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RUNNING TITLE

neonatal TTX inactivation of the ventral subiculum

Latent inhibition-related dopaminergic responses in the nucleus accumbens are disrupted following neonatal transient inactivation of the ventral subiculum.

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ABSTRACT

Schizophrenia would result from a defective connectivity between several integrative regions, as a consequence of neurodevelopment failure. Various anomalies reminiscent of early brain development disturbances have been observed in patients' left ventral subiculum of the hippocampus (SUB). Numerous data support the hypothesis of a functional dopaminergic dysregulation in schizophrenia. The common target structure for the action of antipsychotics appears to be a subregion of the ventral striatum, the *dorsomedial shell* part of the nucleus accumbens. Latent inhibition, a cognitive marker of interest for schizophrenia, has been found to be disrupted in acute patients. The present study set out to investigate the consequences of a neonatal functional inactivation of the left SUB by tetrodotoxin (TTX) in 8-day-old rats for the latent inhibition-related dopaminergic responses as monitored by *in vivo* voltammetry in freely moving adult animals (11 weeks) in the left *core* and *dorsomedial shell* parts of the nucleus accumbens in an olfactory aversion procedure. Results obtained during the retention session of a three-stage latent inhibition protocol showed that the postnatal unilateral functional blockade of the SUB was followed in pre-exposed TTX conditioned adult rats by a disruption of the behavioral expression of latent inhibition and induced a total and a partial reversal of the latent inhibition-related dopaminergic responses in the *dorsomedial shell* and *core* parts of the nucleus accumbens respectively. The present data suggest that neonatal inactivation of the SUB has more marked consequences for the dopaminergic responses recorded in the *dorsomedial shell* part than in the *core* part of the nucleus accumbens. These findings may provide new insight into the pathophysiology of schizophrenia.

Keywords: neonatal functional inactivation; neurodevelopment; conditioned olfactory aversion; nucleus accumbens; *in vivo* voltammetry; schizophrenia.

INTRODUCTION

Schizophrenia is a very complex and disabling neuropsychiatric disease, the pathophysiology of which is not yet fully understood. According to several recent proposals, the psychic disintegration characteristic of this disease would result from a defective connectivity between various integrative regions having a neurodevelopmental origin (Weinberger and Lipska, 1995; Bullmore *et al*, 1997; Friston, 1998; Lewis and Levitt, 2002; Sawa and Snyder, 2002; Arnold *et al*, 2005; Foucher *et al*, 2005). Thus, the ventral subiculum of the hippocampus (SUB) seems to be subjected to neurodevelopmental abnormalities in schizophrenia, as suggested by the cytoarchitectural and cortical morphometric aberrations reported by several authors, particularly in the left hemisphere (Arnold *et al*, 1995; Arnold, 2000; Rosoklija *et al*, 2000; Law *et al*, 2004). Data obtained over the past three decades also support the hypothesis of a functional dysregulation of the mesostriatal dopaminergic neurons in schizophrenia (Swerdlow and Koob, 1987; Harrison, 1999; Carlsson *et al*, 2001), consistent with a cortico-subcortical disconnectivity (Kegeles *et al*, 2000). Moreover, it would appear that the common target structure for the action of antipsychotics is a subregion of the ventral striatum, the *dorsomedial shell* part of the nucleus accumbens (Deutch and Cameron, 1992, Robertson and Fibiger, 1992, Jennings *et al*, 2006).

Latent inhibition, a cognitive marker of interest for schizophrenia, has been found to be disrupted in non-treated patients (Baruch *et al*, 1988; Gray *et al*, 1992, 1995; Lubow *et al*, 2000; Rasclé *et al*, 2001). Latent inhibition is a behavioral phenomenon characterized by a decrease or even disappearance of the conditioned response (CR) normally observed during the retention session when the conditional stimulus (CS) is first pre-exposed, by itself, before the conditioning session (Lubow and Moore, 1959). Many studies aimed to identify the neurobiological substrates underlying normal and disrupted expression of latent inhibition (see Lubow, 1989; Weiner, 2003). Differential involvement of the dopaminergic neurons innervating the *core* and *dorsomedial shell* parts of the nucleus accumbens in latent inhibition has been highlighted by *in vivo* methods in animal studies (Young *et al*, 1993; Murphy *et al*, 2000; Jeanblanc *et al*, 2002). The *core* and *dorsomedial shell* dopaminergic responses seem to be regulated differently in adult animals by the entorhinal cortex (ENT) and the SUB (Jeanblanc *et al*, 2004; Peterschmitt *et al*, 2008). Moreover, the functional inactivation of the two left parahippocampal regions (ENT, SUB) appears sufficient to impair the latent inhibition-related dopaminergic responses in the left hemisphere (Jeanblanc *et al*, 2004; Peterschmitt *et al*, 2005; 2008). These findings are consistent

with previous anatomofunctional (Louilot and Le Moal, 1994; Louilot and Choulli, 1997) and behavioral data (Besson and Louilot, 1995) showing a left predominance for temporo-accumbal dopaminergic regulation and aversive dopaminergic responses.

Recently, we found that the neonatal functional blockade of the left ENT abolished the latent inhibition-related dopaminergic responses in the left *core* and left *dorsomedial shell* parts of the nucleus accumbens (Peterschmitt *et al*, 2007). Given all the aforementioned data, this raises the question of the consequences of neonatal transient inactivation of the left SUB for latent inhibition-related dopaminergic responses in the two subregions of the nucleus accumbens. During development, impulse electrical activity appears to be crucial for shaping connections once developing fibres reach the target structure (Stryker and Harris, 1986; Katz and Shatz, 1996; Frotscher *et al*, 2000; Hutchins and Kalil, 2008). Tetrodotoxin (TTX) is a potent and specific voltage-gated Na⁺ channel blocker which interrupts neuronal activity (Mosher, 1986). In the present study, the transient neonatal blockade of the SUB was achieved by local microinjection of TTX in 8-day-old rats. This particular time point is critical for the neurodevelopment of rats, comparable in humans to the middle of the second trimester of gestation (Clancy *et al*, 2001), which is considered to be a time window of great vulnerability for developing schizophrenia (see e.g. Weinberger and Lipska, 1995; Bullmore *et al*, 1997; Arnold, 2000; Falkai *et al*, 2000; Lewis and Levitt, 2002). Latent inhibition-related dopaminergic changes in the left *core* and left *dorsomedial shell* subregions of the nucleus accumbens were monitored in freely moving adult animals (11 weeks) using *in vivo* voltammetry in a three-stage latent inhibition paradigm with an aversive conditioned olfactory procedure (Jeanblanc *et al*, 2002).

MATERIALS AND METHODS

Animals and surgery

Subjects

Female rats (Sprague-Dawley, Janvier, Le Genest, France) obtained at 14 days gestation were housed individually at 22 ± 2° C on a 12-hour light/12-hour dark cycle (lights on at 8 am) and fed *ad libitum*. At birth, the size of the litters was limited to 12 animals by killing off supernumerary pups with a lethal intraperitoneal (i.p.) injection of pentobarbital. The day of birth was defined as postnatal day 0 (PND0). On PND8, rat pups underwent a microinjection of either phosphate-buffered saline (PBS) in the left SUB (control animals) or of TTX in the left SUB

(experimental animals). On PND21 the neonatal rats were weaned. On PND56 the male animals were individually housed in plexiglas cages under a reversed lighting cycle (lights on between 11 pm and 11 am) at $22 \pm 2^\circ$ C, with free access to food and water. A total of 131 Sprague-Dawley male rats were used. On PND70 the male rats were implanted with a microsystem for monitoring behavior and dopaminergic responses, in parallel. All experimental procedures were conducted in accordance with European Community guidelines for the care and use of experimental animals (Council Directive 86/609/EEC) and authorized by the French Ministry of Agriculture (Authorization 67-244).

Neonatal TTX inactivation

Neonatal surgery was performed on PND8 (weight 19.1 ± 0.4 g). Pups were anaesthetized by gas anaesthesia by vaporizing isoflurane (Forene®, ABBOTT, Rungis, France) with an anaesthesia vaporiser (Univentor 400, Univentor®, Zetjun, Malta) connected to an air pump (Dymax 30, Charles Austen Pumps®, Byfleet, UK) and released through a specially adapted gas anaesthesia mask. Induction was obtained with a concentration of $\sim 3.8 - 4$ % isoflurane in air. The pups were then immediately placed on a specially adapted stereotaxic apparatus (Unimécanique®, Epinay/Seine, France) (incisor bar set at 2.8 mm below the interaural line). Anaesthesia was maintained throughout surgery with ~ 2 % isoflurane in air (Flecknell, 1987). Neonatal microinjection of either PBS or TTX was performed by means of a stainless steel guide cannula (30 gauge, 12.5 mm length, Small Parts, Miami, USA) implanted in the SUB at coordinates 1.1 mm anterior to the interaural line (AP), 4.25 mm lateral to the midline (L), and 4.35 mm below the cortical surface (H). TTX (100 μ M) was dissolved in PBS (NaCl 8 g/l, KCl 0.2 g/l, MgCl₂, 6H₂O 0.1 g/l, KH₂PO₄ 0.2 g/l, Na₂ HPO₄, 2H₂O 1.15 g/l, pH: 7.4). PBS and TTX were infused locally in a total volume of 0.3 μ l over a period of 2 min 15 s by means of an infusion pump (Razel, Stamford, CT, USA). Following the microinjection, the cannula was left in place for 4 min to allow PBS and TTX to diffuse in the SUB. It is worth noting that the amount of TTX microinjected in the SUB (100 μ M x 0.3 μ l), about 10 ng, is similar to that reported in the literature (Zhuravin and Bures, 1991; Ambrogi Lorenzini *et al*, 1997), and that different authors (Rothfeld *et al*, 1986; Zhuravin and Bures, 1991) showed that the blockade effects of a local microinjection of 10 ng TTX last for 24 or 48 h. To identify the microinjection site in the SUB, the PBS and TTX solutions were both tinted with Evans Blue (Sigma, France), a vital dye

described as remaining visible in the cerebral tissue several weeks after injection (Martin and Ghez, 1993; Peterschmitt *et al*, 2007; Meyer *et al*, 2009). Each rat pup was identified by means of small three-digit ear tags (Ref 52-4716, Harvard Apparatus, Les Ulis, France). After the surgical intervention, the neonates were returned to their mothers. Male rat pups from the same litter, microinjected with either PBS or TTX, were placed in the same cage.

Microsystem implantation

Animals microinjected neonatally with either PBS or TTX were implanted in adulthood at PND70 (weight 400 ± 25 g) with a specially designed microsystem (Unimécanique, Epinay/Seine, France) (Louilot *et al*, 1987) following anaesthetization by intraperitoneal injection (i.p.) of chloral hydrate (400 mg/kg) and after being placed in a stereotaxic frame (incisor bar set at 3.3 mm below the interaural line) (Unimécanique, Epinay/Seine, France). Male rats were implanted either in the left *core* part of the nucleus accumbens at coordinates 10.2 mm (AP), 1.8 mm (L) and 6.9 mm (H), or in the left *dorsomedial shell* part of the nucleus accumbens at coordinates 10.2 mm (AP), 1.0 mm (L) and 6.2 mm (H) (Paxinos and Watson, 2009). After surgery animals were given one week to recover.

Behavioral analysis

Behavioral procedure

The behavioral procedure begins 7 days after the microsystem implantation in the adult animals, so at PND77. The behavioral and voltammetric analyses were performed in parallel. All experiments were carried out during the dark phase of the light/dark cycle. Latent inhibition was measured in a conditioned olfactory aversion procedure in a three-stage latent inhibition paradigm, with banana odor (amyl acetate, Prolabo, Strasbourg, France) as the conditional olfactory stimulus (CS), as previously reported (Jeanblanc *et al*, 2002; Peterschmitt *et al*, 2007; Meyer *et al*, 2009, Louilot *et al*, 2010). The latent inhibition paradigm consists of three sessions conducted at 72 hour intervals. During the 1st (pre-exposure) session, animals were placed in the experimental cage (24cm wide x 27cm long x 44cm high) for 1 hour before being exposed for 2 hours to banana odor, a novel olfactory stimulus. During the 2nd (conditioning) session, 3 days later, the animals were placed in the test cage for 1 hour before being exposed to banana odor for a further hour, at the end of which they received either an i.p. injection of 2% of body weight of

NaCl (0.9%) (control animals) or an i.p. injection of 2% of body weight of a toxic substance inducing nausea (LiCl 0.15 M) (conditioned animals) (Garcia *et al*, 1985). They were then kept in the test cage for a further hour. During the last (test) session, 3 days later, the animals were again exposed to the conditional olfactory stimulus (banana odor) for one hour. Non pre-exposed animals, on the other hand, were only subjected to the last two sessions (conditioning and test sessions). All pre-exposed and non pre-exposed animals were microinjected at PND8 with either PBS or TTX in the SUB. After the neonatal intervention they were randomized to 2 pre-exposed control groups (pre-exposed PBS-NaCl; pre-exposed TTX-NaCl), 2 pre-exposed conditioned groups (pre-exposed PBS-LiCl; pre-exposed TTX-LiCl), 2 non pre-exposed control groups (non pre-exposed PBS-NaCl; non pre-exposed TTX-NaCl) and 2 non pre-exposed conditioned groups (non pre-exposed PBS-LiCl; non pre-exposed TTX-LiCl).

Behavioral data analysis

The animals' position in the experimental cage was monitored using a small infrared camera (Ref. 51.8050, CA-H34C, Selectronic, Lille, France) inserted into the ceiling of the cage and connected to a video monitor and video tape. The olfactory stimulus was fed into the cage through a hole in the wall adjacent to the cage door. Attraction or aversion to the banana odor was evaluated by measuring the amount of time spent near the olfactive source. The floor of the cage was divided empirically into two virtual zones. One zone, containing the hole, was a semi-circle covering 35% of the total surface area. The rest of the floor made up the second zone. Behavioral analysis was performed over 10-min periods. It was assumed that an animal moving randomly in the cage should spend 35% (210 sec) of the 10-min period in the area containing the hole. Results are expressed as mean \pm standard error to the mean (S.E.M) of the time spent near the hole.

Voltammetric analysis

Voltammetric procedure

The electrochemical procedures were those previously described (Gonzalez-Mora *et al*, 1991; Louilot *et al*, 1991). A standard three-electrode potentiostatic setting was used with working, reference and auxiliary electrodes. The working electrodes were pyrolytic carbon fibre microelectrodes (12 μ m diameter, 500 μ m length, ref AGT 8000, SEROFIM, Gennevilliers, France) which were pretreated electrochemically according to Gonon *et al* (1984). Differential

normal pulse voltammetry (DNPV) (see O'Neill *et al*, 1998 for review), combined with the computerized waveform analysis of the catechol peak, was used to establish the selective detection of the extracellular dopamine levels in the *core* and *dorsomedial shell* parts of the nucleus accumbens (see Gonzalez-Mora *et al*, 1991). DNP voltammograms were recorded every minute. The parameters of the voltammetric apparatus (Biopulse, Tacussel, Villeurbanne, France) were as follows: scan range -240 mV, +200 mV; scan rate 10 mV/s; potential step 4 mV; pulse period 400 ms; pre-pulse duration +120 ms; pulse duration 40 ms; pulse amplitude 40 mV.

Voltammetric data

The average amplitude of the last 10 peaks (last 10 minutes) of dopaminergic obtained during the control period (variation of voltammetric signal less than 10%) was calculated for each animal and set at 100%. Voltammetric variations, recorded minute by minute, are expressed as percentages (mean \pm S.E.M.) of the mean values determined before exposure to the olfactory stimulus. Only variations obtained every 2 minutes are shown on the graphs.

Statistics

Statistical analysis was performed using a multifactorial ANOVA analysis with repeated measurements on the factor time. Only between-subject ANOVAs are shown. Between-subject grouping factors were conditioning with 2 levels (NaCl, LiCl), microinjection with 2 levels (PBS, TTX), and pre-exposure with 2 levels (pre-exposed, non pre-exposed). The dependent variables were the time spent near the hole for the behavioral study and the dopaminergic variations for the voltammetric study. Contrast analysis of the ANOVA was used to test specific hypotheses (see Rosenthal and Rosnow, 1985; Rosenthal *et al*, 2000). Statistical significance for all analyses was set at $P < 0.05$.

Histology

At the end of each experiment, the rats underwent electrocoagulation (Louilot *et al*, 1985). They then received a lethal i.p. injection of pentobarbital and were intracardially perfused, first with NaCl 0.9 % and then with a 4 % paraformaldehyde solution. Their brains were quickly removed from their skull. The brain sections were stained with Thionin Blue so that the position of the recording sites in the left *core* and the left *dorsomedial shell* parts of the nucleus accumbens (Fig.1, upper sections) could be verified. The neonatal microinjection site in the left

SUB was histologically checked with the vital dye Evans Blue and Neutral Red staining of the SUB sections (Fig.1, lower sections). Paxinos and Watson's atlas (2009) was used as a reference.

Histology (Fig.1)

The qualitative macroscopic observations of brain sections at the level of the SUB or the nucleus accumbens reveal no gliosis or anatomical differences between the animals microinjected at PND8 with PBS or TTX in the SUB.

Retention session: Behavioral study (Fig. 2)

The overall ANOVA performed on the 131 animals for the 10-min period preceding the presentation of the banana odor, revealed no significant effect in the time spent near the hole for the conditioning factor (NaCl/ LiCl), microinjection factor (PBS/ TTX), pre-exposure factor (non pre-exposed/ pre-exposed) or different interactions (conditioning x microinjection, microinjection x pre-exposure, pre-exposure x conditioning, conditioning x microinjection x pre-exposure).

As regards pre-exposed PBS-control (n=16) and non pre-exposed PBS-control (n= 17) groups (Fig. 2A), the time spent in the area near the hole exceeded the limit of 210 s for the whole hour following exposure to the olfactory stimulus. For pre-exposed TTX-control (n=14) and non pre-exposed TTX-control (n=16) animals (Fig. 2B), the time spent in the semi-circle also exceeded the limit of 210 s for the first 50 min after presentation of the banana odor. As regards the two PBS-conditioned groups, the profiles of the pre-exposed and non pre-exposed animals differ (Fig. 2A'). For the pre-exposed PBS-conditioned group (n=20), the time spent in the area near the olfactory stimulus exceeded the limit of 210 s for the whole hour. By contrast, the time the non pre-exposed PBS-conditioned group (n=15) spent in the semi-circle was well below 210 s for the first half-hour. Regarding the pre-exposed TTX-conditioned (n=18) and non pre-exposed TTX-conditioned groups (n=15) (Fig. 2B'), the profiles of the amount of time spent in the semi-circle in the first half-hour were comparable, with values below the limit of 210 s.

The ANOVA for the full hour after the animals' exposure to the olfactory stimulus yielded significant main effects of conditioning (NaCl/ LiCl) ($F_{(1,123)} = 18.87$; $p < 0.0001$) and injection (PBS/TTX) ($F_{(1,123)} = 4.59$; $p < 0.05$), as well as significant interactions of pre-exposure

x conditioning ($F_{(1,123)} = 7.05$; $p < 0.01$), of injection x pre-exposure ($F_{(1,123)} = 4.68$; $p < 0.05$) and of conditioning X injection X pre-exposure ($F_{(1,123)} = 9.81$; $p < 0.01$), whereas no significant effects were found for the pre-exposure factor (non pre-exposed/ pre-exposed) and the injection X conditioning interaction.

Contrast analysis of ANOVA was used to test the following specific hypotheses: 1) conditioning (NaCl/LiCl) was followed by statistically different responses in non pre-exposed and pre-exposed groups; 2) Behavioral responses differed significantly in pre-exposed PBS-conditioned and non pre-exposed PBS-conditioned animals on the one hand and pre-exposed PBS-conditioned and pre-exposed TTX-conditioned animals on the other hand.

ANOVA contrast analysis for the time spent near the hole for the 60 min following stimulus presentation highlighted a significant conditioning effect for the non pre-exposed PBS groups (non pre-exposed PBS-control / non pre-exposed PBS-conditioned) ($F_{(1,123)} = 23.11$; $p < 0.00001$), non pre-exposed TTX groups (non pre-exposed TTX-control/ non pre-exposed TTX-conditioned) ($F_{(1,123)} = 4.44$; $p < 0.05$), and pre-exposed TTX groups (pre-exposed TTX-control/ pre-exposed TTX-conditioned) ($F_{(1,123)} = 6.73$; $p < 0.05$), whereas it revealed no significant conditioning effect (NaCl/LiCl) for the pre-exposed PBS groups (pre-exposed PBS-control/ pre-exposed PBS-conditioned). A clear statistical pre-exposure effect was found for the pre-exposed PBS-conditioned group (pre-exposed PBS-conditioned/ non pre-exposed PBS-conditioned) ($F_{(1,123)} = 25.75$; $p < 0.00001$), and a significant effect of the neonatal injection was obtained for the pre-exposed TTX-conditioned group (pre-exposed PBS-conditioned/ pre-exposed TTX-conditioned) ($F_{(1,123)} = 18.18$; $p < 0.0001$).

The ANOVA for the 1st 30 min after the animals' exposure to the olfactory stimulus showed significant main effects of conditioning (NaCl/ LiCl) ($F_{(1,123)} = 76.74$; $p < 0.000001$), injection (PBS/TTX) ($F_{(1,123)} = 13.99$; $p < 0.001$) and pre-exposure (non pre-exposed/ pre-exposed) ($F_{(1,123)} = 13.14$; $p < 0.001$), as well as significant interactions of pre-exposure x conditioning ($F_{(1,123)} = 17.1$; $p < 0.0001$), of injection x pre-exposure ($F_{(1,123)} = 5.01$; $p < 0.05$) and of conditioning X injection X pre-exposure ($F_{(1,123)} = 24.18$; $p < 0.00001$), whereas no significant effects were found for the injection X conditioning interaction. No statistical results were obtained for the last 30 min of the hour following presentation of the olfactory stimulus.

Contrast analysis of ANOVA was used to test the following specific hypotheses: 1) conditioning (NaCl/LiCl) was followed by statistically different responses in non pre-exposed

and pre-exposed groups; 2) Behavioral responses differed significantly in pre-exposed PBS-conditioned and non pre-exposed PBS-conditioned animals, on the one hand, and pre-exposed PBS-conditioned and pre-exposed TTX-conditioned animals, on the other hand.

ANOVA contrast analysis for the time spent near the hole in the 1st 30 min following presentation of the stimulus revealed a significant conditioning effect for the non pre-exposed PBS groups (non pre-exposed PBS-control/non pre-exposed PBS-conditioned) ($F_{(1,123)} = 62.91$; $p < 0.000001$), non pre-exposed TTX groups (non pre-exposed TTX-control/non pre-exposed TTX-conditioned) ($F_{(1,123)} = 22.98$; $p < 0.000001$), and pre-exposed TTX groups (pre-exposed TTX-control/pre-exposed TTX-conditioned) ($F_{(1,123)} = 31.43$; $p < 0.000001$), but no significant conditioning effect (NaCl/LiCl) for the pre-exposed PBS groups (pre-exposed PBS-control/pre-exposed PBS-conditioned). A clear statistical pre-exposure effect was found for the pre-exposed PBS-conditioned group (pre-exposed PBS-conditioned/non pre-exposed PBS-conditioned) ($F_{(1,123)} = 58.91$; $p < 0.000001$), and a significant effect of the neonatal injection was obtained for the pre-exposed TTX-conditioned group (pre-exposed PBS-conditioned/pre-exposed TTX-conditioned) ($F_{(1,123)} = 47.36$; $p < 0.000001$).

To summarize, pre-exposed PBS-conditioned animals microinjected at PND8 in the left SUB differ significantly from non pre-exposed PBS-conditioned animals. For pre-exposed TTX-conditioned animals and non pre-exposed TTX-conditioned animals, however, similar results were obtained.

Retention session: Voltammetric results (Fig. 3)

Only animals with implantation sites clearly located in the *core* part (Fig. 1A) or *dorsomedial shell* part (Fig. 1B) of the nucleus accumbens were considered for the voltammetric analysis.

Dopaminergic variations recorded in the *core* part of the nucleus accumbens (Fig. 3A)

With respect to the PBS groups (Fig. 3A), after the banana odor was presented there was a rapid increase in the dopaminergic responses of the pre-exposed PBS-control animals during the 1st half-hour of the retention session. The dopamine signal then continued to rise until the end of the hour (58th min). In the case of the non pre-exposed PBS-control animals, the dopaminergic responses recorded in the *core* part of the nucleus accumbens increased gradually after the olfactory stimulus was presented and until the end of the hour. With the pre-exposed PBS-

conditioned animals, the dopaminergic responses rose fairly sharply following presentation of the stimulus, before peaking in the 46th min. With the non pre-exposed PBS-conditioned animals, there were small dopaminergic variations which oscillated around the baseline and peaked in the 60th min.

As regards the TTX groups (Fig. 3A), presentation of the olfactory stimulus in the case of the pre-exposed TTX-control animals was followed by a rise in the dopamine levels which peaked towards the end of the hour, in the 56th min. In the case of the non pre-exposed TTX-control animals, dopaminergic variations were characterized by a steady increase following exposure to the banana odor, reaching a maximum at the end of the hour. The dopaminergic changes in respect of the two TTX-conditioned groups differed. With the pre-exposed TTX-conditioned animals, the dopamine signal increased gradually, peaking first in the 10th min and then oscillating above the baseline for the rest of the hour. A maximal value was obtained in the 44th min. With the non pre-exposed TTX-conditioned animals, the dopamine signal fluctuated around the basal level, peaking in the 48th min.

The overall ANOVA performed for the dopaminergic variations in the *core* subregion (n=47) with respect to the full hour following exposure to the olfactory stimulus highlighted significant differences with the dopaminergic variations for the conditioning factor (NaCl/ LiCl) ($F_{(1,39)} = 14.20$; $P < 0.001$), as well as the pre-exposure factor (non pre-exposed/ pre-exposed) ($F_{(1,39)} = 9.04$; $P < 0.005$), but revealed no significant effect for the microinjection factor (PBS / TTX). The various interactions (conditioning x microinjection, microinjection x pre-exposure, pre-exposure x conditioning or conditioning x microinjection x pre-exposure) were not found to be statistically different.

Contrast analysis of ANOVA was used to test the ensuing specific hypotheses: 1) A significant effect of conditioning (NaCl/ LiCl) is observed for the pre-exposed groups; 2) A significant effect of pre-exposure (non pre-exposed/pre-exposed) is observed for the PBS-conditioned and the TTX-conditioned groups. Contrast analysis of ANOVA for dopaminergic variations recorded in the *core* part of the nucleus accumbens for the whole hour following presentation of the olfactory stimulus revealed significant effects of the conditioning factor (NaCl/ LiCl) for pre-exposed TTX groups (pre-exposed TTX-control/ pre-exposed TTX-conditioned) ($F_{(1,39)} = 5.41$; $P < 0.05$). Conversely, no significant effect of the conditioning factor was observed for the pre-exposed PBS animals (pre-exposed PBS-control/ pre-exposed PBS-

conditioned) ($F_{(1,39)} = 0.27$; ns). The contrast analysis showed a significant effect of the pre-exposure factor for the PBS-conditioned animals (pre-exposed PBS-conditioned/ non pre-exposed PBS-conditioned) ($F_{(1,39)} = 7.06$; $P < 0.05$), but no statistical effect for the TTX-conditioned animals (pre-exposed TTX-conditioned/non pre-exposed TTX-conditioned) ($F_{(1,39)} = 1.72$; ns).

In summary, the dopaminergic variations recorded in the *core* part of the nucleus accumbens in respect of the pre-exposed PBS-control animals and pre-exposed PBS-conditioned animals increased in a similar way following the CS presentation (banana odor). Increases in the pre-exposed PBS-conditioned animals were significantly different from the dopaminergic variations recorded in the non pre-exposed PBS-conditioned animals. Regarding the pre-exposed TTX-conditioned animals, the dopaminergic responses were less pronounced and significantly different from the increases observed in the pre-exposed TTX-control animals, but not statistically different from those observed in the non pre-exposed TTX-conditioned animals.

Dopaminergic variations recorded in the *dorsomedial shell* part of the nucleus accumbens (Fig. 3B)

With respect to the PBS groups (Fig. 3B), exposure to the olfactory stimulus in the case of the pre-exposed PBS-control group was followed during the retention session by a rapid increase in the dopaminergic signal until the end of the hour, peaking in the 60th min, whereas in the case of the non pre-exposed PBS-control animals, variations of dopamine increased moderately throughout the hour following presentation of the banana odor, peaking in the 14th min. In respect of the pre-exposed PBS-conditioned animals, dopaminergic levels remained fairly stable and oscillated around the basal level. The non pre-exposed PBS-conditioned animals were characterized by a marked dopaminergic increase which peaked in the 48th min.

As regards the TTX groups (Fig. 3B), a moderate and steady increase was observed in the case of the pre-exposed TTX-control group for the whole hour following presentation of the olfactory stimulus, with a maximal value attained in the 44th min. With the non pre-exposed TTX-control animals, the extracellular dopaminergic levels increased only slightly after the olfactory stimulus presentation, reaching a maximal value in the 48th min. With the pre-exposed TTX-conditioned animals, the dopaminergic responses remained close to the baseline for the first

15 min. Then, the dopamine signal rose sharply and remained fairly stable, peaking in the 56th min. The non pre-exposed TTX-conditioned animals displayed a marked increase, peaking in the 56th min.

The overall ANOVA carried out for the full hour after the animals' exposure to the olfactory stimulus showed no significant difference in the dopaminergic variations in the *dorsomedial shell* subregion (n=44) for the conditioning factor (NaCl/ LiCl), pre-exposure factor (non pre-exposed/ pre-exposed), microinjection factor (PBS/ TTX) or microinjection x pre-exposure interaction and the microinjection x conditioning interaction. This analysis highlighted a significant difference for the pre-exposure x conditioning interaction ($F_{(1,36)} = 19.16$; $P < 0.0001$) and the conditioning x microinjection x pre-exposure interaction ($F_{(1,36)} = 4.59$; $P < 0.05$).

The following specific hypotheses were then tested with ANOVA contrast analysis: 1) A significant effect of the conditioning (NaCl / LiCl) is observed for the non pre-exposed and pre-exposed groups; 2) The dopaminergic variations recorded in the *dorsomedial shell* part of the nucleus accumbens for pre-exposed PBS-conditioned animals are significantly different from, on one hand, those recorded for non pre-exposed PBS-conditioned animals and, on the other hand, those observed for pre-exposed TTX-conditioned animals.

Contrast analysis of ANOVA for the dopaminergic variations recorded in the *dorsomedial shell* part of the nucleus accumbens for the whole hour following exposure to the banana odor revealed significant effects of the conditioning factor for the non pre-exposed PBS groups (non pre-exposed PBS-control/ non pre-exposed PBS-conditioned) ($F_{(1,36)} = 7.75$; $P < 0.01$), and non pre-exposed TTX groups (non pre-exposed TTX-control/ non pre-exposed TTX-conditioned) ($F_{(1,36)} = 5.15$; $P < 0.05$) and a significant effect of the conditioning factor for pre-exposed PBS animals (pre-exposed PBS-control/ pre-exposed PBS-conditioned) ($F_{(1,36)} = 14.81$; $P < 0.0005$), but not for pre-exposed TTX animals (pre-exposed TTX-control/ pre-exposed TTX-conditioned). The contrast analysis also revealed a significant effect of the pre-exposure factor for PBS-conditioned animals (pre-exposed PBS-conditioned/ non pre-exposed PBS-conditioned) ($F_{(1,36)} = 14.22$; $P < 0.001$) and of the microinjection factor for the pre-exposed-conditioned groups (pre-exposed PBS-conditioned/ pre-exposed TTX-conditioned) ($F_{(1,36)} = 4.51$; $P < 0.05$).

To sum up, the dopaminergic variations recorded during the retention session in the *dorsomedial shell* part of the nucleus accumbens following intervention in the left SUB at PND8

amounted to moderate increases for the non pre-exposed PBS-control and non pre-exposed TTX-control groups, and marked increases for the non pre-exposed PBS-conditioned and non pre-exposed TTX-conditioned groups. Unlike in non pre-exposed PBS-conditioned animals, in pre-exposed PBS-conditioned animals the dopamine signal oscillated around the baseline. For the pre-exposed TTX-conditioned animals, the dopamine increase is not significantly different from that observed in the non pre-exposed TTX-conditioned group.

DISCUSSION

The present study was designed to investigate the consequences of neonatal functional inactivation of the left ventral SUB by TTX in 8-day-old rats for latent inhibition-related dopaminergic responses monitored in grown-up rats in the left *core* and left *dorsomedial shell* parts of the nucleus accumbens in an olfactory aversion procedure. The postnatal unilateral (left) functional blockade of the SUB was followed in pre-exposed TTX-conditioned adult rats by a disruption of latent inhibition behavior and induced a partial and total reversal of the latent inhibition-related dopaminergic responses in the *core* and the *dorsomedial shell* parts of the nucleus accumbens respectively.

The neonatal TTX inactivation of the left SUB induced no significant behavioral and dopaminergic differences in adulthood between non pre-exposed PBS and non pre-exposed TTX animals. In respect of the two postnatal microinjections (PBS or TTX), the non pre-exposed control (NaCl) and non pre-exposed conditioned (LiCl) groups displayed attraction or aversion towards the olfactory stimulus respectively. These responses were similar to those previously obtained using the same conditioning procedure in adult animals free of neonatal intervention (Jeanblanc *et al*, 2002, 2004; Peterschmitt *et al*, 2008) and suggest olfactory perception is not impaired following neonatal TTX inactivation of the SUB. Regarding pre-exposed PBS animals, the behavioral and dopaminergic responses for pre-exposed PBS-control and pre-exposed PBS-conditioned animals are consistent with the latent inhibition responses reported in the same paradigm in adult animals without neonatal PBS microinjection (Jeanblanc *et al*, 2002, 2004; Peterschmitt *et al*, 2008). Thus, it appears that the neonatal intervention in the SUB does not, by itself, induce any significant effects on the different responses observed in adult animals.

Pre-exposed TTX-conditioned adult rats displayed typical aversive responses corresponding to a complete disappearance of the latent inhibition behavior. This is in keeping

with recent observations (Meyer *et al*, 2009). The present results show that TTX inactivation of the left SUB at PND8 induces a differential reversal of the latent inhibition-related dopaminergic responses in the two subregions of the nucleus accumbens. In the *core* part of the nucleus accumbens, dopaminergic responses in pre-exposed TTX-conditioned animals were in-between those obtained in non pre-exposed TTX-conditioned animals and pre-exposed PBS-conditioned animals. By contrast, in the *dorsomedial shell* part of the nucleus accumbens, dopaminergic changes were similar in pre-exposed TTX-conditioned animals and non pre-exposed TTX-conditioned rats and characteristic of aversion. Contrary to the SUB neonatal inactivation, our previous data showed that the neonatal inactivation at PND8 of the left ENT, which is closely interconnected with the SUB (van Groen and Lopes da Silva, 1986; Naber *et al*, 2000; van Groen *et al*, 2003; O'Mara, 2005), induced a total disappearance of the typical latent inhibition-related dopaminergic responses in both the *core* and *dorsomedial shell* parts of the nucleus accumbens (Peterschmitt *et al*, 2007). These data argue in favor of an anatomical specificity of the neonatal TTX inactivation in the SUB as compared to the same inactivation of the ENT (Peterschmitt *et al*, 2007; Meyer *et al*, 2009).

No macroscopic anatomical changes are observed in adult animals subjected to postnatal TTX microinjection in the left SUB. The neonatal blockade at PND8 may affect subicular developing fibres inasmuch as in the second week after birth the afferent and efferent connections of the SUB are still maturing (Schlessinger *et al*, 1975; Singh, 1977a, b; Fricke and Cowan, 1978). The establishment of neural networks depends in part on electrical activity involved in neuronal survival (Farber and Olney, 2003), synaptic proliferation (Marty *et al*, 2000), axons' myelination (Barres and Raff, 1993; Demerens *et al*, 1996; Zalc and Fields, 2000), the rearrangement of synaptic connections in the target structures (Stryker and Harris 1986, Katz and Shatz, 1996; Hutchins and Kalil, 2008) and the maturation of dendritic spines (Drakew *et al*, 1999, Frotscher *et al*, 2000). The transient functional disconnection of the SUB in the critical two-week period after birth (Clancy *et al*, 2001) may thus, by a combination of the aforementioned mechanisms, permanently affect communication with structures directly connected to the SUB, in particular the nucleus accumbens (Brog *et al*, 1993; Groenewegen *et al*, 1987, 1999, Mulder *et al*, 1998). Indeed, the dopaminergic changes in pre-exposed TTX-conditioned animals in both the *core* and *dorsomedial shell* parts of the nucleus accumbens after SUB inactivation at PND8 are different from those observed after SUB blockade in adult animals

before the pre-exposure session in previous experiments in our laboratory (Peterschmitt *et al*, 2008). There is no direct demonstration in the present paper of the specificity of the developmental aspect of SUB neonatal inactivation, but the differences in dopaminergic variations obtained for different ages of TTX microinjection (present study, Peterschmitt *et al*, 2008) are compatible with neurodevelopmental consequences of SUB postnatal inactivation.

The disappearance of the behavioral expression of latent inhibition after neonatal inactivation of the SUB is comparable to that observed after transient TTX blockade of the SUB at pre-exposure in adult animals (Peterschmitt *et al*, 2008). Those results have been interpreted as a consequence of the failure of the SUB to encode the CS-related information during the first session (see Peterschmitt *et al*, 2008, for discussion). The present results could have a similar explanation. A permanent dysfunction of the SUB following neonatal inactivation would prevent the learning and memorization of the information about the CS in the pre-exposure stage. Thus, after neonatal SUB inactivation it is only the association between the CS and the aversion acquired during the conditioning stage which would be retrieved during the retention stage. This may explain the aversion behavior in pre-exposed TTX-conditioned animals during the retention phase.

The present data suggest neonatal inactivation of the SUB has more serious consequences for the dopaminergic responses recorded in the *dorsomedial shell* part of the nucleus accumbens than those recorded in the *core* part of the nucleus accumbens. Projections from the SUB to the two subregions of the nucleus accumbens have been described, although the SUB appears to reach predominantly the *dorsomedial shell* subregion (Brog *et al*, 1993; Groenewegen *et al*, 1987, 1999, Mulder *et al*, 1998). Regulation of dopamine release and metabolism in the nucleus accumbens by SUB projections has been reported for both the *core* and *dorsomedial shell* parts in adult animals (Louilot and Le Moal, 1994; Blaha *et al*, 1997; Legault and Wise, 1999; Legault *et al*, 2000; Taepavarapruk *et al*, 2000, Floresco *et al*, 2001; Cano-Cebrian *et al*, 2003). Previous data showed that unlike the SUB, neonatal blockade of the ENT induced a complete disruption of the typical latent inhibition-related dopaminergic responses in both the *core* and in the *dorsomedial shell* parts of the nucleus accumbens (Peterschmitt *et al*, 2007). The ENT sends projections both to the *core* and *dorsomedial shell* parts of the nucleus accumbens (Totterdell and Meredith, 1997). Therefore, a tentative explanation for the present dopaminergic changes recorded in the *core* could be that the control exerted by the SUB on dopamine release in the *core*

part of the nucleus accumbens following neonatal inactivation is defective but partially offset by the intact ENT projections. Such a compensatory mechanism would not exist for the *dorsomedial shell* subregion which receives equivalent innervation from the SUB and the ENT (Brog *et al*, 1993; Groenewegen *et al*, 1987, 1999, Mulder *et al*, 1998; Totterdell and Meredith, 1997).

The relationships between the behavioral responses and the dopaminergic variations in the two parts of the nucleus accumbens could be considered in the context of the switching model of latent inhibition proposed by Weiner (2003), which was developed initially on the basis of lesion results which showed that latent inhibition expression was spared after *core* lesions but disrupted after *shell* lesions (Tai *et al*, 1995; Weiner *et al*, 1996; 1999; Jongen-Rêlo *et al*, 2002; Gal *et al*, 2005, Pothuizen *et al*, 2005; but see Pothuizen *et al*, 2006). According to this model, the *core* part of the nucleus accumbens is involved in a switching mechanism between responding according to the CS-reinforcement associations acquired during conditioning and responding to the CS-no event associations acquired during pre-exposure; the *shell* inhibiting this mechanism. Further, the two behavioral responses (conditioned response or latent inhibition response) are associated in this model with no initial increase in dopamine and a rapid increase in dopamine release respectively in the *core* subregion (Weiner, 2003; 2010; see also Cassaday and Moran, 2010). This hypothesis would be consistent with the dopaminergic variations observed in the non pre-exposed PBS-conditioned or non pre-exposed TTX-conditioned animals which showed an aversion towards the CS and dopaminergic changes in pre-exposed PBS-conditioned animals which displayed a behavioral preference towards the CS. However, the present results obtained in pre-exposed TTX-conditioned animals, which showed intermediate dopaminergic changes between the two latter groups but which showed a withdrawal behavior, are not easily interpreted with the proposed switching model alone. One interpretation would be that a given increase of the dopamine level in the *core* part would be necessary to switch the responding according to the CS-no event associations acquired during pre-exposure. The present data could also be tentatively interpreted as reflecting a functional complementarity between the *core* and anterior dorsal striatal subregions (see Voorn *et al*, 2004). After neonatal blockade of the SUB, an absence of dopaminergic changes in the anterior part of the dorsal striatum in pre-exposed TTX-conditioned animals has recently been reported (Meyer *et al*, 2009).

Regarding the *dorsomedial shell* part of the nucleus accumbens, no increase in dopamine was obtained in the present study in the pre-exposed PBS-conditioned group displaying latent

inhibition, whereas dopamine levels were increased for the three other conditioned groups displaying aversion towards the CS. These variations in conditioned animals seem fairly compatible with the switching model proposed by Weiner (see Weiner, 2003; 2010), according to which inhibition of the switching leading to latent inhibition manifestation is thought to correspond to a decrease in dopamine levels in the *dorsomedial shell*, whereas disinhibition of the switching mechanism and disruption of latent inhibition would correspond to a rise in dopamine levels in this *shell* subregion. However, the possibility that the dopaminergic changes in the *dorsomedial shell* may partly reflect the salience of the stimulus acquired in a given situation (see for review Horvitz 2000; Young *et al*, 1998; 2005) cannot be ruled out, and this suggestion would be consistent with the increases in the dopamine signal obtained in 1) the four control groups showing an approach towards the stimulus and 2) the non pre-exposed PBS-conditioned, non pre-exposed TTX-conditioned and pre-exposed TTX-conditioned groups which exhibit an aversive behavior.

To conclude, the present experimental data support the view that early functional impairment of the SUB results in a disappearance of the normal expression of the latent inhibition phenomenon and latent inhibition-related dopaminergic responses in the *core* and *dorsomedial shell* subregions of the nucleus accumbens. They could open up new prospects for modelling the pathophysiology of schizophrenia. Not only have SUB anomalies reminiscent of neurodevelopmental failures been described in schizophrenia (Arnold *et al*, 1995; Arnold, 2000; Rosoklija *et al*, 2000; Law *et al*, 2004), but also a reduction in latent inhibition has been reported in neuroleptic-naïve patients with schizophrenia (Baruch *et al*, 1988; Gray *et al*, 1992, 1995; Lubow *et al*, 2000 Rasche *et al*, 2001). Moreover, the *dorsomedial shell* part of the nucleus accumbens seems to be the target structure for the common action of neuroleptics (Deutch and Cameron, 1992, Robertson and Fibiger, 1992, Jennings *et al*, 2006), this being consistent with enhanced dopaminergic transmission in the *dorsomedial shell* in acute patients. Thus, it could be tentatively suggested that an increased release of dopamine in this accumbal subregion may result in a disinhibition of the switching mechanism and the reduction of latent inhibition observed in patients with schizophrenia.

DISCLOSURE/CONFLICT OF INTEREST

The authors have no conflicts of interest to report, nor any involvement to disclose, in matters relating to the experiments presented in this manuscript.

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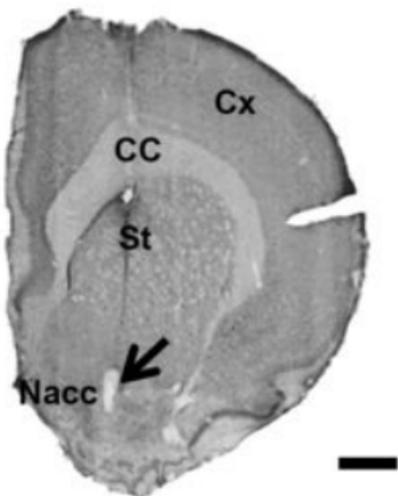
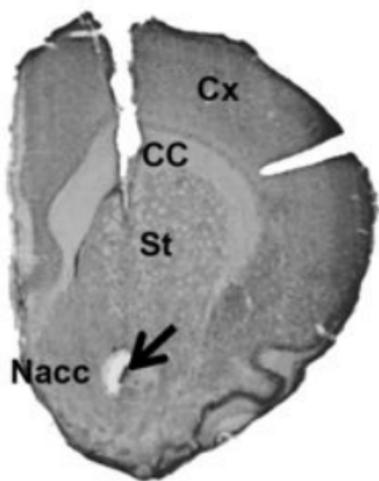
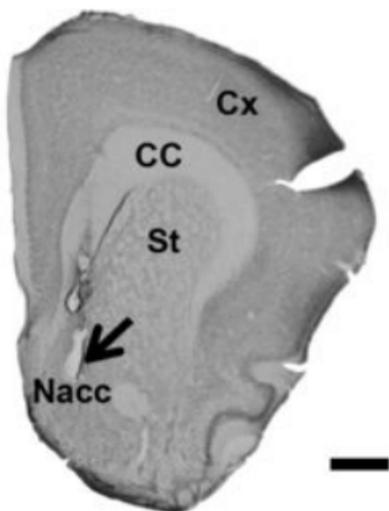
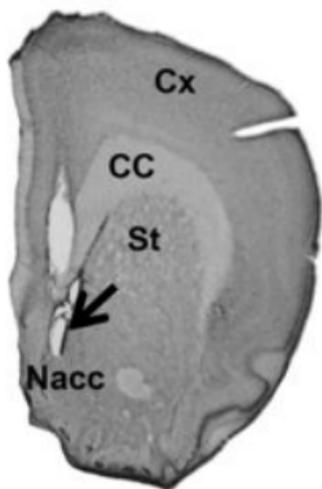
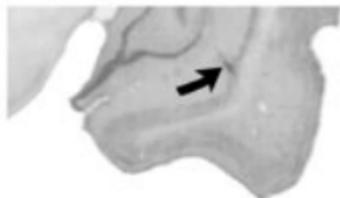
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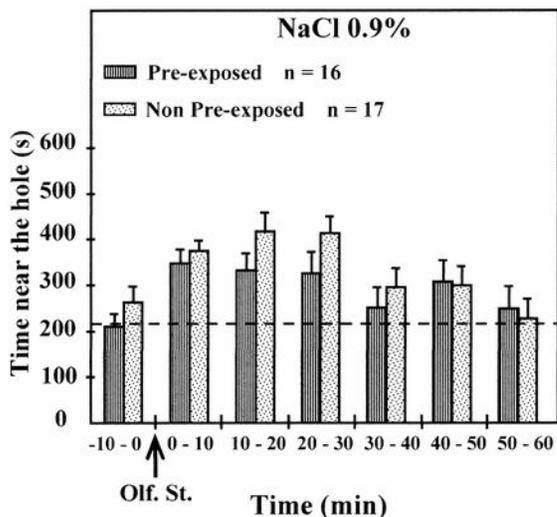
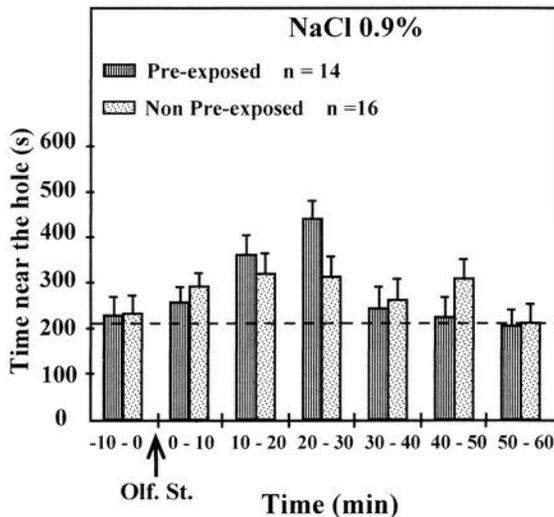
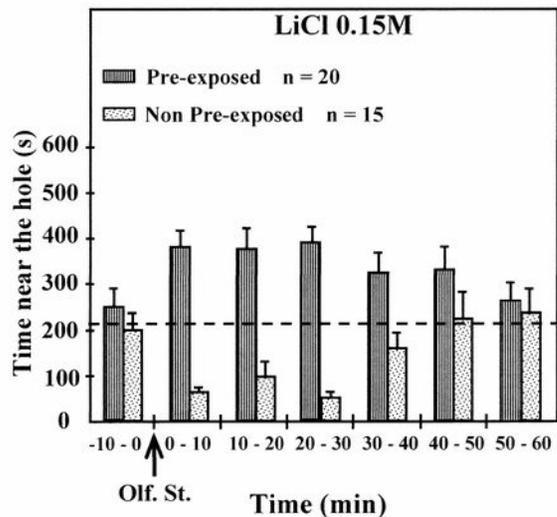
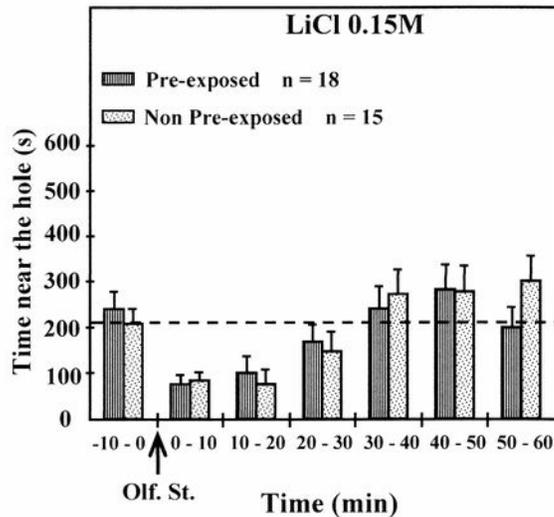
Figure 1. Typical recording sites in the (A) *core* or (B) *dorsomedial shell* part of the left nucleus accumbens (Nacc) (upper sections), and (C) typical injection sites in the left SUB (lower sections) of adult animals neonatally microinjected at PND8 with PBS (left column) or TTX (right column). The recording sites were visualized by means of electrocoagulation at the end of the experiment (arrows); sections were stained using Thionin Blue coloration. The centre of the microinjection site in the left SUB (lower sections) was identified by means of Evans Blue track (arrows) and the sections were stained using Neutral Red coloration. Scale bar = 1 mm.

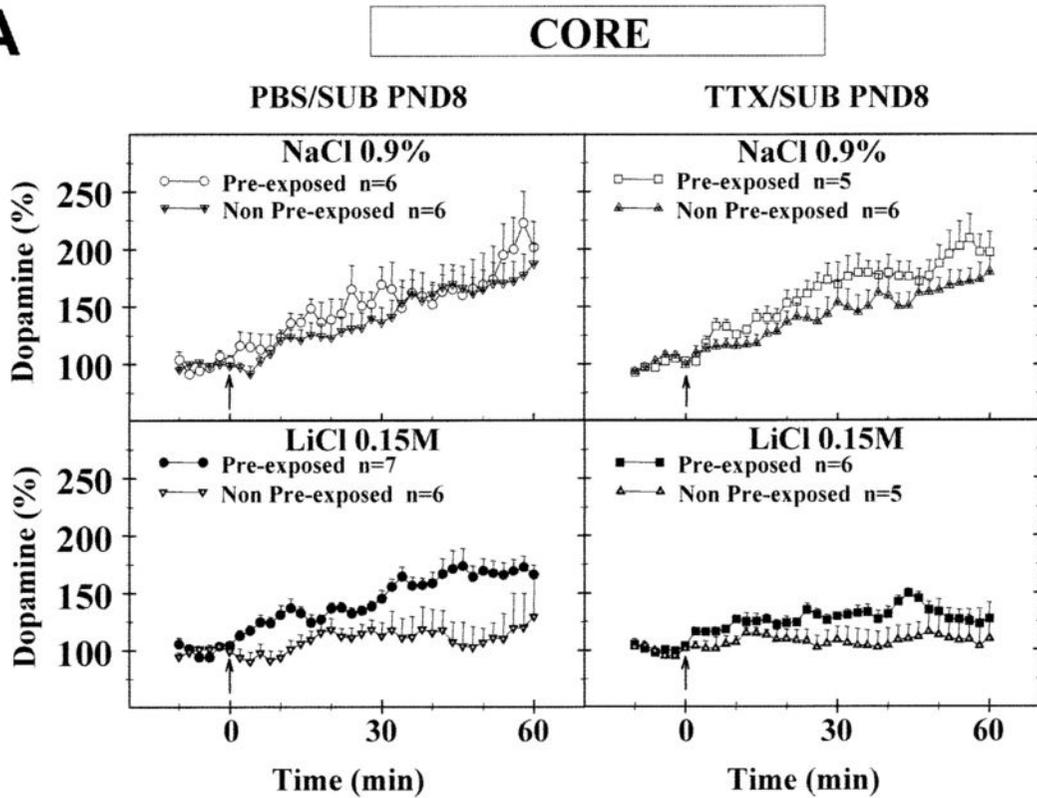
Cx : cortex, CC ; corpus callosum ; St.: striatum.

Figure 2. Time spent near the hole during the test session in pre-exposed (hatched bars) and non pre-exposed (spotted bars) adult animals microinjected in the left SUB with either PBS (A and A') or TTX (B and B') at PND8 of the neonatal period. The arrows indicate when the olfactory stimulus (banana odor) was presented. During the conditioning session, banana odor was associated in control animals with an i.p. injection of NaCl (0.9%) and in conditioned animals with an i.p. injection of the same volume of LiCl (0.15 M). The dashed line represents 35% of one 10-min period (210 s), corresponding to neutral distribution of the rats in the cage. n represents the number of animals per group.

Figure 3. Dopaminergic variations in the (A) *core* and (B) *dorsomedial shell* parts of the nucleus accumbens recorded during the test session in adult pre-exposed and adult non pre-exposed animals after transitory blockade of the left SUB by TTX at PND8 of the neonatal period. Extracellular dopaminergic levels were assessed using DNPV and computer-assisted numerical analysis in freely moving rats. Voltammograms were recorded every minute. Only mean values and SEM corresponding to two scans are presented. Where no SEM is indicated, the size is less than the radius of the symbol. The arrows indicate presentation of the olfactory stimulus (banana odor). n represents the number of rats per group. Results were analyzed by factorial ANOVA.

A**B****C**

A **PBS/SUB PND8****B** **TTX/SUB PND8****A'** **PBS/SUB PND8****B'** **TTX/SUB PND8**

A**B**