



Research report

Imipramine reverses alterations in cytokines and BDNF levels induced by maternal deprivation in adult rats

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H I G H L I G H T S

- ▶ Effects of imipramine in maternally deprived (MD) rats.
- ▶ Imipramine presented antidepressant-like effects.
- ▶ Imipramine regulated the pro-inflammatory cytokines in MD rats.
- ▶ Imipramine regulated BDNF levels in MD rats.

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A B S T R A C T

A growing body of evidence is pointing toward an association between immune molecules, as well brain-derived neurotrophic factor (BDNF) and the depression. The present study was aimed to evaluate the behavioral and molecular effects of the antidepressant imipramine in maternally deprived adult rats. To this aim, maternally deprived and non-deprived (control group) male rats were treated with imipramine (30 mg/kg) once a day for 14 days during their adult phase. Their behavior was then assessed using the forced swimming test. In addition to this, IL-10, TNF- α and IL-1 β cytokines were assessed in the serum and cerebrospinal fluid (CSF). In addition, BDNF protein levels were assessed in the prefrontal cortex, hippocampus and amygdala. In deprived rats treated with saline was observed an increase on immobility time, compared with non-deprived rats treated with imipramine ($p < 0.05$). Deprived rats treated with saline presented a decrease on BDNF levels in the amygdala ($p < 0.05$), compared with all other groups. The IL-10 levels were decreased in the serum ($p < 0.05$). TNF- α and IL-1 β levels were increased in the serum and CSF of deprived rats treated with saline ($p < 0.05$). Interestingly, imipramine treatment reversed the effects of maternal deprivation on BDNF and cytokines levels ($p < 0.05$). Finally, these findings further support a relationship between immune activation, neurotrophins and the depression, and considering the action of imipramine, it is suggested that classic antidepressants could exert their effects by modulating the immune system.

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1. Introduction

Stressful situations during early life have been linked to the development of individual differences in vulnerability to mood disorders, such as depression, throughout life [1–3]. To explain how this alteration could come about during adulthood, the maternal deprivation model has been proposed. According to this model, early adverse experiences would lead to behavioral and

neurobiological alterations in adulthood [4,5]. In addition, during postnatal development, maternal separation induces a response characterized by reduced activity and other depressive-related behaviors [6,7].

Recently, evidence has pointed that depression can be related to changes in the immune system. This is illustrated by the fact that patients with depression presented elevated levels of proinflammatory cytokines, for example, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [8–10]. Also, it was shown an increase of chemokines, adhesion molecules and prostaglandins in the peripheral blood [11,12]. Furthermore, antidepressant drugs are reported to display anti-inflammatory effects [13–15].

As well as the actions of the immune system, several studies have shown a relationship between neurotrophins and depression. Brain derived-neurotrophic factor (BDNF) is a neurotrophin that plays an important role in the growth and differentiation of new neurons and synapses [16]. Moreover, altered BDNF expression or polymorphism in this protein has been related in both patients and in the animal model of depression [17–19]. Moreover, drugs used to treat depression have presented positive effects on BDNF levels [19–22]. More recently, studies also showed that BDNF participates of the inflammatory pain processes [23,24]. Exposure to early life stress leads to neural loss in brain areas, such as the prefrontal cortex, hippocampus and amygdala, which are regions involved with emotion and memory [25,26]. Also, these brain areas are implicated with both pathophysiology and treatment of depression [20,27].

Studies showing the effects of antidepressant drugs on behavior, inflammatory system and neurotrophins in maternally deprived rats have not been characterized. As animal models are important tools for understanding the neurobiology of depression, to understand the mechanism of action of existing drugs and to find new targets, the present study was aimed at evaluating the effects of the classic antidepressant imipramine on behavior, cytokines and BDNF levels in adult rats submitted to maternal deprivation.

2. Experimental procedures

2.1. Animals

Pregnant female Wistar rats (age of 3 months, weight of 250–280 g) were obtained from the UNESC (Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil) breeding colony. All animals were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). The environment temperature ($22 \pm 1^\circ\text{C}$) was kept constant. Pregnant females were individually housed with sawdust bedding and ad libitum access to food and water. During the deprivation maternal were used male and female pups. Litters were culled to eight pups per dam, four males and four females ($\pm$). The day of delivery was marked as day zero, and on post natal day 1 (PND-1) a maternal deprivation protocol was applied to 50% of the male pups from days 1–10 after birth; the other males were used as controls. Animals were weaned at the age of 21 days, and housed in regular cages, with 5 animals to a cage. Females were donated to the UNESC colony for other research purposes. The males were used in the present experiments. There were two experimental groups: (1) control (non-deprived), which received no treatment whatsoever; (2) deprived, which were submitted to maternal deprivation as described. All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Usage of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNec) recommendations for animal care, and also with approval by the local Ethics Committee under protocol number 12/2010.

2.2. Maternal deprivation protocol

Female Wistar rats were maintained in individual boxes until their delivery day. Deprivation was carried out for 180 min a day from PND-01 to PND-10. The deprivation protocol consisted of removing the mother from the residence box and taking her to another room. Pups were maintained in their home cage (grouped in the nest in the presence of maternal odor). We prefer this maternal deprivation protocol because it does not require manipulation of the pups [4,28]. At the end of each daily deprivation session, the mothers were returned to their home boxes; this procedure was carried out during the light part of the cycle, between 8:00 a.m. and 2:00 p.m. At the same time and place had litters that were not deprived of maternal care. Control rats remained in their resident boxes together with their mothers throughout. Only on PND-11, were the boxes cleaned normally again, according to the laboratory routine. On PND-21 the animals were weaned, and males were

randomized in the following experimental groups: (1) non-deprived + saline; (2) non-deprived + imipramine; (3) deprived + saline; and (4) deprived + imipramine, and maintained in groups of 5 in 50 cm $\times$ 25 cm $\times$ 40 cm plastic boxes with a stainless steel lids, with food and water ad libitum, as per all the other animals in our animal housing facility.

2.3. Drugs and treatment

Imipramine (a tricyclic antidepressant) was obtained from Novartis Pharmaceutical Industry (Brazil) and a dose of 30 mg/kg was injected intraperitoneally once a day, over a period of 14 days, after the animals had reached the age of 3 months. We used the treatment for 14 days and the dose of 30 mg/kg because in previous studies from our group, the treatment with imipramine by this period and dose presented antidepressant effects in the forced swimming test [17,29,30]. The treatments were administered at a volume of 1 mL/kg. To develop this study we employed 48 animals ($n = 12$ each) separated in four groups, as follows: (1) non-deprived + saline; (2) non-deprived + imipramine; (3) deprived + saline; (4) deprived + imipramine. One hour after the last injection, the rats were tested using the forced swim test.

2.4. Forced swimming test

The forced swimming test was conducted according to previous reports [31–33]. The test involves two individual exposures to a cylindrical tank filled with water, in which rats cannot touch the bottom of the tank or escape. The tank is made of transparent Plexiglas, 80 cm tall, 30 cm in diameter, and filled with water ($22\text{--}23^\circ\text{C}$) to a depth of 40 cm. On the 13th day of chronic treatment with imipramine, 1 h after drug treatment, the rats were individually placed in the cylinder containing water for 15 min (pre-test session). On the 14th day, the rats received the last intraperitoneal drug treatment, and after 1 h they were again subjected to the forced swimming test for a 5 min session (test session) and the immobility time of rats was recorded in seconds. Immediately after the forced swimming test, under anesthesia, blood and CSF were collected for subsequent analyses of cytokine levels ($n = 3\text{--}5$). The peripheral blood was collected by cardiac puncture, and CSF was drawn, by direct puncture of the cisterna magna with an insulin syringe (27 gauge 31/20 length). Individual samples of CSF that presented visible blood contamination were discarded. The rats were then killed by decapitation, the skulls were immediately removed and the prefrontal cortex, hippocampus and amygdala were quickly isolated by hand dissection using a magnifying glass and a thin brush. The dissection was based on the histological distinctions described by Paxinos and Watson [34], the brain tissue was then used for BDNF analysis ($n = 6\text{--}8$).

2.5. BDNF analysis

BDNF levels in the prefrontal cortex, hippocampus and amygdala were measured by anti-BDNF sandwich-ELISA, according to the manufacturer's instructions (Chemicon, USA). Briefly, the rat's prefrontal cortex, hippocampus and amygdala were homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM (EGTA). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluents, and the standard curve ranged from 7.8 to 500 pg/mL of BDNF. The plates were then washed four times with sample diluent and a monoclonal anti-BDNF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin-enzyme, substrate and stop solution, the amount of BDNF was determined by absorbance in 450 nm. The analytical sensitivity and detection limits were as follows: sensitivity = 7.8 pg/mL; range of detection = 7.8–500 pg/mL; intra-assay variation: $\pm 3.7\%$ (125 pg/mL); inter-assay variation: $\pm 8.5\%$ (125 pg/mL). We have added the sentence in the methods. The standard curve demonstrates a direct relationship between Optical Density (OD) and BDNF concentration. Total protein was measured by Lowry's method using bovine serum albumin as a standard, as previously described by Lowry et al. [35].

2.6. Cytokine analysis

The serum and CSF were diluted in extraction solution containing PBS. The concentration of cytokines (IL-1 β , IL-10 and TNF- α) were determined by ELISA (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate. Briefly, the capture antibody (13 mL, contains 0.1% sodium azide) was diluted in phosphate-buffered saline (PBS), added to each well and left overnight at 4°C . The plate was washed four times with PBS and 0.05% Tween 20% (Sigma, St. Louis, MO, USA). The plate was blocked with 1% bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS and 0.05% Tween 20%. The samples and standards were added and the plate was incubated overnight at 4°C . After washing the plate, a detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plate was incubated for 2 h at room temperature. After washing the plate, streptavidin (DuoSet R&D Systems, Minneapolis, MN, USA) was added and the plate was incubated for 30 min. Finally, a color reagent, phenylenediamine (Sigma, St. Louis, MO, USA) was added to each well and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped with the

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