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Research article

Long-term effects of prenatal stress and diazepam on D2 receptor expression in the nucleus accumbens of adult rats

S. Lakehayli[a,*], N. Said[a], M. El Khachibi[b], M. El Ouahli[c], S. Nadifi[b], F. Hakkou[a], A. Tazi[a]

[a] Laboratory of Pharmacology, Faculty of Medicine and Pharmacy of Casablanca, 19 Rue Tarik Bnou Ziad, Casablanca, Morocco
[b] Genetics and Molecular Pathology Laboratory, Faculty of Medicine and Pharmacy of Casablanca, 19 Rue Tarik Bnou Ziad, Casablanca, Morocco
[c] Sultan My Slimane University, Fac Sciences & Tecniques Beni-Mellal, Life Sciences, Morocco

HIGHLIGHTS

- Prenatal stress has long lasting effects on the dopaminergic system in the nucleus accumbens.
- Offspring from prenatally stressed dams exhibited significantly elevated *Drd2* mRNA expression.
- Repeated adult diazepam exposure down-regulated *Drd2* expression and prevented the effect of prenatal stress.

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ABSTRACT

Early life stress during the gestational period alters specific neuronal circuits leading to behavioral alterations later in life. In the present study, we assessed the effects of prenatal stress and repeated benzodiazepine administration on dopamine receptor 2 expression in the nucleus accumbens of adult offspring.

Our results show elevated *Drd2* expression levels in the nucleus accumbens (NAcc) of prenatally stressed rats compared to control subjects, while repeated diazepam administration in adulthood down-regulated *Drd2* expression and prevented the effect of prenatal stress. These observations suggest that prenatal stress may induce permanent alterations in the corticolimbic pathway implicated in drug-seeking behavior.

1. Introduction

It is well known that dopamine exerts its functions via binding to DA receptors, which are divided into two classes (D1-like and D2-like receptors) on the basis of their biochemical and physiological effects [1]. The D2 receptors are the most abundant subtypes of D2-like receptors critically involved in drug addiction and the reward pathway [2]. Many studies have shown that stress alters dopaminergic function and elicits specific responses depending on the type and duration of aversive stimulation [3–5].

Benzodiazepines are some of the most commonly prescribed medications used to treat anxiety and insomnia. Despite the strong anxiolytic potential, chronic benzodiazepine treatment elicits adaptive responses in the central nervous system, seen behaviorally as functional tolerance and physical dependence [6]. Furthermore, benzodiazepines modulate chloride ion flux through $GABA_A$ receptor channels leading to an overall hyperpolarization of GABA interneurons and a decrease in their activity. As a consequence, a reduction in the release of GABA induces a disinhibition of dopamine (DA) neurons and produces a strong increase in extracellular DA in the mesolimbic reward circuit [7].

Based on the specific involvement of DA system in drug-seeking behavior, it was important to evaluate the long-lasting effects of prenatal stress on this system in adult life. In this report, we aimed to examine whether prenatal stress exposure may have a detrimental impact on the dopamine receptor 2 expression and its response to chronic benzodiazepine administration. We therefore, exposed female rats to chronic stress during their last ten days of pregnancy and measured the levels of D2-like receptors by real time PCR in the nucleus accumbens of adult offspring.

* Corresponding author. Tel.: +212 22471289; fax: +212 22267091.
E-mail address: Lakehayli.sara@gmail.com (S. Lakehayli).

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Table 1
Primer sequences used for semi-quantitative real-time PCR.

Gene	Gene bank	Primer sequences (5′–3′)	Amplification size
Hprt	NM_012583.2	Fwd: GACCGGTTCTGTCATGTCG Rev:ACCTGGTTCATCATCACTAATCAC	61
Drd2	NM_012547	Fwd AAGCGCCGAGTTACTGTCAT Rev GGCAATGATACACTCATTCTGGT	111

2. Materials and methods

2.1. Animals

Experiments were carried out in male and female Wistar rats (Laboratory of Pharmacology Casablanca), weighing 200–250 g. Rats were housed three per cage and allowed free access to food and water. Animals were handled daily for 7 days before each experiment. Constant temperature (22 ± 1 °C) and lighting conditions (12 L:12 D cycle) were maintained in the housing room. All experiments were approved by the Ethical Committee for biomedical research of the Faculty of Medicine and Pharmacy of Casablanca, Morocco (Comité d'Éthique pour la Recherche Biomédicale de Casablanca: CERBC).

2.2. Drugs

Diazepam (Roche, Morocco), was obtained as solution (1 g/100 ml) and diluted in 0.9% NaCl. Animals received saline (0.9% NaCl) or drug (Diazepam: 2.5 mg/kg) injections, as appropriate in a volume of 5 ml/kg body weight of animal.

2.3. Prenatal stress procedure

Prenatal stress was conducted as previously described [8]: Pregnant female rats were assigned randomly to prenatal stress (PS) and control (Ctrl) groups. Stress was performed each day of the last ten days of pregnancy in which the neural development of the fetus is supposed to occur in rats [9].

Stressed dams were taken to an experimental box with a grid floor that allowed delivering daily 80 electric shocks (0.5 mA, for 5 s, 1–2 min apart) on a random basis during 100-min sessions carried out between 08:00 and 16:00 h. Control females were left undisturbed in their home cages. After birth, the litter sizes were recorded and adjusted to the same litter size (8 pups per litter). All offspring were fostered by their own mothers. The pups were weaned at 21 days of age and housed in groups of three per cage. A total of 5 randomly selected litters per group was used in this study. A maximum of three pups per litter was used for each experimental group to avoid any litter effect [10]. The experiments were carried out during the light phase of the light-dark cycle.

2.4. Injection treatments

Adult control (Ctrl) and PS offspring (80 days of age) were weighed and randomly assigned to either a saline or diazepam (DZP) group ($n = 6$ for each group). Saline groups received four daily intraperitoneal (i.p) injections of NaCl 0.9%, while the DZP groups received four daily i.p injections of diazepam (2.5 mg/kg).

2.5. Tissue collection

Adult treated and untreated rats were euthanized by decapitation 24 h after the last injection. Brains were quickly removed and placed on ice. The nucleus accumbens was dissected out from this section, a rat brain atlas [11] being used for reference. Two coronal cuts were made at right angles to the axis of the brain. The first cut was made 1 mm anterior to the optic chiasma while the second cut was 1.5 mm anterior to the first cut. The nucleus accumbens (NAcc) was dissected, frozen on dry ice and stored at −80 °C until molecular analysis.

2.6. Real-time PCR analysis

Total RNA was extracted from frozen tissues using Trizol (Invitrogen) according to manufacturer's instructions. RNA concentration and quantified using the NanoVue™ Plus Spectrophotometer (GE Healthcare, UK). RNA extracts were kept frozen until use.

For RT-PCR, cDNAs were obtained by reverse transcription from 2 μg of RNA using 4 μl of M-MLV reverse transcriptase (Invitrogen), 1 μl random Examer (Invitrogen), 10 nM dNTP (Invitrogen) and 1 μl de RT Superscript at 200 U/μl (Invitrogen, Morocco).

From resulting cDNAs (2 μl per sample), each sequence of interest was amplified in a final volume of 25 μl of a commercial reaction mixture, containing: 5× reaction buffer, 1.5 mM MgCl2, 50 μM primers, 0.25 U Taq polymerase (BIOLINE, LONDON, UK) and 50 ng of cDNA.

Thermal cycling parameters were: 35 cycles of DNA denaturation (5 min at 95 °C), primer hybridization (30 s at 95 °C), elongation (30 s at 72 °C) and a final elongation step (7 min at 72 °C). PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. Each sample was analyzed in triplicate and to ensure measurements form different PCR runs were cross-comparable. Real time PCR was performed using real time PCR Applied Biosystem FAST 7500 apparatus and Syber Green according to manufacturer's protocol.

Relative quantification was used to determine fold changes (control vs PS), using the ΔΔCT method. Primer sequences are shown in Table 1. *Hprt* was used as a housekeeping gene.

2.7. Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA), with group (control vs PS) and treatment (vehicle vs diazepam) as between-subject variables, followed by student's *t*-test. Significance was set at $p < 0.05$.

3. Results

As shown in Table 2, no differences in litter sizes, number of males and females per litter and male/female ratio were found between prenatally stressed and non-stressed animals ($p > 0.05$).

Two-way analysis of variance showed a significant stress/treatment interaction (Fig 1: $F_{(3,20)} = 32.108$; $p < 0.001$).

Table 2
Litter parameters analyzed in control and prenatally stressed litters.

Parameter	Control	Prenatal stress
Litter size	9.6 ± 0.74	10.2 ± 0.37
Number of males per litter	5.2 ± 0.31	5.0 ± 0.22
Number of females per litter	4.4 ± 0.51	5.2 ± 0.37
Male/female ratio	1.17 ± 0.08	0.99 ± 0.11

Data are expressed as mean ± SEM of PS ($n = 10$) and control ($n = 10$) litters.