



SK channel blockade reverses cognitive and motor deficits induced by nigrostriatal dopamine lesions in rats

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Abstract

Parkinson's disease has traditionally been viewed as a motor disorder caused by the loss of dopamine (DA) neurons. However, emotional and cognitive syndromes can precede the onset of the motor deficits and provide an opportunity for therapeutic intervention. Potassium channels have recently emerged as potential new targets in the treatment of Parkinson's disease. The selective blockade of small conductance calcium-activated K⁺ channels (SK channels) by apamin is known to increase burst firing in midbrain DA neurons and therefore DA release. We thus investigated the effects of systemic administration of apamin on the motor, cognitive deficits and anxiety present after bilateral nigrostriatal 6-hydroxydopamine (6-OHDA) lesions in rats. Apamin administration (0.1 or 0.3 mg/kg i.p.) counteracted the depression, anxiety-like behaviors evaluated on sucrose consumption and in the elevated plus maze, social recognition and spatial memory deficits produced by partial 6-OHDA lesions. Apamin also reduced asymmetric motor deficits on circling behavior and postural adjustments in the unilateral extensive 6-OHDA model. The partial 6-OHDA lesions (56% striatal DA depletion) produced 20% decrease of iodinated apamin binding sites in the substantia nigra pars compacta in correlation with the loss of tyrosine hydroxylase positive cells, without modifying apamin binding in brain regions receiving DAergic innervation. Striatal extracellular levels of DA, not detectable after 6-OHDA lesions, were enhanced by apamin treatment as measured by *in vivo* microdialysis. These results indicate that blocking SK channels may reinstate minimal DA activity in the striatum to alleviate the non-motor symptoms induced by partial striatal DA lesions.

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Introduction

Parkinson's disease (PD) is mainly defined by tremor, rigidity and bradykinesia, classically emerging when 58–64% of the substantia nigra pars compacta (SNc) dopaminergic (DA) neurons have degenerated. Extensive neuropsychiatric features, including apathy, depression, anxiety, mood fluctuations, cognitive impairment, are now increasingly recognized in PD. These non-motor symptoms (NMS) may appear early in the course of the disease and often precede motor symptoms (Chaudhuri

et al., 2011). Understanding the mechanisms underlying the occurrence of these early non-motor symptoms in rodent models of PD may thus help to find new therapeutic treatments.

Recently, potassium channels have emerged as potential new targets in the treatment of PD (Wang et al., 2008). Small-conductance calcium-activated potassium channels (SK) produce the medium afterhyperpolarization phase (mAHP) which contributes to the control of action potential frequency and of the pacemaker firing of neurons (Bond et al., 2005; Adelman et al., 2012). SK1-3 subunits are widely expressed in limbic structures and in the basal ganglia (Stocker and Pedarzani, 2000). In particular, SK3 channels control the timing and stability of the endogenous pacemaker activity of DA neurons in the SNc and the ventral tegmental area (VTA) (Seutin et al., 1993; Wolfart et al., 2001). SK channel blockade by apamin, a neurotoxin isolated from bee venom, promotes irregular firing and bursting activity, *in vitro* (Shepard and Bunney, 1988; Wolfart et al., 2001;

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Kim et al., 2007) and *in vivo* in DA neurons (Waroux et al., 2005; Herrik et al., 2010). SK channels are also involved in synaptic plasticity. Apamin facilitates long-term potentiation and encoding of memory traces (Messier et al., 1991; Stackman et al., 2002; Mpari et al., 2008). Moreover, SK3-deficient mice exhibit antidepressant-like behavior associated with elevated striatal extracellular DA levels (Jacobsen et al., 2008). Taken as a whole, these findings suggest that pharmacological blockade of SK channels could not only reverse parkinsonian motor deficits by promoting DA neuronal activity but may also affect cognitive and neuropsychiatric symptoms observed in early PD. To test this hypothesis, we examined the effects of apamin, in rats with partial bilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal dopaminergic pathway on emotional (anxiety, depression) and cognitive (short-term social recognition, short-term spatial and non-spatial memory) behaviors. In the same PD model, we analyzed iodinated apamin autoradiography in brain structures innervated by the mesocorticolimbic and nigrostriatal DA pathways and measured the effects of systemic apamin administration on striatal extracellular DA concentration. Apamin action on motor symptoms was assessed in unilateral 6-OHDA lesioned animals. We show that apamin reduced non-motor and motor deficits in the two models of PD by enhancing DA activity in the nigrostriatal pathway.

Material and methods

Animals

Male Wistar rats (280–300 g; Charles River Laboratories, France) were housed in groups of two and supplied with food and water *ad libitum*, in a temperature-controlled room (24 °C) on a 12:12 h dark–light cycle (lights on at 07:00). For the social recognition test, juvenile male Wistar rats (3 wk) were used. All efforts were made to minimize the number of animals used and maintain them in good general health, in accordance with the European Communities Council Directive (2010/63/UE).

Stereotaxic surgery and drug treatment

Rats were anaesthetized with ketamine (5%, Virbac, France) and medetomidine (1 mg/ml, Janssen) injected subcutaneously (0.33 ml/kg). They were placed in a stereotaxic apparatus (David Kopf instruments, USA) with the incisor bar positioned 3.3 mm below the interaural line. 6-OHDA hydrobromide (Tocris Bioscience, UK) or vehicle (NaCl 0.9 in 0.1% ascorbic acid) solution was infused bilaterally into the striatum (12 µg/3 µl/side; anteroposterior (AP)+1.0, lateral (L)±3.0 and dorsoventral (DV)–5.5 mm from bregma) or unilaterally into the SNc (8 µg/4 µl; AP –5.2, L+2.1 and DV –7.6 mm from bregma; Paxinos and Watson, 2007). These two lesion models allowed producing either bilateral partial or unilateral extensive DA striatal depletion modeling early or late stage

of Parkinson's disease, respectively. Sham rats received the vehicle solution only. 6-OHDA or vehicle solution was injected at a flow rate (0.33 µl/min) controlled by a micropump (CMA/100; CMA Micro-dialysis, Sweden) using a 10 µl Hamilton microsyringe connected by a Tygon catheter (0.25 mm i.d.) fitted to the 30-gauge stainless steel injector needles. In the experiment, 3 min were allowed for diffusion of the toxin. For the microdialysis experiments, intracerebral guides and dummy cannula were bilaterally implanted in the striatum 500 µm posterior to the 6-OHDA injection sites during the same surgery. All animals were allowed to recover for 2 wk.

Drug treatment

Apamin (0.1 or 0.3 mg/kg, dissolved in NaCl 0.9%, Genepep, France) was injected intraperitoneally (i.p.) 30 min before each test, except for the social recognition task (30 min before the second exposure) (Mpari et al., 2005). Systemic administration of apamin was chosen because it was found to cross the blood–brain barrier, although weakly (Habermann and Cheng-Raude, 1975). Central action of apamin was evidenced in previous studies demonstrating improved object memory encoding, visuospatial memory and contextual fear memory after i.p. administration at a similar doses range (Messier et al., 1991; Ikonen et al., 1998; Van der Staay et al., 1999; Stackman et al., 2002). The animals were allocated to different subgroups (vehicle-sham, apamin-sham (0.1 or 0.3 mg/kg), vehicle-lesioned, apamin-lesioned (0.1 or 0.3 mg/kg)). Each subgroup was tested once in the elevated plus maze, social interaction and object recognition every 4 d in a counterbalanced manner between post-lesion days 14 to 25. The unilateral 6-OHDA group was tested in the cylinder test then in the rotameter.

Behavioral procedures

All behavior experiments were carried out in dim light (8 lumens), video-recorded (Viewpoint Inc., France) and scored later by an experimenter blind to treatment.

Elevated plus maze test

The elevated plus maze was performed to assess anxiety-related behavior (Pellow et al., 1985) over a 5-min period. Each rat was placed in the central area of a plus maze with two open arms and two enclosed arms (50×10 cm, height: 30 cm), raised to a height of 70 cm above the floor. The percentage of time spent in open *vs.* closed arms was used as an index of anxiety level, whereas locomotor activity was measured by the total distance covered in the entire maze.

Sucrose preference test

The lack of sucrose consumption is a model of anhedonic-related state (Henningsen et al., 2009). On day one, rats were familiarized to drinking from two water-bottles.

On day two, 0.5% sucrose solution was introduced in one bottle. On day three, the bottles were reversed. This precludes any effect of neophobia, artefactual bias toward any one side and perseveration. The animals were then liquid deprived for 14 h. On day four, rats were exposed for 1 h to two bottles (filled with water or 0.5% sucrose solution) 30 min after apamin or vehicle injection. Sucrose preference was expressed as the percentage of sucrose intake over total liquid intake in g. Rats that did not drink during the sucrose preference test were excluded from analysis.

Social interaction

Short-term social memory was evaluated with the social interaction test (Prediger et al., 2006). Adult male rats spend a great amount of time investigating novel juveniles. In contrast, rats re-exposed to the same juvenile 30 min after an initial exposure display little investigatory behavior. All rats were habituated for 60 min to the testing room and juveniles housed in individual cages for 30 min prior to the experiment. The adult rats were then allowed to habituate for 10 min to the square open field (50×50 cm) containing a small cage with stainless steel railings (10×10 cm) in its center. After placing the juvenile in the small cage, the number of adult-initiated contacts with the juvenile was recorded for 5 min. The adult rat was then removed from the open field, kept in an individual cage for a 30-min delay period and then re-exposed to the same juvenile for 5 min.

Object recognition

Spatial memory and non-spatial novelty reaction were assessed with the object recognition test (De Leonibus et al., 2007). Animals were submitted to seven consecutive, 6-min sessions (S), interspaced by 3-min intervals, in a square open field (100×100 cm) with 30 cm-high white plastic walls. During S1, the rats explored and acclimatized to the empty open field which allowed to record baseline locomotor activity and prevent neophobic reactions. In S2–S4, five objects were positioned into the open field (habituation phase). All objects were at a similar distance from the wall with the exception of the fifth object placed in the center. During the *spatial-test session* (S5), the object configuration was modified by displacing two objects. In S6, the objects configuration was kept as in S5. In the *novel object-recognition session* (S7), one of the familiar non-displaced objects was replaced by a novel object. Object exploration was measured by the mean time (in s) spent by the animals in contact with the different objects. The evaluation of the subjects' reaction to a spatial change was measured by the difference in time spent exploring the displaced and non-displaced objects between S4 and S5 (*spatial exploration index*). In S7, the contact duration of the substituted object (new) and non-substituted objects (old) evaluated the *novel object-recognition*.

Cylinder test

The degree of forepaw asymmetry induced by unilateral 6-OHDA lesion was assessed by placing the rats in a transparent Plexiglas cylinder (20 cm diameter; 30 cm height) for 10 min (Schallert et al., 2000). Unilateral 6-OHDA lesioned animals preferentially use their ipsilateral forepaw to the lesion to adjust their posture on the cylinder wall instead of using contralateral or the two forepaws (double contact). The scores were expressed as a percentage of the total number of wall-contacts.

Apomorphine-induced circling

Unilateral 6-OHDA lesioned rats were tested in automated rotameter cylinders (TSE, Bad Homburg, Germany). The turning behavior (defined as a full 360° rotation of the body axes) was measured after apomorphine hydrochloride i.p. injection (0.1 mg/kg, Sigma-Aldrich, St-Quentin Fallavier, France). The total number of net rotations (contralateral minus ipsilateral rotations) was recorded for 60 min.

Brain tissue preparation

Rats were deeply anesthetized with pentobarbital injection and killed by decapitation. Brains were immediately removed and rapidly frozen on powdered dry ice and stored to –80 °C. Coronal brain sections (20 μm) were cut at –20 °C with a Leica CM3050 S Cryostat, mounted on Superfrost® Plus slides and stored at –80 °C.

Apamin-binding autoradiography

Brain sections of vehicle-sham and bilaterally 6-OHDA-lesioned rats were incubated with *radioiodinated* apamin (PerkinElmer) (25 pM) as previously described (Mourre et al., 1986). For the detailed protocol of apamin-binding experiments, see Supplement 1.

Microdialysis and high-performance liquid chromatography experiments

Microdialysis experiments were carried out on freely moving rats. The rats were first acclimatized to the microdialysis bowls for 30 min. CMA 11 dialysis probes with a 1-mm-long dialysis membrane (CMA/Microdialysis, USA) were then slowly lowered into the striatum extending 1 mm below the implanted guide-cannula. The probes were perfused at a constant flow of 2 μl/min (CMA/100 high-precision pump) with artificial cerebrospinal fluid containing (in mM) NaCl=147; KCl=2.7; CaCl₂=1.2; MgCl₂=0.85 (pH 7.4). Dialysates were collected every five min (10 μl by sample) on a Univentor refrigerate microfraction collector (Phymep, France). After a 2-h equilibrium period, baseline samples were collected and apamin (0.3 mg/kg) or vehicle was injected i.p. The dialysate collection lasted for 120 min afterwards.

Microdialysis samples were stored at -80°C until analysis. Dopamine was detected by high-performance liquid chromatography (HPLC) with electrochemical detection using an Alexys 100 LC-EC system (Decade, Antec, The Netherlands) (Ikarashi et al., 1985). The system consisted of a pump (model LC 100), a refrigerated automatic injector (AS 100), a reverse-phase analytical column (1 mm ID, 50 mm length) maintained at 35°C with a regulated oven and an electrochemical detector equipped with an analytical cell (type VT-03, Antec). Chromatograms were collected and treated with integration software (ALEXYS 21, The Netherlands). The mobile phase [phosphoric acid (50 mM), citric acid (50 mM), KCl (8 mM), EDTA (0.1 mM), methanol (12.5%), and OSA (500 mg/l) (pH 6)] was delivered by a pump at a flow rate of 50 ml/min. The working electrode potential was +300 mV and the limit of detection was 100 pA/V. The running time for each determination was 10 min.

To check the accuracy of the probe placement and lesion extent, frozen brains were sectioned (coronal, $20\ \mu\text{m}$) through the striatum and stained with Cresyl violet or processed for [^3H]-mazindol binding (see Supplement 2). Rats with incorrect probe placements or DA lesion were removed from the analysis.

Lesion verification

To evaluate the extent of the lesion, binding of tritiated-mazindol to dopamine uptake sites in the striatum was measured on autoradiographic films according to the procedure described by Javitch et al. (1985). In addition, immunohistochemical staining of tyrosine hydroxylase-positive cells in the substantia nigra and ventral tegmental area of sham and 6-OHDA-lesioned rats was performed to measure the DA cell loss. See Supplement 2 for the technical details.

Statistical analysis

In all analyses, values are presented as mean \pm S.E.M. Effects of DA lesion and apamin treatment on behavioral performances of the different groups or on variations of apamin binding were tested by means of one-way analysis of variance (ANOVA) or two-way ANOVA and followed by adapted *post-hoc* tests between groups where $p < 0.05$. Linear regression (Graphpad Prism6) was used for correlation analysis between TH immunohistochemistry and apamin binding data. Student's *t* test was used to analyze the effect of 6-OHDA lesion and the apamin effect on DA concentrations.

Results

Apamin reverses the anhedonic and anxiety-like behavior induced by bilateral nigrostriatal DA lesions

We examined the effect of apamin in two behavioral tests indexing anhedonic or anxiety-like behavior after

6-OHDA nigrostriatal lesions. The DA denervation was partial (56% DA depletion) and restricted to the dorsal striatum (Supplementary results and Fig. S1). The partial striatal DA depletion did not induce bradykinesia or any major motor disabilities. During training, no difference in the total liquid intake was found between sham and 6-OHDA rats (Fig. 1a). On test day, however, the sucrose preference was drastically reduced in 6-OHDA-lesioned rats compared with sham rats showing the emotional alterations associated with partial striatal DA depletion. The ANOVA revealed a group by treatment interaction ($F_{2,39}=4.48$, $p < 0.05$) with a significant main effect of lesion ($F_{1,39}=8.61$, $p < 0.01$) and apamin treatment ($F_{2,39}=4.15$, $p < 0.05$). 6-OHDA-lesioned rats drank significantly less sucrose solution than sham rats. Apamin treatment reversed this effect as seen by the increased sucrose consumption of 6-OHDA-lesioned groups treated with apamin in comparison with vehicle (Tukey's test, $p < 0.05$). The sucrose preference in sham rats was not significantly modified by apamin.

The effects of apamin were then tested in sham and lesioned animals in the elevated plus maze. As shown in Fig. 1b, 6-OHDA lesion decreased the time spent on open arms and increased the time spent on closed arms ($F_{1,17} \geq 5.17$, $p < 0.05$). Administration of apamin at 0.1 or 0.3 mg/kg did not modify the overall percentage of time spent on open and closed arms in sham animals. In 6-OHDA treated rats, apamin, regardless of the dose, increased the time spent on open arms and decreased the time spent on closed arms, when compared with vehicle ($F_{2,26} \geq 3.47$, $p < 0.05$, followed by significant Tukey's test, $p < 0.05$). No significant difference of the total time spent in the maze was found between groups (Table S1), showing that the anxiolytic action of apamin was not produced by a change of exploratory behavior.

Apamin reverses the cognitive deficits induced by bilateral nigrostriatal DA lesions

To investigate short-term recognition memory in 6-OHDA-lesioned animals, we tested the ability of adult rats to recognize a juvenile congener after a second exposure interspaced by a 30-min interval. At the first presentation, the contact number was similar in all groups (Fig. 2a). The sham groups clearly identified the juvenile, as found by a decreased number of contacts between the second and first presentation of the same juvenile regardless of the apamin dose ($F_{1,27}=30.87$, $p < 0.01$). In contrast, 6-OHDA-lesioned rats treated with vehicle showed a similar number of contacts during the two presentations (Fig. 2a). In 6-OHDA-lesioned rats, apamin 0.1 and 0.3 mg/kg significantly restored the recognition of the juvenile ($F_{1,27}=23.90$, $p < 0.01$) without affecting locomotor activity (Supplementary Table S1).

We then carried out a series of experiments to assess the role of apamin on visuospatial memory and novel object recognition processing. Objects exploration during

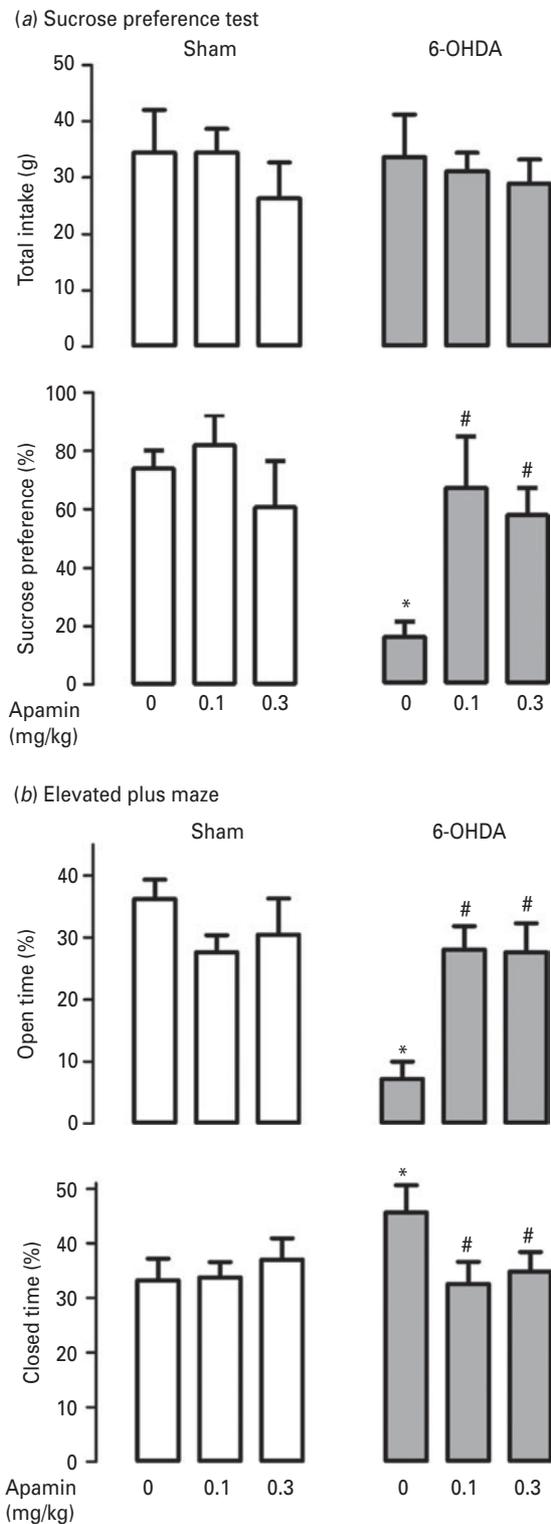


Fig. 1. Apamin reversed the increase of anxiety and anhedonic-like behaviors observed in 6-hydroxydopamine (6-OHDA)-lesioned animals. (a) Sucrose preference test. During the test, the total liquid intake of the six groups: vehicle sham ($n=9$), apamin-sham (0.1 mg/kg ($n=6$) or 0.3 mg/kg ($n=6$), vehicle-6-OHDA ($n=9$), apamin-6-OHDA (0.1 mg/kg ($n=7$) or 0.3 mg/kg ($n=8$)) was similar. The sucrose preference was expressed as percentage of sucrose intake over total liquid intake during the test. Apamin (0.1 and 0.3 mg/kg)

sessions two to four was not different between sham and 6-OHDA groups treated with vehicle (Fig. 2b). All groups tended to decrease their object exploration (contact duration) across session ($F_{2,14} \geq 3.81$, $p < 0.05$). Thus, neither 6-OHDA lesions nor apamin treatment impaired their capacity to explore the objects. Sham groups, exposed to a new spatial configuration of the objects, displayed a high difference of spatial exploration between the displaced objects and non-displaced objects. This indicates that rats discriminated and reacted to the spatial change ($F_{1,14} \geq 5.78$, $p < 0.05$) (Fig. 2b). In contrast, 6-OHDA-lesioned animals re-explored the displaced and non-displaced objects to a similar extent and displayed a significantly lower spatial exploration index compared with the sham group ($F_{1,14} = 5.27$, $p < 0.05$). Apamin 0.1 or 0.3 mg/kg restored the spatial exploration index in 6-OHDA-lesioned rats indicating a recovery of spatial discrimination. This effect cannot be attributed to a change of locomotor activity induced by DA depletion since there was no difference between the two groups (Supplemental Table S1). In the non-spatial recognition context, the contact duration with a novel object was statistically longer than with the older objects ($F_{1,14} \geq 39.99$, $p < 0.01$) demonstrating the same ability of sham and 6-OHDA-lesioned rats to detect and react to novel objects (Fig. 2b). This was not modified by apamin treatment.

Apamin reverses motor impairment induced by unilateral nigrostriatal DA lesions

We then examined the impact of apamin on motor function. Unilateral 6-OHDA-lesioned rats were thus tested for forelimb asymmetry using the cylinder test. Sham animals explored the cylinder with their forelimbs making over 50% of double contacts on the cylinder wall (Fig. 3a). In contrast, vehicle-injected 6-OHDA rats reduced double and contralateral contacts and predominantly increased ipsilateral contacts to the lesion side ($F_{1,18} \geq 4.42$, $p < 0.05$). The total number of contacts did not vary between groups. Although apamin did not alter forelimb use pattern in sham rats, it partially relieved the deficits of 6-OHDA-lesioned rats in comparison with vehicle-injected 6-OHDA rats ($F_{2,25} \geq 3.92$, $p < 0.05$) reaching significance level at 0.3 mg/kg (Tukey's test, $p < 0.05$). Apomorphine-induced rotational asymmetry

counteracted the anhedonic behavior of 6-OHDA-lesioned rats. (b) Elevated plus maze task. Percentage of exploration time on the open (open time) and closed (closed time) arms of the six groups: Vehicle sham ($n=10$), apamin-sham (0.1 mg/kg ($n=10$) or 0.3 mg/kg ($n=9$)), vehicle-6-OHDA ($n=9$), apamin-6-OHDA (0.1 mg/kg ($n=10$) or 0.3 mg/kg ($n=10$)). Apamin reversed the anxiety-like behavior produced by 6-OHDA lesions. Values are shown as mean \pm S.E.M. One-way analysis of variance (ANOVA) * $p < 0.05$, in comparison with vehicle-sham group; # $p < 0.05$, in comparison with vehicle-6-OHDA group.

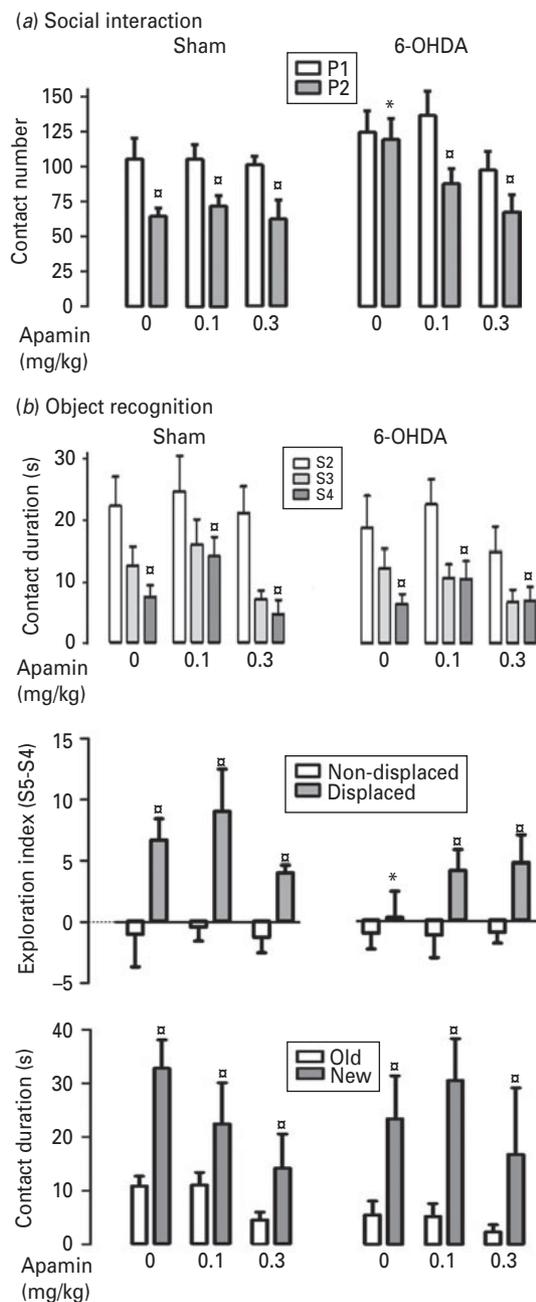


Fig. 2. Apamin rescued the recognition memory deficits of 6-hydroxydopamine (6-OHDA)-lesioned rats. (a) Short-term social interaction test. Number of contacts of the 1st presentation (P1) and the 2nd presentation (P2) to a same juvenile rat for the six groups: vehicle-sham ($n=10$), apamin-sham (0.1 mg/kg ($n=10$) or 0.3 mg/kg ($n=10$)), vehicle-6-OHDA ($n=10$), apamin-6-OHDA (0.1 mg/kg ($n=10$) or 0.3 mg/kg ($n=10$)). The P2 contact number was lower than the P1 contact number for all groups except for the vehicle-6-OHDA group. Apamin (0.1 and 0.3 mg/kg) counteracted the social recognition impairment induced by 6-OHDA. Values are shown as mean \pm S.E.M. One-way analysis of variance (ANOVA), $^*p < 0.05$, in comparison with vehicle-sham group; $^{**}p < 0.05$, in comparison between P1 and P2 contact numbers. (b) Object recognition measured in the six groups: vehicle-sham ($n=8$), apamin-sham (0.1 mg/kg ($n=8$) or 0.3 mg/kg ($n=8$)), vehicle-6-OHDA ($n=8$), apamin-6-OHDA

was then tested in the same groups of rats. Apamin dose-dependently reduced apomorphine-induced rotations for the 60-min test ($F_{1,17}=4.82$, $p < 0.05$) and had no effect on sham animals (Fig. 3b). A significant reduction of the peak effect of apomorphine was produced by apamin 0.3 mg/kg (Tukey's test, $p < 0.05$).

Distribution of apamin binding sites in the mesocorticolimbic and nigrostriatal pathways

SK channels are widely expressed in midbrain DA neurons and in regions innervated by the mesocorticolimbic and nigrostriatal dopaminergic pathways (Stocker and Pedarzani, 2000). To determine if the bilateral DA nigrostriatal lesions could modify the density of apamin binding sites in these structures, we examined their regional distribution in 6-OHDA and sham subjects (Fig. 4a). No variation of apamin binding levels was found in all the structures analyzed between the two groups (Supplemental Table S2), except in the SNc where a significant decrease was observed in the 6-OHDA groups (6.70 ± 0.19 and 5.38 ± 0.11 kbq/g tissue equivalent, sham and 6-OHDA group respectively, -20% , Student's t test $p < 0.001$). We then quantified at midbrain level the DA neuronal loss using tyrosine hydroxylase (TH) immunohistochemistry and analyzed the apamin binding in relation to the lesion extent. Bilateral 6-OHDA nigrostriatal lesions induced a 38% decrease of TH immunopositive cells in the SNc and no change in the VTA (Fig. 4b, c). A significant correlation was found between the apamin binding level and TH immunolabeling in the SNc ($p < 0.05$) but not in the VTA of 6-OHDA rats (Fig. 4d). These data indicate that the decrease of apamin binding is correlated to DA neuronal degeneration of the SNc, but not of the VTA.

Apamin increases dopamine concentrations in the striatum following bilateral nigrostriatal DA lesions

After 6-OHDA lesion, the basal extracellular DA levels were below the detection sensitivity in the striatum and

(0.1 mg/kg ($n=9$) or 0.3 mg/kg ($n=8$)). Values are shown as mean \pm S.E.M. (Top) Habituation phase. Contact duration (in s) with objects from sessions (S) 2 to 4. Object familiarization was evaluated by the difference of contact duration between S4 and S2. ANOVA, $^{**}p < 0.05$, comparison between S4 and S2 for each group. (Middle) Spatial recognition. The spatial exploration index is the difference in contact duration between S5 and S4 for displaced or not displaced objects. 6-OHDA-lesioned rats did not react to spatial object changes contrary to sham rats. Apamin (0.1 and 0.3 mg/kg) restored the object discrimination. ANOVA, $^{**}p < 0.05$ in comparison between displaced and non-displaced objects for each group, $^*p < 0.05$ in comparison with vehicle-sham group. (Bottom) Non-spatial novelty recognition. Contact duration for old and new objects (S7). No change was observed, regardless of the lesion or apamin treatment. ANOVA, $^{**}p < 0.05$ comparison between old and new objects for each group.

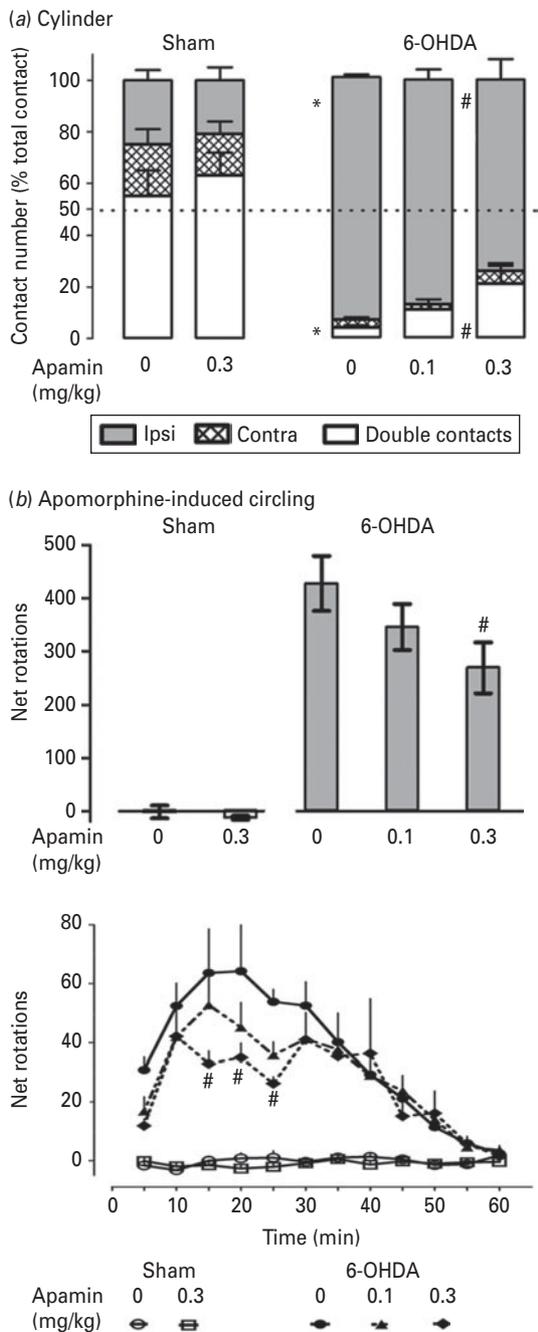


Fig. 3. Apamin decreased the sensorimotor deficits induced by unilateral 6-hydroxydopamine (6-OHDA) lesions. Five groups of rats were tested: vehicle-sham ($n=10$), apamin-sham (0.3 mg/kg) ($n=10$), vehicle-6-OHDA ($n=10$), and apamin-6-OHDA (0.1 ($n=9$) or 0.3 mg/kg ($n=9$)). (a) Cylinder test. Three types of forepaw contacts on cylinder wall were recorded: ipsilateral, contralateral (to the lesion side) and double contacts. Apamin 0.3 mg/kg increased double and reduced ipsilateral contacts of the 6-OHDA-lesioned rats. ANOVA, $*p<0.05$ in comparison with vehicle-sham group, $^{\#}p<0.05$ in comparison with vehicle-6-OHDA group. (b) Apomorphine-induced circling. (Top) Total net rotations (contralateral minus ipsilateral turns) for the 60-min testing. Apomorphine (0.1 mg/kg, s.c.) induced contralateral rotations in 6-OHDA-lesioned rats only. Apamin 0.3 mg/kg decreased the total number of net rotations in the 6-OHDA-lesioned

significantly different from DA levels of the sham group (Student's t test, $p<0.01$, Fig. 5). The microdialysis probes were found to be located within the core of the 6-OHDA lesion in the striatum where DA concentrations were no more detectable (Supplementary Fig. S2). At a dose of 0.3 mg/kg, apamin did not modify extracellular DA levels of the sham group. Interestingly, apamin administration induced a significant striatal increase of extracellular DA levels in the 6-OHDA group (Student's t test, $p<0.01$).

Discussion

The results of the present study show that apamin, a selective SK channel blocker, reduces anhedonia and anxiety and preserves short-term social and spatial memories in an animal model of early PD induced by partial and bilateral nigrostriatal 6-OHDA-lesion. Apamin was also found to reduce motor symptoms after extensive unilateral 6-OHDA lesions. We further demonstrated that the partial loss of DA neurons in the SNc was correlated with a decrease of apamin-binding sites in the SNc but not in the unaffected VTA nor in the projecting areas of midbrain DA pathways. Moreover, apamin increased extracellular DA levels in the striatum of partial 6-OHDA-lesioned animals.

Partial 6-OHDA lesions induce non-motor symptoms

Non-motor symptoms in PD are common and overshadowed by motor symptoms (Dubois and Pillon, 1997; Ferrer et al., 2012; Pagonabarraga and Kulisevsky, 2012). In the present study, the bilateral injection of 6-OHDA in the dorsal striatum induced an average of 50–60% DA striatal depletion and a 38% DA cell loss in the SNc, reproducing the early stage of symptomatic PD. These nigrostriatal DA lesions induced major emotional deficits expressed by increased levels of anxiety in the elevated plus maze and anhedonia in the sucrose preference test, consistent with recent studies in rodent models of early PD (Branchi et al., 2008; Tadaiesky et al., 2008; Bonito-Oliva et al., 2013; Drui et al., 2013). High prevalence of depression and anxiety, often associated with anhedonia, may also predate motor signs in Parkinsonian patients (Dagher and Robbins, 2009; Nègre-Pagès et al., 2010; Blonder and Slevin, 2011). In addition, partial striatal DA depletion produced cognitive deficits. It selectively impaired short-term spatial memory but did not affect novel object discrimination (non-spatial information) and affected short-term social interaction memory. This points to a critical

group. One-way analysis of variance (ANOVA), $^{\#}p<0.05$ compared to vehicle-6OHDA group. (Bottom) Time-course of rotational asymmetry following apomorphine injection. The apamin action was observed during the peak effect of apomorphine (Tukey's test, $^{\#}p<0.05$ compared to vehicle-6-OHDA group).

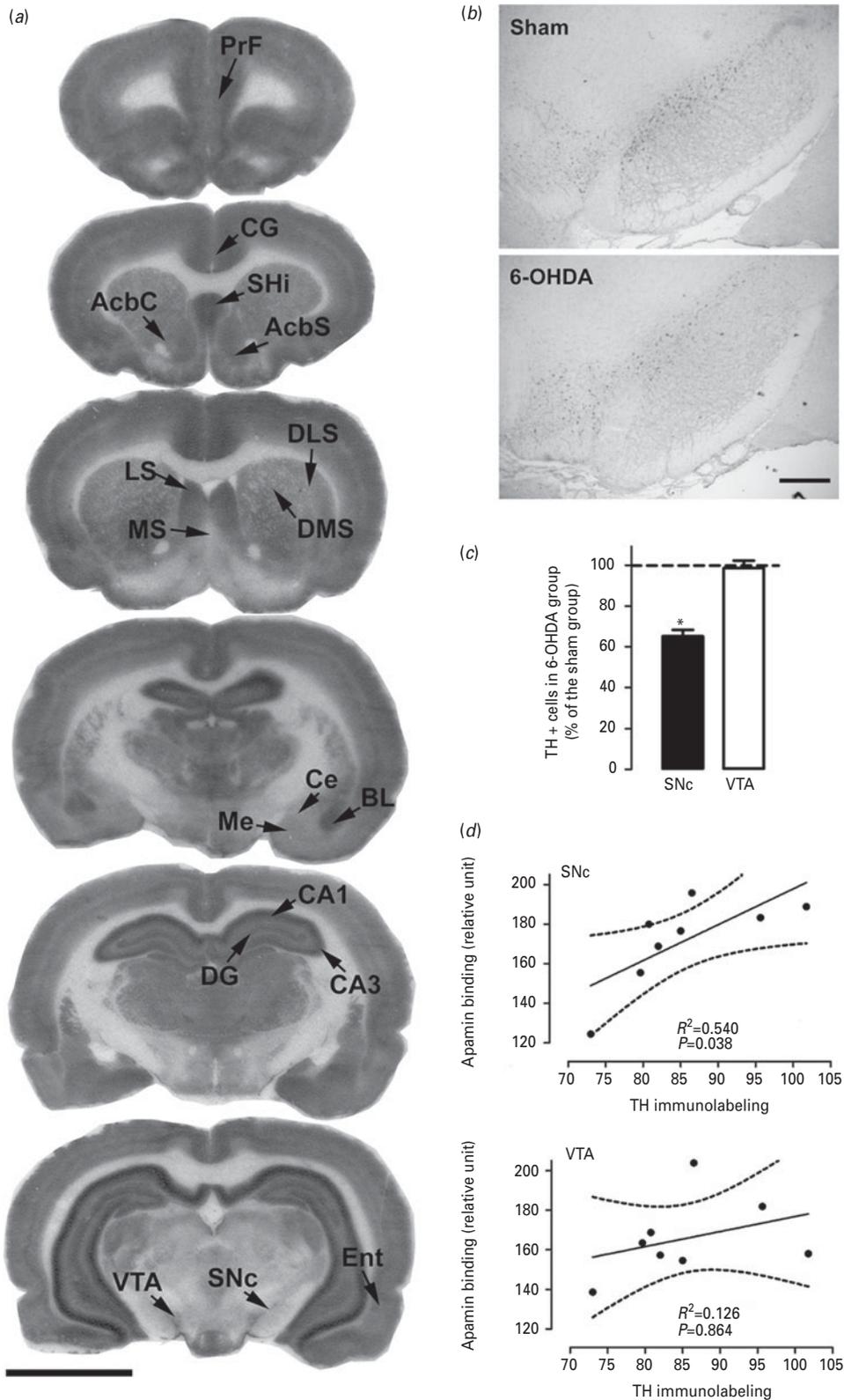


Fig. 4. Apamin binding in dopamine (DA) nigrostriatal and mesolimbic structures and tyrosine hydroxylase (TH) immunolabeling in the substantia nigra pars compacta (SNc). (a) Examples of apamin binding autoradiograms of coronal sections in the rostro-caudal axis of a substantia nigra pars compacta (6-OHDA)-lesioned brain. The dark areas indicate high grain densities, i.e. high binding levels. Bar=5 mm. (b) Illustration of the typical TH immunolabeling in the SNc of representative sham and 6-OHDA-lesioned rats. Bar=1 mm. (c) Quantification of TH labeling in the SNc and the ventral tegmental area (VTA) expressed as percentage of TH positive cells of the sham group. Note that the TH positive cell number in the SNc of 6-OHDA-lesioned rats significantly decreased compared to that of sham rats (* $p<0.05$). (d) Linear regression between TH immunolabeling and apamin

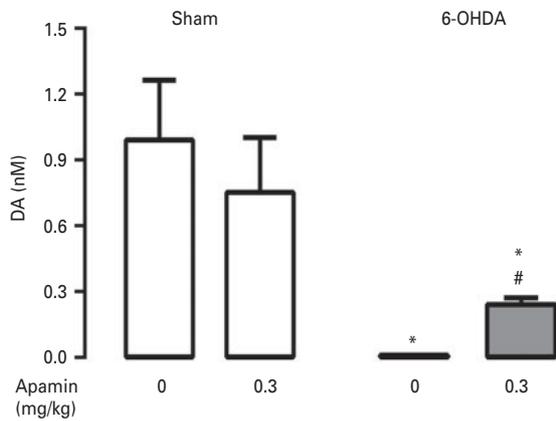


Fig. 5. Effects of 6-hydroxydopamine (6-OHDA) nigrostriatal lesions and apamin on extracellular dopamine (DA) levels in the dorsal striatum. Extracellular DA levels, expressed as the mean (nM) \pm S.E.M., were measured in the striatum of the vehicle-sham ($n=4$), apamin-sham ($n=4$), vehicle-6-OHDA lesioned ($n=8$), or apamin-6-OHDA lesioned ($n=8$) groups for 120 min. Apamin 0.3 mg/kg or vehicle was injected i.p. after baseline dialysate collections and its effect measured after 30 min. Apamin increased DA concentration in 6-OHDA-lesioned rats which was previously undetectable. * $p<0.05$ in comparison with vehicle-sham group, Student's t -test, # $p<0.05$ in comparison with vehicle-6-OHDA group, Student's t -test.

role of the striatum in spatial navigation and short-term memory processes as recently emphasized (Braun et al., 2012; Brooks and Dunnett, 2013). Restricted bilateral 6-OHDA lesions to the SNc, but not the VTA, induce profound motivational and emotional impairment (Drui et al., 2013). In line with this, a reward-related learning procedure tested in PD patients showed that fMRI signals, which may reflect phasic DA activity, were impaired in the dorsal striatum but preserved in the ventral striatum (Schonberg et al., 2010). These studies highlight the important contribution of the nigrostriatal dopaminergic system to these NMS before the onset of any obvious motor signs, although damage to the serotonergic, cholinergic and norepinephrine systems may also contribute to the expression of the NMS at various stages of the disease (Chaudhuri et al., 2011).

Apamin reverses non-motor and motor symptoms in the 6-OHDA model of PD

In this early model of PD, we found that SK channel blockade by apamin fully reverses the anxiety-like and

anhedonia phenotype. In addition, apamin prevented short-term social recognition and visuospatial memory deficits. Importantly, apamin did not impair locomotor and exploratory behavior in the various tests, demonstrating the specific effect of apamin on these non-motor symptoms. Apamin did not modify the reactivity of sham animals towards novelty. Therefore, the effects of apamin cannot be attributed to a general inability to react to novel information but were specific to emotional and spatial memory impairment. Apamin did not modulate anxiety or social interaction in sham animals either. This is consistent with previous studies showing that apamin at doses below 0.4 mg/kg do not have major effect on spontaneous behavior (Ikonen et al., 1998; van der Staay et al., 1999).

Different sites of action in the brain including the amygdala, hippocampus and the striatum might be responsible for the rescue of mood and cognitive performance after a systemic administration of apamin. Indeed, SK2 potassium channel overexpression in the basolateral amygdala reduces anxiety (Mittra et al., 2009) and enhancement of SK channel activity with the channel activator CyPPA (Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine) reduces the abnormalities of amygdala-dependent fear memory caused by repeated stress (Atchley et al., 2012). On the other hand, apamin has minimal effects on basolateral amygdala neuronal activity (Faber and Sah, 2002) and apamin had no effect on anxiety in sham animals in the present study. Furthermore, we found no change of SK channel density in the VTA or in the basolateral amygdala of DA lesioned rats. The reduction of anxiety and anhedonia induced by apamin in our partial PD model may thus involve a specific action of apamin on nigrostriatal DA neurotransmission rather than a direct effect on basolateral amygdala neurons. DA concentration is indeed increased by apamin treatment in the striatum of lesioned rats. Consistent with this, SK3-deficient mice show enhanced DA neurotransmission in the striatum, improving DA neuronal excitability that is accompanied by an antidepressant-like phenotype (Jacobsen et al., 2008).

Various studies underscore the importance of SK channels in mnesic processes (for reviews, Faber, 2009; Adelman et al., 2012). The blockade of SK channels by apamin facilitates the encoding of long-term memory in hippocampal and non-hippocampal paradigms (Fournier et al., 2001; Mpari et al., 2008). Inversely, SK2 overexpression or SK channels activation by CyPPA impairs

binding levels in the SNc and VTA. Each point corresponds to one of the eight 6-OHDA lesioned rats. A positive correlation was found in the SNc but not in the VTA. Therefore, the decrease of apamin binding is proportional to DA neuron degeneration in the SNc. AcbC, Accumbens nucleus, core; AcbS, Accumbens nucleus, shell; BL, Basolateral amygdaloid nucleus; CA1-CA3, CA1-CA3 hippocampal field; Ce, Central amygdaloid nucleus; CG, Cingulate cortex; DG, Dentate gyrus; DLS, Striatum, dorso-lateral part; DMS, Striatum, dorso-medial part; Ent, Entorhinal cortex, ventral part; LS, Lateral septal nucleus; Me, Medial amygdaloid nucleus; MS, Medial septal nucleus; PrF, Prefrontal cortex; SHi, Septohippocampal nucleus; SNc, Substantia nigra, pars compacta; VTA, Ventral tegmental area.

learning and long-term potentiation (Hammond et al., 2006). In addition, SK channels have been recently associated with the induction of striatal long-term depression underlying memory encoding (Hopf et al., 2010). However, some aspects of working/short-term memory are disrupted in SK3-deficient mice (Jacobsen et al., 2009) but were enhanced by apamin in a spatial delayed task (Brennan et al., 2008). In our study, the short-term spatial learning deficit following striatal DA depletion was restored by apamin treatment. Indeed, DA in the striatum plays a critical role in the acquisition of spatial information as evidenced by a negative correlation between striatal DA tissue levels and the re-exploration of displaced objects in a similar task (De Leonibus et al., 2007). SK channels play a major role in controlling the timing and stability of DA neurons endogenous pacemaker activity (Seutin et al., 1993; Wolfart et al., 2001; Waroux et al., 2005). Blockade of SK channels enhances the neuronal excitability after the action potential and may therefore increase extracellular DA concentration in the striatum, nucleus accumbens and prefrontal cortex (Steketee and Kalivas, 1990; Dawson and Routledge, 1995). The present study extends these findings by showing that in striatal DA-depleted animals, a significant level of extracellular DA level is reinstored in the striatum by apamin at a dose alleviating the motor and non-motor symptoms. Conversely, positive modulation of SK channels reduces DA release in cultured DA neurons of rats (Herrik et al., 2012). In partially DA lesioned animals, blockade of SK channels in the remaining midbrain DA neurons could compensate for the loss of DA activity in the dorsal striatum. Our results support this idea, since the loss of TH immunoreactive neurons is correlated with a similar decrease of SK channel protein level in the substantia nigra. We cannot exclude, however, that apamin could reinforce the synaptic corticostriatal glutamatergic activity, essential for processing spatial contextual information, by inhibiting the negative feedback loop of SK channels on NMDA receptors of the dorsal striatum and DA midbrain neurons, as found by others (Ngo-Anh et al., 2005).

In the extensive hemiparkinsonian model of PD, apamin action cannot be mediated by SK channels expressed on dopaminergic neurons, mostly degenerated. For instance SK currents play a fundamental role in the autonomous activity of the subthalamic nucleus by opposing the transition from tonic single-spike activity to burst firing in the glutamatergic neurons (Hallworth et al., 2003). This could be critical in parkinsonism as abnormal firing of the subthalamic nucleus is thought to contribute to the expression of PD symptoms. Indeed, high-frequency stimulation of the subthalamic nucleus ipsilateral to the lesion produces a similar effect to that of apamin on both circling behavior (Darbaky et al., 2003) and forelimb asymmetry in the cylinder (Jouve et al., 2010). We recently found that apamin intracerebral infusion in the subthalamic nucleus potentiated the motor symptoms

of 6-OHDA lesioned rats, whereas its intranigral infusion reduced it (M. Amalric, unpublished results), pointing to a critical role of these channels in the modulation of basal ganglia regions in parkinsonian conditions.

In conclusion, the present data demonstrate that SK channels play an important role in regulating cellular mechanisms of emotional behaviors and spatial memory mediated by the basal ganglia. The positive action of apamin on non-motor symptoms of PD in partially lesioned subjects may result from the increase of striatal extracellular DA release induced by SK channel blockade in the remaining nigral DA neurons. SK channels may therefore represent potential targets in the treatment of motor, emotional and cognitive deficits in the early stages of Parkinson's disease. The therapeutic window for SK channel blockers might be relatively narrow, however, as SK channel hypofunction may also induce psychotic symptoms, as recently shown in SK3-deficient mice (Soden et al., 2013).

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Supplementary material

For supplementary material accompanying this paper, visit <http://dx.doi.org/10.1017/S1461145714000236>.

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