume of preserved striatum was calculated by delimiting negative areas and the entire striatum and multiplying these measurements by the thickness of the slices and the intersectional distance. Values were expressed as a percentage of the TH-negative volume versus the entire volume of the IL striatum.

Changes in the density of dopaminergic neurons and axodendritic network were evaluated through quantifying the TH-ir neuronal density in the entire SN and TH-ir axodendritic network density (ADN) in the SN reticulata (SNr). Data were expressed as the percentage of neurons or ADN presents on the lesioned side versus the non-lesioned hemisphere.

The study of the topological distribution was performed following the previously described approach [20,21].

*Biochemical analysis*

*Western blotting*

After behavioral test, 5 rats of each group were decapitated to obtain fresh brain tissue. Ipsi- and contralateral (CL) striatum and SN were collected by microdissection and quickly frozen. Proteins were
isolated and the concentration of soluble proteins was quantified by the Bio-Rad Protein Assay (Ref: 500-0006, Bio-Rad Laboratories) based on Bradford's method.

As previously described [14,15], for each sample 20 mg of proteins was loaded into polyacrylamide CRITERION TGX 12% gels (Bio-Rad Laboratories Inc., Spain) for electrophoresis and then transferred to a PVDF membrane in a Trans-Blot Turbo Transfer System (Bio-Rad, USA) for 7 min. Membranes were incubated with the following primary antibodies: rabbit anti-Phospho-AKT (Ser 473) (1:1000), rabbit anti-AKT (1:1000), rabbit anti-Phospho-p44/42 MAPK (extracellular signal-regulated protein kinases 1 and 2 [ERK 1/2]) (Thr202/204) (1:1000), rabbit anti-P44/42 MAPK (ERK 1/2) (1:1000) (all of them from Cell Signaling Technology Inc. USA), rabbit anti-caspase 3 (H-277) (1:1000) (Santa Cruz Biotechnology Inc., Spain), rabbit anti-β-Actin (1:2000) (Sigma-Aldrich, Spain) and rabbit anti-Beta-Tubulin (1:1000) (Novus Biologicals, USA) at 4 °C overnight. Afterwards they were incubated with anti-rabbit IgG peroxidase conjugated secondary antibodies (1:2000) (Sigma-Aldrich, Spain) for 2 hours at RT and immunoblots were developed with an enhanced chemiluminescence kit (GE Healthcare Life Science, UK). The luminescence of the reaction product was detected in a personal scanner, LI-COR C-DiGit (LI-COR, Bonsai Advanced Technologies SL, Spain), and visualized bands were analyzed with Image Studio Lite 4.0 software (LI-COR, Bonsai Advanced Technologies SL, Spain). β-Actin and β-tubulin were used as loading controls.

**Statistical analysis**

All results were expressed as the mean ± SE (standard error). Statistical analysis was developed with GraphPad Prism (v 5; GraphPad Software, Inc., USA) and SPSS Statistics (v 20; IBM Corporation, Armonk, NY, USA). Prior to the analysis, the Shapiro–Wilk test was used to assess the normal distribution of the samples, and Levene's test was used to determine the homogeneity of variance. Mann-Whitney Utest was performed to assess differences between groups and within groups in the monitoring analysis. The ROC (Receiver Operating Characteristics) curve was performed and the AUC (Area Under the Curve) was calculated in order to measure the accuracy of the model which are a metrics for checking classification models, in this case: Saline group and 6-OHDA group. The behavioral data, stereology and densitometry results were analyzed by means of the two-tailed unpaired Student's t test to compare differences between groups. A one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test was used to test the differences between rostro-caudal gradients within each experimental group. The correlation was examined by Pearson product-moment correlation coefficient. P values < 0.05 were considered statistically significant.

**Results**

**Behavioral evaluation**

The monitoring of EE cages allows obtaining data about activity and centroid features (speed, acceleration and entropy) during the light/dark cycle. Remarkably, 6-OHDA-lesioned rats decreased significantly the number of activity areas during the dark phase, which is the active period of the rodent
circadian cycle, comparing to saline group (***p < 0.0001, Mann-Whitney U-test). Moreover, the 6-OHDA group also showed a significant hyperactivity during the light phase (resting period) in comparison with control rats which may be related to a sleep behavior disruption (***p < 0.0001, Mann-Whitney U-test; Fig. 2a, b). Overall, these results suggested that animals from the 6-OHDA group switched their transitional rhythms respect to the control group. Furthermore, the effect of 6-OHDA on the disruption of circadian rhythms was also supported by a significant difference in the activity between both light and dark phases which was also seen in saline group (###p < 0.0001 activity area mean in light cycle vs activity area mean in dark phase within the same group, Mann-Whitney U-test; Fig. 2b).

In line with these results, although saline group showed more remarkable changes between dark and light cycles in terms of speed and acceleration than 6-OHDA group, entropy was significantly higher in the 6-OHDA group (6-OHDA group vs saline group, ***p < 0.0001, Mann-Whitney U-test; Fig. 2c-e). The fact that entropy was higher supported and confirmed the incipient sleep pathological condition (Fig. 2e). Both groups also showed significant differences between both dark and light phases in all the examined parameters related to the centroid evolution indicating different behaviors in both phases according to the light or dark period (###p < 0.0001 within the same group, Mann-Whitney U-test; Fig. 2c-e. Besides, the 6-OHDA effect on the acceleration and speed in both phases pointed to a difficulty in the movement noticed in both phases.

Thus, this is a model that allows integrating information about both dark and light phases.

Accordingly, the efficiency of this model was confirmed by the results obtained by the ROC (Receiver Operating Characteristics) curve and its AUC (Area Under the ROC Curve) which measure the accuracy of the model by Logistic Regression. In the ROC curve the true positive rate (Sensitivity) is represented in function of the false positive rate (1-Specificity) for different operating points, features of the system, where an AUC of 100 % would represent the highest specificity and sensibility of the model to separate both groups. Our data gave a ROC curve (each point on the curve represents a sensitivity / specificity) that reached a significant AUC of 0.854 giving 85 % of sensibility and specificity to this model (85% ±0.0047, p = 0; Fig. 3).

In addition to these results, the visual observation of subclinical features showed, for instance, no changes in body weight between both groups; but from the second day post-toxin injection these rats showed orchitis (enhanced testicles size) and constipation (reduced consistence of fecal bolus) (Data not shown). On the other hand, minimal motor deficits were observed in the amphetamine test. Although animals increased significantly the number of IL turns per minute (tpm) respect to control group, (1.93 ± 0.39 tpm vs 0.32±0.076 tpm; t (17) = 4.292, p = 0.0005, unpaired Student's t-test), no rats turned more than 3 turns per minute (1-3 tpm; Additional file 1: Figure S1). Therefore, a prodromal stage would be supported by the mild presence of motor symptoms.

**Morphological evaluation**
Once confirmed the functional changes displayed by 6-OHDA group two weeks after 6-OHDA administration, we also assessed whether 6-OHDA exerted a histological effect that we had previously observed 3 weeks after 6-OHDA administration [14,15]. Remarkably, in this study we also included a control group (saline-lesioned rats) unlike our previous studies about 6-OHDA-induced preclinical model in which we considered as control the CL hemisphere in order to evaluate the histological and molecular changes.

6-OHDA-induced mild nigrostriatal dopaminergic degeneration after short time of evolution

Analysis of TH-immunostained sections from the striatum and SN confirmed that, at this survival time, 6-OHDA induced a moderate TH-ir fiber loss either in the striatum or in the SN (Fig. 4). In addition, dopaminergic neurons in the SN were not notably reduced (Fig. 4e-g).

Rostro-caudal study of TH-ir in the SN indicated that the number of the survival TH-ir neurons as well as the TH-ir axodendritic network (ADN) in 6-OHDA group increased rostro-caudally. Interestingly, one-way ANOVA followed by Turkey post hoc test indicated significant differences (*p < 0.05) between rostral and caudal levels within the 6-OHDA group regarding the number of TH-ir neuronal cells at the rostral sections vs. 70.12 ± 7.03 % of TH-ir neuronal cells at the caudal sections; p = 0.031). However, regarding the number of TH-ir of ADN no significant differences were found between rostral and caudal sections (52.24 ± 6.32 % of TH-ir ADN at the rostral sections vs. 76.86 ± 5.29 % of TH-ir ADN at the caudal section; One-way ANOVA, F(2, 9) = 3.800, p= 0.063; Fig. 4g, h).

Correlations between motor and morphological changes

The number of IL amphetamine-induced rotations was strongly correlated with the percentage of TH-ir neuronal cell loss (r = -0.94) and it was statistically significant (*p ≤ 0.05) (Fig. 5b). On the other hand, the TH-ir terminal loss either in striatum or in SN also showed a correlation with the increase in the number of IL rotations per minute close to be statistically significant ( r= -0.84, p = 0.073 for TH-ir IL striatal volume and r = -0.93, p = 0.065 for TH-ir AND; Fig. 5a, c).

Biochemical analysis

In order to assess changes in apoptosis, the expression of caspase-3 in neuronal cells was morphologically evaluated by double immunostaining against NeuN (nuclear marker of neuronal cells) and caspase-3. In addition, caspase-3 levels were also biochemically analyzed by western blot in the striatum and SN (Fig. 6).

Double immunofluorescence revealed higher caspase-3 immunoreactivity and lower NeuN immunoreactivity in 6-OHDA-lesioned rats compared to saline control group in the IL striatum as well as in the IL SN. In fact, 6-OHDA induced a remarkable apoptosis in neuronal cells due to a considerable amount of NeuN positive cells co-localized with caspase-3 in 6-OHDA group (Fig. 6a, c).
Furthermore, immunoblot results were consistent with the outcomes showed by immunostaining. The expression of caspase-3 was upregulated in 6-OHDA-lesioned rats in the striatum and in SN following a similar pattern (127.9 ± 5.1 % and 127.9 ± 26.08 % respectively), while caspase-3 activation was barely evidenced in saline control group (103.8 ± 7.6 % in the striatum and 90.7 ± 7.57 % in the SN). However, statistically significant differences between both groups were only found in the striatum ($t_{(7)} = 2.426$, $p = 0.045$, unpaired Student’s $t$ test; Fig. 6b, d).

However, no significant changes were seen in the pro-survival signaling protein levels (AKT and ERK) between 6-OHDA and saline groups 2 weeks postinjection, despite both proteins were downregulated in the 6-OHDA group (Additional file 2: Figure S2).

**Discussion**

In the present study, we achieved to monitor the alteration of the circadian cycle in a preclinical model with an automatized and non-invasive method. Indeed, our results evidenced that two weeks after the administration of 6-OHDA in the striatum of adult male rats despite inducing only a subclinical motor deficit, other physiological impairments associated with the premotor phase of PD were found. These were related to mild morphological and molecular changes in the dopaminergic system.

Remarkably, our results suggested that 6-OHDA-lesioned rats showed sleep behavioral disruption as an effect of dysfunctional circadian clock. Circadian disruption consists in the alteration at the level of the daily rest/activity rhythm [8] and involves to multiple systems, thereby some authors speculate that circadian disfunction can worsen the progression of PD [7, 9, 22]. Thus, the use of PD models that reproduce sleep and circadian abnormalities as well as the design of systems that aid to collect data about the evolution of the activity changes in circadian rhythms as we described in this study represent a good strategy for predicting PD prior to the clinical motor deficits manifestation.

In particular, our findings indicated that 6-OHDA-lesioned rats switched their circadian rhythms respect to the control animals reflecting alterations in the temporal patterning of sleep as was previously described in PD patients [9]. Thus, we can speculate that this change in the pattern of rest/activity seen in response to 6-OHDA may be similar to the insomnia at night or the hypersomnia during the daytime experimented by PD patients [7]. Behavioral changes related to circadian cycles were already described in the 6-OHDA model. In line with our results, Sakata and collaborators found alterations in the sleep behavior in both, light and dark phases. They reported after bilateral injection of 6-OHDA into the ventral tegmental area (VTA) a decrease in REM sleep during the light phase contrary to the dark phase, in which they observed an increase in REM sleep and in non-rapid eye movement (NREM) as well as a reduction in spontaneous activity [23].

The tendency to decrease the activity during the dark phase in response to 6-OHDA was also in accordance with other studies, some of them suggested that 6-OHDA-lesioned rats may show slightly
motor impairment and more diurnal activity during the first week after 6-OHDA administration into the SN [24]. While other studies did not detect activity change during the light phase [25].

In addition, 6-OHDA-induced activity changes were also supported by the reduction of speed and acceleration during the light/dark cycle even in the light phase where 6-OHDA-lesioned rats were more active than control rats. These findings may indicate that 6-OHDA-lesioned animals started to present difficulty in the movement. Thus, the assumption of the scarcely motor symptoms as a consequence of circadian disruption reinforces our hypothesis about 6-OHDA enables to induce mild motor symptoms in this model and although they are almost undetectable by conditional motor test. Early motor impairment might be detected by a sophisticated continuous monitoring system like the system we propose.

On the other hand, due to Shannon entropy is often considered as one of the classic and most natural way to measure the expected value (average) of the information in a dysfunctional signal [17, 18], the increase of entropy exhibited by 6-OHDA-lesioned rats confirmed the incipient sleep behavioral disorder. Moreover, the reliability of this model was also confirmed by the high sensibility and specificity that we found allowing us the correct screening of both animal groups. Taken altogether, we propose the sleep disruption as a promising biomarker to be addressed using the 6-OHDA model in the reported conditions

Concerning the motor evaluation developed with amphetamine test, the mild increased number of IL rotations was related to a mild degree of denervation in the dorsal subregion of the striatum and with the subsequent mild loss of dopaminergic terminals and neuronal cells in the SN. Interestingly, this correlation pointed out to the accurately or sensitivity of the amphetamine test predicting the dopamine decline [26]. Moreover, the mild motor deficit displayed by 6-OHDA-lesioned rats may result in the circadian locomotor activity reduction previously reported in the monitoring analysis.

The loss of dopaminergic neurons or fibers found in this model was around 45–55%, indicating that the degree of lesion was lower than the results reported 3 weeks after lesion [14, 15]. Thus, the dopamine depletion did not reach the required threshold to develop motor symptoms [13, 27]. However, this loss of dopamine could be sufficient to induce disruption of the circadian rhythms contrary to other studies which suggested a higher dopaminergic neuronal loss (about 60%) in order to detect disruption of circadian locomotor [24]. In addition, results indicated that a selective vulnerability to the toxin could be observed, pointing out to a dopamine decline topologically distributed in the SN, in concordance with our previous works [20, 21]. In fact, most of the dopaminergic neurons and dopaminergic terminals remained in the caudal axis, decreasing the survival towards rostral axis.

Regarding the mechanisms may underlay the early 6-OHDA-induced neurodegenerative effect on the dopaminergic system; we tested the effect of this toxin over apoptotic cascade and the survival signaling pathways in the striatum and SN. The toxicity mediated by 6-OHDA was able to activate the proapoptotic pathway in neurons increasing caspase-3 levels in the striatum and SN. Indeed, the most remarkable outcomes were found in the striatum where the toxin was injected. These findings supported the capacity of 6-OHDA to trigger the activation of caspase-3 for inducing the cell death through the production of
ROS leading to the apoptosis in agreement with other studies [28]. Thus, the early reduction of ROS production could be a strategy to avoid proapoptotic events and the subsequent cell death.

AKT signaling pathway as well as ERK activation play a pivotal role in the regulation of cellular survival and in the inhibition of cell death [29]. However, the effect of 6-OHDA two weeks after administration was not sufficient to induce remarkable changes either in AKT or ERK expression in the nigrostriatal pathway. However, our previous studies have shown that the activation of both survival pathways were reduced significantly 3 weeks after 6-OHDA administration [15]. Thereby, although proapoptotic events were evident two weeks after lesion, additional time will be required in order to detect changes in survival pathways.

Finally, for the present study the use of EE cages was only with the purpose of monitoring the behavior and assessing functional parameters. In fact, we propose a novel tracking system that allows monitoring animals in their home cages automatically detecting and categorizing behaviors without conditioning their regular environment. Interestingly, our observations indicated that monitored EE cages provided novel additional information for screening changes in the circadian function by monitoring parameters of circadian locomotor activity rhythms (activity area, speed and acceleration, among others) automatically during the light/dark cycle. Since there are discrepancies concerning to the parameters and methods for the evaluation of the active state of the animals [7], this monitoring system may represent a promising strategy offering a plethora of different activity parameters to assess along with other additional information about the state of the animal during the light/dark cycle.

## Conclusions

In conclusion, we provide evidences about the suitability of the 6-OHDA-induced model reproducing the prodromal sleep disruption and we implemented a novel system to monitor behavior changes providing an optimal system to test different neuroprotective / neuroregenerative strategies.

## Abbreviations

- **ADN**: Axodendritic network density
- **CL**: Contralateral
- **EE**: Enriched environment
- **ERK**: Extracellular signal-regulated kinase
- **GFAP**: Glial fibrillary acidic protein
- **IL**
Declarations

Ethics approval and consent to participate

All the experimental protocols were approved by the Ethical Committee and Animal Welfare (CEEA) of the University of the Basque Country (CEEA/M20/2016/176) following the Spanish Royal Decree RD 1201/2005, European Directive 2003/65/EC and the European Recommendation 2007/526/EC on the protection of animals used for scientific purposes.

Consent for publication

Not applicable
Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no competing interests.

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Author contributions

C.R. designed the studio, performed the experiments, analyzed the results, prepared the figures and wrote the manuscript. K.L.D.I., E.F. and P.M.C. analyzed the data from the monitored-EE cages and prepared Fig. 2, 3 and the supplementary Figs. 1 and 2. J.A.R.O., T.M. and C.M. performed the stereotaxic lesions. L.C.G. carried out the GFAP staining and the quantification of GFAP immunoreactivity. H.C. designed the monitored-EE cages. L.U. designed the studio, provided laboratory resources and reviewed the manuscript. J.V.L. conceived and designed the studio, supervised the work, provided funding, reviewed and corrected the manuscript. All authors approved the final version of this manuscript.

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Supplemental Figure Legends

**Additional file 1: Figure S1. Evaluation of motor symptoms.** Graph shows the ipsilateral turns per minute (tpm) in both groups two weeks after 6-OHDA or saline solution administration. (***p < 0.001, unpaired Student’s t-test).

**Additional file 2: Figure S2. Western blot analysis showing AKT and ERK ½ downregulation after 6-OHDA administration in both stratum and SN.** (a, b) Detection by western blot and quantification of levels of phosphorylated and total forms of AKT in the striatum (a) and SN (b). (c, d) Detection by western blot and quantification of levels of phosphorylated and total forms of ERK ½ in the striatum (c) and SN (d). The phosphorylated form of AKT or ERK ½ was normalized respect to the total form respectively and densitometric results are expressed as the % of p-AKT/AKT or p-ERK/ERK 1/2 ratio in the ipsilateral side from the striatum or SN respect to the contralateral one. 6-OHDA, 6-hydroxydopamine; SN, substantia nigra.

Figures
Figure 1

Data acquisition, pre-processing and analysis workflow.
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Figure 2

Circadian rhythm disruption in 6-OHDA-induced rats. (a) Diagram illustrating the motion activity signal in 6-OHDA (upper panel) and saline (bottom panel) groups. Blue: motion activity signal, Green: smoothed signal for control group, Red: smoothed signal for 6-OHDA group, Yellow: light level. Graphs depict significant activity changes (b), the centroid features including the mean of centroid speed (c), the mean of centroid acceleration (d) and the entropy mean (e) between 6-OHDA and saline groups during the
light/dark cycle and within the same group. Data are expressed as mean ± SEM. General analysis was performed by considering 12 hours dark/light periods. Statistical differences appear (p < 0.05) for Mann-Whitney U-test between both groups and within the same group for both light and dark phases (6-OHDA group vs saline group, ***p < 0.001; and light cycle vs dark cycle within the same group, ###p < 0.001).

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**Figure 3**

ROC curve for linear Regression. Modelling for 6-OHDA and saline groups
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**Figure 4**

TH-ir loss in the 6-OHDA-induced preclinical model. (a, b) Rostro-caudal distribution of coronal sections corresponding to both striatum and SN immunostained with tyrosine hydroxylase (TH) in saline and 6-OHDA groups. (c, d) Graphs show the effects of 6-OHDA on the loss of the TH-ir volume in the striatum comparing to saline group (***p < 0.001, unpaired Student's t-test.) and no significant changes were found along the rostro-caudal axis. (e, f) TH-ir neuronal density and TH-ir ADN density decrease significantly following 6-OHDA administration comparing to saline group in the SN (***p < 0.001, unpaired Student's t-test). (g, h) Topological analysis shows a selective vulnerability in the SN to 6-OHDA decreasing the TH-ir neuronal and the TH-ir ADN density rostro-caudally (*p < 0.05 rostral section vs. caudal section within 6-OHDA group, One-way ANOVA). Data are presented either as the percentage of TH-ir ipsilateral striatal volume compared to the total ipsilateral one (c, d) or as the percentage of TH-ir neuronal density or TH-ir ADN density (e-h) remaining in the ipsilateral side respect to the contralateral side. Scale bar: 2 mm (a), 1 mm (b). TH-ir, tyrosine hydroxylase immunoreactivity; 6-OHDA, 6-hydroxydopamine; ADN, axodendritic network; SN, substantia nigra.
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Figure 5

Morpho-functional correlation between amphetamine-induced rotations and dopaminergic neurons in SN in addition to dopaminergic terminals in both striatum and SN after 6-OHDA administration. (a) Amphetamine-induced IL rotations correlate negatively with the % of TH-ir striatal volume IL, (b) also with the % of TH-ir neuronal density and (c) with the % of TH-ir ADN density. Significance. *p < 0.05, IL rotations vs TH-ir neurons. SN, substantia nigra; 6-OHDA, 6-hydroxydopamine; TH-ir, tyrosine hydroxylase immunoreactivity; IL, ipsilateral.
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Figure 6
6-OHDA-induced apoptosis is analyzed by immunofluorescence (a and c) and western blot (b and d) in both striatum and SN. (a) Representative immunofluorescent images for caspase-3 (red), NeuN (green) and merge images illustrating the ipsilateral striatum from saline and 6-OHDA groups. Scale bar = 25 μm. (b) Western blot analysis of caspase-3 levels in the striatum reveals a significant increase in 6-OHDA group comparing to saline group (*p < 0.05, unpaired Student's t-test). (c) Representative immunofluorescent images for caspase-3 (red), NeuN (green) and merge images illustrating the ipsilateral SN from saline and 6-OHDA groups. Scale bar = 25 μm. (d) Western blot analysis of caspase-3 levels in the SN. Actin levels are used as loading control. Densitometric results are normalized with actin and they are set as the % of the caspase-3/actin ratio in the ipsilateral hemisphere respect to the contralateral one. 6-OHDA, 6-hydroxydopamine; SN, substantia nigra.
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**Supplementary Files**

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