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Immunocytochemical Expression of Dopamine-related Transcription Factors Pitx3 and Nurr1 in Prenatally Stressed Adult Rats

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Rats exposed to different types of stress during the last week of pregnancy produce offspring that show severe anomalies in neural development and brain morphology. We have previously reported that prenatal stress (PS) induced by immobilization increases D2-type dopamine (DA) receptor levels in the adult offspring, with a concomitant reduction in DA release in prefrontal cortex after amphetamine stimulation. Recently, two transcription factors, Nurr1 and Pitx3, have been identified that are expressed at critical moments of DA neuron differentiation. Their genetic expression is activated immediately after these neuron determinations and maintained through adult life. Nurr1 regulates several proteins that are required for dopamine synthesis and regulation, and Pitx3 is specifically involved in the terminal differentiation and maintenance of dopamine neurons. By means of an immunocytochemistry approach, we studied the expression of Nurr1 and found an ubiquitous distribution in cerebral cortex, hippocampus, thalamus, amygdala, and midbrain, whereas Pitx3 remains restricted to the mesencephalic DA neurons such as substantia nigra and ventral tegmental area. Our results show that the expression of both Nurr1 and Pitx3 increased in prenatally stressed adult offspring in the ventral tegmental area, whereas no changes were observed in the substantia nigra area. It might be hypothesized that the increase of the specific dopaminergic transcription factors might be a compensatory mechanism to counteract the reduction in dopamine levels previously observed as a consequence of prenatal stress.

Key words: restraint stress; ventral tegmental area; substantia nigra

It has been demonstrated that prenatal stress (PS) exerts a strong impact on fetal brain development in rats (Weinstock, 2001). Rats exposed to different types of stress during the last week of pregnancy produce offspring that show delays in motor development, impaired adaptation to stressful conditions, altered sexual behavior, and learning deficits (Weinstock, 2001, 2002; Huizink et al., 2004). In addition, the offspring display anomalies in neuronal development and brain morphology, as well as changes in cerebral asymmetry that persist into adulthood (Fride and Weinstock, 1989). Several of these alterations have been attributed to changes in D2-type dopamine (DA) neurotransmission induced by PS that could be the neurochemical basis for the development of neuropsychiatric disorders, including attention-deficit/hyperactivity disorder (ADHD), depression, and schizophrenia (Huizink et al., 2004).

Forebrain DA system plays a decisive role in regulating important neuronal functions like motor integration, decision making, and behavior as well as mediating learning and memory functions (Finlay and Zigmond, 1997). The normal expression of DA receptors is influenced by in utero experience and is vulnerable to environmental changes because PS can impair DA neurotransmission and metabolism (Fride and Weinstock, 1989; Diaz et al., 1995, 1997). It is also known that PS alters DA neurotransmission, evidenced by a higher DA turnover in prefrontal cortex and a lower turnover in striatum and nucleus accumbens (Fride and Weinstock, 1988; Alonso et al., 1997), resulting in an increased number of DA D2-type receptors and a decrease in D3-type receptors in nucleus accumbens (Henry et al., 1995). We have previously shown that restraint stress

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applied to the mother in the last week of gestation induce increased levels of DA D2 receptor in medial prefrontal cortex, dorsal frontal cortex, and CA1 hippocampus, as well as in the nucleus accumbens core in adult offspring (Berger et al., 2002), with a concomitant reduction in DA release after amphetamine stimulation in prefrontal cortex (Katunar et al., 2008). It is known that normal rats and humans display cerebral asymmetries that are important for brain organization, and this lateralization is necessary for control of various behavioral functions (Denenberg, 1981). We found that PS increased D2 receptors both in the left and right sides of nucleus accumbens core (NAcC), but the left–right asymmetries observed in the control groups were selectively lost in adult rats exposed to PS (Adrover et al., 2007). Medial caudate putamen showed an increased of D2 receptors after PS in both hemispheres and asymmetries in both control and stressed groups.

The physiological role and clinical relevance of mesodiencephalic DA (mdDA) neurons are well recognized in schizophrenia, in addictive behavioral disorders (Egan and Weinberger, 1997; Swanson et al., 1998), and in Parkinson's disease. mdDA neurons are located in the ventral midbrain and comprise substantia nigra (SN) and ventral tegmental area (VTA). Differentiation and anatomical localization of mdDA neurons are dependent on the action of various diffusible factors and transcription factor (TF), including Nurr1 (nuclear receptor–related factor 1; NR4A2), Pitx3 (a member of the pituitary homeobox family), Lmx1b (member of the LIM homeodomain family), and Engrailed 1 and 2 (En1 and En2) (Lin and Rosenthal, 2003; Simeone, 2005). Nurr1 is a nuclear receptor of the steroid/thyroid hormone receptor superfamily essential for the differentiation of midbrain DA neurons (Jankovic et al., 2005). This TF activates transcription of the rate enzyme in catecholamine biosynthesis, tyrosine hydroxylase (TH), dopamine transporter (DAT), and the vesicular monoamine transporter 2 (VMAT2) genes (Jankovic et al., 2005), and it is required for Ret expression in developing midbrain DA neurons. Because Ret is the signal-transducing subunit of the glial-derived neurotrophic factor receptor complex, it has been suggested that Nurr1 might be involved in the responsivity of midbrain DA neurons to neurotrophic factors (Wallen et al., 2001). However, Nurr1's role in the mature midbrain is less clear, although data suggest that its expression may be required for proper neurotransmission and maintenance of adult DA neurons (Jankovic et al., 2005).

The gene encoding Pitx3 contains a bicoid-related homedomain and is expressed almost exclusively in midbrain DA cells (Smidt et al., 1997). Loss of Pitx3 expression in mice results in the loss of the SN subset of mdDA neurons (Nunes et al., 2003; van den Munckhof et al., 2003) and in overall reduced locomotor activity but does not alter DAT and VMAT2 in the residual TH-positive neurons (Smidt et al., 2004). Its expression is, at the brain level, confined to mdDA neurons and starts at embryonic day 11 and is maintained throughout adult life in both rodents and humans (Smidt et al., 1997). Extra neural expression of Pitx3 was shown in the developing lens of the eye (Semina et al., 1997). The current data suggest that Nurr1 and Pitx3 control different aspects of midbrain DA neurons differentiation and probably survival: Pitx3 might be important for development and/or maintenance of SN DA neurons, whereas Nurr1 influences overall midbrain DA neurotransmission (Chung et al., 2005; Simeone, 2005). Lmx1 is an essential factor for DA neuron development, which is not required for TH expression but is necessary for Pitx3 expression (Smidt et al., 2000). The TFs En1 and En2 are expressed in midbrain DA neurons from early development to adulthood (Simon et al., 2001) and are required for their survival because it was shown that mdDA neurons lacking En1 and En2 died via apoptosis in vivo and in primary culture (Alberi et al., 2004). The midbrain expression of the above described TFs persists in adult life, suggesting that they might be important for maintenance and plasticity of the DA neuron phenotype in the adult midbrain.

PS produces various impairments in the dopaminergic system in several forebrain areas that receive inputs from the mdDA at a developmental stage when these TFs are very active. Because these TFs persist through adult age, we evaluated the expression of Pitx3 and Nurr1 in mesencephalic areas; we used adult offspring whose mothers were subjected to a restraint model of PS in the last week of gestation.

MATERIALS AND METHODS

Animals

Virgin female Wistar rats weighing 250 g were obtained from highly inbred rats from our own animal facility at the University of Buenos Aires. Vaginal smears were collected daily for 8 days before mating to determine the stage of the estrus cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats weighing 400 g were introduced for mating. Vaginal smears were taken the next 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 hr, off at 18:00 hr) and a 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*. Care was taken to minimize the number of animals used.

PS

Pregnant dams ($n = 6$) were randomly assigned to PS or control groups. Animals in one group served as controls and were left undisturbed in the home cage; animals in the other group were subjected to restraint stress. Rats were transferred to an experimental room, where the stressor was applied. Pregnant animals were placed individually in a transparent plastic restrainer fitted closely to body size for 45-min periods three times a day (09:00 hr, 12:00 hr, and 17:00 hr) between days 14 and 21 of pregnancy. This type of stress was

chosen because it has an indirect influence on the fetus via a direct stress on the mother (Ward and Weisz, 1984; Maccari et al., 1995). The sessions were performed in a lighted environment. No other subjects were present in the experimental room during the stress exposure. At the end of the stress session, the animals were returned to the animals housing room and were then individually housed with free access to food and water. On the day of parturition, litter characteristics were recorded and litters were culled to 10 pups, maintaining a similar number of males and females when possible. Physical landmarks for both litters were reported in Berger et al. (2002). Briefly, no significant differences were found in the length of gestation, litter size, or body weight of pups. No missing limbs or gross malformations were apparent in any of the newborn pups. Six litters were maintained for each experimental group. To prevent litter effects, a maximum of two male pups from each litter were tested at postnatal day 60.

Western Blot Analysis

The extracts were obtained by a modification of the method described by Dignam et al. (1983). Briefly, dissected midbrain areas were homogenized by syringe passing in buffer A (20 mM Tris HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium O-vanadate, 0.1 mM ammonium molybdate, 1 mM phenymethylsulfonyl fluoride [PMSF]). After 10 min of centrifugation at 600*g,* the supernatant was saved as the cytoplasmic fraction. The pellet was then resuspended in the same initial volume in buffer B (20 mM HEPES, pH 7.9, 1.5 mM Mg Cl_2, 0.2 mM EGTA, 25% glycerol, 20 mM KCl, 1 mM DTT, and 0.2 mM PMSF), and buffer C (20 mM HEPES, pH 7.9, 1.5 mM Mg Cl_2, 0.2 mM EGTA, 25% glycerol, 1.2 M KCl, 1 mM DTT, and 0.2 mM PMSF) was added to reach a final concentration of 0.4 M KCl. The nuclear fraction was kept on ice for 30 min with agitation. Finally, the suspension was centrifuged at 25,000*g,* and the supernatant was saved as the nuclear fraction.

Samples containing 100 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide; Laemmli, 1970) blotted onto nitrocellulose membranes and subsequently probed with polyclonal anti-Pitx3 (gift of Dr. Marten Smidt), dilution 1/500, and anti- Nurr1 (Santa Cruz Biotechnology, cod sc-991), dilution 1/500, followed by a secondary horseradish peroxidase–conjugated antibody. The bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL)

Fixation and Tissue Processing

At postnatal day 60, four control and four exposed rats (all individuals derived from different litters) were deeply anesthetized with 300 mg/kg of chloral hydrate (Mallinckrodt). They were perfused through the cardiac left ventricle, initially with 15 ml of a cold saline solution containing 0.05% w/v $NaNO_2$ plus 50 IU of heparin and subsequently with 150 ml of a cold fixative solution containing 4% paraformaldehyde and 0.25% v/v glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and kept in the same cold fixative solution for 2–4 hr. After that, brains were washed three times in cold 0.1 M phosphate buffer, pH 7.4, containing 5% w/v sucrose, and left in this washing solution for 18 hr at 4°C. Brains were thereafter embedded in sucrose 30% w/v and stored at −18°C until the sectioning procedure. Coronal brain sections (thickness, 40 μm for light microscopy) were cut with a cryostat. The sections were stored at −20°C in 0.1 M phosphate buffer, pH 7.4, with 50% w/v glycerol added as a cryoprotector until their use in immunocytochemical studies.

Immunocytochemistry

Brains sections of both control and PS rats were selected according to anatomical landmarks corresponding to the plates 36–39 of the Paxinos and Watson (1986) rat brain atlas.

The sections were simultaneously processed in the free-floating state. To inhibit endogenous peroxidase activity, tissue sections were previously dehydrated, treated with 0.5% v/v H_2O_2 in methanol for 30 min at room temperature, and rehydrated. Brain sections were treated for 1 hr with 3% v/v normal goat serum in phosphate-buffered saline (PBS) to block nonspecific binding sites. After two rinses in PBS plus 0.025% v/v Triton X-100 (PBS-X), sections were incubated for 48 hr at 4 hr with primary antibodies to Pitx3 (1/500, rabbit, gift of Dr. Marten Smidt) (Smidt et al., 2000) and Nurr1 (1/500 Santa Cruz, Biotechnology). After five rinses in PBS-X, sections were incubated for 1 hr at room temperature with biotinylated secondary antibodies diluted 1:200. After further washing in PBS-X, sections were incubated for 1 hr with streptavidin–peroxidase complex diluted 1:200. Sections were then washed five times in PBS and twice in 0.1 M acetate buffer, pH:6 (AcB), and development of peroxidase activity was carried out with 0.035% w/v DAB (3,3′-diaminobenzidine hydrochloride) plus 2.5% w/v nickel ammonium sulfate and 0.1% v/v H_2O_2 dissolved in AcB. After the enzymatic reaction step, sections were washed three times in AcB and once in distilled water. Finally, sections were mounted on gelatin-coated slides, air dried, and coverslipped with Permount for light microscope observations. The antibody and the streptavidin complex were dissolved in PBS containing 1% v/v normal goat serum and 0.3% v/v Triton X-100, pH 7.4.

Statistical Analysis

Individual experiment was composed of three to five tissue sections from each animal. Five to 10 fields were measured for each brain area in each section of each animal. Differences among the means were statistically analyzed by one-way analysis of variance and Student-Newman-Keuls posttest. Statistical significance was set to $P < 0.05$.

RESULTS

The ability of the antibodies to recognize the TF proteins was examined by immunoblots performed on membranes prepared from nuclear and cytoplasmic extracts obtained from midbrain. As shown in Figure 1, anti-Nurr1 antibody detected a prominent band at an approximate molecular mass of 66 kDa. The intensity of the nuclear band is higher than the cytoplasmic band. Anti-Pitx3 antibody show three bands at 45, 67, and 108 kDa in the cytoplasmic extract and two bands at 45 and 67 kDa in the nuclear extracts.

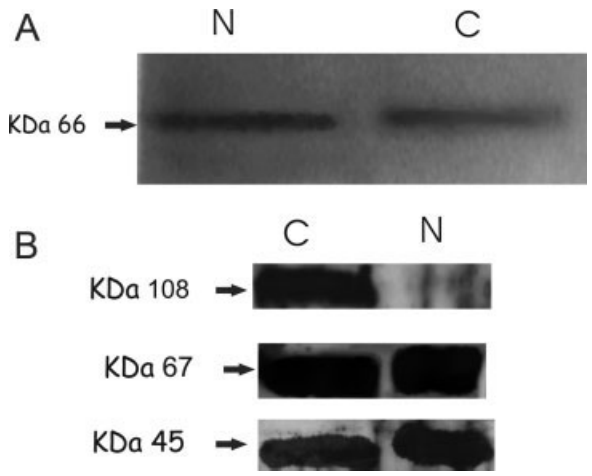

Fig. 1. Western blot analysis of nuclear and cytoplasmic extracts obtained from midbrain with (**A**) anti-Nurr1 and (**B**) anti-Pitx3. Each sample (100 μg of protein) was applied to 12% SDS-PAGE, transferred to nitrocellulose by Western blot, and probed with the antibodies.

TABLE I. Regional Expression of Nurr1 and Pitx3 Protein in Rat Brain*

Brain region	Nurr1	Pitx3
Cortex		
Cerebral		
Entorhinal	+++	−
Temporal	+++	−
Occipital	+++	−
Hippocampus		
CA1	++	−
CA2	++	−
CA3	+++	−
Dentate gyrus	+++	−
PoDG	+	−
Red nucleus		
RPC	++	−
RMC	++	−
Amygdala		
AhiPM	++	−
PMCO	++	−
Midbrain		
VTA	+++	+++
SNc	+++	+++
SNr	+++	+++

*Distribution of Nurr1 and Pitx3 protein by immunocytochemistry in the adult rat brain. Relative abundance was assessed by estimating both staining and cell density. Signals are indicated as negative (−), weak (+), moderate (++), or strong (+++). AhiPM, amydalohippocampal area, posteromedial part; CA, Ammon's horn; PMCO, posteromedial cortical amygdaloid nucleus; PoDG, polymorphic layer of the dentate gyrus; RMC; red nucleus, magnocellular part; RPC; red nucleus, parvocellular part; SNc and SNr; substantia nigra, pars reticulata, and compacta; VTA, ventral tegmental area.

The immunocytochemical expression of Pitx3 and Nurr1 were analyzed in brain slices of control and prenatally stressed adult offspring. Nurr1 exhibited a wide distribution showing higher level of signal in entorhinal, temporal, and occipital cortex, dentate gyrus, and CA3 areas of the hippocampus, SN, and VTA. Lower levels of immunoreactivity (IR) were observed in amygdaloid areas, red nucleus, and CA1 and CA2 areas of the hippocampus. The immunocytochemical expression of the TF Pitx3 was restricted to SN (both pars reticulata and compacta) and to VTA, showing an intense signal (Table I).

Nurr1 expression in extra mesencephalic structures is shown in Figure 2. Nurr1 IR show a similar appearance in area 1 of the temporal cortex (Fig. 2A) and in the posteromedial cortical amygdaloid nucleus (Fig. 2D), with an intense nuclear labeling of neuronal cell bodies with scarce immunostained fibers. Figure 2B shows the dentate gyrus crest with a densely packed Nurr1-labeled granule cell layer and, as expected, no immunostained dendrites projecting into the molecular layer. The polymorphic cell layer shows moderately stained neurons with a distinct nuclear label, most probably corresponding to mossy or multipolar cells. Figure 2C shows a section of the parvocellular part of the red nucleus with moderately labeled large cells with a multipolar shape and medium rounded cells.

Labeled neurons with anti-Nurr1 in mesencephalic structures were found throughout the substantia nigra pars reticulata (SNr), substantia nigra pars compacta (SNc), and VTA. Nurr1 IR was mainly associated to neuronal cell bodies with intense nuclear labeling both in SN and VTA. This label was not homogeneous throughout the nucleus; it presented a patchy (caplike) structure closely apposed to the nuclear envelope. The cytoplasm showed a lighter staining, and only rarely we could observe immunostained fibers in VTA, although moderate fiber staining was observed in SN (Fig. 3C). Mesencephalic areas were quantified showing that prenatally stressed rat brains manifest an increased number of nucleus per unit area in VTA (14 %) but not in SN compared with control rats (Fig. 4).

Pitx3 label was restricted to SNc, SNr, and VTA. In these areas, the IR was also mainly associated to neuronal cell bodies, but the nuclear label was more homogeneously distributed than Nurr1 and less intense. No fiber staining was observed in these areas (Fig. 5). The quantification of the label showed similar values in SN both in controls and prenatally stressed rats. However, in VTA, the number of nucleus per unit area showed a 95% increase in prenatally stressed rats compared with controls (Fig. 6).

DISCUSSION

In the present study, we have assessed the expression of dopamine-related TFs Nurr1 and Pitx3 in brains of adult offspring of gestationally stressed dams. Main results show that Nurr1 is widely distributed in various forebrain and midbrain areas, whereas Pitx3 is restricted to mesencephalic structures. The immunocytochemical expression of both TFs is significantly increased in VTA,

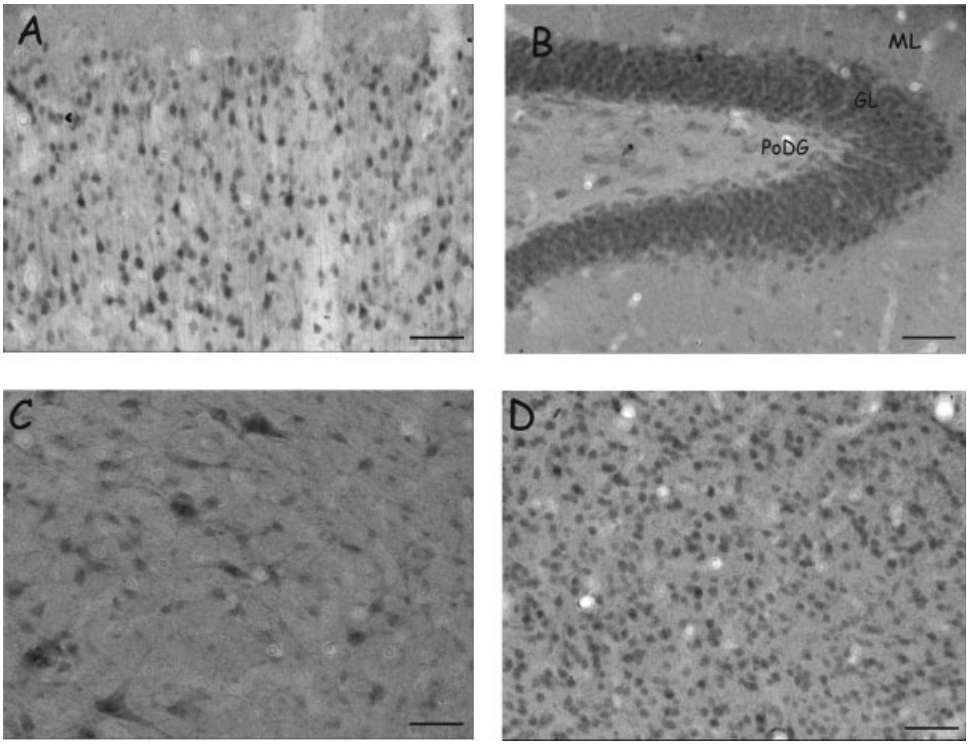

Fig. 2. Photomicrographs showing immunostaining with anti-Nurr1 in (**A**) area 1 of the temporal cortex, (**B**) dentate gyrus of the hippocampus, (**C**) parvicellular part of the red nucleus, and (**D**) posteromedial cortical amygdaloid nucleus. Scale bars = 50 μm. PoDG, polymorphic layer of the dentate gyrus; GL, granule cell layer of the dentate gyrus; ML, molecular layer of the dentate gyrus.

whereas no changes were found in SN of adult prenatally stressed offspring.

The commercial Nurr1 antibody showed a prominent band at an expected approximate molecular mass of 66 kDa (Ojeda et al., 2003; Volpicelli et al., 2004; Xing et al., 2006) in the nuclear extract of a mesencephalic homogenate by Western blot analysis. A lighter signal was detected in the cytoplasmic extract in agreement with the cytoplasmic immunocytochemical staining observed in all areas studied. Even though Pitx3 has a predicted molecular weight of approximately 32 kDa (Semina et al., 1997), the polyclonal antibody against Pitx3 in this study recognizes multiple bands both in nuclear and cytoplasmic extracts. To our knowledge, no other study has shown the Western blot analysis of this antibody; therefore, simple speculation might indicate that the 45-kDa band correspond approximately to the polypeptide and that the 67- and 108-kDa bands might be representing a posttranslational modification such as glycosylation, or even a dimer in the case of the 108-kDa band. Alternatively, the 108-kDa fraction might be the native polymer synthesized in the cytoplasm and thereafter fragmented into two bands of 67 and 45 kDa that enter to the nucleus.

Few studies have shown Nurr1 protein expression by immunocytochemistry because most studies have described Nurr1 mRNA distribution (Backman et al., 1999; Baffi et al., 1999; Wallen et al., 2001; Xiao et al., 1996). Ojeda et al. (2003) reported the immunocytochemical distribution of Nurr1 showing a close correspondence with our results with high level of expression in cortex, hippocampus, and midbrain. In turn, the protein distribution described in both studies is in agreement with the Nurr1 mRNA distribution reported in the studies just mentioned. The restricted distribution in VTA and SN of Pitx3 protein paralleled the distribution found by in situ hybridization (Smidt et al., 1997) and immunocytochemistry (Korotkova et al., 2005).

The midbrain structures give rise to two of the four major subsystems of DA neurons: the nigrostriatal and the mesocorticolimbic pathways. The nigrostriatal pathway originates in the SN and projects to the dorsal and lateral striatum, regulating motor control. The mesocorticolimbic system arises from neuronal cell bodies in the VTA and projects to certain limbic system areas (i.e., nucleus accumbens septi and lateral septal nuclei of the basal forebrain and amygdala, hippocampus, and entorhinal cortex) and to the prefrontal neocortex. This pathway has been indicated as involved in motivation and in emotional and reward behavior (Schultz, 1997). Interestingly, the increase in D2 DA receptors we previously reported in PS rats was observed in corticolimbic areas innervated by VTA, but not in the striatum innervated by SN (Berger et al., 2002). In other words, considering the two dopaminergic pathways that originate in mesencephalic structures, only the VTA–corticolimbic pathway is impaired after the prenatal insult, whereas the SN–striatum remains unaltered.

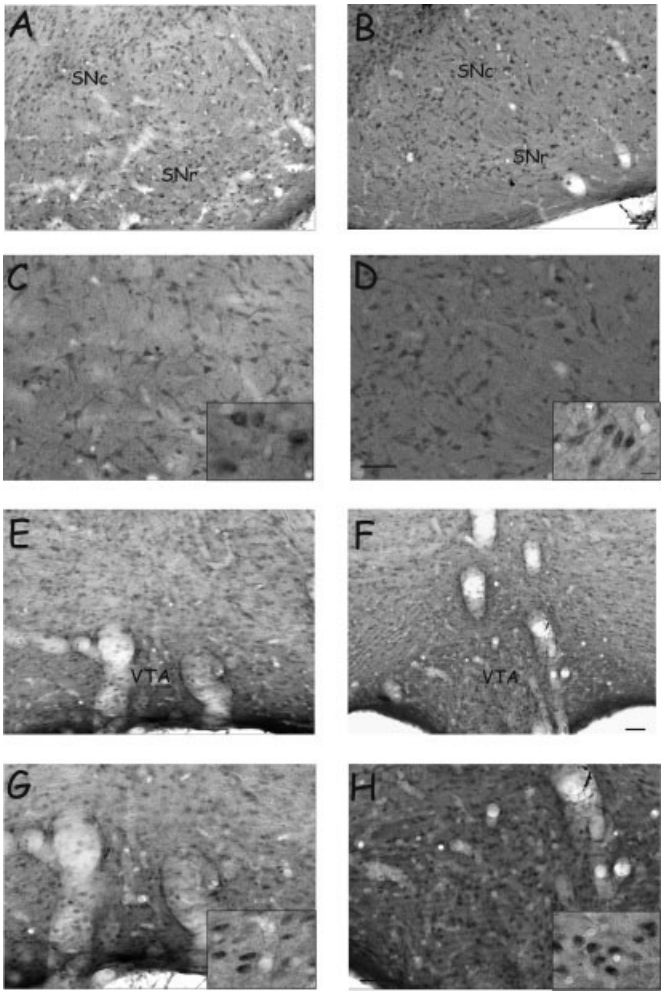

Fig. 3. Photomicrographs showing immunostaining with anti-Nurr1 antibody in mesencephalic structures. Substantia nigra (**A–D**) and ventral tegmental area (**E–H**). A, C, E, G: control. B, D, F, H: prenatal stress. Scale bars = 50 μm in photomicrographs; 12.5 μm for insets.

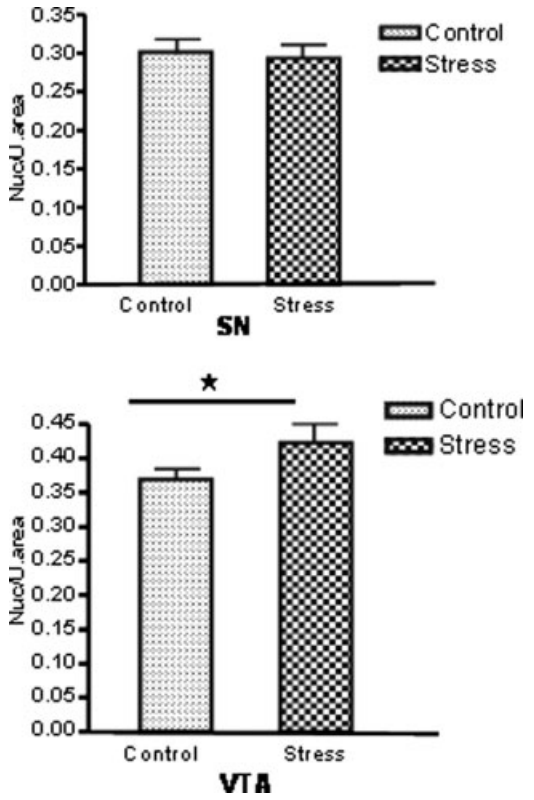

Fig. 4. Quantitative analysis of the number of nucleus per unit area of Nurr1 IR neurons in VTA and SN, both in control and prenatally stressed rats. Values are reported as mean ± SEM. $\star P < 0.05$. SN, substantia nigra; VTA, ventral tegmental area.

The corticolimbic system is considered to be of particular interest for the pathophysiology of idiopathic psychiatric disorders including psychoses and mania, as well as in schizophrenia and ADHD (Biederman, 2005), which have been traditionally related to dopaminergic mesolimbic and mesocortical pathways. The most accepted hypothesis regarding the pathogenesis of schizophrenia and ADHD may be a disorder of neural development (Koenig et al., 2002; Lewis and Levitt, 2002). Nowadays, most models that explain the cause of schizophrenia propose interactive effects between multiple susceptibility genes and environmental factors. In this regard, Lewis and Levitt (2002) pointed out that many environmental events occur during the prenatal or perinatal periods. In this context, it is of particular relevance that an insult exerted indirectly through the mother on the developing fetus brain might be impairing the TFs mostly related to the survival and maintenance of dopaminergic neurons such as Nurr1 and Pitx3.

It has been demonstrated that Nurr1 regulates several proteins that are required for dopamine synthesis and regulation such as TH, VMAT2, DAT, and RET receptor tyrosine kinase (cRET) (Smidt and Burbach, 2007). In has been described that mesodiencephalic neurons are not dependent on Nurr1 for development but need Nurr1 for maintenance and transmitter synthesis and release.

Although the Pitx3 gene is expressed in all mdDA neurons (Smidt et al., 1997, 2000), results obtained from aphakia mutant mice that are $Pitx3^{-/-}$ and lack SNc neurons has induced researchers to suggest that Pitx3 is involved in the terminal differentiation and/or early maintenance of SNc (Smidt and Burbach, 2007). In addition, Pitx3 has been related to TH expression because the promoter region of TH has potential type binding elements for Pitx3 at a region different from Nurr1 binding. Moreover, Pitx3 expression begins concurrently with TH expression (Messmer et al., 2007). However, Maxwell et al. (2005) reported that Pitx3 function may display subregional specificity because their

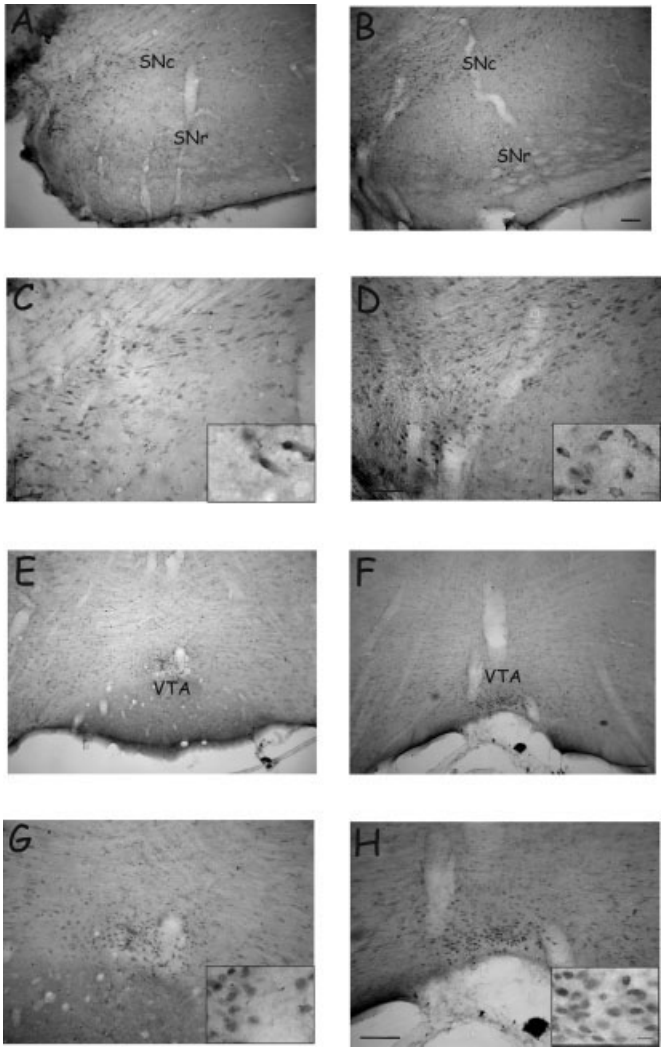

Fig. 5. Photomicrographs showing immunostaining with anti-Pitx3 antibody in substantia nigra (**A–D**) and in ventral tegmental area (**E–H**) A, C, E, G: control. B, D, F, H: prenatal stress. Scale bars = 50 μm in photomicrographs; 12.5 μm for insets. SNc, substantia nigra; pars compacta, SNr, sustantia nigra; pars reticulata; VTA, ventral tegmental area.

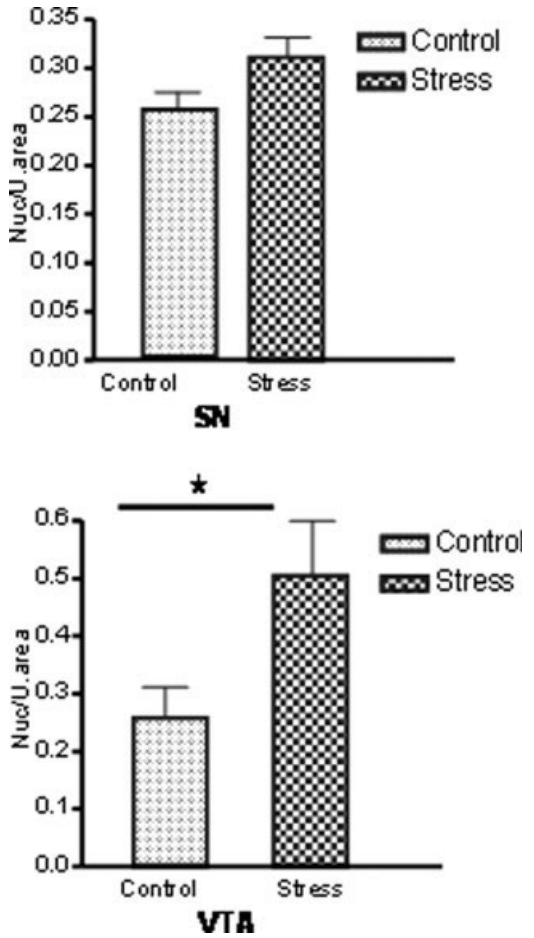

Fig. 6. Quantitative analysis of the number of nucleus per unit area of Pitx3 IR neurons in VTA and SN, both in control and prenatally stressed rats. Values are reported as mean ± SEM. $\star P < 0.05$. SNc, substantia nigra; pars compacta; SNr, substantia nigra; pars reticulata; VTA, ventral tegmental area.

data suggest that Pitx3 is required specifically by the SNc neurons for initiation and maintenance of TH expression.

In our PS model, dopamine levels were found to be decreased in prefrontal cortex dialysates of prenatally stressed rats when stimulated by amphetamine (Katunar et al., 2008). These might be indicating that the synaptic vesicle content of dopamine in prefrontal cortex nerve endings of prenatally stressed offspring might be diminished in relation to control rats, and consequently releases less dopamine to the synaptic cleft when stimulated by amphetamine. In turn, dopamine D2 receptors are up-regulated (Berger et al., 2001) to compensate the impairment in DA stimulation. Interestingly, it has been recently demonstrated that the dopaminotrophic properties of dopamine D2 receptors during development might be exerted through the activation of Nurr1 via extracellular signal-regulated kinase signaling (Chung et al., 2005). Although the authors did not study adults, they speculate that Nurr1 might promote the survival of mature dopaminergic neurons via a similar mechanism. Much work is still needed especially at early stages of development, but it is tempting to speculate, as depicted in Figure 7, that PS might be impairing the synthesis of TH in the perinatal period, thereby diminishing dopamine synthesis. The increase of Nurr1 and Pitx3 in VTA in adult stages of prenatally stressed offspring observed in this study might be induced by the increase in dopamine D2 receptors we previously observed, indicating a compensatory mechanism designed by the system to up-regulate TH and increase dopamine synthesis. Additionally, the area selectivity of the increase of the TFs toward VTA (and not SN) and the mesolimbic pathway probably indicates that an insult received during

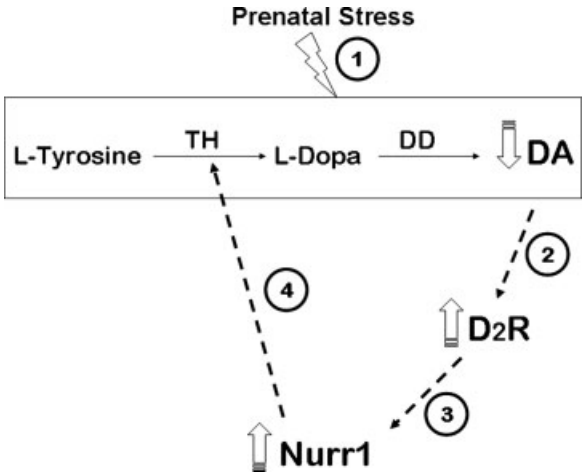

Fig. 7. Suggested mechanism of action of Nurr1 over the dopaminergic pathway. Prenatal stress exerts an inhibitory effect on general levels of dopamine at some unknown stage of dopamine biosynthesis (**1**, Katunar et al., 2008). Low levels of dopamine produces an up-regulation of dopamine D2 receptors (**2**, Berger et al., 2002), which in turn activates Nurr1 (**3**, Chung et al., 2005). High levels of Nurr1 might up-regulate TH expression (**4**, Smidt and Burbach, 2007) and eventually DAT and VMAT2, which will increase dopamine levels in an attempt to compensate for the dysbalance produced by prenatal stress. TH, tyrosine hydroxylase; DD, dopa decarboxylase.

the prenatal period will exert motivational, emotional, and reward behavior impairments in the adult life, rather than motor impairments mostly related to SN and the mesostriatal pathway.

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