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Activity-Dependent Regulation of the Dopamine Phenotype in the Adult Substantia Nigra: Prospects for Treating Parkinson's Disease

Mal Horne, Kate Lord and Tim Aumann

*Florey Neuroscience Institutes,
The University of Melbourne,
Australia*

1. Introduction

The motor symptoms of Parkinson's disease (PD) are caused by degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and the resulting depletion of DA signaling in their target structure, the dorsal striatum (the nigrostriatal pathway). PD motor symptoms are successfully alleviated by systemic administration of a blood-brain barrier permeable DA precursor (levodopa) or DA receptor agonists (Olanow et al. 2001), however these treatments are marred by side-effects in some patients (Wood 2010), and increasingly unreliable response and shortened duration of effect coupled with the emergence of dyskinesias in most patients (Stocchi et al. 2010, Stocchi & Marconi 2010). These problems probably arise from loss of physiological storage, release and reuptake of nigrostriatal DA and ensuing down-stream changes in post-synaptic signaling, and from increased DA signaling in structures outside the nigrostriatal pathway, especially when D2 agonists are used. Targeted (nigrostriatal) reconstruction of physiological DA signaling, aimed at restoring nigrostriatal DA transmission to at least the level at disease outset, ought to alleviate PD motor symptoms without side-effects. However, attempts to achieve this by replacing SNc DA cells through transplantation or endogenous repair are often hampered by poor acquisition and maintenance of the DA phenotype in the microenvironment of the adult SNc (Brundin et al. 2000, Courtois et al. 2010, Torres et al. 2005, Bauer et al. 2000).

There is evidence that expression of tyrosine hydroxylase (TH, the rate-limiting enzyme in DA synthesis) by adult SNc and midbrain neurons is pliable. TH is down-regulated in cells that survive exposure to neurotoxins [6-hydroxy-dopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)], and can be up-regulated again, presumably in these same cells, by glial-derived neurotrophic factor (GDNF) (Bjorklund *et al.* 1997, Bowenkamp *et al.* 1996, Gash *et al.* 1996, Sauer & Oertel 1994). There is also a degree of spontaneous recovery in the number of TH immunoreactive (TH+) SNc cells following 6-OHDA, which occurs coincidentally with a decrease in the number of SNc cells that are not immunoreactive against TH (TH-) (Stanic *et al.* 2003), implying acquisition of DA phenotype by extant cells. The DA phenotype of SNc neurons also appears to be regulated in an activity-dependent way. Reduced SNc TH expression following striatal infarct is blocked by

intraventricular administration of a GABA_A receptor agonist (Soriano *et al.* 1997, Yamada *et al.* 1996); SNc TH mRNA is elevated followed systemic administration of nicotine or α -7 nicotinic acetylcholine receptor agonists (Serova & Sabban 2002); and we recently reported that the number of SNc TH⁺ cells in adult mice can be increased or decreased by direct brain infusions of drugs targeting SNc neuronal activity (Aumann *et al.* 2011, Aumann *et al.* 2008). These data are consistent with the possibility that the DA phenotype of adult SNc neurons can be gained or lost in an activity-dependent way.

Understanding how the DA phenotype of neurons in the adult SNc is regulated will highlight strategies and molecular (drug) targets to facilitate DA phenotype acquisition and maintenance by either transplanted or endogenous cells. The aims of the experiments described in this report were to determine whether infusions of drugs that increase the number of SNc DA (TH⁺) cells in normal mice: (1) increase the number of these cells in a 6-OHDA rodent model of PD; and (2) restore motor deficits.

2. Methods

2.1 Animals

All experimental procedures on animals were approved by the Florey Neuroscience Institutes animal ethics committee and conform to the National Health and Medical Research Council of Australia's published code of practice.

Male C57Bl/6J mice and male Sprague-Dawley rats were used for this study. Throughout the experiments all animals were housed in a climate-controlled (22°C) room on a 12/12hour light/dark cycle with ad libitum access to food (standard rodent chow) and water.

2.2 6-OHDA lesion

Unilateral 6-OHDA lesions were made in the mice and rats when they were 8-weeks old. We aimed for partial (40-60%) loss of SNc DA neurons because another form of compensatory plasticity (i.e. spouting of surviving nigrostriatal projections) in the nigrostriatal system is hampered with lesions >75% (Stanic *et al.* 2003). Mice were anesthetized with an i.p. injection of 5% chloral-hydrate in sterile phosphate buffered saline, whereas rats were anesthetized with 1-2% isoflurane in air. Their heads were secured in a stereotaxic frame and a midline incision made over the skull. A small (2-3mm diameter) hole was drilled through the skull overlying the left (mice) or right (rats) SNc with a dental burr. In mice, 1.5µg/µl 6-OHDA (Sigma-Aldrich) in distilled H₂O with 0.2mg/ml ascorbic acid was prepared on the morning of the lesions and kept on ice until injected to minimize oxidation. 1.6µl of the 6-OHDA solution was injected slowly (1.0µl/min.) through a 26gauge sterile needle at stereotaxic coordinates: 3.0mm posterior to Bregma, 1.5mm lateral to the midline, 4.0mm deep. In rats, two 1.0µl injections of 2.0µg/µl 6-OHDA in distilled H₂O with 0.2mg/ml ascorbic acid were made through a glass micropipette at stereotaxic coordinates: (1) 3.7mm anterior to Lambda, 1.7mm lateral to the midline, 8.1mm deep; and (2) 3.7mm anterior to Lambda, 2.1mm lateral to the midline, 7.5mm deep. At the completion of each injection the injection needle was left in situ for 2mins. to allow toxin diffusion then the needle was slowly withdrawn to minimize toxin backtracking up the needle track. The skin

was sutured and antiseptic applied, an anti-inflammatory [3mg/kg Meloxicam (Metacam®), s.c.] was administered, and the animal was left to recover in a warmed cage.

2.3 Osmotic minipump infusions

Some weeks following the 6-OHDA lesion, when SNc TH+ cell loss was maximal, a cannula [ALZET® (Cupertino, CA, USA) or PlasticsOne® (Roanoke, VA, USA)] was implanted into the midbrain or striatum on the same side as the lesion, through which a drug (or vehicle) was infused continuously via an osmotic pump (ALZET® model #1002 in mice & model #2006 in rats) for a period of time (see Results section for specific timings). To prime the pumps prior to implantation they were filled with drug (or vehicle) and immersed in 37°C sterile saline overnight. The next day animals were anaesthetized and prepared as described above. The cannula was implanted at the following stereotaxic coordinates: (1) For SNc infusions in mice 3.0mm posterior to Bregma, 1.5mm lateral to the midline, 4.0mm deep; (2) For SNc infusions in rats 3.7mm anterior to Lambda, 1.9mm lateral to the midline, 7.8mm deep; (3) For striatal infusions in rats 0.5mm anterior to Bregma, 3.0mm lateral, 3.5mm deep. The cannula was glued to the skull with dental cement and the attached pump was placed in a subcutaneous 'pocket' created in the interscapular region.

In rat experiments the period of drug delivery was extended beyond the lifetime (6 weeks) of the first implanted pump by replacing it with a new filled pump. This was done by anaesthetizing the rat with 1-2% isofluorane in air, making a small incision in the skin overlying the old pump, clamping the vinyl tube closed, removing the old pump (and flow moderator), replacing it with a new primed pump (and new flow moderator), releasing the clamp and suturing the skin. The implanted cannula remained undisturbed throughout this procedure.

2.4 Behavior

The behavioral studies described below were performed on cohorts of animals typically comprising 12 animals, 6 treated with drug and 6 with vehicle. Each behavioral test was performed at the same time of day within each cohort and each animal was studied concurrently. If availability of equipment prevented this, animals were studied consecutively in a different pseudo-randomized order and equipment was thoroughly cleaned with 80% ethanol after each animal to remove any distracting odors.

2.4.1 Rotational response to amphetamine

Animals were injected intraperitoneally with 5mg/kg amphetamine and immediately placed into a cylindrical chamber (17cm diameter by 17cm high for mice & 31cm diameter by 31cm high for rats). In some rat experiments rotational behavior was also assessed for 20mins. prior to amphetamine. The behavior of each animal in its chamber was recorded on videotape for at least 1hour following amphetamine. On days where amphetamine was administered, no other behavioral tests were performed.

Rotational behavior was measured off-line using Ethovision® XT animal tracking software (Noldus Information Technology, Wageningen, Netherlands). One rotation was defined as a cumulative 360° change in heading direction of the animal's centre of mass, with no time

limit. If an animal interrupted a turn in one direction with a turn in the other of more than 90°, the cumulative change in the first direction was reset to zero.

2.4.2 Locomotor cells

Locomotion was measured using photo-optic locomotor cells (Truscan Photobeam; Coulbourn Instruments, Allentown, PA, USA), which automatically detect horizontal- (locomotor) and vertical-plane (rearing) movements. Cell dimensions were 42cm wide x 42cm deep x 38cm high. Each rat was placed into a locomotor cell (6 rats at a time) for 30mins. in a quiet room with low-level light.

2.4.3 Cylinder test

Lateralized forelimb movement deficits were assessed using the cylinder test. This test was always performed in conjunction with the locomotor cells – half the cohort was run in the locomotor cells while the other half was run in the cylinder test, then vice versa (in a different pseudo-randomized order each time). Each rat was placed in a clear plastic cylinder (19cm diameter and 26cm high) standing vertically on a bench (1 rat at a time). A video camera recorded behavior for 10mins. in a quiet room with low-level light. Whilst in the cylinder the rats reared up to explore the only route of escape, supporting themselves against the cylinder wall with their left and/or right forelimbs. Two mirrors were positioned behind the cylinder to observe forelimb movements obscured by the rat's body. The number of times each forelimb touched the wall of the cylinder over the 10mins. was counted by an observer without prior knowledge of the treatment received.

2.4.4 Corridor test

Lateralized motor or attention deficits were assessed using the corridor test. No other test was performed on the day of the corridor test. Three days prior to the corridor test, rats were food-deprived by rationing chow at the rate of 2.5g/100g body weight. Two rats were run concurrently (in a different pseudo-randomized order each time). Each rat was placed in a corridor measuring 240cm long by 7cm wide by 21cm high, enclosed at each end but open at the top. Food (chocolate rice-puff cereal) was available *ad libitum* at multiple, evenly spaced points (13cm apart) along the left and right sides of the corridor. A video camera recorded their behavior for 10mins. in a quiet room with low-level light. Whilst in the corridor the rats explored freely along its length including sniffing and eating the cereal at their leisure. The number of times each rat sniffed at or ate cereal on their left and right sides was recorded by an observer without prior knowledge of the treatment received.

2.5 Immunohistochemistry

At the end of the experiment animals were killed with an overdose of anesthetic (sodium pentobarbitone, 100mg/kg, i.p.). Before their heart stopped beating the animals were perfused transcardially with warm (37°C) heparinized (0.1%) 0.1M phosphate buffered saline (PBS) followed by cold (4°C) 4% paraformaldehyde plus 0.2% picric acid in 0.1M phosphate buffer (PB). The brains were removed and placed at 4°C in PBS with 20% sucrose. Once equilibrated with 20% sucrose in PBS, the brains were frozen and 16µm thick coronal sections were cut through the striatum and SNc. Sections were collected directly onto glass

slides coated with 0.1% chrome alum and 1% gelatin in distilled H₂O and stored at -80°C until immunohistochemical processing.

For TH immunohistochemistry, mounted sections were post-fixed in 10% neutral buffered formalin (5min.), incubated for 10min. in blocking solution (0.1M PBS, 0.3% Triton X-100 & 3% normal goat serum), then for 72hours at 4°C in rabbit anti-TH primary antibody (1:1500, Chemicon, Temecula, CA, USA) with 0.3% Triton X-100 and 1% normal goat serum in PBS. This was followed by 2hours incubation at room temperature in a biotinylated secondary antibody (1:1000, goat anti-rabbit IgG, Dako, Denmark). Next, sections were incubated for 1hour in 0.02% avidin peroxidase with 0.75% Triton X-100 in PBS at room temperature, then in cobalt- and nickel-intensified diaminobenzidine (DAB) for 20mins., then 3% hydrogen peroxide was added to the DAB solution for a further 2mins. to complete the chromagen reaction. Rinses (3 x 5min. each) in PBS were performed between each step. Sections were counterstained with 1% neutral red for 8min., washed in H₂O, dehydrated in a series of graded ethanol solutions (50-100%) and cleared in X3B before being coverslipped with a polystyrene mounting medium.

For DAT immunohistochemistry the protocol was the same as for TH immunohistochemistry with the following exceptions. Prior to incubation in blocking solution an antigen retrieval step was performed in which the sections were heated in 0.2% citrate buffer (pH 6) and allowed to cool at room temperature for 30min. Sections were incubated in a blocking solution (5% normal goat serum, 0.3% Triton X-100 in 0.1M PBS) for 10min. The primary antibody was mouse anti-DAT (1:3000, Chemicon) and the biotinylated secondary antibody was sheep anti-mouse (1:600, Chemicon). No counterstain was performed.

2.6 Stereology

To estimate the numbers of TH⁺ and TH⁻ neurons in the SNc following 6-OHDA lesion and drug treatment, a fractionator sampling design was used, as previously described in detail (Parish *et al.* 2001), using a stereology program (Stereo Investigator, MicroBrightField, VT, USA) attached to a microscope. The SNc was delineated at its anatomical boundaries with reference to rodent brain atlases (Franklin & Paxinos 2008, Paxinos & Watson 2007) and based on high cell-packing density, cell morphology, and TH immunoreactivity (Nelson *et al.* 1996). Counts of TH⁺ and TH⁻ SNc neurons (glia were excluded on the basis of soma diameter <5µm) within a counting frame (45µm x 35µm) were made at regular pre-determined intervals (x=145µm, y=145µm) using a 60x oil-immersion objective lens. The cell nucleus was the counting unit. TH⁺ cells were immunoreactive against TH and TH⁻ cells were not immunoreactive against TH but counterstained. Every 5th section of the series was analyzed (i.e. 80µm apart) and the volume of the SNc was estimated according to Cavalieri's method (Gundersen *et al.* 1988), which estimates on the basis of area, section thickness and distance between sections. Cells on the treated and contralateral (internal control) sides of each brain were always counted consecutively to ensure consistency of cell classifications. Cell count in the lesioned and treated SNc was expressed as a proportion of cell count in the contralateral internal control SNc in each animal. Cell counts were performed by an experienced SNc stereologist without prior knowledge of the treatment received.

3. Results

3.1 Midbrain infusions of SK channel agonists

3.1.1 1-EBIO in mice

The number of SNc TH⁺ cells in normal adult mice increased by ~500 following a 2 week infusion of 100μM 1-EBIO (or 30μM riluzole) into midbrain (Aumann et al. 2008, Aumann et al. 2011). To examine whether midbrain 1-EBIO infusion can also increase SNc TH⁺ cells in a PD model, we repeated this in adult mice with prior depletion of TH⁺ SNc cells by 6-OHDA.

Eight-week old mice received a single injection of 6-OHDA directly into the left SNc. Two weeks later [when TH⁺ SNc cell loss is maximal (Aumann et al. 2008)] an infusion cannula was implanted into the left SNc, to allow continuous delivery of vehicle, 100μM 1-EBIO or 200μM 1-EBIO by osmotic pump for a further 2 weeks. The number of TH⁺ cells in the lesioned then treated SNc [expressed relative to the number of TH⁺ cells in the contralateral (internal control) SNc] is shown in figure 1. Data from individual mice are represented by the square symbols and the mean of each treatment group by the horizontal line. Note: (1) the relative tightness of the number of TH⁺ SNc cells in the vehicle-treated group, highlighting the consistency of the effect of our 6-OHDA injections across different mice; (2) the much greater variability in the drug-treated groups, indicating 1-EBIO has a restorative effect in some mice, even at 100μM; and (3) the increasing restorative effect with increasing dose of 1-EBIO. Unfortunately, although there is a net (average) increase in the number of TH⁺ SNc cells from ~50% (vehicle) to ~80% (200μM 1-EBIO) of the normal number of cells, this difference is not significant ($p=0.068$, one-way ANOVA) due to high variability in the 1-EBIO-treated groups.

We also examined the density of immunoreactivity for the DA transporter (DAT) in the striatum in a cohort of vehicle-treated and 200μM 1-EBIO-treated mice to examine whether 1-EBIO improved striatal DA innervation following 6-OHDA depletion. Although there was a trend for DAT density to be higher in the 1-EBIO-treated group, and specifically in the dorsal striatum, not the ventral striatum, this difference was not statistically significant (data not shown).

3.1.2 Riluzole in mice

Riluzole is approved for the treatment of amyotrophic lateral sclerosis and is reported to affect neuronal excitability through a number of different ion-channels (e.g. voltage-activated Na⁺, glutamate receptors and SK channels). It activates SK channels at concentrations $\geq 3\mu\text{M}$ (Cao et al. 2002, Grunnet et al. 2001) and at 30μM (direct injection) it increases the number of TH⁺ SNc cells in normal mice (Aumann et al. 2011). We therefore tested its ability to increase the number of TH⁺ SNc in the PD model. Note that riluzole protects SNc neurons from MPTP and 6-OHDA in animal models of PD (Barneoud et al. 1996, Bezard et al. 1998) but does not provide benefit in PD patients, either early (Jankovic & Hunter 2002) or late (Braz et al. 2004) in disease, or in Parkinson plus disorders (Bensimon et al. 2009). To our knowledge riluzole has not been considered as a neuro-restorative agent in PD, such as we are proposing here (i.e. replenishing SNc DA cells by DA phenotype recruitment). We also note that the doses of riluzole used so far in clinical trials for PD have all been relatively low (around 3μM) rather than the 30μM, which we have shown increases the number of SNc TH⁺ cells in normal mice (Aumann et al. 2011). Therefore, any restorative benefit of riluzole through DA phenotype recruitment may have been missed.

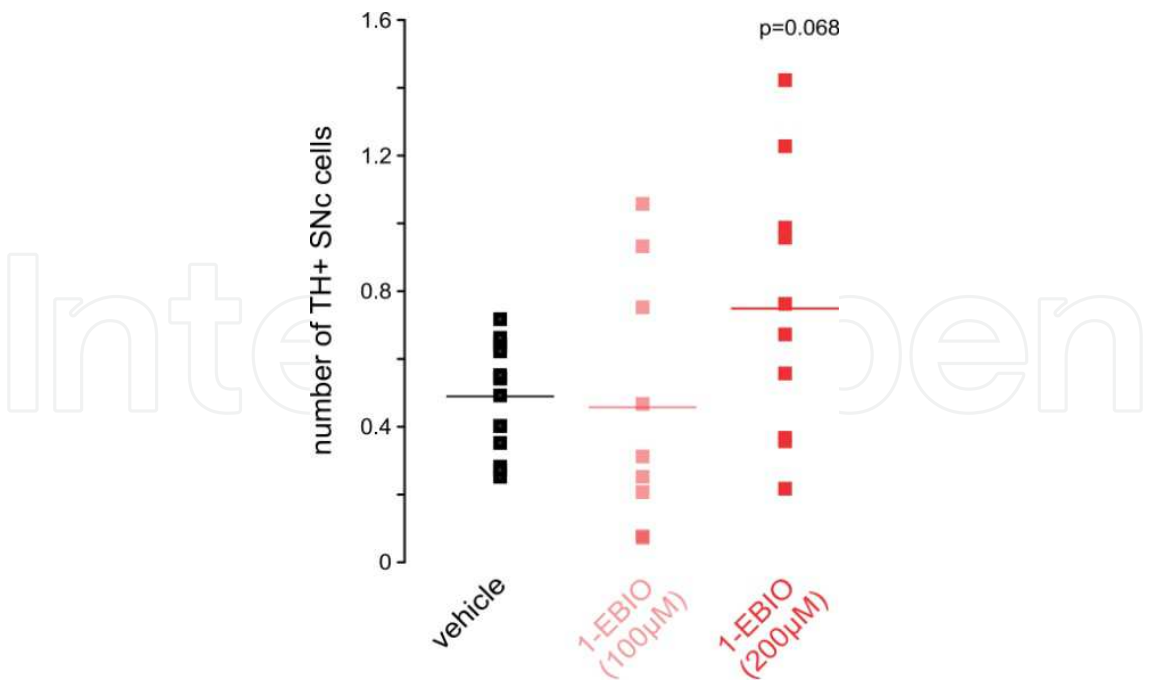


Fig. 1. Effect of the SK channel agonist 1-EBIO on the number of TH+ SNc cells in 6-OHDA-lesioned adult mice. 6-OHDA was injected into the left SNc of 8-week old male C57Bl/6J mice to reduce the number of SNc TH+ cells to ~50% of normal (see vehicle-treated mice, black symbols). Two weeks later a cannula was implanted into the left SNc, through which 1-EBIO or vehicle was infused (from an attached minipump) continuously for a further 2 weeks. The number of TH+ cells in the left and right SNc of each mouse was estimated using unbiased stereology by an observer who was unaware of the treatment received. The data for each mouse (filled squares) are expressed as the ratio of the number of TH+ SNc cells on the lesioned and treated (left) side relative to the internal control (right side). The mean of each treatment group is represented by a horizontal line. Lesion and vehicle-treated mice (n=15, black symbols) have a ~50% reduction, on average, in the number of TH+ SNc cells. Treatment with 100µM 1-EBIO (n=9, pale red symbols) appears to have had a beneficial effect (see text) in some mice, but there was no change on average. Treatment with 200µM 1-EBIO (n=10, red symbols) appears to have had an even stronger beneficial effect in some mice, resulting in an average increase in SNc TH+ cells to ~80% of normal. Note, however, that this increase was not statistically significant (p=0.068, one-way ANOVA).

We tested the neuro-restorative effects of orally administered riluzole on motor symptoms and TH+ SNc cell number in a mouse PD model. Three weeks after 6-OHDA SNc lesion, mice were administered riluzole in their drinking water for 2 weeks. Both 3µM and 30µM riluzole doses were tested. Note these were the projected daily systemic concentrations based on the average daily volume of drinking water consumed and body-weight of each mouse. Every 3 days following the lesion and throughout the treatment period, the rotational behavior of the mice in response to amphetamine (5mg/kg i.p.) was measured (figure 2A & A'). Following 6-OHDA lesion and before treatment began (figure 2A), the relative number of ipsiversive rotations (rotations toward the side of the lesion) increased progressively to reach a maximum by 12 days [p=0.035 (time), two-way ANOVA]. Presumably this reflects gradual depletion of DA in the left striatum caused by progressive degeneration of DA neurons in the left SNc. The relative number of contraversive rotations

(rotations away from the side of the lesion) remained constant throughout this experiment (data not shown). There was no significant difference in rotational behavior amongst the 3 treatment groups in this post-lesion and pre-treatment period [figure 2A, $p=0.107$ (treatment)], nor was there a treatment by time interaction [figure 2A, $p=0.981$ (treatment $\times$ time)]. Drug (or vehicle) treatment began 18 days following 6-OHDA (figure 2A'). Despite this, the rotational phenotype of the mice in each treatment group remained unchanged throughout the treatment period [figure 2A'; $p=0.923$ (treatment), $p=0.986$ (time), $p=0.949$ (treatment $\times$ time), two-way ANOVA]. Thus, riluzole had no effect on 6-OHDA-induced motor dysfunction in this experiment.

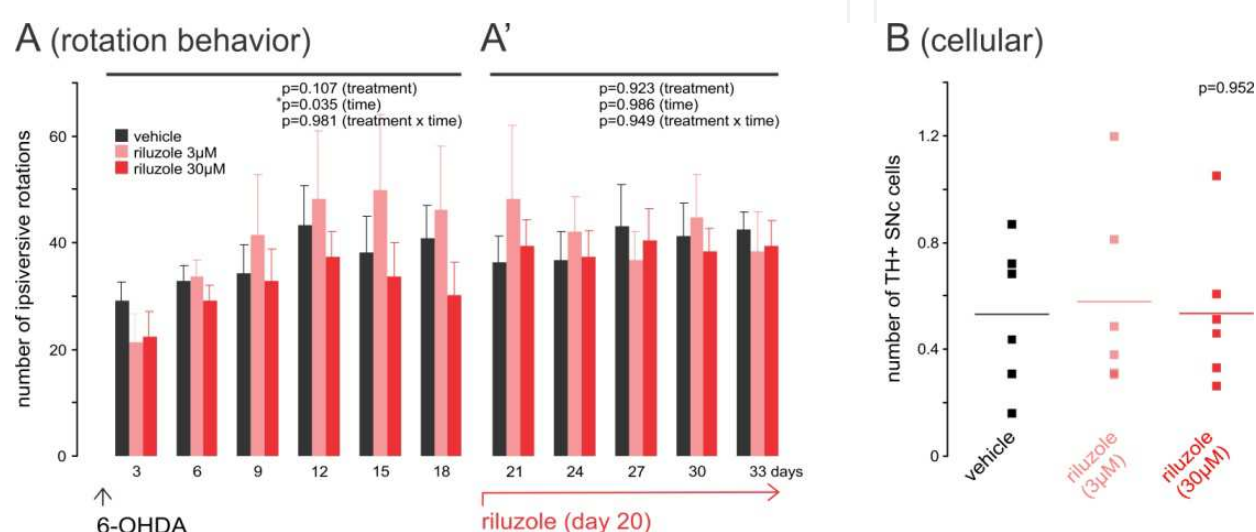


Fig. 2. Effects of the SK channel agonist riluzole on rotational behavior in response to amphetamine and the number of TH+ SNc cells in 6-OHDA-lesioned adult mice. 6-OHDA was injected into the left SNc of 8-week old male C57Bl/6J mice to reduce the number of SNc TH+ cells to ~50% of normal (see vehicle-treated mice, black symbols in B). (A & A') Every 3rd day for the next 18 days the rotational behavior in response to amphetamine was examined. The relative mean $\pm$ SE number of ipsiversive rotations performed over a 25 minute interval, beginning 25 minutes following amphetamine injection, is plotted over time for each treatment group. Note, the software used to count rotations was not as accurate as manual counting or other methods (e.g. rotometers); therefore the number of rotations here should not be compared with data collected using these other methods. However, the software consistently counted rotations at different times; therefore any changes in number of rotations accurately reflect behavioral changes. (A) 6-OHDA resulted in progressive increase in the number of ipsiversive rotations over time ($p=0.035$, two-way ANOVA). No differences in number of contraversive rotations occurred over this same period (data not shown). (A') Over days 20-33, vehicle (black bars), 3μM riluzole (pale red bars), or 30μM riluzole (red bars) was administered to the mice in their drinking water. During this time there was no change in the number of ipsiversive (or contraversive, data not shown) rotations by treatment or by time (two-way ANOVA). (B) At the end of day 33, there were no differences in the number of TH+ cells in the 6-OHDA-lesioned SNc across the three treatment groups ($p=0.952$, one-way ANOVA). See figure 1 legend for details about how these data are represented.

Following riluzole treatment, we estimated the total number of TH+ SNc cells and measured the density of DAT immunoreactivity in the dorsal striatum in these mice. No differences were found across treatment groups in the number of TH+ SNc cells (figure 2B; $p=0.952$, one-way ANOVA) or the density of DAT immunoreactivity in the dorsal striatum (data not shown).

3.1.3 1-EBIO in rats

We repeated the 200 μ M 1-EBIO infusion experiment in mice detailed above in unilateral 6-OHDA-lesioned rats, because motor impairments can be more accurately quantified in rats using locomotor cells, the cylinder test, and rotational response to amphetamine. The timecourse of this experiment was longer than the mouse experiment because it takes longer for SNc DA cell degeneration to occur following 6-OHDA, and because we wanted to see whether a treatment period longer than we used in mice (2 weeks) produced any effect.

In summary, there were no effects of 200 μ M 1-EBIO infusion on motor behavior or number of TH+ SNc cells following 6-OHDA lesion in rats. The rotational behavior in response to amphetamine is plotted against time in figure 3A. This shows that following 6-OHDA, there was a gradual increase in the ratio of ipsiversive:contraversive rotations, as expected, which peaked at 4 weeks. Two weeks later (i.e. 6 weeks following lesion) half the rats were implanted with osmotic pumps infusing 200 μ M 1-EBIO directly into the lesioned midbrain, and the other half were implanted with osmotic pumps infusing vehicle. Following the onset of drug (or vehicle) treatment, there was a trend (non-significant) for animals receiving 200 μ M 1-EBIO to show improvement in their rotational response at 9 & 11 weeks (3 & 5 weeks after treatment onset; figure 3A). However, no such trend was apparent at 13 & 15 weeks (7 & 9 weeks after treatment onset; figure 3A). A similar scenario, including a non-significant trend for improvement at 9 & 11 weeks, was also observed in motor behavior during the cylinder test (data not shown).

At the experiment end-point (15 weeks after lesion), the number of SNc TH+ cells was similar in 1-EBIO- and vehicle-infused rats (figure 3B).

3.2 Striatal infusions of quinpirole

While infusion of SK channel agonists (1-EBIO or riluzole) into the normal adult mouse SNc recruited ~500 more TH+ SNc cells (Aumann et al. 2008, Aumann et al. 2011), infusion of the D2 DA receptor agonist quinpirole into the normal adult mouse striatum was far more potent in this respect; recruiting ~3000 new TH+ SNc cells (Aumann et al. 2011). We therefore examined the behavioral and cellular effects of striatal quinpirole infusion in 6-OHDA-lesioned rats.

Four different behaviors were measured before and after unilateral 6-OHDA lesion of the SNc and during treatment (100nM quinpirole or vehicle). These are plotted in figure 4A-D. While there was no obvious effect of lesion or treatment on locomotor and rearing behavior (figure 4A-B), an asymmetry in left/right forelimb use (cylinder test and corridor test) and possibly also left/right side attention (corridor test) was evident following lesion (figure 4C-D). The lesion was made in the right SNc in this experiment, resulting in left forelimb

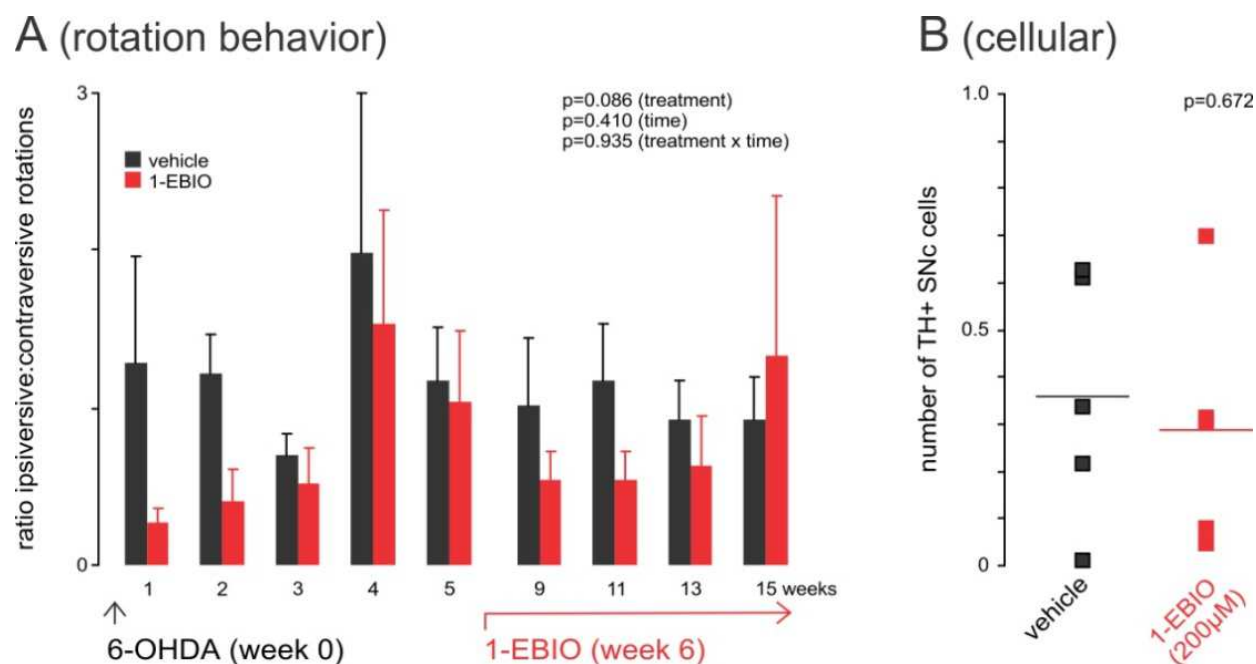


Fig. 3. Effects of the SK channel agonist 1-EBIO (200μM) on rotational behavior in response to amphetamine and the number of TH+ SNc cells in 6-OHDA-lesioned adult rats. (A) 6-OHDA was injected into the right SNc of 8-week old male Sprague-Dawley rats to reduce the number of SNc TH+ cells to ~35% of normal (see vehicle-treated rats, black symbols in B). Over the ensuing 15 weeks the rotational behavior in response to amphetamine was examined. The relative mean $\pm$ SE ratio of ipsiversive-to-contraversive rotations performed over a 60 minute interval, beginning immediately following amphetamine injection, is plotted over time for vehicle-treated (black bars) and 1-EBIO-treated (red bars) rats. 6-OHDA resulted in progressive increase in the ratio of ipsiversive:contraversive rotations from weeks 1-5, although this was not evident in the vehicle-treated group and was not statistically significant in either group. At week 6, 200μM 1-EBIO (or vehicle) infusion directly into the right SNc commenced. There was a non-significant trend for 1-EBIO to improve the rotational bias at 9 & 11 weeks, but not at 13 & 15 weeks. There were no effects of treatment or time over the course of this experiment (two-way ANOVA). (B) At the end of week 15, there was no difference in the number of TH+ cells in the 6-OHDA-lesioned SNc between vehicle- (black symbols) and 1-EBIO-infused (red symbols) rats ($p=0.672$, t-test). See figure 1 legend for details about how these data are represented.

akinesia (and possibly left side attention deficit), evidenced by increases in right:left forelimb use in figure 4C and right:left feeding in figure 4D. There was a degree of normalization of these asymmetries following treatment onset (week 12), however there were no differences in the extent of these normalizations in quinpirole- versus vehicle-infused rats (figure 4C-D).

At the experiment end-point the number of SNc TH+ cells was not different between quinpirole- and vehicle-infused rats (figure 4E).

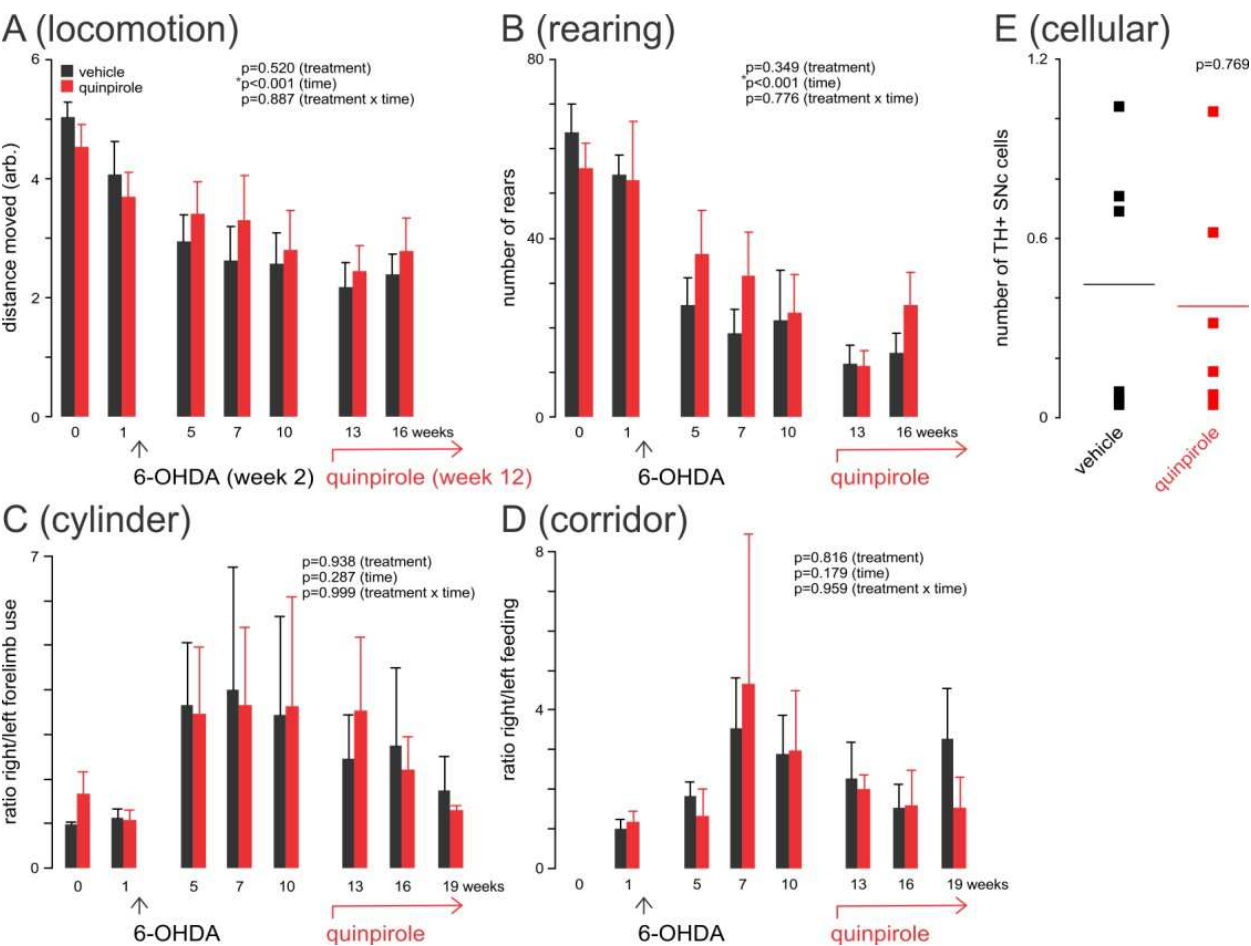


Fig. 4. Effects of the D2 DA receptor agonist quinpirole on motor behavior and the number of TH+ SNc cells in 6-OHDA-lesioned adult rats. (A-D) 6-OHDA was injected into the right SNc of 8-week old male Sprague-Dawley rats to reduce the number of SNc TH+ cells to ~50% of normal (see vehicle-treated rats, black symbols in E). Over the ensuing 19 weeks locomotion (A), rearing (B), right/left forelimb use in the cylinder test (C) and right/left feeding in the corridor test (D) were examined. At week 12, 100nM quinpirole (or vehicle) infusion directly into the right dorsal striatum commenced. The mean $\pm$ SE of each of the behavioral measures is plotted over time and treatment (vehicle = black, quinpirole = red). 6-OHDA resulted in an increased ratio of right/left forelimb use in the cylinder test (C) and of right/left side feeding in the corridor test (D). There were no effects on locomotion (A) and rearing (B) beyond the expected habituation over time. Quinpirole had no effect over vehicle on any of the behaviors examined (two-way ANOVAs). (E) At the end of week 19, there was no difference in the number of TH+ cells in the 6-OHDA-lesioned SNc between vehicle- (black symbols) and 1-EBIO-infused (red symbols) rats ($p=0.769$, t-test). See figure 1 legend for details about how these data are represented.

4. Discussion

These studies were carried out because administration of 1-EBIO, riluzole and quinpirole to normal mice leads to a robust, rapid and reproducible increase in DA cells in the SNc

(Aumann et al. 2011, Aumann et al. 2008). However, these agents failed to alleviate motor deficits and failed to consistently recruit “new” TH+ SNc cells in mice or rats with prior 6-OHDA-induced depletion of SNc DA neurons. The following discussion focuses on caveats of this conclusion, and on what the present experimental outcomes might reveal about the underlying biology. We conclude that these agents warrant further experimentation, perhaps using animal models that more faithfully recapitulate slow nigrostriatal degeneration in PD.

The first caveat is technical and around drug delivery. Given the credible increase in number of SNc TH+ cells brought about by 2-weeks 1-EBIO infusion in some 6-OHDA-lesioned mice (figure 1), we opted for a longer infusion in the rat experiments, expecting cell recruitment would improve. However, the opposite was true. After 9-weeks 1-EBIO infusion in 6-OHDA-lesioned rats, there was no difference in behavior (week 15 in figure 3A) or in the number of SNc TH+ cells (figure 3B), compared with vehicle infusion. However, there was evidence of some behavioral improvement, and presumably nigrostriatal cell recovery, earlier in the course of the 1-EBIO infusion (weeks 9 & 11 in figure 3A). Why then did this potential improvement lapse at weeks 13 & 15? Degradation of the drug is not the explanation because a new pump with a fresh supply of 1-EBIO was introduced at week 12. However, it could have been due to inadvertent and premature termination of drug delivery around week 12. At the experimental endpoint (week 15) it was noted that the majority of pumps were disconnected from their cannula; this was not the case when the pumps were replaced (week 12). Unfortunately we cannot know precisely when this detachment, and therefore cessation of drug infusion, occurred. However, if it was around week 12, it is possible 1-EBIO was having a beneficial effect. Therefore we believe the experiment should be repeated. If future experiments reveal 1-EBIO does facilitate recruitment of new SNc DA neurons and improves motor symptoms in 6-OHDA-lesioned rodents, the present data indicate that these benefits are acutely dependent on the presence of the drug, and therefore continuous drug delivery will be necessary to maintain them.

A second caveat relates to lesion size. In the mouse 1-EBIO experiments (figure 1), lesions were similar in size, evidenced by the relatively tight cluster of data from vehicle-treated mice (black symbols, figure 1). Therefore it is likely that: (1) lesions in the animals receiving 1-EBIO infusion were also of similar size; and (2) 1-EBIO treatment is recruiting new SNc DA neurons in some 6-OHDA lesioned mice. We propose that responsiveness to 1-EBIO may be determined by lesion size, with large 6-OHDA lesions being relatively unresponsive compared to smaller lesions. Our reasoning for this is discussed later. Suffice to point out here that in the 1-EBIO rat experiment (figure 3) [and in the quinpirole rat experiment (figure 4)], lesion size was much more variable than in the 1-EBIO mouse experiments. Moreover, the size of the lesions was $\geq$ the upper range of mouse lesions [i.e. $<40\%$ of TH+ cells remaining, figure 3B (and 4E)] in a significant proportion of rats. Therefore it may be that many of the rats in these experiments were unable to respond to 1-EBIO (or quinpirole) because their lesions were too big (and possibly also too rapid, see discussion below).

A less likely caveat in our opinion is that rats are less able to respond to 1-EBIO or striatal quinpirole than mice. The ability of the rodent SNc to spontaneously compensate following 6-OHDA insult, and in different ways (e.g. recruitment of TH+ SNc cells or sprouting of surviving nigrostriatal axons), is no different in mice versus rats in our experience.

The caveats discussed above are tantalizing and the importance of a positive outcome justifies further experiments that attend to: (1) ensuring drug delivery remains patent; (2) whether continual drug delivery is necessary to maintain the recruited population of DA neurons; (3) whether lesion size (and rate) are confounding factors in the responsiveness to treatment; and (4) whether there are species differences.

We will now turn to discussion of what the present results might reveal about the underlying biology. There is an extensive literature showing that expression of TH in cells is regulated by changes in membrane potential (neuronal activity) and intracellular Ca^{2+} . We recently reported that this is true also in the SNc of normal (i.e. unlesioned) adult mice (Aumann et al. 2011, Aumann et al. 2008). When drugs targeting the activity of SNc neurons are infused into the brain for 2 weeks, both the amount of TH protein/SNc cell and the number of SNc TH+ cells are altered (Aumann et al. 2011, Aumann et al. 2008). Specifically, our data show that when the number of TH+ cells increases, the TH immunoreactivity of each cell decreases, and vice versa. Also, when the number of TH+ cells increases, the number of SNc cells that are not TH immunoreactive (TH-) decreases by the same amount, and vice versa [i.e. the net number of SNc cells (TH+ & TH- combined) does not change]. From a broad perspective these data indicate that TH expression in adult SNc neurons is activity-dependent. More closely they imply a homeostatic mechanism(s) regulating DA neurotransmission in the striatum, but acting at the levels of TH expression and DA phenotype recruitment/loss in SNc. We propose that this homeostatic mechanism(s) operates at two levels. At the level of an individual SNc cell, TH expression is activity- and Ca^{2+} -dependent and is brought about by altered Ca^{2+} -dependent DA gene expression. At the level of nigrostriatal circuitry, striatal DA receptor signaling and feedback circuitry from the striatum to the SNc operates to control the number of SNc DA cells.

The rapid (within 2 weeks) recruitment/loss of the DA phenotype by SNc cells in normal mice (Aumann et al. 2011, Aumann et al. 2008) implies the existence of a population of relatively mature (but not DA) neurons located in and around SNc that can be recruited into and out of the DA population. In other (unpublished) studies we have evidence that significant numbers of new neurons (NeuN+) are generated in the adult mouse midbrain [from Nestin+ (but BrdU-) neural precursor cells], many (~420 over an 8 week period) of which end up in SNc, see also (Shan *et al.* 2006). A very small number of these new midbrain neurons express TH, i.e. demonstrate a capacity to acquire the DA phenotype, but so far these have been observed around the ventral midline and in the ventral tegmental area (VTA), not in SNc. It could be that these newborn NeuN+ but TH- SNc neurons are the same cells in which the DA phenotype can be recruited should appropriate signals arrive. We believe that one of these signals is a change in their electrical activity, such as occurs when 1-EBIO is infused into the midbrain or quinpirole is infused into the striatum.

In this context, it is relevant to consider what might have happened to this putative DA phenotype recruitment when confronted with rapid depletion of SNc DA neurons following direct 6-OHDA injection. Evidence from our laboratory and others (Sauer & Oertel 1994) shows that following 6-OHDA, and in the absence of any further treatment or manipulation, the number of SNc TH+ cells spontaneously recovers toward normal, while at the same time the number of SNc TH- cells declines (Stanic et al. 2003). This suggests DA phenotype

recruitment, possibly as part of a process that has directed the homeostasis described above toward repair. The failure of our attempts to facilitate this recruitment using 1-EBIO or quinpirole in the present study might be due to depletion of the population of recruitable cells by this spontaneous recovery. It also indicates the rate of replenishment of the recruitable population (i.e. neurogenesis) is not very high, since not enough were generated within the timeframe of our experiments (4-17 weeks) to significantly impact the TH+ population. This would fit with the consensus in the literature that the rate of adult midbrain neurogenesis is not very high (Shan et al. 2006, Zhao *et al.* 2003) or zero (Aponso *et al.* 2008, Chen *et al.* 2005, Cooper & Isacson 2004, Frielingsdorf *et al.* 2004, Lie *et al.* 2002, Peng *et al.* 2008, Yoshimi *et al.* 2005). This point brings us to another caveat of the present experiments, which is the very rapid depletion of SNc DA neurons by 6-OHDA injection may not be the best PD model in which to study activity-dependent DA phenotype recruitment. Perhaps a model in which the rate of degeneration is much slower, which better mimics the situation in PD, would provide enough time for the recruitable population of cells to be sustained.

5. Conclusion

In summary, our working model is: (1) The nigrostriatal DA pathway is under homeostatic control to maintain a constant level of striatal DA signaling; (2) This is achieved at the level of individual SNc cells via activity- and Ca^{2+} -dependent alterations in DA (TH) gene expression; (3) It is also achieved at the level of D2 DA receptor-mediated striatonigral feedback circuitry (the indirect pathway) leading to changes in the number of SNc DA cells; (4) A population of relatively mature NeuN+ but TH- cells located in and immediately surrounding SNc is available to be rapidly recruited into and out of the DA population; (5) This recruitable population of cells can be rapidly depleted (i.e. recruited into the DA population) in response to perturbations of the system (e.g. altered SNc neuronal activity, nigrostriatal DA signaling, or 6-OHDA), but is only slowly replenished by neurogenesis. The implication of this homeostasis for nigrostriatal DA cell-replacement therapies is that any manipulation designed to increase the number of SNc DA cells is likely to be offset by a homeostatic response. The effects of infusing D2 agonists into the striatum suggest that striatal DA signaling may be a factor. Thus in PD or its models the effect of homeostatic control may be difficult to predict. On the other hand, pharmaceuticals that increase SNc DA cells in normal mice fail to do so in 6-OHDA-lesioned rodents, possibly because the population of recruitable cells is also extensively depleted. Thus, researchers looking to help develop cell-replacement therapies to treat the motor symptoms of PD should consider the effects their interventions might have on nigrostriatal DA homeostasis because: (1) homeostatic responses may be confounding interpretation of the effects of their interventions; and (2) homeostatic responses may need to be addressed also, as part of an overall strategy to increase cell-based nigrostriatal DA transmission.

Future work should therefore aim to better understand these homeostatic responses by: (1) identifying downstream signaling pathways mediating activity- and Ca^{2+} -dependent changes in DA gene expression; (2) identifying mechanisms of SNc DA phenotype recruitment; (3) identifying the phenotype of recruitable cells; (4) characterizing the ontogenesis of newborn SNc neurons; and (5) investigating mechanisms regulating the rate

of SNc neurogenesis. Progress in these areas promises to be vital for better treating the motor symptoms of PD, but will also be relevant for other disorders involving dysfunctional midbrain DA signaling (e.g. attention deficit hyperactivity disorder, schizophrenia, drug addiction), and for adult neurogenesis and brain plasticity more generally.

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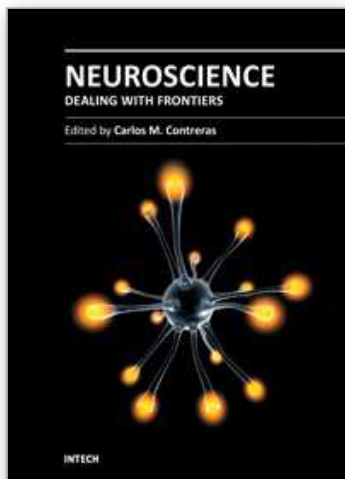
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The Neuronal Doctrine recently reached its 100th year and together with the development of psychopharmacology by the middle of 20th century promoted spectacular developments in the knowledge of the biological bases of behavior. The overwhelming amount of data accumulated, forced the division of neuroscience into several subdisciplines, but this division needs to dissolve in the 21st century and focus on specific processes that involve diverse methodological and theoretical approaches. The chapters contained in this book illustrate that neuroscience converges in the search for sound answers to several questions, including the pathways followed by cells, how individuals communicate with each other, inflammation, learning and memory, the development of drug dependence, and approaches to explaining the processes that underlie two highly incapacitating chronic degenerative illnesses.

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Phone: +86-21-62489820
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