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Ontogenetic Expression of Dopamine-Related Transcription Factors and Tyrosine Hydroxylase in Prenatally Stressed Rats

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Abstract The development of the central nervous system can be permanently affected by insults received during the perinatal period, predisposing the organism to long-term behavioral and neurochemical abnormalities. Rats exposed to different types of stress during the last week of gestation produce offspring that show several alterations, many of which have been attributed to changes in dopamine (DA) neurotransmission that could serve as the neurochemical basis for the development of neuropsychiatric disorders. Employing an immunocytochemical approach, we studied the expression levels of two transcription factors Nurr1 and Pitx3 which are expressed at critical moments of DA neurons differentiation as well as the expression of the rate limiting enzyme in DA synthesis, tyrosine hydroxylase (TH) in mesencephalic areas of the brains of prenatally stressed (PS) offspring at different postnatal ages. Main results show that stress exerted to the gestant mother produces permanent effect in the ontogenetic expression of key factors related to the DA metabolism mainly in the ventral tegmental area (VTA) of the mesencephalon. The immunocytochemical expression of the transcription factor Nurr1 shows an increase at postnatal days (PNDs) 7, 28, and 60 whereas Pitx3 shows a decrease at PND 28 and an increase at 60 PND. The rate limiting step in DA synthesis, the enzyme TH shows a decrease at PND 7 to reach control levels at PNDs 28 and 60. The increase of TFs might be up-regulating TH in order to restore DA levels that were previously seen to be normal before puberty. The area selectivity of the increase of the TFs toward VTA and the mesolimbic pathway indicates that an insult received during the prenatal period will exert mainly motivational, emotional, and reward behavior impairments in the adult life.

Keywords Nurr1 · Pitx3 · Ventral tegmental area · Substantia nigra · Mesolimbic pathway

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Introduction

Early-life environmental factors may cause structural and functional changes in the central nervous system (CNS), which persist for the entire lifespan and affect synaptic plasticity, behavior, and responses to stress (Casolini et al. 2007). The study of the biochemical and molecular consequences of stress has become increasingly interesting because exposure to stressful situations may be involved in the development of specific neuropathologies, such as anxiety and depressive disorders (Kendler et al. 1999). In rats, development of the CNS can be durably altered by deleterious perinatal environment, predisposing the organism to long-term behavioral abnormalities (Chapillon et al. 2002; Maccari et al. 2003; Weinstock 2005). 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 deficit (Weinstock 2001, 2002; Huizink et al. 2004). Several of these alterations have been attributed to changes in dopamine (DA) neurotransmission (Fride and Weinstock 1989; Diaz et al.

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1995, 1997) evidenced by a higher DA turnover in prefrontal cortex and a lower turnover in striatum and nucleus accumbens (NAc) (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 NAc (Henry et al. 1995). These changes could serve as the neurochemical basis for the development of neuropsychiatric disorders, including attention-deficit hyperactivity disorder (ADHD), depression, and schizophrenia (Weinstock 2001; Huizink et al. 2004). Prenatal restraint stress in rats is a validated model of early stress that has been shown to produce permanent behavioral and neurobiological consequences (Maccari and Morley-Fletcher 2007; Darnaudery and Maccari 2008).

The catecholamine DA is an important regulator of many neural functions, including motor integration, neuroendocrine hormone release, cognition, emotive behaviors, and reward (Schultz 1997). Midbrain DA neurons, with their cell bodies localized in substantia nigra (SN) project to the striatum and receive innervations from multiple structures in the diencephalon and telencephalon. The ascending nigrostriatal pathway regulates motor control and its degeneration in humans is associated with Parkinson's disease. Neurons from the ventral tegmental area (VTA) project to the limbic system and cortex, and are involved in emotional and reward behaviors and in motivation (Schultz 1997). Disturbances in this system have been associated with schizophrenia, addictive behaviors disorders, and ADHD (Swanson et al. 1998).

At the biochemical level, DA is generated from the amino acid tyrosine in two enzymatic steps. Tyrosine hydroxylase (TH) (EC 1.14.16.2) (Nagatsu and Ichinose 1999; Verheij and Cools 2008) is a key enzyme in the synthesis converting tyrosine to levodopa (L-DOPA) which is then converted further to DA by aromatic L-amino acid decarboxylase (AADC). The function of TH enzyme is regulated by several mechanisms including an activation by tetrahydrobiopterin cofactor and protein phosphorylation and an inactivation by DA and catecholamine end-products (Nagatsu and Ichinose 1999; Fujisawa and Okuno 2005). Upon release from the presynaptic terminal into the synaptic cleft, DA neurotransmission is terminated by uptake into the presynaptic DA fibers. The physiological role and clinical relevance of dopaminergic neurons are well-recognized and has been the object of intense investigations.

The precise anatomical localization and functional differentiation of DA neurons in the mammalian brain is achieved through the action and gradient disposition of various diffusible factors. It has recently been identified two transcription factors (TFs), Nurr1 and Pitx3 which are expressed at critical moments of DA neurons differentiation. Nurr1 (also known as NR4A2) is a transcription factor (TF) in the orphan nuclear receptor family (1). Nurr1 forms a highly conserved subfamily of nuclear orphan receptors together with Nur77 (NR4A1) and Nor1 (NR4A3). Nuclear receptors are ligand-regulated transcription factors that bind steroid hormones, thyroid hormone, retinoids, and other small lipophilic signalling molecules (Mangelsdorf et al. 1995). Nuclear receptors are ligand-inducible TFs that bind to DNA and modulate expression of target genes (Aranda and Pascual 2001). However ligand for Nurr1 and several additional orphan receptors remain unknown. Analyses of Nurr1-null mice have revealed the roles of this TF in the generation, maturation, migration, striatal target innervation, and survival of DA neurons in the developing midbrain (Saucedo-Cardenas et al. 1998; Wallen et al. 1999). However, Nurr1 expression is not confined to the developing midbrain. Within the CNS, Nurr1 is expressed in a number of areas including the cortex, hippocampus, brain stem, and spinal cord (Perlmann and Wallen-Mackenzie 2004). Furthermore, transcriptosome analyses revealed that Nurr1 targets genes involved in axonal outgrowths and guidance, general cell survival and neuron differentiation (Hermanson et al. 2006; Luo et al. 2007; Sousa et al. 2007; Volpicelli et al. 2007), suggesting a broad spectrum of Nurr1 roles in brain development. Their genetic expression is activated immediately after these neurons determination and maintained throughout adult life. Nurr1 activates transcription of the rate enzyme in catecholamine biosynthesis, TH, dopamine transporter (DAT), and the vesicular monoamine transporter 2 (VMAT2) genes. Nuclear receptors are interesting proteins for several reasons. First, they are excellent tools for understanding the way in which genes are regulated, since small molecule ligands can be used to switch these transcription factors between active and inactive states. Second, classical nuclear receptor signalling pathways, e.g., steroid hormone and retinoid receptors, influence many biological pathways important in development and adult physiology. Accordingly, their significance in disease is critical.

The gene encoding the Pitx3 contains a bicoid-related homedomain and is expressed almost exclusively in midbrain DA cells (Smidt et al. 1997). Its expression within the brain, starts at embryonic day 11.5 and is maintained throughout adult life in both rodents and humans (Smidt et al. 1997). The mechanism by which Pitx3 influences the development of this specific mesodiencephalic DA (mdDA) subpopulation is still unknown. Extra neural expression of Pitx3 was shown in the developing lens of the eye (Semina et al. 1997).

Previous studies in our laboratory have shown that adult offspring of stressed rats exhibits higher levels of DA receptors in frontal cortex, NAc core, and hippocampus (Berger et al. 2002) concomitantly with an astroglial hypertrophy and a reduced dendritic arborization with

synaptic loss in several brain areas (Barros et al. 2006). We also found that prenatal stress (PS) increased D2 receptors both in the left and right sides of NAc but the left–right asymmetries observed in the control groups were selectively lost in adult rats exposed to PS. Medial caudate putamen (CPu-M) showed an increased of D2 receptors after PS in both hemispheres and asymmetries in both control and stressed groups (Adrover et al. 2007). We also observed a decrease of amphetamine stimulated DA dialysate in prefrontal cortex of PS rats at postnatal day (PND) 60 compared with controls (Katunar et al. 2008). After amphetamine or nicotine stimulation, DA levels were higher at PND 60 in NAc shell (Silvagni et al. 2008).

Employing an immunocytochemistry approach, we have recently shown that the expression of Nurr1 presents a ubiquitous distribution in cerebral cortex, hippocampus, thalamus, amygdala, and midbrain whereas Pitx3 remains restricted to the mesencephalic DA neurons such as SN and VTA. The expression of both Nurr1 and Pitx3 increased in PS adult offspring in the VTA area, whereas no changes were observed in SN areas (Katunar et al. 2009).

In the present work we have extended this study to different postnatal ages where we have studied the immunocytochemical expression of Nurr1 and Pitx3 in SN and VTA at PNDs 7, 28, and 60 of PS rats. In addition, we measured the expression levels of TH at the same postnatal ages. We anticipate that by studying the ontogeny of the main dopaminergic transcription factors and the rate limiting step enzyme in the synthesis of DA, we will approach to a better understanding of the impairments previously found in the levels of DA and DA receptors in the mesocorticolimbic pathways of the PS offspring.

Materials and Methods

Animals

Virgin females 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 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 outline in the NIH *Guide for the Care and Use of Laboratory Animals*. Care was taken to minimize the number of animals used.

Prenatal Stress

Pregnant dams ($n = 6$) were randomly assigned to PS or control groups. One group served as control and was left undisturbed in the home cage; the other group was subjected to restraint stress. Rats were transferred to an experimental room, where the stressor was applied. Pregnant females were placed individually in a transparent plastic restrainers fitted closely to body size for 45 min periods 3 times per day (9:00 h, 12:00 h and 17:00 h) between days 14 and 21 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 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 litter body weight. 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 for each experimental condition.

Fixation and Tissue Processing

At PND 60, four control and four exposed rats (all individuals derived from different litters) were deeply anaesthetized 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 I.U. 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 h. After that, brains were washed 3 times in cold 0.1 M phosphate buffer, pH 7.4, containing 5% w/v sucrose, and left in this washing solution for 18 h at 4°C. Brains were thereafter embedded in sucrose 30% w/v and stored at −18°C until sectioning procedure. Coronal brain sections (thickness 40 μm for light microscopy) were cut using 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 h with 3% v/v normal goat serum in phosphate buffer saline (PBS) to block non-specific binding sites. After two rinses in PBS plus 0.025% v/v Triton X-100 (PBS-X), sections were incubated for 48 h at 4°C with primary antibodies to Pitx3 (1/500, rabbit, gift from Dr. Marten Smidt (Smidt et al. 2000) and Nurr1 (1/500 Santa Cruz, Biotechnology) and anti-TH (1/100 MAB 31, Chemicon). After five rinses in PBS-X, sections were incubated for 1 h at room temperature with biotinylated secondary antibodies diluted 1:200. After further washing in PBS-X, sections were incubated for 1 h with streptavidin–peroxidase complex diluted 1:200. Sections were then washed 5 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 3,3′-diaminobenzidine hydrochloride (DAB) plus 2.5% w/v nickel ammonium sulphate and 0.1% v/v H_2O_2 dissolved in AcB. After the enzymatic reaction step, sections were washed 3 times in AcB and once in distilled water. Finally, sections were mounted on gelatine-coated slides; air dried and cover slipped using Permount for light microscope observations. The antibody as well as the streptavidin complex was dissolved in PBS containing 1% v/v normal goat serum and 0.3% v/v Triton X-100, pH 7.4.

Image Analysis

Tissue images were obtained through an Axiophot Zeiss light microscope equipped with a cooled Olympus Q5 camera on line with an OPTIMAS 6.0 image analyzer. The relative number of immunopositive nucleus and cells per unit area were analyzed in SNc and VTA.

Statistical Analysis

Five to nine separate immunohistochemical experiments were run for each primary antibody. Individual experiment was composed of at least three to five tissue sections from each animal for each group. For SNc and VTA measurements coronal sections at posteriority from Bregma 5.60 to 5.80 according to the atlas of Paxinos and Watson (1986) were selected and the fields measured were randomly chosen from the areas shaded in Fig. 1. Four to five fields were measured from these areas for each brain area in each section of each animal.

Differences among the means were statistically analyzed by using two-way ANOVA. Results from treatments showing significant overall changes were subjected to post hoc Tukey tests with significance for $P < 0.05$. Interanimal differences in each group, as well as interexperimental differences, were not statistically significant.

Results

The immunocytochemical expression of Pitx3, Nurr1, and TH were analyzed in brain slices of control and PS offspring at different PNDs (7, 28, and 60). Labeled neurons with anti-Nurr1 and anti-Pitx3 in mesencephalic structures were found throughout the SNr, SNc, and VTA at all ages tested. Nurr1 immunoreactivity was mainly associated to neuronal cell bodies with intense nuclear labeling both in SN and VTA. Figure 2 shows the immunocytochemical expression of Nurr1 and Pitx3 in VTA of control offspring at PNDs 7, 28, and 60. At PND 7 (Fig. 2a) the label is diffuse and even the shape of the VTA neurons are not yet well defined as observed in PND 28 (Fig. 2b) and PND 60 (Fig. 2c). At these two ages, the label is clearly distributed around the nucleus in defined rounded or pyramidal shaped cells. Control Pitx3 immunoreactivity (Pitx3-ir) (Fig. 2d–f) was mainly associated to small round neuronal cells bodies but the nuclear label was more homogeneously distributed than Nurr1 and more intense, specially at PND 7. No fiber staining was observed in any of the areas analyzed (SNc and VTA) and at all ages tested. Although not shown, the

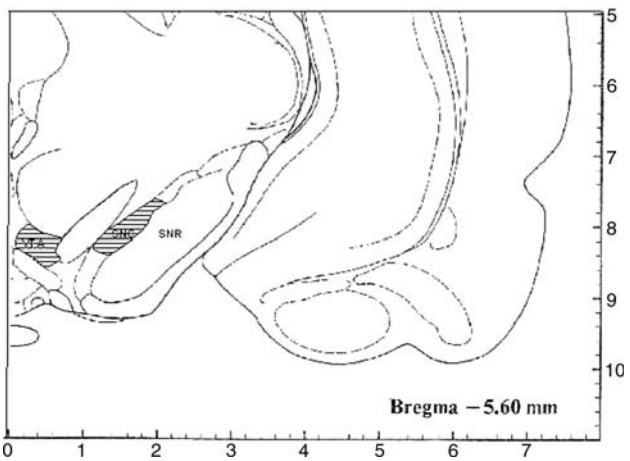

Fig. 1 Schematic representation of a section at 5.60 posterior to the bregma, according to Paxinos and Watson (1986), showing the shaded areas of SNc and VTA that were selected for measurements. *SNc* substantia nigra compacta, *VTA* ventral tegmental area

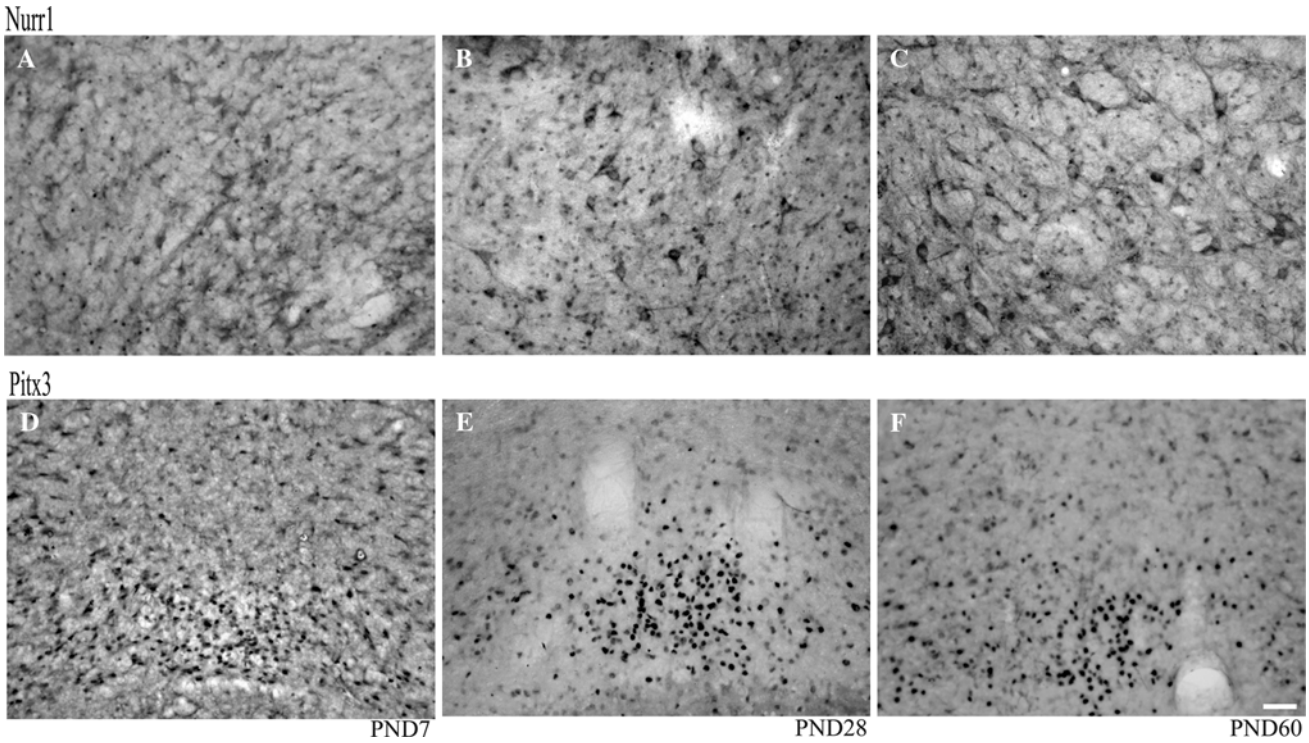

Fig. 2 Photomicrographs showing immunostaining with anti-Nurr1 (**a**–**c**) and anti-Pitx3 (**d**–**f**) antibody in control VTA at different postnatal ages, PND 7 (**a**, **d**), PND 28 (**b**, **e**), and PND 60 (**c**, **d**). *Scale bar* = 100 μm. *VTA* ventral tegmental area

morphology of the immunostained neurons in the PS offspring for Nurr1 and Pitx3 was very similar to control neurons depicted in Fig. 2.

Figure 3 shows the quantification of Nurr1 immunostaining showing that, at all ages tested, PS rats present a significant increase in the number of nucleus per unit area in VTA but not in SNc when compared to control rats. Figure 4 shows the quantification of Pitx3-ir. In SNc area, there were no significant differences in Pitx3-ir between control and PS rats (Fig. 4a, c, e). In VTA, there ware no differences in the number of nucleus per unit area at early stages (PND 7) between control and PS rats (Fig. 4b), however, there was a significant decrease in PS rats at PND 28 (Fig. 4d), and a significant increase in the number of nucleus per unit area at PND 60 compared with control groups (Fig. 4f).

The immunocytochemical distribution of TH in mesencephalic structures can be observed in Fig. 5. TH immunoreactivity (TH-ir) was associated mainly to SNc and VTA (Fig. 5a–c). At a higher magnification, it could be observed that TH-ir in SNc was particularly associated to the cytoplasmatic structures and even some positive immunostained fibers can be observed across the area of interest. There is a macroscopically detectable age-related reduction in the TH immunoreactivity staining in the coronal sections of the SNc and VTA of control rats. A significant reduction in neuronal density was observed in the VTA and SNc region at PND 7 when PS rats were compared to control rats (Fig. 6a, b). However, there are no significant changes at PNDs 28 and 60 (Fig. 6c–f) in the number of TH-ir neurons in both areas in PS rats compared to controls.

Figure 7 shows the immunocytochemical expression of TH in VTA at PND 7. A scarce number of neurons are readily seen in the PS image (B) when compared to control (A). The temporal courses of Nurr1, Pitx3, and TH in SNc are depicted in Fig. 8, employing the data presented in Figs. 3, 4, and 6. In control rats, Nurr1-ir show an age-related increase that is significant between PNDs 28 and 60. PS rats also show a temporal increase that is significantly different between PNDs 7 and 60 and between PNDs 28 and 60. Pitx3 shows a decrease between PNDs 7 and 28 only in control rats. In control rats, TH shows a significant decrease between PNDs 7 and 60 and between PNDs 28 and 60.

The temporal courses of Nurr1, Pitx3, and TH in VTA are depicted in Fig. 9, employing the data presented in Figs. 3, 4, and 6. While Nurr1 positive neurons show an age-related increase in the control group, Pitx3 and TH expression shows an age-related decrease. In all three cases, the temporal courses of the immunocytochemical expression in the PS rats differ from their respective control course.

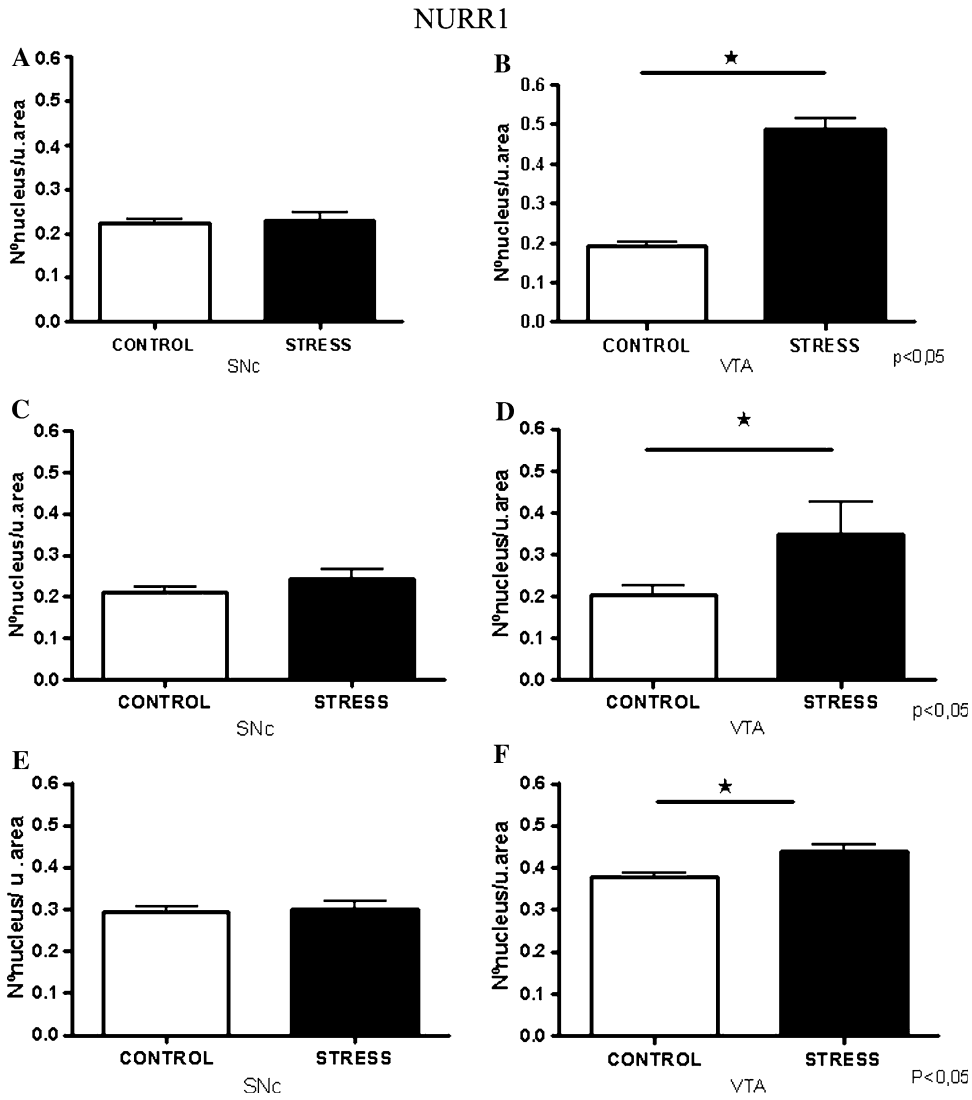

Fig. 3 Quantitative analysis of the number of nucleus per area of Nurr1-ir in SNc (**a**, **c**, **e**) and VTA (**b**, **d**, **f**) both in control and PS rats at PND 7 (**a**, **b**), PND 28 (**c**, **d**), and PND 60 (**e**, **f**). Values are reported as mean ± SEM. * $P < 0.05$. *SNc* substantia nigra compacta, *VTA* ventral tegmental area

It is interesting to point out that both in VTA and SNc in control rats Nurr1-ir shows a significant age-related increase while Pitx3 and TH show an age-related decrease.

Discussion

Main findings of this study show that stress exerted to the gestant mother produces permanent effect in the ontogenetic expression of key factors related to the DA metabolism in the VTA of the mesencephalon. The immunocytochemical expression of the transcription factor Nurr1 shows an increase at PNDs 7, 28, and 60 whereas Pitx3 shows a decrease at PND 28 and an increase at PND 60 in the PS offspring. The rate limiting step in DA synthesis, the enzyme TH shows a decrease at PND 7 to reach control levels at PNDs 28 and 60.

It is interesting to point out that the ontogenetic expression of Pitx3 in the control group of rats show a significant decrease with age whereas Nurr1 shows an increase that is significant between PNDs 28 and 60. To our knowledge no other study has shown the postnatal age-related expression of these TFs in normal rats. Although still a matter of study, most authors agree that at earlier stages of embryonic development Nurr1 is crucial for determining the neurotransmitter phenotype of mdDA neurons and that at later stages it has a role in survival, migration, and target innervations (Perlmann and Wallen-Mackenzie 2004). Moreover, it is believed that in adult stages, Nurr1 participates in the maintenance and survival of mdDA neurons (Prakash and Wurst 2006; Alavian et al. 2008). On the other hand, Pitx3 seems to be required for the initiation and/or maintenance of TH transcription and for proper differentiation or maturation of SNc neurons (Prakash and Wurst

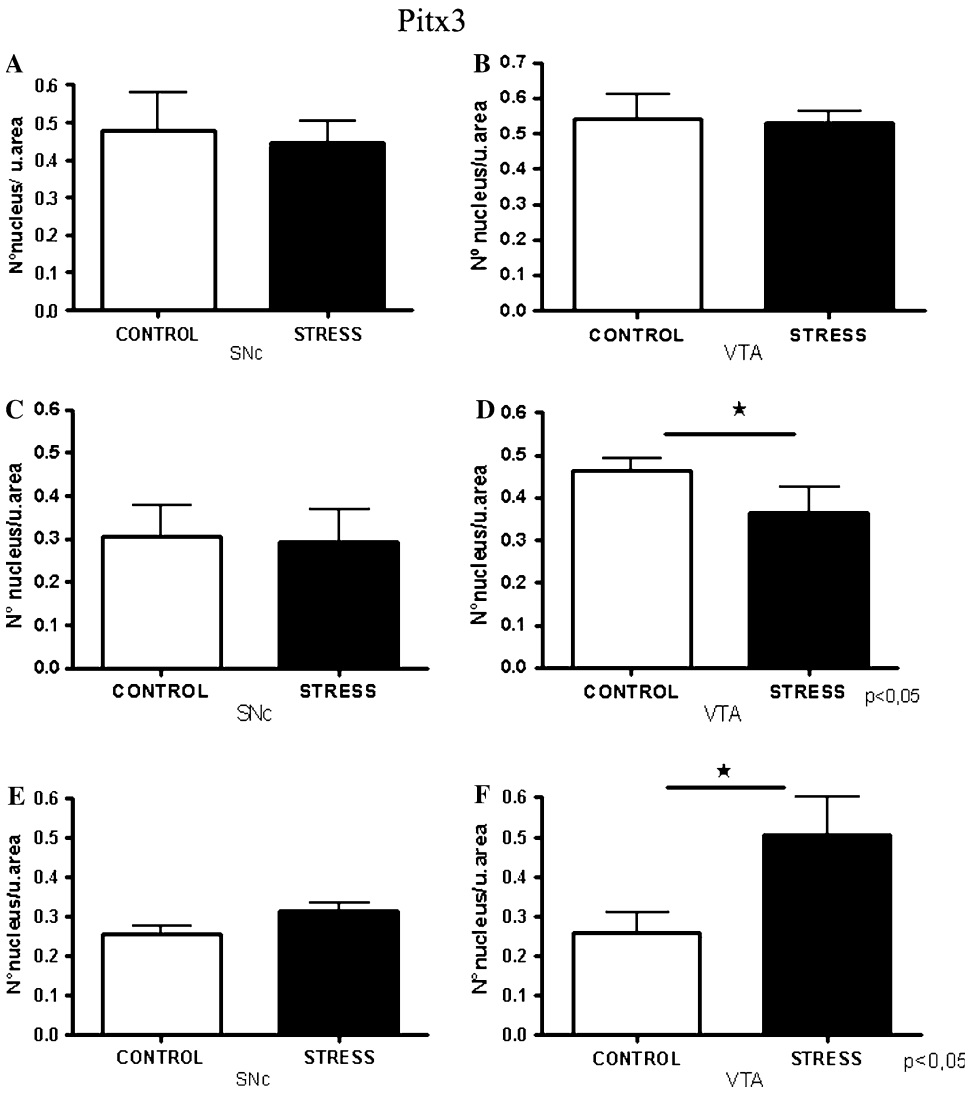

Fig. 4 Quantitative analysis of the number of nucleus per unit area of Pitx3-ir neurons in SNc (**a**, **c**, **e**) and VTA (**b**, **d**, **f**), both in control and PS rats at PND 7 (**a**, **b**), PND 28 (**c**, **d**), and PND 60 (**e**, **f**). Values are reported as mean ± SEM. * $P < 0.05$. *SNc* substantia nigra compacta, *VTA* ventral tegmental area

2006). However the role of Pitx3 in adult is still not clear. The inverted shapes of the control curves of Pitx3 and Nurr1 in this study might be supporting the notion that Pitx3 has a prominent role at early stages in the postnatal development of the mdDA system whereas Nurr1 plays a crucial role in adulthood probably in the maintenance of dopaminergic metabolism through the regulation of the expression of its key enzymes and transporters. Interestingly, a recent report demonstrate that although traditionally assumed to be independent (Smidt et al. 1997; Chung et al. 2005), the developmental pathways of both factors are interconnected (Jacobs et al. 2009). These authors show that Pitx3 modulates the Nurr1 transcriptional complex by decreasing the interaction with the co-repressor SMRT, which acts through histone deacetylases to keep promoters in a repressed deacetylated state.

In the PS rats, Pitx3 expression shows a decrease at PND 28 in VTA to further increase at PND 60 when compared to control offspring. This can be interpreted that the pubertal period might be exerting important challenges in parallel to the gonadal hormones surge, typical of this period. It has been reported that several behavioral and biochemical alterations exerted by PS were seen only after puberty (Henry et al. 1995; Diaz et al. 1997). Previous work from our laboratory (Barros et al. 2002) showed that DA receptor over expression seen in prenatally adult rat brains was not observed before puberty and more recently the amphetamine-induced DA decreased release in PFC of PS rats was observed in PND 60 and not in PND 28 (Katunar et al. 2008). As suggested by Reznikov et al. (2001), the hormone-neurotransmitter imprinting of the neuroendocrine system may be disrupted in offspring that suffered a severe in utero stress. Although not as evident, but Nurr1 expression in the PS rats also shows a decline at PND 28 that recovers after puberty, probably also indicating an altered vulnerability to gonadal hormones.

In control rats, TH immunoreactivity showed an age-related decrease which was already seen to be accentuated

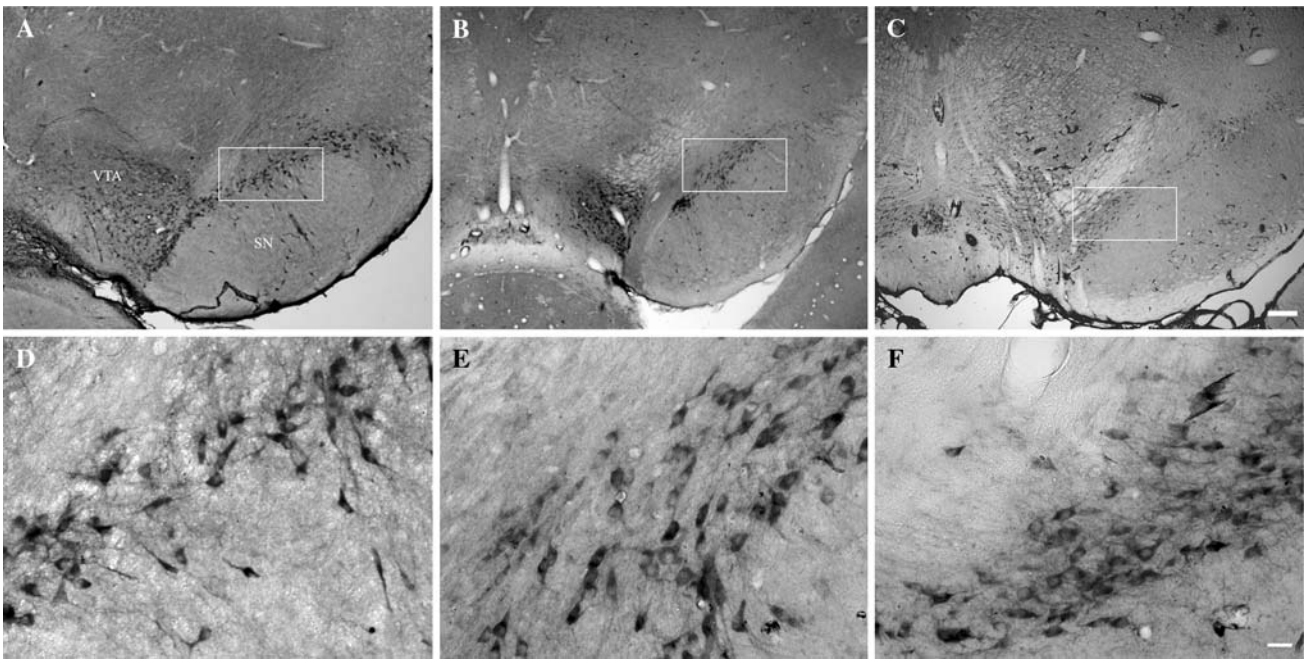

Fig. 5 Photomicrographs showing immunostaining with anti-TH antibody in control mesencephalic structures at PNDs 7, 28, and 60. **a–c** Low magnification micrographs showing full view of SNc and VTA in the left hemisphere of a coronal section. *Scale bar* = 300 μm. **d–f** Higher magnification of the boxed areas in **a–c**, showing TH-ir neurons of the SNc. *Scale bar* = 100 μm. *SNc* substantia nigra compacta

in older to senile rats (Sanchez et al. 2008). In PS rats, a significant decrease was observed at PND 7, but the number of TH positive cells were similar to control group at PND 28 and maintained till PND 60 both in VTA and SNc. The age-related profile of TH expression in control rats shows almost a specular image with the PS profile.

The mechanism by which stress exerted to the mother might be disrupting the normal expression of TH and essential transcription factors of the DA metabolism is still unknown, but it is interesting to point out that the embryonic expression of Nurr1 is initiated at E 10.5 in ventral midbrain and other areas of the developing mouse embryo (Volpicelli et al. 2007), Pitx3 starts later at E11.5 and TH expression in classical dopaminergic groups also starts at 11.5 (Smidt et al. 2003). All these proteins initiate their expression close to the time when the restraint stress is applied to the mother at E14 through E21. There is nowadays a large consensus that the stress-induced maternal increase of glucocorticoids is the main factor that influences fetal brain development (Maccari et al. 2003; Weinstock 2008). Therefore, it is tempting to hypothesize that corticosterone or CRH might be the factors directly affecting the normal embryonic development of TH and the transcription factors in the developing embryos. In fact, it has long been known that glucocorticoid receptor stimulation induces TH gene expression in the peripheral nervous system (Otten and Thoenen 1976), but more recently, Hagerty et al. (2001) have reported the identification of functional glucocorticoid response element in the promoter region of the mouse TH gene. Moreover, McArthur et al. (2005) have shown that perinatal glucocorticoid exposure permanently alters the mesencephalic dopaminergic population in adult rats and produces changes in TH positive cells. However, these authors found an increase of TH immunopositive cells in VTA and SNc in male adult rats. Differences between the accessibility and concentration of endogenous corticosterone induced by stress versus the complex pharmacokynetic of exogenous dexamethasone administered via the maternal drinking water may account for the differences in TH response observed. Alternatively, other effectors might be regulating TH expression such as the nitrergic system. In this regard, Balda et al. (2009) show that neural nitric oxide synthase (nNOS) knockout mice express lower levels of TH-immunoreactive neurons in the VTA area. Since, nNOS is known to be down-regulated by glucocorticoids (Lopez-Figueroa et al. 1998; Schwarz et al. 1998), corticosterone down-regulated nNOS might be inhibiting TH in the early perinatal period.

At the postnatal level, TH expression in the PS rats is low at PND 7 but recovers to normal values in adulthood. Since Nurr1 is known to regulate the expression of TH in adult stages, the sustained increase of Nurr1 in the PS rat might be the mechanism by which TH recovers its normal values in adult PS rats. However, previous work from our laboratory showed that DA levels were decreased at PND 60 in PFC of PS rats upon challenge with amphetamine

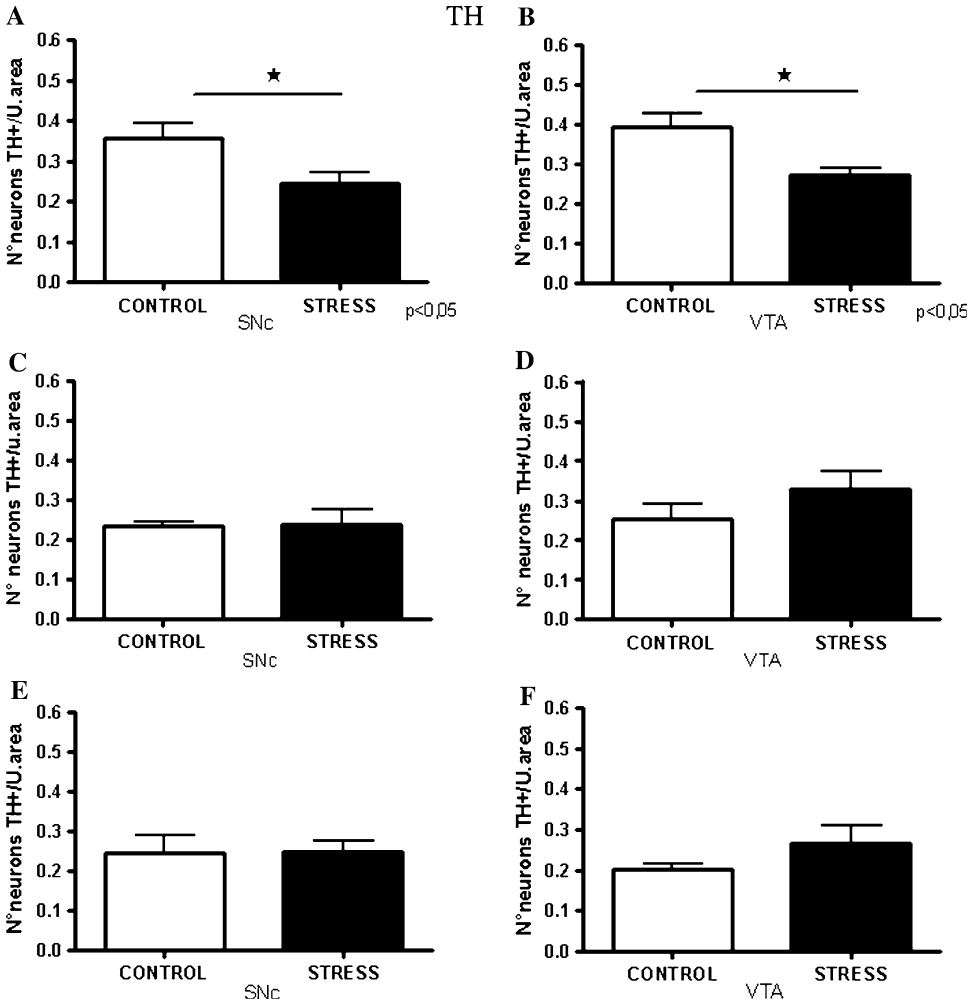

Fig. 6 Quantitative analysis of the number of neurons per unit area of TH-ir in SNc (**a**, **c**, **e**) and VTA (**b**, **d**, **f**), both in control and PS rats at PND 7 (**a**, **b**), PND 28 (**c**, **d**), PND 60 (**e**, **f**). Values are reported as mean ± SEM. * $P < 0.05$. *SNc* substantia nigra compacta, *VTA* ventral tegmental area

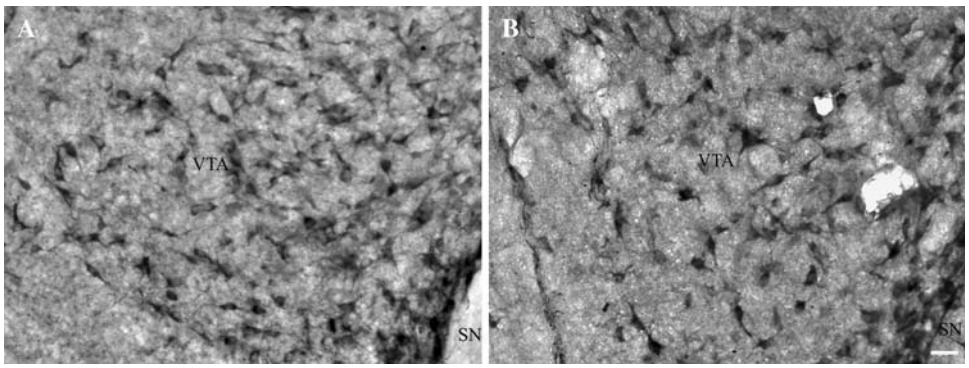

Fig. 7 Photomicrographs showing immunostaining with anti-TH antibody at PND 7 in VTA in control (**a**) and PS (**b**). *Scale bar* = 50 μm. *VTA* ventral tegmental area

(Katunar et al. 2008). DA levels in the PFC are in synaptic terminals of neurons arising in the VTA and are directly depending on TH levels in these neurons (Del Arco and Mora 2009). Since TH is at normal levels in these neurons in spite of the impaired DA levels in PS rats, we speculate that other steps in the DA synthesis and storage metabolism is impaired such as DA transporters VMAT2 or DAT or the degradation machinery (MAO, COMT) (Guillot and Miller 2009) that still justify the decreased levels of DA in cortical structures of adult PS rats.

In a previous paper (Katunar et al. 2009), we discussed the area selectivity of the DA receptors and transcription factors impairment in adult PS rats toward the mesocorticolimbic pathway as opposed to the nigrostriatal system. In the present article, we extend these results by showing that the TFs selectivity toward VTA spans from early-life to adult ages.

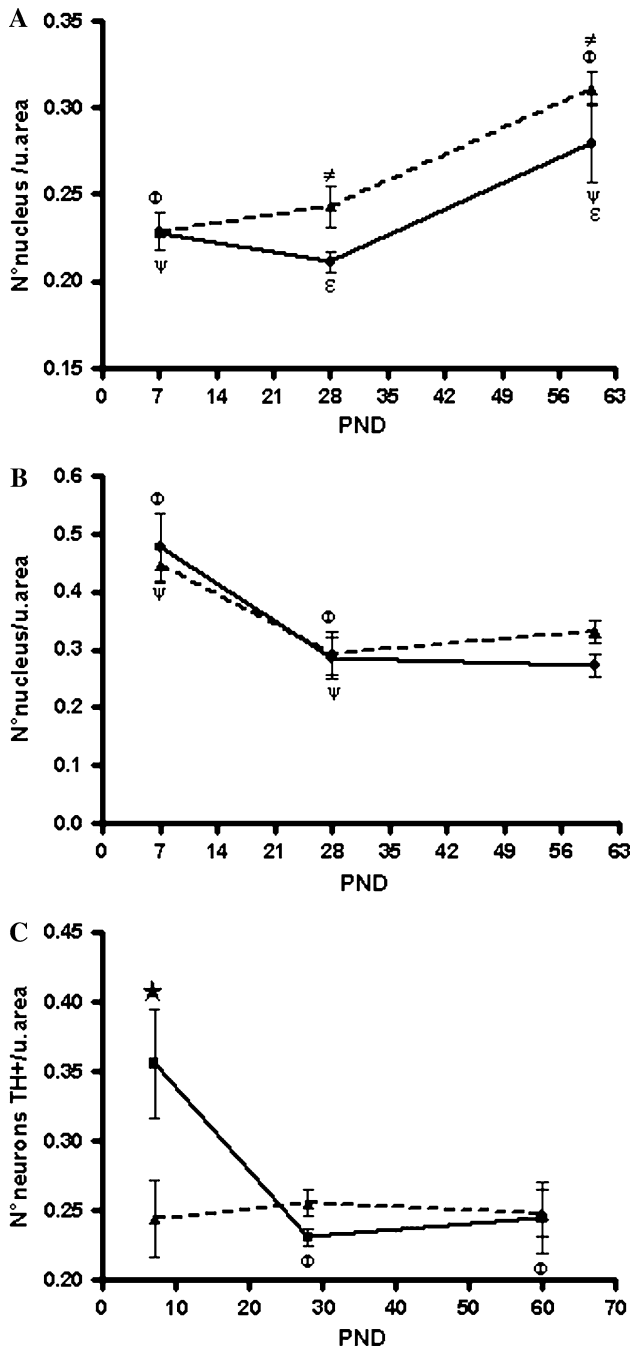

Fig. 8 Time-course of immunocytochemical expression of Nurr1 (**a**), Pitx3 (**b**), and TH (**c**) in control and PS rats in SNc. Values are reported as mean ± SEM. **a** *Control curve*: $^{\psi}$ $P < 0.05$ PND 7 vs. PND 60; $^{\varepsilon}$ $P < 0.05$ PND 28 vs. PND 60. *PS curve*: $^{\Phi}$ $P < 0.05$ PND 7 vs. PND 60; $^{\neq}$ $P < 0.05$ PND 28 vs. PND 60. **b** *Control curve*: $^{\Phi}$ $P < 0.05$ PND 7 vs. PND 28. *PS curve*: $^{\psi}$ $P < 0.05$ PND 7 vs. PND 28. **c** * $P < 0.05$ C vs. PS at PND 7. *Control curve*: $^{\Phi}$ $P < 0.05$ PND 28 vs. PND 60. *SNc* substantia nigra compacta. *Solid line*: C, *dotted line*: PS

The VTA projects to limbic areas like NAc septi, lateral septal nuclei of the basal forebrain, amygdala, hippocampus, entorhinal cortex, and prefrontal neocortex (Schultz 1997).

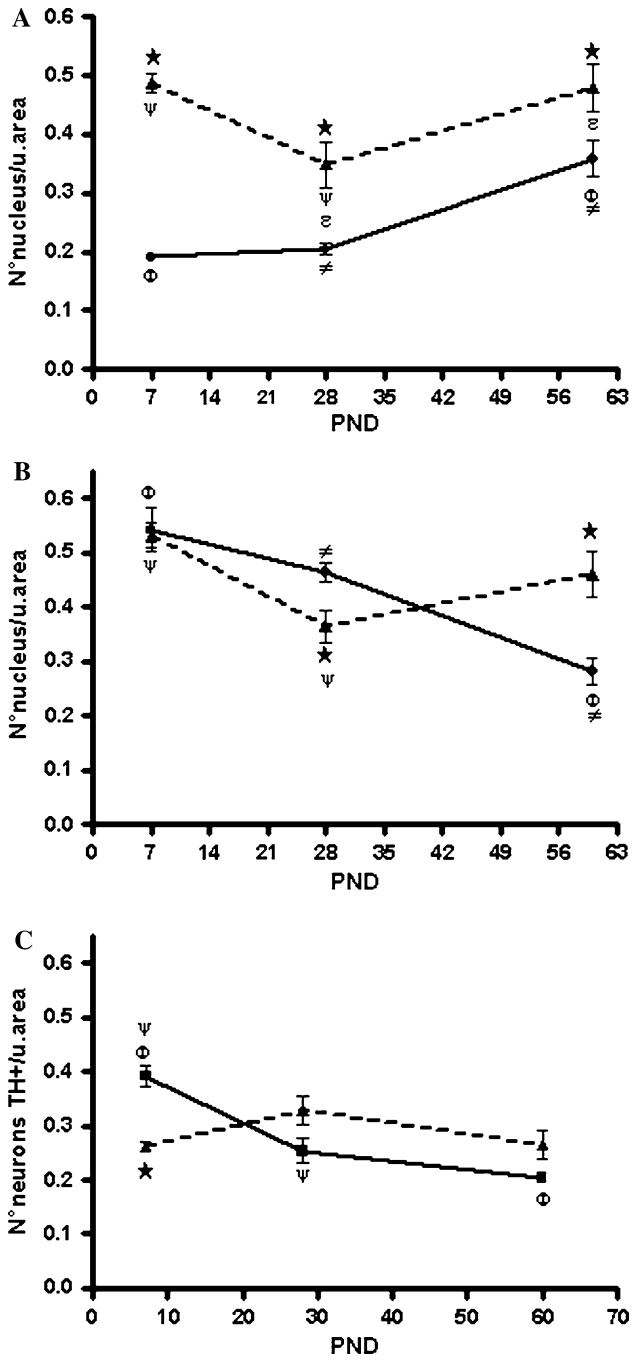

Fig. 9 Time-course of immunocytochemical expression of Nurr1 (**a**), Pitx3 (**b**), and TH (**c**) in control and PS rats in VTA. Values are reported as mean ± SEM. **a** *$P < 0.05$ C vs. PS at PNDs 7, 28, and 60. *Control curve*: $^{\Phi}$ $P < 0.01$ PND 7 vs. PND 60; $^{\neq}$ $P < 0.01$ PND 28 vs. PND 60. *PS curve*: $^{\psi}$ $P < 0.05$ PND 7 vs. PND 28. $^{\varepsilon}$ $P < 0.05$ PND 28 vs. PND 60. **b** * $P < 0.05$ C vs. PS at PND 28. *$P < 0.05$ C vs. PS at PND 60. *Control curve*: $^{\Phi}$ $P < 0.05$ PND 7 vs. PND 60; $^{\neq}$ $P < 0.05$ PND 28 vs. PND 60. *PS curve*: $^{\psi}$ $P < 0.05$ PND 7 vs. PND 28. **c** * $P < 0.01$ C vs. PS at PND 7. *Control curve*: $^{\Phi}$ $P < 0.01$ PND 7 vs. PND 60. $^{\psi}$ $P < 0.05$ PND 7 vs. PND 28. *VTA* ventral tegmental area. *Solid line*: C, *dotted line*: PS

This mesolimbic pathway is associated to motivational, emotional, and reward behaviors, that when disrupted give rise to psychiatric disorders such as schizophrenia. The mesolimbic selectivity in the impairment of TFs after the prenatal insult reported here is in agreement with the neurodevelopmental origin of various psychiatric disorders proposed by various authors (Lewis and Levitt 2002; Boksa and El-Khodor 2003). Conversely, there is no area selectivity of TH impairment. However, in both VTA and SN areas, the decrease is only evident at PND 7 and it levels off at older ages.

As mentioned before, the cell density of each area analyzed was calculated by carefully matching control and PS sections in the same anteroposterior plane to avoid differences in cell distribution along the rostrocaudal axis. Considering the heterogeneous structure of VTA and SN our future work will aim to analyse the nucleus volume in order to calculate the absolute number of cells in case PS could exert changes in the number of cells, distribution and volume of VTA and SN as observed with prenatal dexamethasone treatment (McArthur et al. 2007). This might not be the case in our model of PS since McClure et al. (2004) have shown that mild stress applied to pregnant dams reduces the volume of NAc in the offspring but the cell density is not changed with treatment.

In summary, stress imposed to the pregnant mother exerts an early-life impairment of the regulatory enzyme of DA synthesis and permanent changes in key factors of the formation and maintenance of DA pathways along the life of the offspring. The changes are selectively found mainly in the VTA area of the mesencephalon, indicating a major vulnerability to eventually suffer cognitive, reward, and motivational disorders. Considering these and previous results, we speculate that other steps in the dopaminergic metabolism still need to be explored such as key steps of the DA uptake or catabolism that might shed light to the mechanism by which prenatal insults exert such dramatic alterations in the dopaminergic metabolism of the mesocorticolimbic pathway.

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