Int. J. Devl Neuroscience 30 (2012) 285–291

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form 17 January 2012
Accepted 26 January 2012

Keywords:
Neonatal handling
Reversal learning
Sex-specific
Oxidative stress

ABSTRACT

Early life experiences have profound influences on behavior and neurochemical parameters in adult life. The aim of this study is to verify neonatal handling-induced sex specific differences on learning and reversal learning as well as oxidative stress parameters in the prefrontal cortex and striatum of adult rats. Litters of rats were non-handled or handled (10 min/day, days 1–10 after birth). In adulthood, learning and reversal learning were evaluated using a *Y* maze associated with palatable food in male and female rats. Morris water maze reversal learning was verified in males. Oxidative stress parameters were evaluated in both genders. Male neonatal handled animals had a worse performance in the *Y* maze reversal learning compared to non-handled ones and no difference was observed in the water maze reversal learning task. Regarding females, neonatal handled rats had a better performance during the *Y* maze learning phase compared to non-handled ones. In addition, neonatal handled female animals showed a decreased SOD/CAT ratio in the PFC compared to non-handled females. We conclude that neonatal handling effects on learning and memory in adult rats are sex and task specific. The sex specific differences are also observed in the evaluation of antioxidant enzymes activities with neonatal handling affecting only females.

1. Introduction

Several studies have documented the impact of early life events on neuroendocrine and behavioral status in adulthood (Levine, 1957; Meaney et al., 1996; Padoin et al., 2001). A suitable environment during the neonatal period is essential for a healthy development of neonates, since they are more vulnerable during this period. The hypothalamic–pituitary–adrenocortical (HPA) axis is one of the most important neuroendocrine systems activated in response to actual or presumed environmental challenges. In rats, it has been demonstrated that both prenatal and postnatal factors may influence the development of the HPA axis (Liu et al., 1997; Maccari et al., 2003; Plotsky and Meaney, 1992; Weinstock, 1997). Rats who are briefly and repeatedly separated from their mothers at the beginning of their lives, by a procedure called neonatal handling, show when adults a less pronounced increase in the secretion of adrenal glucocorticoids in response to a variety of stressors (Meaney et al., 1991). They also have attenuated fearfulness (decreased freezing, increased exploration) in novel environments and an increased ingestion of palatable food (Silveira et al., 2004). In addition, we have demonstrated in a previous study that adult female neonatal handled rats have impairment in spatial learning (a sex-specific effect) in the water maze task (Noschang et al., 2010). However, many outcomes concerning neonatal handling and learning and memory are possible dependent on age, sex and task evaluated (Kosten et al., 2007; Meaney et al., 1988; Vallee et al., 1999; Weinberg and Levine, 1977).

Different types of learning involve distinct cerebral structures. The hippocampus and related structures support learning in spatial tasks like water maze (Morris et al., 1982). On the other hand, behavioral flexibility, the ability to adjust responses according to changes in strategies, rules or stimulus-reward contingencies, is mediated by the prefrontal cortex (PFC). This cortical region has been identified as a key structure for reversal learning in many species, including humans, monkeys and rodents (Clarke et al., 2008; Hornak et al., 2004; McAlonan and Brown, 2003). Additionally, it has been suggested that flexible behavior is not supported uniquely by PFC. One brain structure that may interact with PFC to mediate cognitive flexibility is the striatum (Kolb, 1977).

It has been demonstrated that oxidative stress contributes to age-related impairments in learning and memory (Liu et al., 2003). The whole brain is vulnerable to free radicals-induced damage

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doi:10.1016/j.ijdevneu.2012.01.010

because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes (Halliwell and Gutteridge, 2007; Olanow, 1992). Oxidative stress happens when there is an imbalance between antioxidant defenses and oxidative species. In this case, the antioxidant defenses, such as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), are not able to neutralize the reactive species efficiently (Halliwell and Gutteridge, 2007). As consequences, oxidative stress may affect structure and function of different proteins and enzymes; it may also affect membranes, lipids and DNA (Cochrane, 1991). Therefore, function and plasticity of neurons could be changed leading to altered memory processes.

Once we have already observed sex specific differences in spatial learning in adult neonatal handled rats when compared to non-handled ones, we wonder how this early intervention would affect reversal learning associated to food reward. In addition, striatum and PFC are involved in reversal learning, and no study was found evaluating oxidative stress parameters in these structures in adult neonatal handled rats considering sex differences. Therefore, the aim of this study is to evaluate the sex specific differences in reversal learning in adult neonatal handled rats as well as in oxidative stress parameters in the striatum and PFC of these animals.

2. Materials and methods

2.1. Subjects

All animal proceedings were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone from gestational day 18th in home cages made of Plexiglas (65 cm × 25 cm × 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2 °C, cage cleaning twice a week, food and water provided ad libitum. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for handling procedures which were carried out between 10:00 and 15:00 h. Several litters were submitted to the handling procedures in the same day. The researcher changed gloves between the handling procedures of each litter to avoid any kind of odor to be spread from nest to nest. Litters were weaned and separated by sex on postnatal day 21. Animals were maintained in groups from 3 to 5 rats per cage. Experiments were performed when adults, and each experimental group had no more than two animals per litter (around three months old) in the behavioral experiments and only one animal per litter (around four months old) for biochemical measurements. Experiments using males and females were performed separately at different times. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral task was applied (task was performed between 9:00 and 14:00 h).

2.2. Neonatal handling (Silveira et al., 2006)

Non-handled group: Pups were left undisturbed with the dam since birth until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the main researcher.

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper towel. This cage was placed into an incubator set to maintain an ambient temperature at 30–32 °C. After 10 min, pups were returned to their dams. This procedure was performed from day 1 to 10 following birth, and then pups were left undisturbed until the 21st day of life (weaning). It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure of non-handled group was performed to change dirty sawdust.

2.3. Learning and reversal learning evaluation

A *Y* shaped maze with three wood arms (each one measuring 30 cm high × 51 cm long × 12 cm wide) with a 120° angle from each other was used and the behavior was performed under red light. On the first day of training, animals were allowed to freely explore the maze for 5 min. On the following days, they were introduced in the maze facing the wall in the extremity of the arm that was closer to the evaluator. Animals could choose to enter one of the two other arms of the maze, but only one arm had five units of sweet palatable food (Froot Loops®). Once in the maze, animals had 60 s to choose one of the arms. If they did not do it they were returned to their cages. On the other hand, once they chose one of the arms, the other entrance was closed with a door and they could stay in the maze for additional 60 s. This procedure was repeated four times a day for each rat, using a inter-trial interval of around 20 min. Animals were trained in the learning phase to a criterion of four trials with a maximum of one error/day (error was defined by entrance in the arm with no food). After reaching that, the palatable food was switched to the other arm, requiring the subject to reverse what was previously learned (reversal phase). Successful reversal performance was defined as four trials with a maximum of one error/day. The animals were food restricted during this task (receiving about 80% of habitual ingestion of standard lab chow). Results show the number of correct choices (entrance in the arm with food) as well as the number of pellets consumed (corrected by the number of correct choices) in both learning and reversal phases.

2.4. Morris water maze reversal learning

Another set of neonatal handled and non-handled male rats with three months of age were used in this task. The Morris water maze (Morris et al., 1982) acquisition phase was performed as described in detail in Noschang et al. (2010). After seven days of acquisition, reversal learning was performed. Briefly, animals were trained to find the hidden platform now located in a different position from the acquisition phase, during four days (four trials per day). Latency to find the platform was determined in each trial.

2.5. Preparation of the samples for biochemical measurements

Animals were killed by decapitation when they were four months old, and the PFC and striatum were quickly dissected out. The brain structures were stored at −70 °C until analysis, when they were homogenized in 10 vol. (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged (at 960 × *g*) for 10 min at 4 °C and the supernatant was used for the evaluation of reactive species production by the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and the evaluation of antioxidant enzymes activity.

2.5.1. Superoxide dismutase activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux et al. (1995). SOD activity is expressed as U/mg of protein. One unit of SOD causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride under the conditions of the assay.

2.5.2. Glutathione peroxidase activity

GPx activity was determined according to Wendel (1981), with modifications (Noschang et al., 2010). The reaction was carried out at 37 °C, and the activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. GPx activity was expressed as nmol NADPH oxidized per minute per mg of protein.

2.5.3. Catalase activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2) and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25 °C (Aebi, 1984). CAT activity was calculated in terms of micromol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.5.4. Evaluation of reactive species production by the chemical oxidation of DCFH (Lebel et al., 1992)

The samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100 μM) at 37 °C for 30 min. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in the samples. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored using excitation and emission wavelength of 488 and 525 nm, respectively, using a spectrophotometer. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein.

2.5.5. Determination of total thiol (SH) content

This assay is based on the reduction of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) by SH groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm (Riddles et al., 1983).

2.5.6. Protein assay

The total protein concentrations were determined using the method described by Lowry et al. (1951), with bovine serum albumin as standard.