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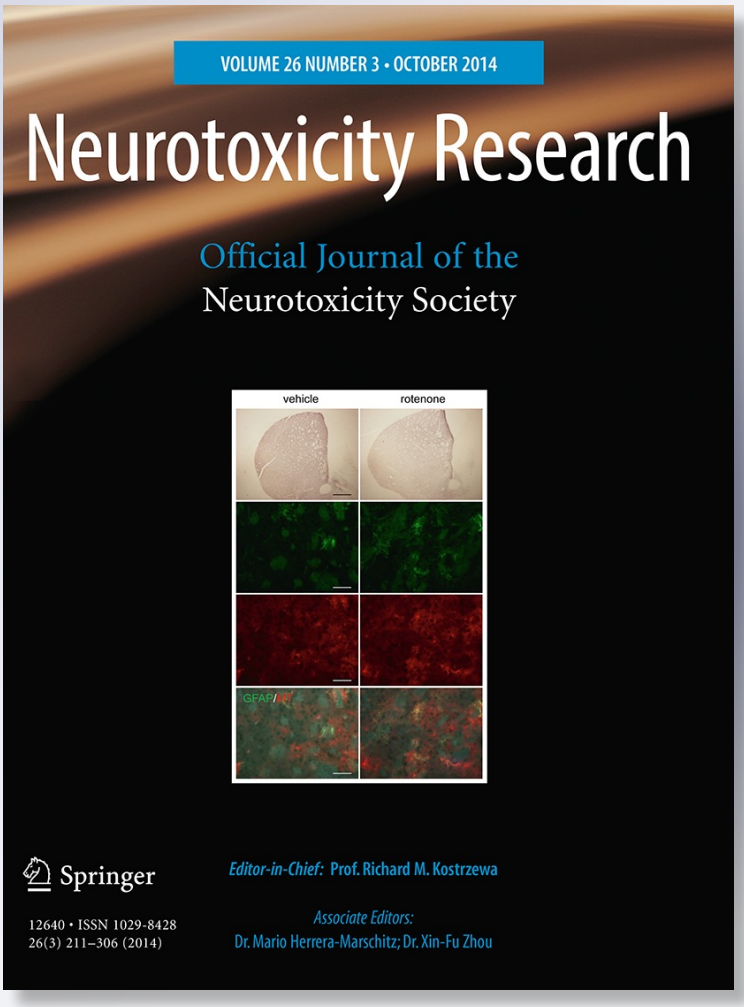

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ORIGINAL ARTICLE

Intrastriatal 6-OHDA Lesion Differentially Affects Dopaminergic Neurons in the Ventral Tegmental Area of Prenatally Stressed Rats

Carlos J. Baier · María Eugenia Pallarés · Ezequiela Adrover · María R. Katunar · Rita Raisman-Vozari · Marta C. Antonelli

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Abstract Exposure to a variety of stressful events during the last week of pregnancy in rats interferes with the correct progeny development, which in turn leads to delays in motor development, impaired adaptation to stressful conditions, altered sexual behaviour, learning deficits, neuronal development and brain morphology. Many of these alterations have been attributed to changes in dopamine (DA) neurotransmission and occur primarily in the mesolimbic system. We found that prenatally stressed offspring showed higher levels of cells expressing tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and that these cells were more susceptible to a neurochemical insult with 6-hydroxy-DA (6-OHDA) in adulthood. Moreover, prenatally stressed rats presented differences in terms of the number and asymmetry of neuronal nitric oxide synthase-expressing cells in the VTA and nucleus accumbens, respectively. Similar to the results described for TH-expressing cells, the nitrergic systems were differentially regulated after 6-OHDA lesion in control and prenatally stressed rats. These results indicated that prenatal stress affects the dopaminergic and nitrergic systems in the mesolimbic pathway. In addition, we propose that the mesolimbic areas are more susceptible than the motor areas to a neurochemical insult during adult life.

Keywords Restraint stress · Limbic areas · Neurochemical insult · Dopamine · Nitric oxide

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Introduction

The midbrain dopaminergic system regulates diverse behavioural and cognitive functions which are critical for the integration of mammalian responses and adaptations to the environment. In humans, dysfunction of the dopaminergic system is associated with the development of several neurological disorders including Parkinson disease (PD), schizophrenia, attention-deficit hyperactivity disorder and depression. It has been demonstrated that prenatal stress (PS) exerts a significant impact on foetal brain development in experimental animal models (Weinstock 2001). Indeed, the last few years have seen an increase in evidence suggesting that exposure to a variety of stressful events during the last week of pregnancy in rats interferes with the correct progeny development, thus leading to delays in motor development, impaired adaptation to stressful

conditions, altered sexual behaviour, and learning deficits (Weinstock 2001, 2008; Huizink et al. 2004; Darnaudery and Maccari 2008). In addition, the offspring display anomalies in neuronal development and brain morphology, as well as changes in cerebral asymmetry which persist into adulthood (Fride and Weinstock 1989). Evidence provided by animal research, as well as retrospective studies in humans, have indicated that exposure to adverse events in early life can alter adult behaviour and neurochemical indicators of midbrain dopamine (DA) activity, suggesting that the development of the DA system is sensitive to disruption if exposed to early stressors (Baier et al. 2012).

Local injections of 6-hydroxy-DA (6-OHDA) represent the traditional method used when it comes to producing neurodegeneration of the DA system. This molecule is transported into the cell bodies and fibres of both dopaminergic and noradrenergic neurons. The intrastriatal injection of 6-OHDA produces a selective and severe degeneration of DA nerve terminals in the striatum as well as a loss of DA cell bodies in the substantia nigra (SN) and to a lesser extent in the ventral tegmental area (VTA; Przedborski et al. 1995; Deumens et al. 2002; Debeir et al. 2005).

The pathogenic mechanism which leads to neuronal loss and neurodegeneration still remains elusive. However, among another candidates, recent data have identified nitric oxide (NO)-derived reactive nitrogen intermediates as critical contributors to protein modification and cell injury (Eve et al. 1998; Ischiropoulos and Beckman 2003). In particular, Kiss et al. (1999) showed that endogenously produced NO may influence the activity of the DA transporter (DAT), the effect of which may have special importance in the regulation of extracellular DA transmitter concentration in the striatum. In turn, basal ganglia NO systems can be selectively regulated in response to changes in dopaminergic input (Eve et al. 1998).

The free radical gas NO, is a highly reactive and short-lived liposoluble molecule which is generated from the amino acid L-arginine by a family of enzymes called NO synthases (NOSs). In the central nervous system, NO is synthesised by neuronal NOS (nNOS) and was originally identified as a neurotransmitter (Bredt and Snyder 1992). Interestingly, NO can lead to neuronal cell death when produced in excess (Dawson et al. 1991; Zhang et al. 1994) or can represent an anti-apoptotic factor (Estevez et al. 1998; Kim et al. 1999; Ha et al. 2003). In reference to NO and 6-OHDA lesion, Barthwal et al. (2001) described the role of NO in the neurotoxic damage induced by 6-OHDA, whilst Singh et al. (2010) demonstrated that NO participates in the neuronal death caused by 6-OHDA. Similarly, Gomes et al. (2008) and Yuste et al. (2012) found that NO inhibitors attenuate the 6-OHDA damage in the nigrostriatal pathway. Treatment of C6 cells with 6-OHDA increased the expression of inducible NOS and the subsequent production of NO (Lee et al. 2011).

The aim of the present study was to investigate whether PS affects the vulnerability of the nigrostriatal dopaminergic neurons to degenerate after an intrastriatal 6-OHDA injection in adult life. The extent of the dopaminergic damage in the nigrostriatal system measured by tyrosine hydroxylase (TH) immunohistochemistry (IHC) was similar in control and prenatally stressed offspring. Unexpectedly, we found a decrease of TH immunoreactivity in the VTA of prenatally stressed offspring, thus prompting us to explore the characteristics of the lesion in this area. Since it has been reported that nNOS participates in the 6-OHDA lesion, we analysed whether or not the neurotoxic insult differentially affects this system in prenatally stressed animals. The results are discussed in the context of the relationship between NO and DA and their vulnerability following exposure to a prenatal insult.

Materials and Methods

Animals

Virgin females Wistar rats weighing 250 g were obtained from an outbred colony of rats from the animal facility at the Facultad de Farmacia y Bioquímica (University of Buenos Aires). Vaginal smears were collected daily for 8 days before mating to determinate the stage of the oestrus cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats weighing 250–300 g were introduced for mating. Vaginal smears were taken on the following morning. The day on which spermatozoa were found in the smear was designated day 1 of pregnancy. A constant light/dark cycle (on at 06:00 h, off at 18:00 h) and the temperature of 21–25 °C were maintained. All procedures were in agreement with the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (Facultad de Medicina, Universidad de Buenos Aires, Argentina). Care was taken to minimise the number of animals used.

Prenatal Procedures

Pregnant dams were randomly assigned to either the control or the PS group and were individually housed with ad libitum access to standard rat chow and water. Control rats were left undisturbed in the home cage, while PS dams were subjected to a restraint stress procedure, which involved rats being transferred to an experimental room where the stressor was applied. Pregnant females were

individually placed into a transparent plastic restrainer fitted closely to body size for three 45 min periods per day (9:00, 12:00 and 16:00 h) between days 14 and 21 of the pregnancy. The restrainer was fitted with ventilation holes, whilst the dimensions needed for a pregnant rat of 350 g involved an internal diameter of 64 mm with an adjustable length of 149–208 mm. This type of stress was chosen because it has an indirect influence on the foetuses via a direct stress on the mother (Ward and Weisz 1984; Maccari et al. 1995). The sessions were performed in a lit environment. No other subjects were present in the experimental room during the stress exposure. At the end of the stress session, the rats were returned to the animal housing room and were then individually housed with free access to food and water. On the day of parturition, litter characteristics were recorded and culled to 10 pups, maintaining similar numbers of males and females wherever possible. The male and female offspring were housed in separate cages, with no more than five pups per cage, and with standard rat chow and water ad libitum. Only male offspring were used in this study, whilst in order to prevent litter effects, a maximum of one–two pups from each litter were tested in each experiment.

6-OHDA Lesion Procedure

The procedure was performed following the protocol described in Debeir et al. (2005). Briefly, control and prenatal stressed males at postnatal day (PND) 75 of age (average weight $\sim$ 320 g) were injected with desipramine (25 mg/kg i.p.) 1 h prior to surgery so as to protect noradrenergic pathways against 6-OHDA neurotoxicity. Fifty minutes later, animals were anesthetised with a Ketamine/Xylazine mixture (50 and 8 mg/kg, respectively) and placed in a small animal stereotaxic apparatus. Following incision of the scalp, a small drill hole was made in the skull and a pulled-glass injection cannula was slowly inserted into the right striatum at the following stereotaxic coordinates: 0.5 mm anterior and 3.5 mm lateral to bregma, −5.0 mm ventral from dura mater. Following this, 6-OHDA hydrobromide (8.75-µg of 6-OHDA in 2-µL of 0.9 % NaCl containing 0.02 % ascorbic acid) was then injected (Debeir et al. 2005). The cannula was left in place for 5 min at the end of the injection to prevent reflux and to allow for toxin diffusion. Control and prenatal stressed animals which received an ipsilateral intrastriatal injection of the vehicle alone (2 µL of 0.9 % NaCl containing 0.02 % ascorbic acid) served as the sham groups. The scalp wound was then sutured, and the animals were housed singly until sacrifice 28 days later. We used $n = 4$–5 rats per condition. As a result, four experimental groups were obtained: (1) control–sham (control rats injected with vehicle), (2) PS–sham (prenatally stressed rats injected with vehicle), (3) control–6-OHDA (control rats injected with 6-OHDA) and (4) PS–6-OHDA (prenatally stressed rats injected with 6-OHDA).

Tissue Preparation

Twenty-eight days following the intrastriatal injection, rats were deeply anesthetised with a Ketamine/Xylazine mixture and perfused through the cardiac left ventricle, initially with 50 mL of cold physiological saline (0.9 % NaCl solution containing 0.05 % w/v $NaNO_2$ plus 50 IU of heparin), followed by a perfusion with 500 mL of fixative (4 % paraformaldehyde in 100 mM phosphate buffer, PBS, pH 7.4). The brains were then removed, post-fixed for 2 h in 4 % paraformaldehyde, cryoprotected by subsequent overnight immersion in 15 and 30 % sucrose, and finally stored at −80 °C until processing for IHC. Twenty series of 25-µm-thick coronal sections for the striatum and 12 for the midbrain were cut on a cryostat. Slices were stored at −20 °C in PBS pH 7.4, with 50 % w/v glycerol added as a cryoprotector until their use in immunohistochemical studies.

Immunohistochemistry

Immunohistochemical reactions were performed on 25-µm-thick free-floating sections encompassing the entire striatum or midbrain. To inhibit endogenous peroxidase activity, tissue sections were treated with 0.3 % v/v H_2O_2 in PBS containing 0.15 % Triton X-100 (PBST) for 30 min at room temperature. Following this, and in order to block non-specific binding sites, brain sections were incubated for 1 h with 5 % v/v normal goat serum in PBST. Sections were incubated overnight at 4 °C with primary antibodies to TH (1:500; US Biological, MA, USA) and nNOS (1/2,000 sc-648, Santa Cruz Biotechnology, CA, USA). After five rinses in PBST, sections were incubated for 1 h at room temperature with biotinylated secondary antibodies diluted 1:200. After further washing in PBST, sections were incubated for 90 min with streptavidin–peroxidase complex (1:200; Sigma, St. Louis, MO, USA). Sections were then washed three times in PBST and twice in 0.1 M acetate buffer, pH 6 (AcB), whilst development of peroxidase activity was carried out with 0.035 % w/v 3,30-diaminobenzidine hydrochloride (DAB, Sigma) plus 2 % w/v nickel ammonium sulphate and 0.1 % v/v H_2O_2 dissolved in AcB. Alternatively, after the incubation with the biotinylated secondary antibody, samples were incubated with avidin–biotinylated horseradish peroxidase complex (Vectastain, ELITE ABC kit, Vector Laboratories, Burlingame, CA, USA). Finally, the substrate H_2O_2 (0.015 %) was added in the presence of 0.05 % DAB in order to visualise the antigen–antibody complex. Following the enzymatic

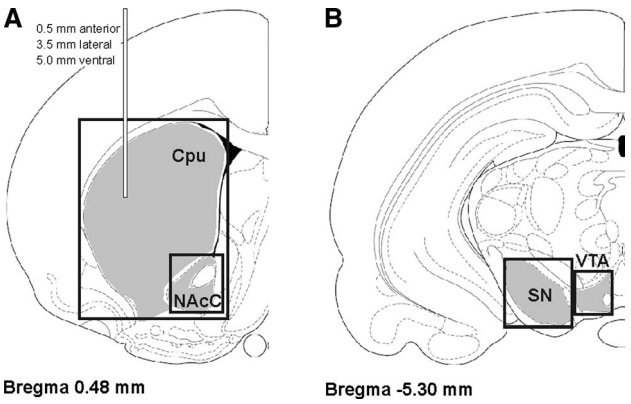

Fig. 1 Representatives coronal sections used for densitometry or cell count analysis of rat brain regions. Samples include 25 µm from **a** +2.52 to −1.56 mm AP, **b** −5.28 to −6.24 mm AP relative to bregma, according to Paxinos and Watson (1986). The *bar* in **a** indicate the location of the cannulae for 6-OHDA injections and the stereotaxic coordinates: 0.5 mm anterior and 3.5 mm lateral to bregma, −5.0 mm ventral from dura mater. *CPu* caudate putamen, *NAcC* nucleus accumbens core, *SN* substantia nigra, *VTA* ventral tegmental area. The areas indicated in *grey* were used for the analysis

reaction step, sections were washed five times in AcB. Finally, sections were mounted on gelatine-coated slides before being air dried and coverslipped using Permount for light microscope observation.

Image Analysis

In order to ensure objectivity, all measurements were performed on coded slides, under blind conditions, carrying out the measurements of immunolabelled brain sections of both controls and prenatally stressed rats with the same standardised observation schedule. A single series of sections from each animal of each group was used for quantification. The optical densities of the TH+ fibres in the striatum were measured using a computerised image analysis system (MCID, Imaging Research, St. Catharines, Ontario, Canada) reading optical density as grey levels. For each brain, the optical density was measured at eight different rostrocaudal levels along the extent of the striatum, from +2.52 to −1.56 mm AP relative to bregma (Paxinos and Watson 1986) (Fig. 1). For each brain level, nonspecific background densities were measured in a region devoid of TH-immunostaining, such as the corpus callosum, and were subtracted from the striatal optical density values. Optical density values for the striatum on the ipsilateral side of lesioned and unlesioned animals were expressed as a percentage of their nonlesioned contralateral side. Stained neurons, TH+ and nNOS+, were counted using a computerised image analysis system. Images were captured from slices using a Zeiss microscope and CCD camera, together with Image Pro-Plus software 9.0 (Media Cybernetics, Inc., Bethesda, MD, USA) or Nikon Eclipse E-600 using a K2E Apogee CCD camera driven by CCDOPS software (Santa Barbara Instrument Group, Santa Barbara, CA, USA). In order to count TH and nNOS-immunoreactive neurons in all of the SN and VTA, sections encompassing rostral, middle, and caudal levels of the whole SN and whole VTA from its rostral to caudal extent were examined. These range from −5.28 to −6.24 mm AP relative to bregma (Paxinos and Watson 1986). For the analysis, we delimited the area according to Paxinos and Watson (1986) (Fig. 1) in each section, whilst the number of neurons obtained was normalised to a fixed size/area (0.5 mm^2).

In order to count nNOS-immunoreactive neurons in the striatum and nucleus accumbens (NAc), we analysed different rostrocaudal levels along the extent of the striatum (from +2.52 to −1.56 mm AP relative to bregma Paxinos and Watson 1986) and the NAc (from +2.76 to +0.76 mm AP relative to bregma Paxinos and Watson 1986), respectively (Fig. 1). For the analysis, the area was delimited according to Paxinos and Watson (1986) in each section (Fig. 1), whilst the number of neurons obtained was normalised to a fixed size/area (0.5 mm^2). The average section thickness was 25 µm. Since measurements were made on every 12th or 20th serial section (i.e. separated by 300–500 µm), it was unlikely that a neuron would be analysed twice.

Statistical Analysis

TH+ and nNOS+ cells counts results were analysed using three way-ANOVA in order to evaluate the differences between prenatal treatment (C, control, or PS), postnatal treatment (sham or 6-OHDA administration), hemisphere (ipsilateral or contralateral) and possible interactions between the three factors. When interactions were found, simple effects two-way or one-way ANOVA analyses were conducted, followed by Tukey post hoc test, for multiple comparison. Visual inspection of histograms, *qq* plots and random distribution of fitted values were checked. All results are presented as mean ± SEM. The observed differences were considered to be statistically significant when $p < 0.05$. n values reported in the figures represent number of litters. Analysis of data was performed using SPSS 13.0 version and Infostat 2013. Optical density measurements at seven different anatomical levels in sham and lesioned animals were compared as above.

Results

Effect of Intrastriatal 6-OHDA Injection on TH-Immunoreactivity in the Striatum of Control and Prenatally Stressed Rats

In order to evaluate whether the nigrostriatal system of the adult prenatally stressed offspring has a differential

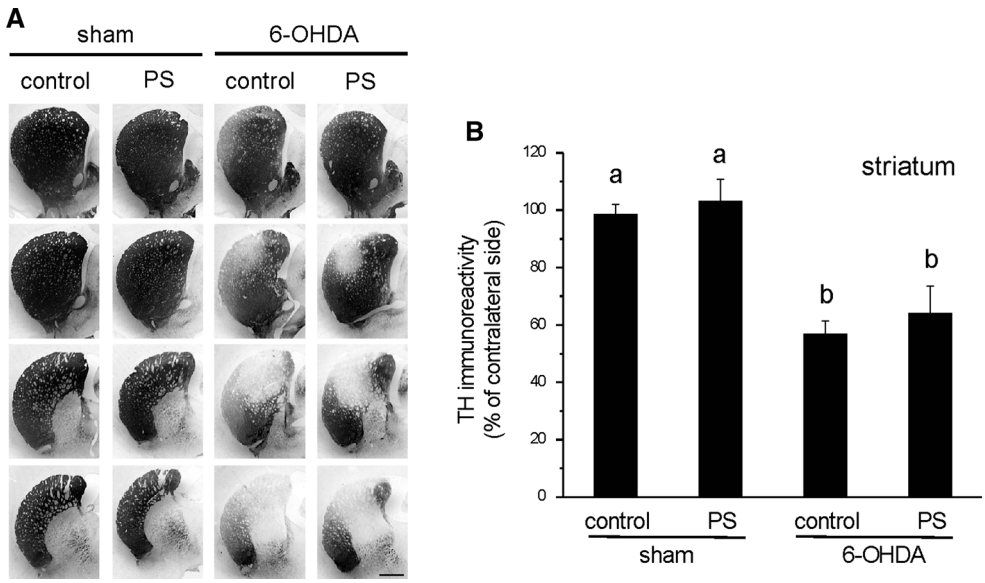

Fig. 2 Effect of intrastriatal 6-OHDA injection on striatal TH+ fibres. **a** Photomicrographs show four sections at equally spaced rostrocaudal ipsilateral levels of the sham (control and PS) and 6-OHDA injected animals (control and PS). *Scale bar* 1 mm. **b** Quantification of striatal TH immunoreactivity by optical density measurements in the whole striatum. Values represent the percentage (mean ± SEM) of the optical density of the contralateral (intact) striatum; $n = 4$–5 litters per condition. Two-way ANOVA followed by main effects analyses: $F(1,16) = 33.54$, $p < 0.0001$ for sham versus 6-OHDA factors. Different *letters* depicts significant differences between groups with $p < 0.0001$

susceptibility to a neurochemical insult we injected 6-OHDA into the striatum of control and prenatally stressed males at PND 75. PS did not interfere with the length of gestation, number, ratio, weight of the pups at birth as well as body gain weight of offspring (Berger et al. 2002; Barros et al. 2004; Pallares et al. 2013). The experimental groups were designated as follows: (1) control–sham (control rats injected with vehicle), (2) PS–sham (prenatally stressed rats injected with vehicle), (3) control–6-OHDA (control rats injected with 6-OHDA) and (4) PS–6-OHDA (prenatally stressed rats injected with 6-OHDA). The extent of DA denervation at 28 days after the unilateral injection of 6-OHDA into the dorsal part of the striatum was analysed by IHC in serial coronal sections throughout the rostrocaudal extent of the structure. Representative images from four different levels of the ipsilateral side of sham and lesioned animals are illustrated in Fig. 2a. The 6-OHDA lesion affected the dorsolateral part of the striatum (Fig. 2a, b). Quantitative analysis revealed a mean TH-immunoreactivity of 56.8 (±4.7) % and 64.0 (±9.5), in control- and PS–6-OHDA rats, respectively, in the ipsilateral lesioned striatum with respect to contralateral striatum, compared with its corresponding sham group (Fig. 2b). We did not found an interaction between prenatal and postnatal factors. As shown in Fig. 2b, TH immunoreactivity levels were not affected by prenatal treatment but decreased after 6-OHDA lesion in both groups [$F(1,16) = 33.54$, $p < 0.0001$ for sham vs. 6-OHDA factors], i.e. PS did not differentially affect the degree of the 6-OHDA lesion in the striatum of prenatally stressed rats.

Evaluation of the Relative Number of TH+ Neurons in the SN and VTA in Control and Prenatally Stressed Rats

TH+ cell loss in whole SN from control and prenatally stressed animals was assessed as described in "Materials and Methods" section. No statistical interaction between pre- and postnatal treatments (PS and 6-OHDA lesion, respectively) weas found, but there is an effect as a consequence of 6-OHDA lesion [$F(1,26) = 11.40$, $p = 0.0023$ for postnatal treatment factor effect]. No effects between hemispheres were found in sham animals (Fig. 3b). After 6-OHDA treatment, as expected, there were a statistically significant differences between hemispheres in 6-OHDA treated rats [$F(1,26) = 35.38$, $p < 0.001$ for ipsi- vs. contralateral hemispheres in 6-OHDA group]. Quantitatively, a mean loss of 48 % (TH+ cells/0.5 mm^2 control–6-OHDA ipsi-/contralateral 65 ± 5/124 ± 5) and of TH 36 % (TH+ cells/0.5 mm^2 PS–6-OHDA ipsi-/contralateral 78 ± 11/122 ± 4) were obtained in controls- and PS–6-OHDA rats, respectively, in the whole ipsilateral side of SN respect to contralateral SN in the same animal (Fig. 3b). Interestingly, we found an effect of treatment in the contralateral hemisphere as a consequence of the 6-OHDA treatment

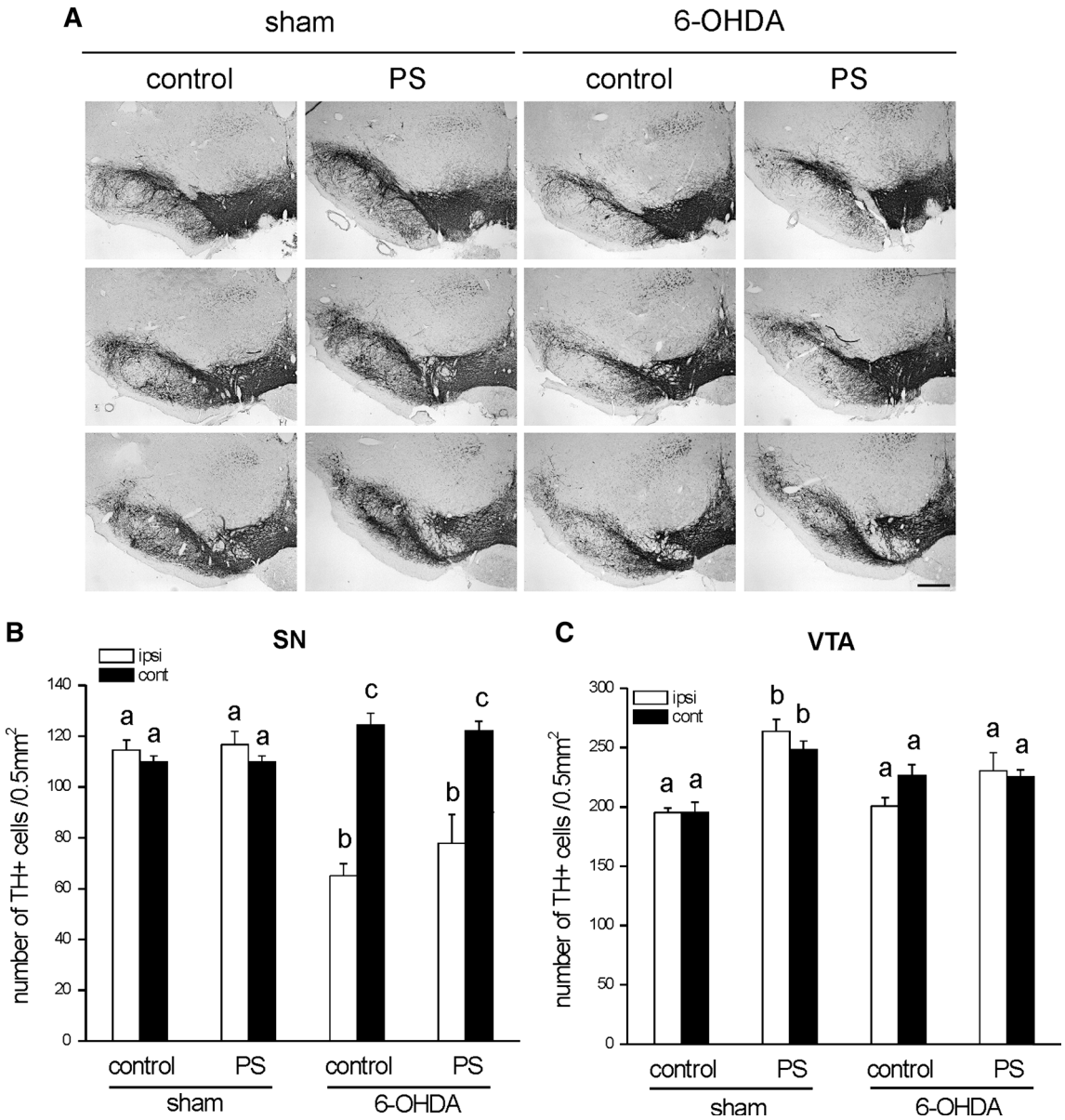

Fig. 3 Effect of intrastriatal 6-OHDA injection on TH+ cells and fibres in the ventral mesencephalon. **a** Photomicrographs show the ipsilateral side at three different levels of the ventral mesencephalon in sham and 6-OHDA-lesioned rats. *Scale bar* 500 μm. **b** Quantification of TH+ neurons of the entire SN. Three-way ANOVA followed by main effects analyses: $F(1,26) = 11.40$, $p = 0.0023$ for postnatal treatment factor effect; interaction between side and postnatal treatment was statistically significant [$F(1,26) = 40.18$, $p < 0.001$ for postnatal treatment vs. hemisphere factors], $F(1,26) = 35.38$, $p < 0.001$ for left versus right hemispheres in 6-OHDA group, $F(1,26) = 4.53$, $p < 0.05$ sham versus 6-OHDA in left hemispheres. **c** Quantification of TH+ neurons of the entire VTA. Three-way ANOVA followed by main effects analyses: interaction between pre- and postnatal treatment [$F(1,43) = 7.79$, $p = 0.0078$ for pre- vs. postnatal treatment factors], $F(1,43) = 22.35$, $p < 0.001$ for control versus PS factors in the sham group, $F(1,43) = 7.778$, $p < 0.01$ for sham versus 6-OHDA factor in the PS group. Values represent (mean ± SEM) TH+ cells/0.5 mm^2; $n = 4$–5 litters per condition. "Ipsi" and "cont" indicate ipsi- and contralateral sides respect to 6-OHDA-injection side, respectively. Different *letters* depicts significant differences between groups

when compared to the sham animals [$F(1,26) = 4.53$, $p < 0.05$ sham vs. 6-OHDA in contralateral hemispheres]. In summary, PS did not affect the extent of the 6-OHDA lesion in the SN.

We also determined the relative TH+ cell number in control and prenatally stressed animals in VTA (Fig. 3a, c). We found that PS–sham offspring showed an increased number of TH+ cells compared with control–sham animals. We found a statistically significant interaction between pre- and postnatal treatment [$F(1,43) = 7.79$, $p = 0.0078$ for pre- vs. postnatal treatment factors]. We did not found an interaction between hemispheres and

postnatal treatment [$F(1,43) = 1.18$, $p > 0.05$]. When we compare the sham animals we found an effect as a consequence of prenatal treatment, i.e., the prenatal stressed rats showed and increased number of TH+ cells in the VTA (TH+ cells/0.5 mm^2 PS–sham ipsi-/contralateral 264 ± 10/248 ± 7) compared with control animals (TH+ cells/0.5 mm^2 control–sham ipsi-/contralateral 196 ± 4/195 ± 9) [$F(1,43) = 22.35$, $p < 0.001$ for control vs. PS factors in the sham group]. Similar to Debeir et al. (2005) an intrastriatal injection of 6-OHDA did not affect the number of TH+ cells in the VTA of the control–6-OHDA group (TH+ cells/0.5 mm^2 control–6-OHDA ipsi-/contralateral 200 ± 7/226 ± 10; Fig. 3c), although 6-OHDA negatively affected the TH+ cell number in prenatally stressed offspring (TH+ cells/0.5 mm^2 PS–6-OHDA ipsi-/contralateral 231 ± 15/225 ± 6), compared with PS–sham group [$F(1,43) = 7.778$, $p < 0.01$ for sham vs. 6-OHDA in the PS group]. In summary, PS increased the population of TH+ cells in the VTA and intrastriatal 6-OHDA lesion reduced this cell population to the levels of the control group (control–sham/6-OHDA).

Evaluation of the Relative Number of nNOS+ Neurons in the Mesolimbic System, in Control and Prenatally Stressed Rats

We analysed the effects of PS on the population of cells which express nNOS in the VTA and the NAc. We found an interaction between prenatal and postnatal treatment [$F(1,16) = 40.08$, $p < 0.001$ for pre- and postnatal factors]. We did not found an interaction between hemispheres and postnatal treatment [$F(1,16) = 0.06$, $p = 0.8033$]. As shown in Fig. 4, the number of nNOS+ cells in VTA increased significantly in PS–sham rats (nNOS+ cells/0.5 mm^2 PS–sham ipsi-/contralateral 170 ± 18/160 ± 5) when compared to the control–sham group (nNOS+ cells/0.5 mm^2 control–sham ipsi-/contralateral 78 ± 1/84 ± 7) [$F(1,16) = 62.06$, $p < 0.001$ for control vs. PS factors in the sham group]. Whilst the control–6-OHDA group showed a slight increase in the number of nNOS+ cells after 6-OHDA treatment (nNOS+ cells/0.5 mm^2 control–6-OHDA ipsi-/contralateral 110 ± 12/111 ± 17) [$F(1,16) = 7.5$, $p < 0.05$ for sham vs. 6-OHDA factor in control group], the PS–6-OHDA group showed the opposite response after 6-OHDA treatment: PS–6-OHDA rats showed a decreased number of nNOS+ cells as a consequence of the intrastriatal 6-OHDA lesion (nNOS+ cells/0.5 mm^2 PS–6-OHDA ipsi-/contralateral 98 ± 7/100 ± 7) [$F(1,16) = 39.175$, $p < 0.001$ for sham vs. 6-OHDA factor in PS group] (Fig. 4b). Following 6-OHDA treatment, control- and PS–6-OHDA rats did not show differences in terms of nNOS+ cell number.

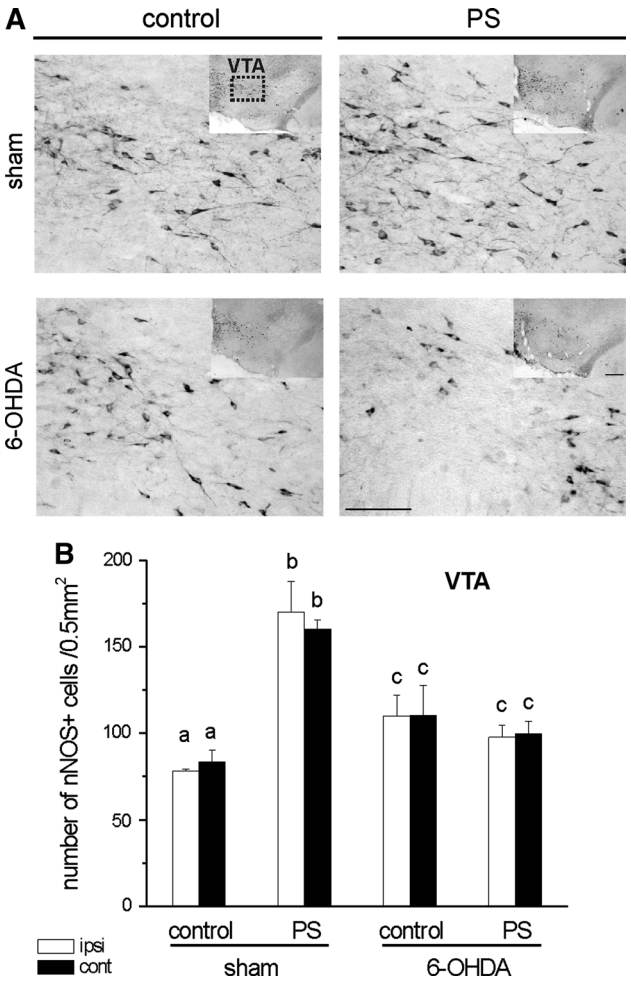

Fig. 4 Relative number of nNOS+ cells in the VTA of *control* and PS rats, sham and treated with 6-OHDA. **a** Representative sections at equally spaced rostrocaudal sides of VTA in control and PS rats, sham or treated with 6-OHDA, respectively. The *inset* corresponds to the same image at low magnification. *Scale bar* 250 μm. **b** Quantification of nNOS+ cells in the VTA of control and PS animals, sham or 6-OHDA lesioned. Three-way ANOVA followed by main effects analyses: interaction between pre- and postnatal treatment [$F(1,16) = 40.08$, $p < 0.001$ for pre- and postnatal factors], $F(1,16) = 62.06$, $p < 0.001$ for control versus PS factor in sham group, [$F(1,16) = 7.5$, $p < 0.05$ for sham vs. 6-OHDA factor in control group], $F(1,16) = 39.175$, $p < 0.001$ for sham vs. 6-OHDA factor in PS group. Values represent (mean ± SEM) nNOS+ cells/0.5 mm^2; $n = 3$ litters per condition. "Ipsi" and "cont" in *bar* graph indicate ipsi- and contralateral sides respect to 6-OHDA-injection side, respectively. Different *letters* depicts significant differences between groups

As shown in Fig. 5, PS–sham rats presented differences related to the number of nNOS+ cells in the NAc core (NAcC), compared with control–sham animals. Interestingly, the control–sham group presented an asymmetrical distribution of nNOS+ cells in the NAcC (nNOS+ cells/0.5 mm^2 control–sham ipsi-/contralateral 10 ± 1/16 ± 2) [$F(1,16) = 18.55$, $p < 0.001$ for ipsi- vs. contralateral

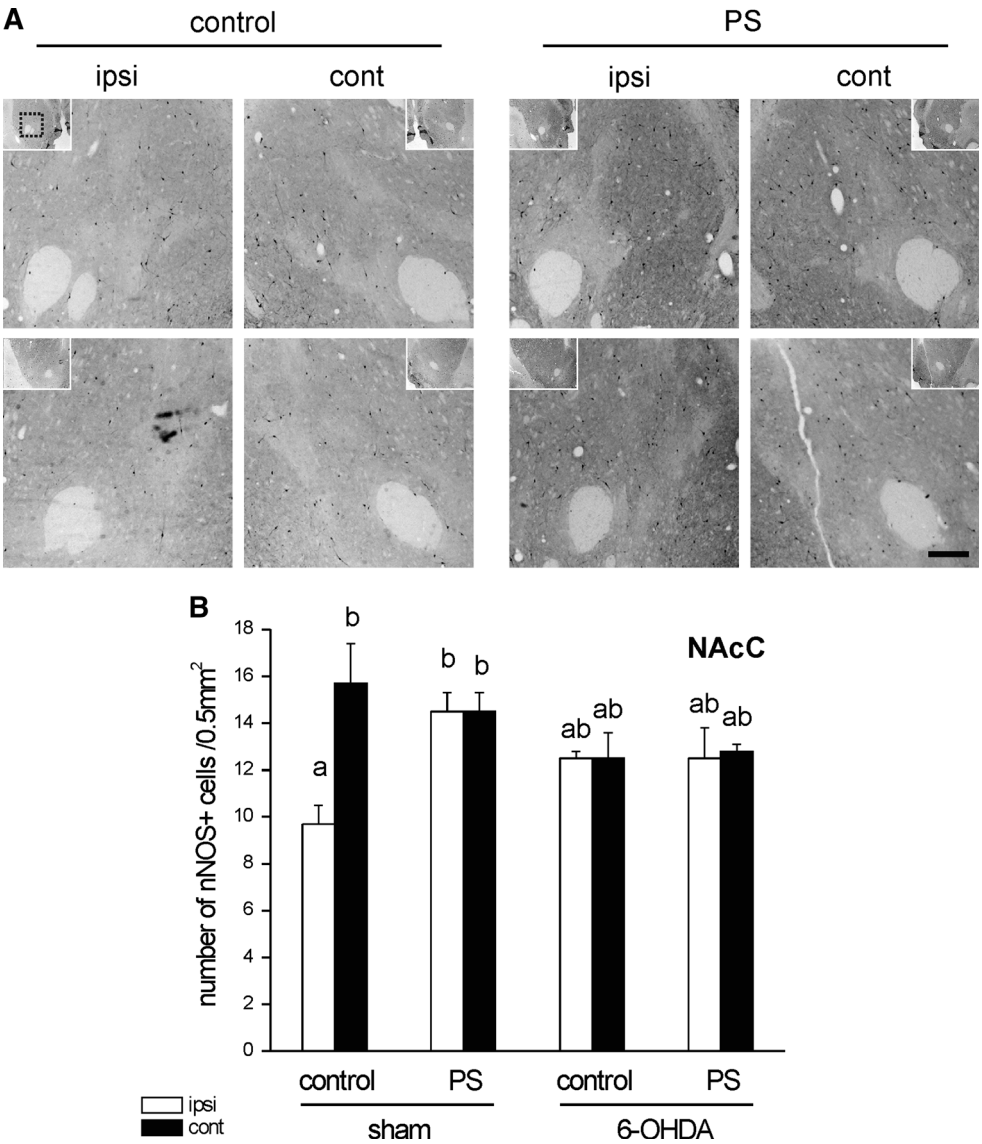

Fig. 5 Relative number of nNOS+ cells in the NAcC of control and PS rats, sham and treated with 6-OHDA. **a** Representative sections at equally spaced rostrocaudal ipsilateral and contralateral sides of NAcC in control and PS rats, sham and treated with 6-OHDA, respectively. The *inset* corresponds to the same image at low magnification. *Scale bar* 250 μm. **b** Quantification of nNOS+ cells in the NAcC of control and PS animals, sham or 6-OHDA lesioned. Three-way ANOVA followed by main effects analyses: $F(1,16) = 18.55$, $p < 0.001$ for contra- versus ipsilateral hemisphere factors in control group, $F(1,16) = 11.6$, $p < 0.001$ for control versus PS factors in the ipsilateral hemisphere, $F(1,16) = 5.32$, $p < 0.05$ for sham versus 6-OHDA factors in the contralateral hemisphere of control group. Values represent (mean ± SEM) nNOS+ cells/0.5 mm^2. $n = 3$ litters per condition. "Ipsi" and "cont" in *bar* graph indicate ipsi- and contralateral sides respect to 6-OHDA-injection side, respectively. Different *letters* depicts significant differences between groups

hemisphere factors in control group]. However, the asymmetry was lost in the PS–sham group (nNOS+ cells/0.5 mm^2 PS–sham ipsi-/contralateral 15 ± 1/15 ± 1) and consequently showed an increased number of nNOS+ cells in the ipsilateral hemisphere compared with the control–sham group [$F(1,16) = 11.6$, $p < 0.001$ for control vs. PS factors in the ipsilateral hemisphere]. Following the intrastriatal 6-OHDA lesion, the contralateral hemisphere of the control–6-OHDA group showed a decrease in the number of nNOS+ cells (nNOS+ cells/0.5 mm^2 control–6-OHDA ipsi-/contralateral 13 ± 0.3/13 ± 1), losing its asymmetry [$F(1,16) = 5.32$, $p < 0.05$ for sham vs. 6-OHDA factors in the contralateral hemisphere of control group]. The number and distribution of nNOS+ cells in the NAcC of 6-OHDA lesioned animals showed no changes between control and prenatally stressed rats. No changes in the number and distribution of nNOS+ cells in the NAc shell were observed in both analysed groups (data not shown).

Additionally, we also analysed the effect of PS on the relative number of cells expressing nNOS in the nigrostriatal pathway. No differences were found between control and prenatally stressed rats. The data are resumed in Table 1.

Discussion

In the present article, we demonstrate that prenatally restrained stressed rats showed an increased number of TH+ cells in the VTA (PS–sham) and that a neurochemical insult during adulthood (PS–6-OHDA) reduced this population to the levels of control cells (control–sham/6-OHDA). Additionally, the population of nNOS neurons was quantitatively different between control- and PS–sham rats in two areas corresponding to the mesolimbic system: NAcC and VTA. Moreover, we found no alterations in the

Table 1 Effect of unilateral striatal 6-OHDA injection on nNOS+ cell/0.5 mm^2 in striatum and SN

Brain areas	Sham				6-OHDA			
	Control		PS		Control		PS	
	Ipsi	Contr	Ipsi	Contr	Ipsi	Contr	Ipsi	Contr
Striatum	10.0 ± 0.7^a	9.5 ± 0.5^a	10.9 ± 0.3^a	10.4 ± 0.2^a	12.4 ± 0.6^b	10.2 ± 0.2^a	13.5 ± 0.4^b	11.1 ± 0.3^a
SN	12.3 ± 1.4^a	11.8 ± 0.9^a	11.6 ± 0.2^a	11.8 ± 0.3^a	16.8 ± 0.5^b	10.6 ± 1.1^a	18.0 ± 0.7^b	10.9 ± 0.6^a

Quantification of nNOS+ neurons in the striatum and SN. Values represent (mean ± SEM) nNOS+ cells/0.5 mm^2; $n = 3$ litters per condition. Three-way ANOVA followed by main effects analyses. In striatum: $F(1,16) = 26.12$, $= 0.0001$ for postnatal treatment factor effect; interaction between side and postnatal treatment was statistically significant [$F(1,16) = 8.55$, $p = 0.0099$ for postnatal treatment vs. hemisphere factors]; $F(1,16) = 27.84$, $p < 0.001$ for ipsi- versus contralateral hemispheres in 6-OHDA group; $F(1,16) = 32.15$, $p < 0.001$ sham versus 6-OHDA in ipsilateral hemispheres. In SN: $F(1,16) = 15.46$, $p = 0.0012$ for postnatal treatment factor effect; interaction between side and postnatal treatment was statistically significant [$F(1,16) = 32.00$, $p < 0.0001$ for postnatal treatment vs. hemisphere factors]; $F(1,16) = 67.01$, $p < 0.001$ for ipsi- versus contralateral hemispheres in 6-OHDA group; $F(1,16) = 46.03$, $p < 0.001$ sham versus 6-OHDA in ipsilateral hemispheres. Different letters depicts significant differences between groups

number of TH+ or nNOS+-expressing cells in the nigrostriatal system. These results were in agreement with previous results from our laboratory showing that prenatal restraint stress affected mostly limbic areas of the offspring rather than motor areas (Baier et al. 2012).

The intrastriatal lesion of 6-OHDA is classically used as a model for PD due to the fact that it primarily produces nigrostriatal neuronal death. The fact that the procedure employed by Debeir et al. (2005) produced minimal lesions to the VTA of undisturbed animals (control–6-OHDA), allowed us to demonstrate the increased sensitivity of TH+ neurons in this area of prenatally stressed offspring (PS–6-OHDA).

Nurr1 and Pitx3, two critical transcription factors regulating the development and maintenance of midbrain dopaminergic neurons, were affected in prenatally stressed adult offspring (Katunar et al. 2009, 2010). It is interesting to note that Katunar et al. (2010) reported that the expression of both Nurr1 and Pitx3 increased in prenatally stressed adult offspring in the VTA, although no changes were observed in the SN. In agreement with this observation, the present work revealed an increased population of TH-expressing neurons in the VTA of prenatally stressed rats (PS–sham) with a differential susceptibility to a neurochemical insult in the adulthood. Similarly, McArthur et al. (2005, 2007) demonstrated that pre- and postnatal exposure to dexamethasone increased the number of TH+ cells in the VTA. A possible mechanism involved in this differential neurotoxicity of 6-OHDA in the VTA of prenatally stressed rats could be explained on the basis of the higher levels of Nurr1 observed in this area (Katunar et al. 2010). Higher levels of Nurr1 might up-regulate TH expression (Smidt and Burbach 2007), and eventually, as we proposed recently (Baier et al. 2012), also increase the expression or activity of DAT and vesicular monoamine transporter 2. We recently found that adult prenatally stressed rats displayed an increased activity of DAT (measured by [^{3}H]DA uptake) in the NAc but not in the striatum (unpublished results). The expression of DAT was not affected in those brain areas (unpublished results). Uptake of 6-OHDA to DA terminals is mediated by DAT or noradrenaline transporter (Simola et al. 2007). Therefore, the neurotoxic effects of 6-OHDA would be facilitated in the VTA of prenatally stressed offspring with a higher activity of DAT.

The results described in the present study suggested the existence of a deregulation between the dopaminergic and the nitrergic system in the VTA of rats which were prenatally stressed. The combined analysis of the results obtained in control offspring showed that the VTA responded to the neurotoxic aggression of 6-OHDA, thus increasing the number of nNOS cells, whose function might be involved in the neuroprotection of TH+ cells. However, this neuroprotective response would be impaired in prenatally stressed offspring. In support of this hypothesis, Klejbor et al. (2004) showed that TH+ and NOS+ neurons interact with each other in the VTA; and nNOS knockout mice expressed lower levels of TH+ neurons in the VTA compared to the wild type counterparts, suggesting an essential role of nNOS in regulating the mesolimbic DA system (Balda et al. 2009).

It is interesting to note that undisturbed animals (control–sham) present an asymmetry in the number of neurons which express nNOS in the NAcC. This asymmetry was lost in PS–sham/6-OHDA as well as in control–6-OHDA groups. Alterations in NAc after intrastriatal injections of 6-OHDA were previously reported (Przedborski et al. 1995). Bilateral injection of 6-OHDA in the ventral striatum of rats modified the DA metabolism at the NAc (Kuter et al. 2011). Unilateral injection of 6-OHDA into the SN

affected the TH immunoreactivity in the NAc (Steiner and Kitai 2001). Gomes and Del Bel (2003) observed changes in the number nNOS cells in the NAc contralaterally to the lesion after unilateral 6-OHDA injection in the medial forebrain bundle. Adrover et al. (2007) described that D2 DA receptors were asymmetrically distributed in the NAcC of the control group but not in prenatally stressed rats. The NO and DA systems are inter-related in the NAcC: Hoque and West (2012) demonstrated that nNOS activity is differentially modulated by DA D1 and D2 receptor activation in distinct subregions of the NAcC. In addition, Ohno et al. (1995) described how NO mediates DA release from NMDA receptor activation in the NAc. Several studies from our laboratory (Berger et al. 2002; Barros et al. 2004; Adrover et al. 2007), as well as the present article, represent a final brain snapshot demonstrating that PS results in a long lasting alteration of the DA and NO systems in the VTA and NAcC of the offspring. These changes could be responsible for the long term behavioural and cognitive impairments observed in prenatally stressed rats (Lemaire et al. 2000; Weinstock 2001, 2008; Kofman 2002; Pardon and Rattray 2008; Hausknecht et al. 2013). More studies will be necessary in order to understand whether the modifications observed in these systems are independent or are consequences of a developmental and complex interrelationship.

Together, the results described in the present article were in agreement with the literature (Kofman 2002; Rice et al. 2007; Markham et al. 2010; Sun et al. 2013; and with previous studies from our laboratory reviewed in Baier et al. 2012). Biochemical alterations in the brain of prenatally restrained stressed rats were mainly observed in limbic areas rather than in motor areas, thus suggesting that these animals could be more prone to developing cognitive, reward and motivational disorders rather than motor-related affections.

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