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PRENATAL RESTRAINT STRESS: AN *IN VIVO* MICRODIALYSIS STUDY ON CATECHOLAMINE RELEASE IN THE RAT PREFRONTAL CORTEX

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Abstract—There is substantial evidence that prenatal exposure to adverse environmental conditions might lead to the psychiatric disorders that can appear in adolescence or in adulthood; vulnerability to drug addiction may increase as well. It is currently accepted that the alteration of catecholamine transmission in the prefrontal cortex plays a prominent role in the etiology of psychiatric disorders. We assessed basal and stimulated dopamine and noradrenaline extracellular concentration in the medial prefrontal cortex by means of microdialysis in awake male adolescent and young adult offspring of rats exposed to restraint stress in the last week of pregnancy. Catecholamine stimulation was obtained by amphetamine or nicotine. We observed that prenatal stress (PNS) did not change dopamine but decreased noradrenaline basal output in both adolescents and adults. Moreover, it decreased amphetamine stimulated dopamine output and increased amphetamine stimulated noradrenaline output. PNS decreased nicotine stimulated noradrenaline (but not dopamine output) in adults, though not in adolescents. These data show that PNS stress modifies prefrontal cortex catecholamine transmission in a complex and age dependent manner. Our results support the view that prenatal stress may be a contributing factor for the development of psychiatric disorders and that its effect may augment drug addiction vulnerability.

Key words: adolescence, amphetamine, dopamine, noradrenaline, nicotine.

It has been widely reported that prenatal stress (PNS) may considerably affect neurobiological development, leading to physical and behavioral abnormalities such as reduced birth weight, altered locomotion, infant retardation, anxiety and sleep disturbances, also in addition to a higher incidence of psychiatric disorders (Weinstock, 2001;Kofman, 2002; Huizink et al., 2004). In particular, adverse life events during pregnancy may be linked to the manifestation of attention deficit with hyperactivity disorder (ADHD), (Clements, 1992; McIntosh et al, 1995; Linnet et al., 2003; Rodriguez and Bohlin, 2005; Grizenko et al., 2008), schizophrenia (Weinstock, 2001; King et al., 2005), depression (Watson et al., 1999; Brown et al., 2000), or Tourette's syndrome (Leckman et al., 1990), although a clear cause-effect relationship has yet to be established because of the diversity of adverse events.

On the other hand, animal studies allow us to identify PNS effects on brain regions, hormones and neuronal circuits, the alteration of which may be the basis for a general susceptibility to psychopathology in humans (as reviewed by Weinstock, 2001, 2008; Kofman, 2002; Huizink et al., 2004). Among brain areas, the prefrontal cortex (PFC) is considered crucial for the expression of the highest-order cognitive abilities but also the most sensitive to the detrimental effects of exposure to stress (Arnsten, 2009). On these grounds, it is conceivable that PNS-related psychiatric disorders might occur as a result of the dysfunctions of catecholamine transmission in the PFC, but this is an issue that requires further investigation.

Early, Fride and Weinstock (1988) reported that PNS elevated DA metabolism in the right PFC, and so altered brain asymmetry. They suggested that these changes may underlie the increased reactivity to anxiety-inducing situations. Later it was found that PNS reduced the concentration of NA and increased that of metabolites in the cerebral cortex and locus coeruleus (LC) of adult rats (Takahashi et al., 1992). A more recent report showed that PNS did not affect NA but reduced DA metabolism in the PFC of 2-month old male rats (Bowman et al., 2004). Moreover, as reported by Berger et al. (2002), PNS increased DA D2-type and glutamate receptors in the PFC. Besides the effects on receptors, catecholamine content and metabolism, PNS can change spine density and dendritic complexity in the prefrontal cortex, in a sex-specific manner (Murmu et al., 2006). There is abundant evidence of an overactive and dysregulated hypothalamic-pituitary adrenal (HPA) axis in PNS animals (Weinstock, 2001; Huizink et al., 2004) and recently it has been posited that there is a strict interaction between the HPA axis and PFC functioning (Radley et al., 2006, 2008). In particular, it has been suggested that the LC may act as an upstream component of a circuit involved in the PFC modulation of HPA responses to acute emotional stress (Radley et al., 2008). Consequently, PFC functions such as working memory and attention may be affected by an altered HPA axis response to stress as a result of altered catecholamine transmission in the PFC.

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Abbreviations: DA, dopamine; HPA, hypothalamic–pituitary adrenal; LC, locus coeruleus; NA, noradrenaline; NAcc, nucleus accumbes; nAChRs, nicotinic acetylcholine receptors; PFC, prefrontal cortex; PNS, prenatal stress; VTA, ventral tegmental area.

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These observations, together with the fact that major psychiatric diseases such as depression, schizophrenia, and ADHD rely on drugs that affect DA and NA transmission, strongly suggest that the evaluation of PNS effects on DA and NA transmission in the PFC may be relevant to understanding its possible role in the aetiology of psychiatric disorders.

The aim of the present study, carried out by microdialysis in freely moving rats, was to ascertain whether PNS alters basal and stimulated DA and NA transmission. Although representative of synaptic concentration, basal release alone may not bring about changes at neuronal level because of the ability of brain circuitry to maintain transmission homeostasis even in the presence of substantial deficits (Bongiovanni and See, 2008). We evaluated whether or not DA and NA extra-cellular concentration (output) in the PFC of PNS rats and controls were differentially affected by amphetamine and by nicotine, two drugs that release DA and NA by impulse independent and dependent mechanisms, respectively. In particular, amphetamine can increase DA and NA output by acting at terminal level by means of a complex mechanism. It primarily involves DA re-uptake and vesicular monoamine transport but also synthesis and degradation (Sulzer et al., 2005). Nicotine produces its reinforcing properties by acting through excitatory nicotinic acetylcholine receptors (nAChRs) at the ventral tegmental area (VTA) level. At this point, nicotine exerts a stimulation of mesocorticolimbic DA neurons with an increase of DA release in the nucleus accumbens (NAcc) shell and in the PFC (reviewed by Markou, 2008). Moreover, nAChRs located in different cells in the PFC, may mediate a cholinergic role in cognition and in particular in attention (reviewed by Poorthuis et al., 2009).

The results of this investigation may therefore provide useful information on the role of PNS in vulnerability to psychostimulants and nicotine abuse but also on catecholamine-nicotine interaction in the PFC. Finally, in order to clarify the role of PNS in the neurobiological changes that occur in the PFC during adolescence, we investigated adolescent and young adult rats. These results, together with those of a parallel investigation of PNS effects in the NAcc (Silvagni et al., 2008), may well provide us with useful information on the importance of age as a factor in the incidence of psychiatric diseases such as ADHD or schizophrenia (Adriani and Laviola, 2004).

EXPERIMENTAL PROCEDURES

Animals

In order to obtain a homogeneous rat population from a standard mating procedure, pregnant Wistar rats weighing 250 g were acquired from Harlan [S. Pietro al Natisone (UD) Italy]. Females were shipped in the first week of pregnancy and housed under standard conditions of temperature and humidity under artificial light (light from 8:00 to 20:00 h) up to the 14th day of pregnancy. These results were generated in three different experimental sessions by stressing a total of 24 pregnant rats that generated 4.17±1.58 males per rat. Twenty-four rats used as a control group generated 3.92±1.66 males per rat (mean±stand. dev.).

PNS procedures

The stress procedure used (Maccari et al., 1995) was as follows. Pregnant female dams were randomly assigned to control or prenatal stress group. The control group was left undisturbed in the home cage; the PNS group was transferred to an experimental room where the selected stress paradigm was applied. Pregnant females were placed individually in Plexiglas, transparent cylindrical restrainers (internal diameter 6 cm) of adjustable length (15–18 cm) fitted closely to body size for three periods (45 min each) per day (9:00, 12:00, 17:00 h) between the 14th and the 21st (included) day of pregnancy. This type of stress [described originally by Ward and Weisz (1984)] was chosen because it influences the fetus indirectly via direct stress on the mother. The stress sessions were performed in a lighted environment with no other subjects in the room. The animals were then individually housed with *ad libitum* access to food and water in their animal housing room.

On the day of parturition, litters were culled to eight pups, maintaining equal numbers of males and females, whenever possible. No differences in litter sizes were found between stressed and non stressed animals. Control or PNS pups were maintained with their biological mother. The offspring were weaned 21 days after birth, and only male offspring were selected for microdialysis studies. A maximum of five male pups were placed in each cage and left undisturbed until they became adolescent (30–35 postnatal days) or young adult (56–63 postnatal days) rats. We included only one or two male pups from each litter in each experimental group to prevent litter effects.

Probe preparation

Concentric dialysis probes (dialyzing portion=3 mm) were prepared with an AN 69 (sodium methallyl sulphate copolymer) dialysis fiber (310 μm outer diameter 220 μm inner diameter; Hospal, Dasco, Italy) as previously reported (Silvagni et al., 2008).

Surgery and experiments

The rats were anesthetized with 100 mg/kg i.p. ketamine (Ketalar; Farmaceutici Gellini, Milan Italy) and 10 mg/kg i.p. xylazine (Sigma, Milano, Italy) and placed in a stereotaxic apparatus. A small hole was drilled on one side of the exposed skull. The probe was implanted vertically in the right medial PFC and then fixed on the skull with dental cement (Shofu CX-plus, GmgH, Ratingen, Germany). The coordinates used [expressed in millimeters from bregma, according to Paxinos and Watson's atlas (2007)] were: anterior 2.8, lateral 0.6 and vertical 3.5, and anterior 3.5, lateral 0.8 and vertical four, for adolescent and adult rats, respectively. Fig. 1 shows the area of implant in a series of coronal sections at the PFC level according to Paxinos and Watson's atlas (2007). Vertical lines have been drawn in each section to reproduce the dialyzing part of the fiber as observed during histological examination. The rats were housed in a transparent (Plexiglas) hemisphere, covered with a top hemisphere, with food and water available.

Experiments were performed in freely moving rats 24 h after the probe implant. Ringer's solution (147 mM NaCl; 2.2 mM $CaCl_2$; 4 mM KCl) was pumped through the dialysis probe at a constant rate of 1 μL/min. Samples were taken and analyzed every 20 min. In each experiment, balanced numbers of PNS and control rats were used. When the basal output of NA and DA reached stable values, rats were given a challenge dose of s.c. amphetamine [0.25 mg/kg (free base)] or nicotine [0.4 mg/kg (free base)] or saline. The NA or the DA output was considered stable when the quantity evaluated through the last sample differed less than 10% from the mean of the last three samples. Stable levels of DA and NA were obtained after 2–3 h of dialysis. Each implanted rat was challenged with a single dose of the test drug only

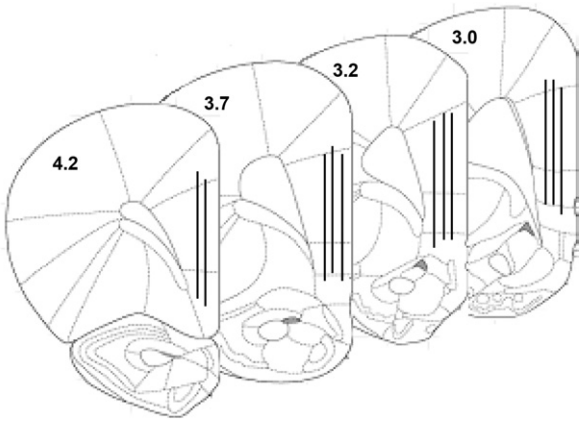

Fig. 1. Schematic representation of the area investigated by a succession of coronal section at the prefrontal cortex level. Vertical lines have been drawn in each section representing some of the traces left by the fibers implanted, as observed by histological examination. The number in each section represents the anteriority from bregma according to Paxinos and Watson's atlas (2007).

once. All animal experimentation was conducted in accordance with the guidelines for care and use of experimental animals in the European Communities Council Directive of 24 November 1986 (86/609/EEC and Italian DL 116 dated 27/01/1992), and approved by the "Comitato Etico, Università di Cagliari."

Analytical procedure

Dialysate samples (20 μL) were injected with no purification into high performance liquid chromatography apparatus equipped with reverse-phase column (C-8 Simmetry, Waters) and a coulometric detector (first electrode +125 mV; second electrode −175 mV (ESA Coulochem II, Bedford, MA, USA)). The mobile phase composition was 0.1 M sodium acetate, 0.3 mM Na_2EDTA, 1.8 mM octanesulfonic acid, 120 ml/L methanol (pH 5.4). The flow rate was set at 0.6 mL/min. The sensitivity of the assay allowed for the detection of 5 fmoles of NA and DA.

Histology

Histological analysis was performed in order to locate the position of the fiber. At the end of the experiment, rats were anaesthetized with chloral hydrate (450 mg/kg i.p.) and killed. After the probes were taken out, the brain was removed and stocked in formaldehyde (10%). Brains were cut on an oscillating microtome (Campden Instuments, Lafayette, IN, USA) in serial coronal slices oriented according to Paxinos and Watson's rat brain atlas (2007). Results from rats implanted outside the PFC were discarded.

Drugs

D-amphetamine sulfate (Sigma, by SALARS, Como, Italy), and (−)-nicotine hydrogen tartrate salt (Sigma, Milano, Italy) were dissolved in saline and injected immediately.

Statistics

Statistical analysis was carried out by STATISTICA (Statsoft, Tulsa OK, USA). Two-way, three-way or four-way analysis of variance (ANOVA) for repeated measures was applied to the data expressed either as a percentage of basal DA or NA concentration. Considering that basal NA levels in PNS rats were significantly lower than controls, the effect of both amphetamine and nicotine on NA output was analyzed and reported in absolute fmoles as well (Panels C and D in figs. 4 and 6). Results from treatments showing significant overall changes were subjected to *a post hoc* Tukey test with significance for $P<0.05$. Basal value was the mean of the last three consecutive samples before treatment.

RESULTS

Basal output of DA and NA

Fig. 2A shows that PNS did not affect basal DA output in either age groups. Basal output of DA (expressed in fmol/20 μL sample, ±SE) in PNS adolescent (19.94±1.43, $n=21$) and PNS adult (22.17±2.09 $n=20$) were not significantly different from those of relative controls (20.84±1.04, $n=21$ and 21.72±1.34 $n=20$, respectively). Two-way ANOVA of DA estimation showed a non significant age ($F_{1,78}=1.38$, $P=0.24$) and PNS effects ($F_{1,78}=0.02$, $P=0.89$).

Fig. 2B shows that basal NA output was lower in both PNS exposed age groups. Basal output of NA (expressed in fmol/20 μL sample, ± SE) in PNS adolescent (22.58±1.47, $n=21$) and in PNS adults (21.63±1.15, $n=20$) were significantly lower than those of relative controls (28.21±1.53, $n=21$ and 31.11±1.7, $n=20$, respectively). Two-way ANOVA of NA estimation showed a significant PNS ($F_{1,78}=26.08$, $P<0.001$) but not age effect ($F_{1,78}=0.41$, $P=0.52$). Post-hoc analysis (Tukey) showed that NA basal output was significantly lower in adolescent and in adult PNS rats as compared with relative controls (adolescents: $P<0.05$; adults: $P<0.005$).

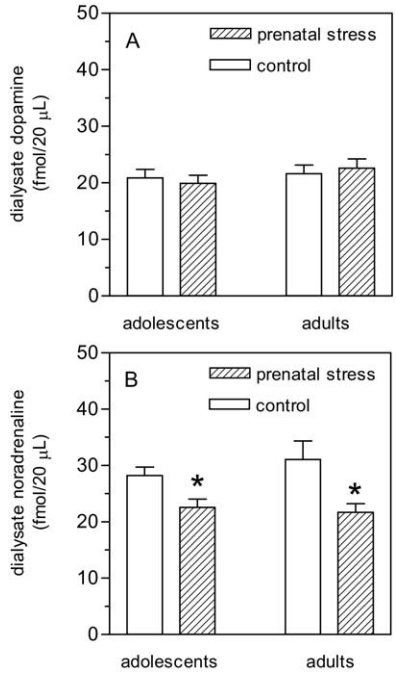

Fig. 2. Basal extracellular concentration of dopamine (panel A) and noradrenaline (panel B) in the medial prefrontal cortex of prenatal stress and control rats. Each column is the mean (±SE) of 21 determinations for each of the two adolescent groups and 20 determinations for each of the two adult groups. * $P<0.05$ from relative control.

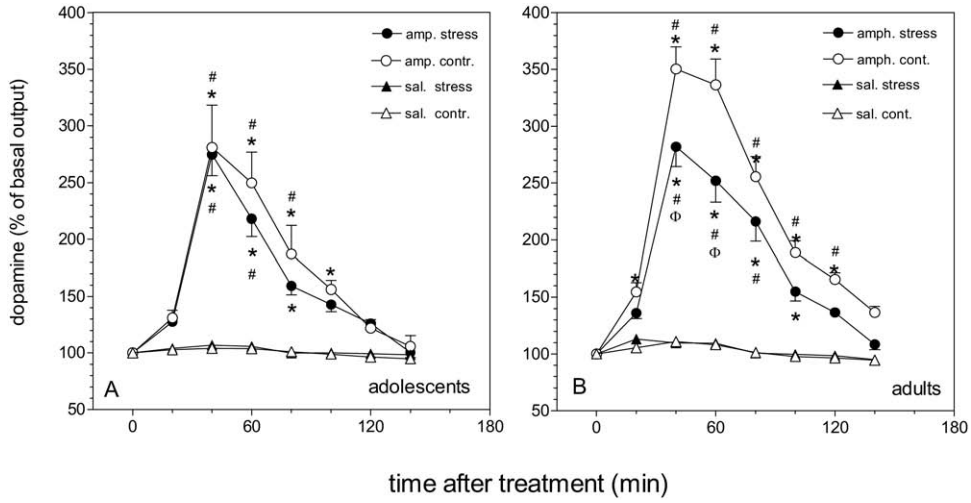

Fig. 3. Effect of amphetamine (0.25 mg/kg s.c.) and saline on dialysate dopamine expressed as a percentage of basal output from the medial prefrontal cortex of adolescent (A) and adult (B) prenatal stress and control rats. Each point is the mean (±SE) of at least five determinations. * $P<0.05$ from basal values; # $P<0.05$ from the corresponding time point of saline in the same experimental group. $^{\Phi}$ $P<0.05$ versus the corresponding time point of amphetamine in controls.

Effect of amphetamine on DA output

Percentage of basal level. Fig. 3A, B show that PNS decreased the amphetamine effect on DA output in adults but not in adolescents. Overall analysis (four-way ANOVA) of amphetamine treatment results showed a significant age effect ($F_{1,46}=13.10$, $P<0.001$), PNS effect ($F_{1,46}=7.31$, $P<0.05$), treatment effect ($F_{1,46}=282.67$, $P<0.001$), time effect ($F_{7,322}=98.86$, $P<0.001$), age×treatment interaction ($F_{1,46}=11.15$, $P<0.005$), PNS×treatment interaction ($F_{1,46}=8.52$, $P<0.01$) but not age×PNS interaction ($F_{1,46}=2.30$, $P=0.13$). Amphetamine (0.25 mg/kg s.c.) maximally increased DA output by 174% and by 181% above basal, in adolescent PNS and control rats respectively, as recorded 40 min after treatment (Fig. 3A). Three-way ANOVA of the results obtained showed a non significant PNS effect ($F_{1,22}=0.73$, $P=0.4$), a significant treatment effect ($F_{1,22}=88.84$, $P<0.001$), a significant time effect ($F_{7,154}=42.34$, $P<0.001$) and a treatment×time interaction ($F_{7,154}=35.92$, $P<0.001$). Fig. 3B shows that amphetamine (0.25 mg/kg s.c.) maximally increased DA output by 182% and by 250% above basal in adult PNS and control rats, respectively, as recorded 40 min after treatment. Three-way ANOVA of the results obtained showed a significant PNS effect ($F_{1,24}=8.73$, $P<0.01$), treatment effect ($F_{1,24}=209.63$, $P<0.001$), time effect ($F_{7,168}=58.67$, $P<0.001$), stress×treatment interaction ($F_{1,24}=10.17$, $P<0.005$) and treatment×time interaction ($F_{7,168}=48.03$, $P<0.001$). Post-hoc analysis (Tukey) showed that dialysate DA was lower in adult PNS rats as compared with control adult rats at 40 and 60 min after amphetamine administration. The above reported results were not affected by DA basal levels.

Effect of amphetamine on NA output

Percentage of basal level. Fig. 4A, B show that PNS increased the amphetamine effect on NA output in adults but not in adolescents. Overall analysis (four-way ANOVA) of NA output after amphetamine treatment showed a significant age effect ($F_{1,46}=4.29$, $P<0.005$), PNS effect ($F_{1,46}=9.13$, $P<0.005$), treatment effect ($F_{1,46}=295.69$, $P<0.001$), time effect ($F_{7,322}=136.96$, $P<0.001$), age×treatment interaction ($F_{1,46}=4.15$ $P<0.05$), PNS×treatment interaction ($F_{1,46}=7.46$, $P<0.01$) but not age×PNS interaction ($F_{1,46}=2,77$, $P=0.10$). Amphetamine (0.25 mg/kg s.c.) maximally increased NA output by 223% and by 195% above basal in adolescent PNS and control rats, respectively, as recorded 40 min after treatment (Fig. 4A). Three-way ANOVA of the results represented in Fig. 4A showed a non significant PNS effect ($F_{1,22}=0.19$, $P=0.66$) but a significant treatment effect ($F_{1,22}=49.95$, $P<0.001$), time effect ($F_{7,154}=26.40$, $P<0.001$) and treatment×time interaction ($F_{7,154}=23.03$, $P<0.001$). Fig. 4B shows that amphetamine (0.25 mg/kg s.c.) maximally increased NA output by 314% and 201% above basal in adult PNS and control rats, respectively, as recorded 40 min after treatment. Three-way ANOVA of the results obtained showed a significant PNS effect ($F_{1,24}=6.81$, $P<0.05$), treatment effect ($F_{1,24}=172.56$, $P<0.001$), time effect ($F_{7,168}=100.34$, $P<0.001$), and stress×treatment×time interaction ($F_{7,168}=4.72$, $P<0.001$). Post-hoc analysis (Tukey) showed that NA output was significantly higher in adult PNS than in control rats in the time interval 40–60 min after amphetamine administration.

Absolute estimation. Fig. 4C, D show that the amphetamine (0.25 mg/kg s.c.) effect on NA output (expressed in fmol/20 μL) was not different in adult PNS rats as compared with controls whereas it was lower in PNS adolescent rats, as compared with controls, due to the lower basal output. Three-way ANOVA of the results represented in Fig. 4C showed a significant PNS effect ($F_{1,22}=5.08$, $P<0.05$), treatment effect ($F_{1,22}=28.35$, $P<0.001$), time effect ($F_{7,154}=30.41$, $P<0.001$) and

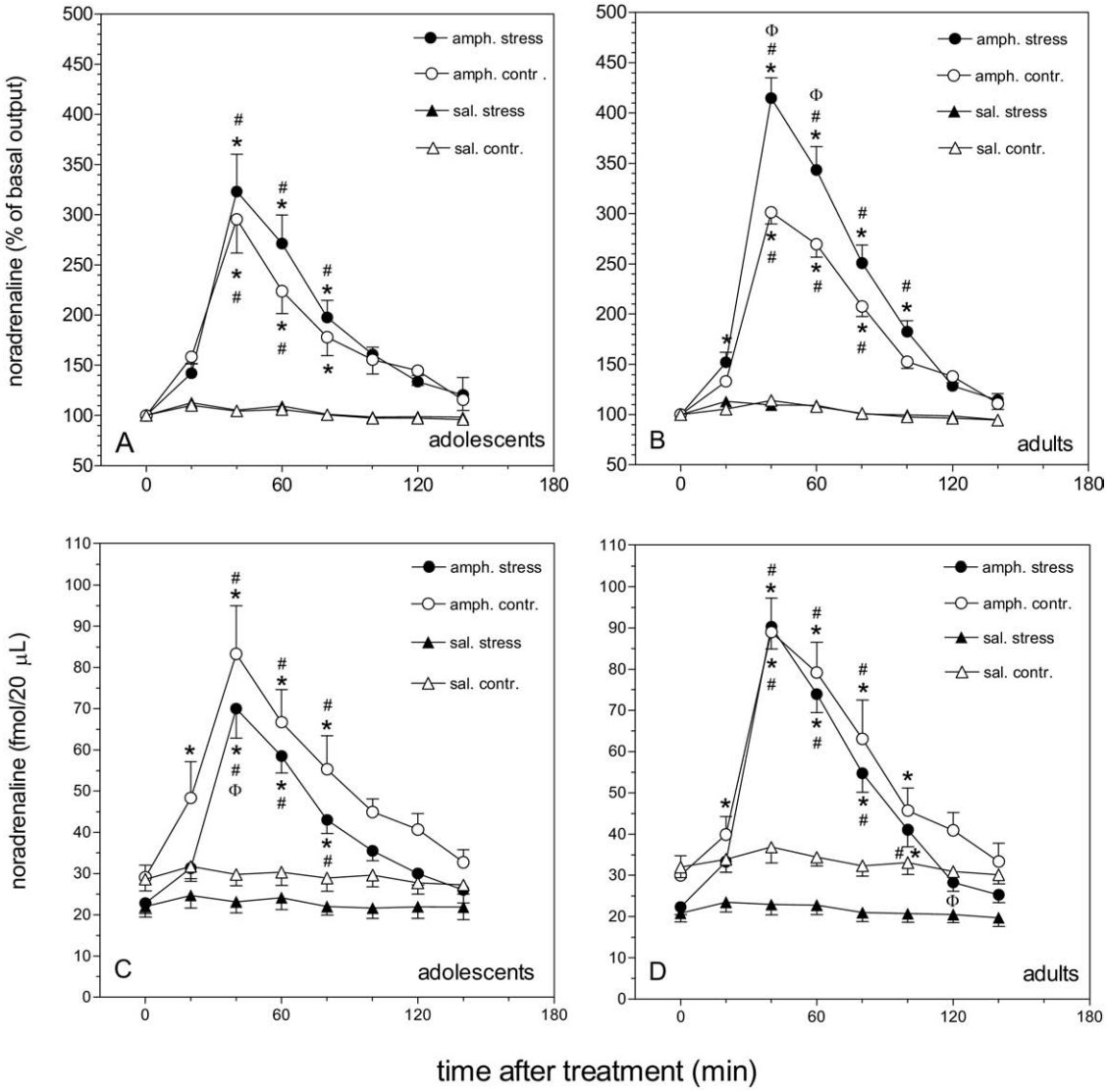

Fig. 4. Effect of amphetamine (0.25 mg/kg s.c.) and saline on dialysate noradrenaline expressed as a percentage of basal output (A, B), or fmol/sample (C, D) from the medial prefrontal cortex of adolescent (A, C) and adult (B, D) prenatal stress and control rats. Each point is the mean (±SE) of at least five determinations. * $P<0.05$ from basal values; # $P<0.05$ from the corresponding time point of saline in the same experimental group. $^{\Phi}$ $P<0.05$ versus the corresponding time point of amphetamine in controls.

treatment×time interaction ($F_{7,154}=26.47$, $P<0.001$). Fig. 4D shows the effect of amphetamine (0.25 mg/kg s.c.) or saline, on NA output (fmoles 20/μL) in adult control and PNS rats. Three-way ANOVA of the results obtained showed a significant PNS effect ($F_{1,24}=4.83$, $P<0.05$), treatment effect ($F_{1,24}=29.72$, $P<0.001$), time effect ($F_{7,168}=95.12$, $P<0.001$), and treatment×time interaction ($F_{7,168}=76.43$, $P<0.001$). Post-hoc analysis (Tukey) showed that NA output was significantly lower in adult PNS than in control rats 120 min after amphetamine administration.

Effect of nicotine on DA output

Percentage of basal level. Fig. 5A, B show that PNS did not change the effect of nicotine on DA output either in adults or adolescent rats. Overall analysis (four-way ANOVA) of DA output after nicotine treatment showed a significant age effect ($F_{1,40}=4.62$, $P<0.05$), treatment effect ($F_{1,40}=63.52$, $P<0.001$), time effect ($F_{7,280}=70.05$, $P<0.001$), and age×treatment×time interaction ($F_{7,280}=5.9$, $P<0.001$) and age×treatment×time interaction ($F_{7,280}=5.9$, $P<0.001$) but not PNS effect ($F_{1,40}=0.51$, $P=0.48$) and age×PNS effect interaction ($F_{1,40}=0.02$ $P=0.88$). Nicotine (0.4 mg/kg s.c.) maximally increased DA output by 69% and 50% above basal in adolescent PNS rats and control rats respectively, as recorded 40 min after the treatment (Fig. 5A). Three-way ANOVA of the results represented in Fig. 5A showed a non significant PNS effect ($F_{1,22}=0.46$, $P=0.50$) but a significant treatment effect ($F_{1,22}=28.23$, $P<0.001$), time effect ($F_{7,154}=39.25$, $P<0.001$), and PNS×treatment×time interaction ($F_{7,154}=2.18$, $P<0.05$). Fig. 5B shows that nicotine (0.4

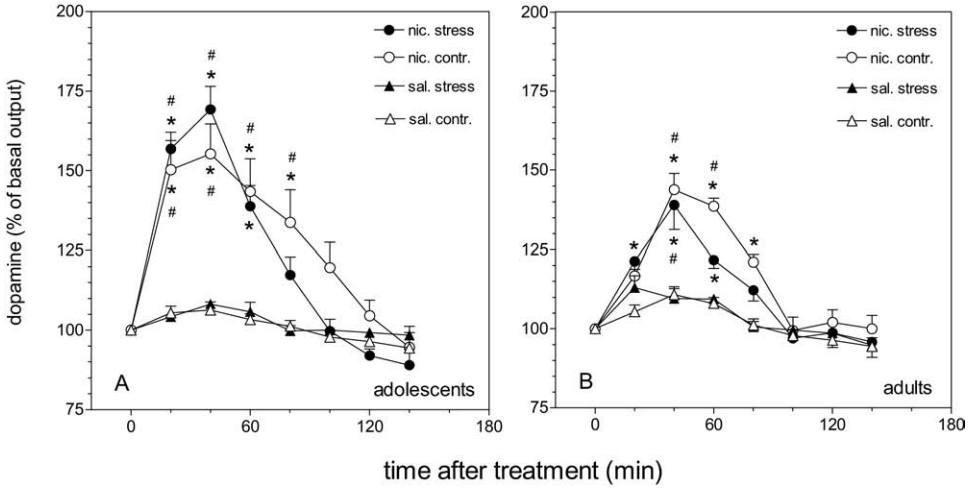

Fig. 5. Effect of nicotine (0.4 mg/kg s.c.) and saline on dialysate dopamine expressed as a percentage of basal output from the medial prefrontal cortex of adolescent (A) and adult (B) prenatal stress and control rats. Each point is the mean (±SE) of at least five determinations. * $P<0.05$ from basal values; # $P<0.05$ from the corresponding time point of saline in the same experimental group.

mg/kg s.c.) maximally increased DA output by 39% and 44% above basal in adult PNS rats and control rats respectively, as recorded 40 min after treatment. Three-way ANOVA of the results obtained showed a non significant PNS effect ($F_{1,18}=0.38$, $P=0.54$) but a significant treatment effect ($F_{1,18}=38.54$, $P<0.001$), time effect ($F_{7,126}=49.92$, $P<0.001$) and treatment×time interaction ($F_{7,126}=11.13$, $P<0.001$). The above reported results were not affected by DA basal levels.

Effect of nicotine on NA output

Percentage of basal level. Fig. 6A, B show that PNS decreased the effect of nicotine on NA output in adults but not in adolescent rats. Overall analysis (four-way ANOVA) of NA output after nicotine treatment showed a non significant age effect ($F_{1,40}=0.24$, $P=0.62$) and PNS effect ($F_{1,40}=0.03$, $P=0.87$), but a significant treatment effect ($F_{1,40}=34.42$ $P<0.001$), time effect ($F_{7,280}=71.28$, $P<0.001$), age×PNS effect×treatment interaction ($F_{1,40}=4.541$, $P<0.05$) and age×PNS effect×treatment×time interaction ($F_{7,280}=2.81$, $P<0.01$). Nicotine (0.4 mg/kg s.c.) maximally increased NA output by 90% and 72% above basal in adolescent PNS rats and control rats respectively, as recorded 20 min after treatment (Fig. 6A). Three-way ANOVA of the results represented in Fig. 6A showed a non significant PNS effect ($F_{1,22}=1.09$, $P<0.30$), but a significant treatment effect ($F_{1,22}=8.16$ $P<0.01$), time effect ($F_{7,154}=25.86$, $P<0.001$) and treatment×time interaction ($F_{7,154}=14.24$, $P<0.001$). Fig. 6B shows that nicotine (0.4 mg/kg s.c.) maximally increased NA output by 28% and 61% above basal in adult PNS rats and control rats, respectively, as recorded 20 min after treatment. Three-way ANOVA of the results obtained showed a significant PNS effect ($F_{1,18}=7.06$, $P<0.05$), treatment effect ($F_{1,18}=42.71$, $P<0.001$), time effect ($F_{7,126}=64.6$, $P<0.001$) and PNS stress×treatment×time interaction ($F_{7,126}=5.26$, $P<0.001$). Post-hoc analysis (Tukey) showed that NA output was lower in PNS than in control rats in the time interval 20–60 min after nicotine administration.

Absolute estimation. Fig. 6C shows that nicotine (0.4 mg/kg s.c.) administration determined a much lower increase of NA output (expressed in fmol/20 μL), in PNS rats as compared with control adult rats. The effect of nicotine was not different in adolescents rats (Fig. 6D). Three-way ANOVA of the results represented in Fig. 6C showed: a significant PNS effect ($F_{1,22}=4.77$, $P<0.05$), a non significant treatment effect ($F_{1,22}=0.47$, $P=0.49$), a significant time effect ($F_{7,154}=13.34$, $P<0.001$) and treatment×time interaction ($F_{7,154}=7.22$, $P<0.001$). Fig. 6D shows the effect of nicotine (0.4 mg/kg s.c.) or saline, on NA output (fmol/20 μL) in adult PNS and control rats. Three-way ANOVA of the results obtained showed: a significant PNS effect ($F_{1,18}=23.24$, $P<0.001$), a non significant treatment effect ($F_{1,18}=1.24$, $P=0.28$), a significant time effect ($F_{7,126}=44.34$, $P<0.001$) and PNS×treatment×time interaction ($F_{7,126}=8.15$, $P<0.001$). Post-hoc analysis (Tukey) showed that NA output was significantly lower in PNS than in control rats in the time interval 0–140 min after nicotine administration.

DISCUSSION

This study shows that the application of repeated restraint stress on pregnant rats determines an alteration of basal NA transmission in the PFC of offspring, as well as an age dependent change of stimulated DA and NA transmission.

The PFC is a key brain region in controlling cognition and emotion. It is strongly influenced by stress which can impair high order abilities such as working memory and attention (Arnsten, 2009). Hains and Arnsten (2008) have argued that the alteration of catecholamine release plays a crucial part in the impairment of PFC functioning. Other research (Sesack and Carr, 2002; Guiard et al., 2008) has shown that the PFC has direct and indirect connections to

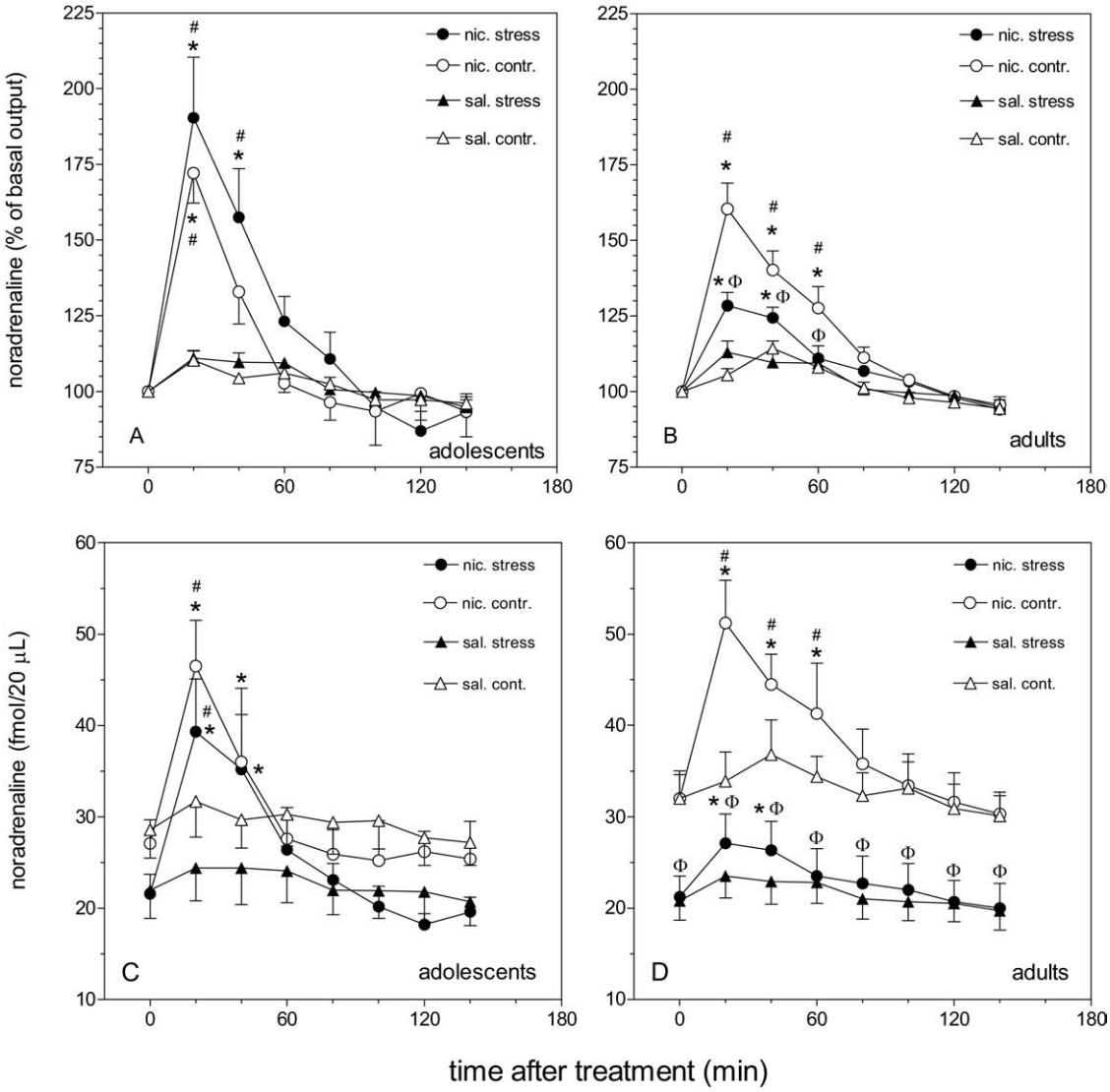

Fig. 6. Effect of nicotine (0.4 mg/kg s.c.) and saline on dialysate noradrenaline expressed as a percentage of basal output (A, B), or fmol/sample (C, D) from the medial prefrontal cortex of adolescent (A, C) and adult (B, D) prenatal stress and control rats. Each point is the mean (±SE) of at least five determinations. * $P<0.05$ from basal values; # $P<0.05$ from the corresponding time point of saline in the same experimental group. $^{\Phi}$ $P<0.05$ versus the corresponding time point of nicotine in controls.

monoamine cell bodies in the LC and in the VTA, where NA and DA neurons innervating the PFC originate. Considering that optimal levels of catecholamine release in the PFC are required for it to fulfil its complex functions (Arnsten, 2009), it does appear conceivable that stress or dysfunctions of stress circuits, altering catecholamine transmission may affect PFC functions. On the other hand, the medial PFC and its NA innervations originating in the LC has a fundamental role in the circuit that mediates emotional stress-induced HPA activation (Urry et al., 2006; Radley et al., 2008).

Although a number of studies have investigated the mechanisms by which PNS determines behavioral and neurochemical changes (Kofman, 2002; Huizink et al., 2004; Weinstock, 2008), a clear picture is not yet available, due to the different types of PNS and animals used (different species, sex and age of offspring). One major difficulty lies in correlating changes that occur at prenatal stage in the developing brain, with the complex neurochemical and behavioral changes that are observed at adolescent or adult life. However, there is a general agreement that PNS causes a dysfunction of the HPA system in offspring and that these effects in the offspring may be linked to the elevated glucocorticoid levels in the mother and in the fetus (Ward and Weisz, 1984). High levels of corticosterone, crossing both the placental and the blood-brain-barrier (Barbazanges et al., 1996; Arishima et al., 1977) can interact with glucocorticoid receptors (Matthews, 2000), potentially altering CNS development and the relationship with HPA at postnatal stage.

While elevated levels of basal corticosterone have been observed in female but not male PNS offspring (see

Huizink et al., 2004), a large body of evidence suggests that PNS rats exhibit a heightened and prolonged response to a variety of stress stimuli (reviewed by Weinstock, 2001, 2008; Kofman, 2002; Huizink et al., 2004). This prolonged response to stress was attributed to a reduced efficacy of the corticosterone feedback mechanism Vallée et al. (1997). As regards CNS stress interaction, it has been reported that tail-pinch stress determines a higher increase of DA release in the right PFC (infralimbic region) and right basolateral amygdala in male rats whereas in female rats, a higher increase was observed in the left side (Sullivan et al., 2009). It has also been suggested that the right PFC is normally dominant in the activation of stress-related systems whereas the left may play a role in countering this activation through processes of interhemispheric inhibition (Sullivan, 2004). If this is the case, it is not surprising that PNS may alter brain asymmetry as previously reported (Fride and Weinstock, 1988; Alonso et al., 1997). However, Kofman (2002) reported that, aside from the higher sensitivity of females, no clear lateralization pattern of PNS effects emerges. Although our study was not specifically concerned with evaluating PNS effects on brain asymmetry, it is worth mentioning that we implanted the probe in the right side of the PFC, which is considered to be more vulnerable to prenatal stress. In point of fact, Fride and Weinstock (1988) showed that only the right PFC presents an increased turnover of DA and Brake et al. (2000) observed a persistent blunting of stress-induced DA release in the right PFC but not in the left side. Since the right hemisphere has been traditionally linked to emotionality, some authors have suggested that this right side vulnerability to prenatal stress may underlie the incapacity of the offspring to cope with emotion-activating situations (Fride and Weinstock, 1988; Alonso et al., 1997).

Basal levels

We observed that basal NA output in both adolescent and adult PNS rats was lower than that of relative controls whereas basal DA output was not significantly different. The noradrenaline innervations of PFC depend on neurons located in the LC (Foote et al., 1983; Van Gaalen et al., 1997) that project to dendrites of both pyramidal cells and interneurons. PFC pyramidal cells project back to the LC providing a circuitry that plays a prominent role in the behavioral response to stress (Arnsten and Goldman-Rakic, 2005) Morilak et al. (2005).

To our knowledge, the effect of PNS on *in vivo* NA levels in the PFC has never been investigated before although we recently reported that PNS increased DA but not NA basal levels in the nucleus accumbens (NAcc) shell of adolescent and adult male offspring (Silvagni et al., 2008). Moreover, Takahashi et al. (1992) reported that NA tissue levels were significantly reduced in cerebral cortex and in the LC of prenatally stressed rats while the concomitant elevated levels of metabolites suggested an increased NA turnover.

Interestingly, prenatal injection of dexamethasone (a synthetic glucocorticoid hormone) increased VTA tyrosine hydroxylase-positive cell numbers by approximately 50% in male and female rats (McArthur et al., 2005). These data would suggest that overactive DA neurons, at VTA levels, determine a higher DA release in the NAcc shell but could also determine a higher inhibition of LC NA neurons (Guiard et al., 2008) with consequent reduction of NA release in the PFC. On the other hand the complexity of the VTA/LC interactions (Guiard et al., 2008) and the unmodified DA output in the PFC we observed in this study, suggest that the role of VTA in PNS effects has yet to be fully clarified.

The fact that either stress or specifically intracebroventricular and LC infusion of corticotropin-releasing factor (CRF) increased DA and NA transmission in the PFC (Shimizu et al., 1994; Gresch et al., 1995; Kawahara et al., 1999), suggests that the results observed might reflect an adaptive response of the catecholamine circuitry to the altered responsiveness of the HPA axis.

Amphetamine effect

In this PFC study, we observed that amphetamine stimulated DA output in adult PNS rats was significantly lower whereas amphetamine stimulated NA output was significantly higher than that observed in the relative controls. In contrast, no effect of PNS on DA and NA stimulated output was observed in adolescent rats.

Amphetamine is a useful tool for investigating brain function because it interacts with brain circuitry on multiple levels acting as a DA, NA and 5-hydroxytryptamine indirect agonist (Carboni and Silvagni, 2004). The main mechanism through which amphetamine enhances catecholamine output is by binding it to the vesicular transporter VMAT2 (Sulzer et al., 2005). The consequent increase of catecholamines in the cytosol, seems to determine the reversed functioning of membrane transporter, causing catecholamine diffusion in the extracellular space (Sulzer et al., 2005). Thus, amphetamine data suggest that PNS effects may influence directly catecholamine circuits by altering the vesicle storing and releasing machinery of DA and NA neurons in a different way. Consequently, PNS may unbalance the delicate relationship between DA and NA transmission in the PFC, which essentially determines the expression of its highly sophisticated functions.

Age differences in the amphetamine effect

The attempt to correlate the different amphetamine response of DA and NA output to the relative basal levels had no grounds in either experimental groups because no difference was found between adolescent and adult basal levels and between the amphetamine response in PNS adolescent rats and that of relative controls. The possible dissociation between basal output and vesicle transmitter content is supported by the observation that a neuron can support basal release at the expense of the transmitter reservoir (Bongiovanni and See, 2008). These authors reported that a tyrosine and phenylalanine free diet determined a reduction of tyrosine and DOPA levels without affecting basal dopamine and noradrenaline levels in the PFC. At the same time, this diet robustly lowered the response of DA as well as of NA to the administration of desipramine. Lastly, the different responsiveness of DA and NA output to amphetamine, observed in adult but not

in adolescent PNS rats, suggests that the neurocircuitry changes occurring in adolescence (Chambers et al., 2003) could expose PNS-caused unexpressed alterations in the catecholamine PFC circuitry, leading to defective PFC maturation. A similar PNS effect in humans might be a contributing factor to the appearance of psychiatric disorders or higher sensitivity to psychostimulants.

Nicotine effect and age differences

Nicotine stimulated DA output in adolescent and adults PNS rats, was not different from that of relative controls. On the other hand nicotine, stimulated DA output was higher in adolescent rats as compared with adults. We investigated the stimulating effect of nicotine on catecholamine transmission in the PFC in order to gain insights into the possible role of PNS in the development of nicotine dependence and in the heavier nicotine addiction that is observed in patients affected by schizophrenia, depression and ADHD; whether or not subjects affected by these disorder smoke to ameliorate their condition implementing a sort of self-medication is a much-debated matter (Gregg et al., 2007). Our results, as regards DA transmission in the PFC, suggest that PNS does not influence the nicotine response whereas the higher response observed in adolescents might be correlated to their higher sensitivity to the effects of nicotine. It might have a contributory role in the strong nicotine addiction that is observed in smokers who start nicotine abuse during adolescence (see the review of O'Dell, 2009). In fact, although nicotine abuse has much in common with other drugs of abuse in that it increases DA output in the NAcc shell (Di Chiara, 2000) or in other brain areas (Carboni et al., 2000), its ability to determine a higher increase of DA in the PFC of adolescents could potentially be correlated to the alteration of the brain maturation process that occurs in adolescent smokers. Consequently this feature may alter the PFC's role in the ability to establish a rational evaluation of smoking even during adult age.

On the other hand, PNS did not alter the response of NA to nicotine in adolescents, though did lower it significantly in adults. Nicotine can increase NA release through nAChRs nicotinic receptors at cell body level or through presynaptic nicotinic receptors localized in noradrenergic terminals (Reuben et al., 2000; Leslie et al., 2002; Amtage et al., 2004; Rossi et al., 2005). These data are only apparently in contrast with the higher nicotine stimulated NA transmission observed in the NAcc of PNS rats (Silvagni et al., 2008), and suggests that PNS may alter nicotine response in several brain areas. In fact NA accumbal innervation originates primarily in the nucleus of tractus solitarius unlike with cortical NA innervation (Berridge et al., 1997; Delfs et al., 1998). This is substantiated by the fact that prenatally stressed rats exhibit modifications in locomotor activity in response to nicotine (Koehl et al., 2000). Lastly, the nicotine effect on DA and NA transmission in the PFC may have a role in the nicotine enhancement of cognition (see the review of Poorthuis et al., 2009) by interfering with the central role of thalamic glutamatergic innervation of the PFC in cognition and nicotine induced glutamate release in the PFC (Gioanni et al., 1999).

CONCLUSION

We have observed that PNS produces complex biochemical changes on DA transmission in the PFC that are very different from those observed in the NAcc shell (Silvagni et al., 2008). Indeed, an increased basal and stimulated DA transmission and an unchanged basal NA output were clearly observed in the NAcc shell. The role of NA in the PFC is expressed through a neuro-modulatory type action with a long onset and protracted effect. This action produces an inhibition on background discharge, increasing the signal–to-noise ratio that helps to filter irrelevant stimuli while enhancing behaviorally significant stimuli (Bjorklund and Lindvall, 1986). This view has been recently expanded by Arnsten (2007), who used experimental evidence on different mammals to propose that NA is crucial for many PFC functions mediated by α_{2A} post-synaptic receptors, i.e. working memory, attention regulation, planning and behavioural inhibition. It is interesting to consider that the blockade of α_{2A} receptors in monkeys re-creates the symptoms of ADHD (Arnsten, 2006), that the ADHD drug atomoxetine, increases NA output in the PFC by blocking NA uptake (Bymaster et al., 2002; Swanson et al., 2005), and also that the long established stimulants amphetamine and methylphenidate (Arnsten and Dudley, 2005) increase NA transmission by acting on both DA and NA transmission. We might therefore suggest that since PNS causes insufficient NA transmission in the PFC of the rat, it may well be implicated in the aetiology of ADHD. This suggestion is supported in a report by Rodriguez and Bohlin (2005) who observed that prenatal stress and smoking were independently associated with symptoms of ADHD in boys especially. The recent evidence from Grizenko et al. (2008) who observed that maternal stress during pregnancy (particular during the last trimester) is correlated with the severity of ADHD symptoms also supports this suggestion.

On the other hand, it is thought that PNS produces depression symptomatology in men (Watson et al., 1999) and the appearance of behavioral features of depression in animals, with regard to both physical (Yang et al., 2006) and psychological PNS (Abe et al., 2007). In particular, it has been observed that PNS can enhance immobility time in the forced swim test and that antidepressant treatment can reverse this increase (Alonso et al., 1994; Drago et al., 1999; Morley-Fletcher et al., 2003, 2004). Moreover, gestational stress in rats has been associated with exaggerated fear of intimidating situations and depressive-like behaviour (Weinstock, 2001). Therefore, considering that a significant portion of antidepressant therapy targets NA transmission, our data also support the view that PNS can be a concurrent factor in the outbreak of depression, even though depression clearly involves many other brain areas and transmitters as well. Finally, the different PNS effects on the changes induced by amphetamine and nicotine on DA and NA transmission in the PFC of adolescent and adult rats suggests that PNS produces a long lasting, wide and complex effect on PFC circuitry which, by interfering with brain maturation, may affect vulnerability to psycho-stimulants.

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