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Sex-related long-term behavioral and hippocampal cellular alterations after nociceptive stimulation throughout postnatal development in rats

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ABSTRACT

Early noxious stimuli may alter the neurogenesis rate in the dentate gyrus and the behavioral repertoire of adult rats. This study evaluated the long-term effects of noxious stimulation, imposed in different phases of development, on nociceptive and anxiety-like behaviors, hippocampal activation, cell proliferation, hippocampal BDNF and plasma corticosterone levels in 40 day-old male and female adolescents. Noxious stimulation was induced by intra-plantar injection of Complete Freund's adjuvant (CFA), on postnatal days (P) 1 (group P1), 8 (P8) or 21 (P21). Control animals were not stimulated in any way. On P21 a subset of animals from each group received BrdU and was perfused on P40 for identification of proliferating cells in the granule cell layer of the dentate gyrus. Another subset of rats was subjected to behavioral testing on P40 and one week later, to magnetic resonance imaging (MRI) acquisition. Noxious stimulation evoked hypoalgesia in adolescents, mainly in females ($P < 0.02$), reflected by greater latency to withdraw the paw and less paw lickings in the hot plate test than controls ($P < 0.001$). It also resulted in more time spent in the open arms, e.g., less anxiety-like behavior than controls ($P < 0.01$), especially in females ($P < 0.01$, compared with males). Proliferative cell rate in the dentate gyrus was the highest in P8 males and females ($P < 0.001$), with males exhibiting more proliferation than females on P1 and P8, which was directly related to the hippocampal levels of BDNF and inversely related to plasma corticosterone. Sex differences were also detected in manganese-enhanced MRI signal, which was more prominent in P1 females than males ($P < 0.01$). This study represents the first step of investigation on the cellular basis of the sex-dependent long-term consequences of nociceptive stimuli in newborns.

1. Introduction

Advances in early postnatal medical care have allowed substantial increase in the survival rate of prematurely-born infants (Stoll et al., 2010). However, stressful routine interventions in intensive care units result in excessive exposure of preterm neonates to multiple invasive procedures (Carbajal et al., 2008; Simons et al., 2003) that may cause long lasting alterations in somatosensory and cognitive processing (Anand, 2000; Grunau et al., 1998, 1994). The mechanism subjacent to such altered processing could be related to reduction of white matter and maturation of subcortical gray matter as has been recently described after procedural pain (Brummelte et al., 2012).

Recent clinical follow up studies have demonstrated that in adulthood, former preterm children display altered pain threshold (Hermann et al., 2006; Hohmeister et al., 2010; Walker et al., 2009)

Abbreviations: CFA, complete Freund's adjuvant; MEMRI, Manganese-enhance MRI; MRI, Magnetic resonance image; P1, postnatal Day 1; P8, postnatal Day 8; P21, postnatal Day 21.

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and decreased modulatory pain mechanisms (Goffaux et al., 2008). Ten year-old former preterm infants rate medical-related pain pictures as more intense that other pain pictures, indicating that they may have more pain memories than age-matched full term infants (Grunau et al., 1998). However, one limitation of the clinical studies is the difficulty to distinguish between long-term effects of pain and confounding factors such as stress, neonatal morbidities and environmental influences (Brummelte et al., 2012).

Newborn rats and humans display similar behavioral repertoire in response to noxious stimulation (Guy and Abbott, 1992; Johnston et al., 1993). Such parallel suggests that functional properties in the nociceptive processing may share similar mechanisms that can be investigated using animal models for nociception. As previously described (Anand et al., 1999), at birth the formation of central nervous system is incomplete in rat pups and corresponds to that of 24 week intra-uterine human preterm neonates, following similar patterns in the development of pain system (Fitzgerald and Anand, 1993). The first postnatal week in newborn rat pups corresponds to human premature infants from 24 to 36 weeks of gestation (Kim et al., 1996; Wilson, 1995), offering a suitable condition to model and compare preterm to full term infants subjected to noxious stimulation.

The involvement of hippocampal formation in cognitive tasks has been recognized for decades (Milner, 1972; Squire, 1982). It has been proposed that differences in dentate granule cell proliferation affect the formation of temporal associations during acquisition and retention (Aimone et al., 2006). In adult animals, while dentate neurogenesis rate is positively related to better performance in cognitive tasks, it is negatively related to stress and anxiety (for review, see Schoenfeld and Gould (2012)). However, there is limited information regarding the effects of neonatal nociception on neurogenesis and its role on subsequent pain threshold and anxiety-like behavior. Recently it was demonstrated that the natural ability of dentate granule cells to proliferate from birth until adulthood may be affected by early neonatal nociceptive stimulation. In response to inflammatory nociceptive stimulus applied on postnatal day 1 (P1), hippocampal dentate granule cell layer displays greater elevation in mitotic rate during adolescence (Leslie et al., 2011), which raises questions about the role of hippocampus in long-term nociceptive processing.

The sex of the patient and/or experimental animal may be an important modulator of the long term effects of neonatal pain and stress. Recent experimental studies have addressed these sex differences in behavioral expression after early postnatal noxious stimulation (LaPrairie and Murphy, 2007, 2010; Negrigo et al., 2011) and sex-specific behavioral alterations between preterm and age-matched infants (Guinsburg et al., 2000).

Considering the abovementioned evidence, the purpose of the present study was to investigate the consequences of noxious stimulus applied in three different phases of development on hippocampal cell proliferation and activation, hippocampal BDNF levels, anxiety-like behavior and baseline corticosterone plasma levels of male and female adolescent rats.

2. Methods

This was an experimental, randomized, controlled and blind study. Protocol approval was granted by the Independent Ethics Committee of the Universidade Federal de São Paulo (approval #1378/09). All efforts were made to minimize animal suffering and to reduce the number of animals to the minimum necessary.

2.1. Subjects

Time-pregnant Wistar rats were obtained on the 14th day of gestation from the Center for Development of Animal Models of this University. Dams were housed individually in plastic cages and placed in the animal facility, equipped with an automatic temperature control system (23 ± 2 °C), ventilation, and a 12-h light–dark cycle (lights on at 7:00 AM). All litters were studied from the first day of life (P1) on, reared identically, weaned at P21, and from then on, housed in same-sex groups of 8. All possible care was taken in order to avoid stress to the dams and litters during the experimental protocol.

One hundred and thirty four pups (67 males and 67 females) underwent noxious stimulation, whereas 46 pups were not submitted to any noxious manipulation. A subset of 96 animals was used both for the behavioral and MRI experiments and 36 animals were used in the study of hippocampal cell proliferation and differentiation. Additional 61 animals were used for determination of hippocampal BDNF (final $N = 53$) and plasma corticosterone levels (final $N = 61$). Observers who were blind to the treatment of neonatal rats performed and evaluated the behavioral testing and other measurements. Weight gain was measured with an electronic scale (sensitivity 0.1 g) on P1, P21, P40 and no differences could be detected among groups, as previously reported by our group (Leslie et al., 2008). Vaginal smears were conducted in order to determine the phase of the estrous cycle, and all samples were taken between 08:00 and 09:00 h. All females that were already cycling were excluded from study.

2.2. Noxious stimulation

The protocol for noxious stimulation of the rats was previously described (Leslie et al., 2011). Briefly, on postnatal days 1, 8 or 21 rat pups received a single intraplantar injection of an inflammatory agent, the complete Freund's adjuvant (CFA; 25 μl, Sigma, Saint Louis, MI, US) into the left hindpaw and were returned to their home-cage. These groups were named P1, P8 and P21, respectively. Control (CTL) animals were not injected but were handled at the same time as experimental pups. These ages were chosen because P1 corresponds to 24 weeks of human gestation (extreme prematurity) (Anand et al., 1999), whereas P8 would correspond approximately to 38–40 weeks of intra-uterine age; finally, day 21 was included as a control group for painful stimulation during later development. The experimental design is summarized in Fig. 1. All animals in a single litter were assigned to a particular group (manipulated at P1, P8, P21 or not stimulated), to avoid the influence that handling of pups at different ages could have on maternal care towards the remainder of the litter. Moreover, within each litter, pups were randomly assigned to different experiments (behavior and MRI analysis or dentate cell proliferation/differentiation) to avoid litter effects.

2.3. Nociceptive and anxiety measurements

Behavioral tests (hot plate and elevated plus maze tests) results are based on data from 19 animals for P1 group (11 males, 8 females); 26 animals for P8 group (12 males, 14 females); 31 animals for P21 (13 males, 18 females) group and 20 CTL animals (10 males, 10 females).

The nociceptive test consisted of placing the rat on a hot plate (52.5 °C) and measuring the latency for and the number of lickings of the hindpaw (either one). To avoid potential tissue damage, a 20 s automatic termination of the heat stimulus was established if no paw withdrawal occurred. This cut off time (20 s) was then considered as the maximal latency. The hot plate test data was averaged from three trials with 15 min intervals. The testing apparatus was cleaned between sessions. The observer was blind to treatment condition during testing.

The elevated plus maze apparatus consisted of a central platform (5 × 5 cm) with two open arms (50 cm long, 10 cm wide and 0.5 cm high borders) and two closed arms (same dimensions as the open arms with 40 cm high walls) elevated 50 cm above the ground. Rats were placed on the central platform facing the open arm and were observed during 5 min. The numbers of entries into the open and closed arms as well as the time spent in each arm were recorded.

2.4. Manganese-enhanced MRI (MEMRI) procedures

MEMRI acquisitions were performed in a subset of animals after the behavioral tests: 10 animals for P1 group (5 males, 5 females); 13 animals for P8 group (5 males, 8 females); 16 animals for P21 group (7 males, 9 females) and 10 animals for CTL (5 males and 5 females) group. Detailed MEMRI procedure has been previously published elsewhere (Malheiros et al., 2012). Briefly, $MnCl_2$ (60 mg/kg, i.p.) was administered to all animals; the solution consisted of 100 mM $MnCl_2{-}4H_2O$ (Sigma, Saint Louis, MI, US) in 400 mM bicine buffer, pH 7.4. In order to investigate the pattern of cellular activation in the dorsal hippocampus following nociceptive stimulation, all animals including controls, received a single injection of CFA (25 μl) in the left hindpaw 12 h after manganese chloride injection and two hours before MRI brain scanning. MRIs were obtained in a 2 T/30 cm horizontal superconducting magnet 85310HR (Oxford Instruments, Abingdon, UK) interfaced to a Bruker Avance AVIII console (Bruker-Biospin, Ettlingen, GE) running Paravision 5.1 software (Bruker, Ettlingen, GE). A Double Crossed Saddle radiofrequency coil (Papoti, 2006) was used as a head probe in animals anesthetized with ketamine/xylazine (95/12 mg/kg, i.p.). A T_1-weighted 3D FLASH (Fast Low Angle SHot) sequence (TR = 200 ms, TE = 5.8 ms, flip angle = 90°, 4 averages, 40 min/animal) was acquired. A volume of 40 × 40 × 11.2 mm^3 was covered with 192 × 192 × 16 points, producing a spatial resolution of 208 × 208 × 700 μm^3. All images were normalized to the same window and level settings.

MRI data were analyzed using the Paravision 5.1 software. One author (JMM), blind to the group's identity, manually outlined the region of interest (ROI)