- We examined the interaction between MS and NHI on emotionality and markers of plasticity.
- Both MS and NHI increased anxiety levels, but only NHI induced depression-like behavior.
- Maternal separation did not further increase emotionality in HI-treated rats.
- Both MS and NHI decreased synaptophysin levels in dentate gyrus and CA3 hippocampal areas.
- BDNF expression in CA3 was decreased only in the HI animals that were maternally-separated.

Maternal separation prior to neonatal hypoxia-ischemia: Impact on emotional aspects of behavior and markers of synaptic plasticity in hippocampus

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Abbreviations

BDNF: Brain-derived neurotrophic factor; EPM: Elevated plus maze; FST: Forced swimming test; MS: Maternal separation; NHI: Neonatal hypoxia-ischemia; NMS: No maternal separation; OFT: Open field test; PND: Postnatal day; SYN: Synaptophysin

Abstract

Exposure to early-life stress is associated with long-term alterations in brain and behavior, and may aggravate the outcome of neurological insults. This study aimed at investigating the possible interaction between maternal separation, a model of early stress, and subsequent neonatal hypoxia-ischemia on emotional behavior and markers of synaptic plasticity in hippocampus. Therefore, rat pups (*N*=60) were maternally separated for a prolonged (MS 180min) or a brief (MS 15min) period during the first six postnatal days, while a control group was left undisturbed. Hypoxia-ischemia was applied to a subgroup of each rearing condition on postnatal day 7. Emotional behavior was examined at three months of age and included assessments of anxiety (elevated plus maze), depression-like behavior (forced swimming) and spontaneous exploration (open field). Synaptic plasticity was evaluated based on BDNF and synaptophysin expression in CA3 and dentate gyrus hippocampal regions. We found that neonatal hypoxia-ischemia caused increased levels of anxiety, depression-like behavior and locomotor activity (ambulation). Higher anxiety levels were also seen in maternally separated rats (MS180min) compared to non-maternally separated rats, but prolonged maternal separation prior to HI did not potentiate the HI-associated effect. No differences among the three rearing conditions were found regarding depression-like behavior or ambulation. Immunohistochemical evaluation of synaptophysin revealed that both prolonged maternal separation (MS180min) and neonatal hypoxia-ischemia significantly reduced its expression in the CA3 and dentate gyrus. Decreases in synaptophysin expression in these areas were not exacerbated in rats that were maternally separated for a prolonged period prior to HI. Regarding BDNF expression, we found a significant decrease in immunoreactivity only in the hypoxic-ischemic rats that were subjected to the prolonged maternal separation paradigm. The above findings suggest that early-life stress prior to neonatal hypoxia-ischemia leads to significant alterations in synaptic plasticity of the dorsal

hippocampus during adulthood, but does not exacerbate HI-related changes in emotional behavior.

Keywords: anxiety, BDNF, depression-like behavior, neonatal stress, neonatal hypoxia-ischemia, synaptophysin

1. Introduction

Traumatic life events during childhood can exert a profound and long-lasting effect on brain, both at structural and functional levels. Individuals who endured abuse, neglect or significant loss are at high risk for developing anxiety or depression (Gibb et al., 2007; Heim et al., 2010; Springer et al., 2007), as well as cognitive impairments (Bremner et al., 2003; Bücker et al., 2012; Mills et al., 2011; Nixon et al., 2004; Syal et al., 2014). In addition, childhood adversities have been associated with dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Ehlert, 2013) and decreased gray matter volume in limbic regions (Lim et al., 2014; Van Dam et al., 2014).

Animal models of postnatal stress have also demonstrated the detrimental impact of early-life adversity on brain and behavior. Maternal separation (MS), a well-established model of postnatal stress, induces short or long-term changes in the stress reactivity system as indicated by the potentiated HPA axis response to subsequent stressors (Knuth and Etgen, 2007; Lippmann et al., 2007; McCormick et al., 1998; Veenema et al., 2006). Furthermore, it enhances manifestations of anxiety and depression-like behaviors (Fabricius et al., 2008; Lambás-Señas et al., 2009; Rüedi-Bettschen et al., 2005; Tata, 2012), and impairs spatial learning and memory (Aisa et al., 2009b, 2007; Tata et al., 2015) during adulthood. These behavioral effects tend to be mediated by structural and synaptic changes (Bock et al., 2005; Eiland and McEwen, 2012; Oomen et al., 2010; Pascual and Zamora-León, 2007), as well as alterations in neurotrophin levels and neurogenesis (Aisa et al., 2009a; Andersen and Teicher, 2004; Lippmann et al., 2007; MacQueen et al., 2003; Marais et al., 2008; Roceri et al., 2004).

The experimental procedure of MS is employed during the first postnatal weeks. This period is considered critical for brain development since many structures, including the hippocampus, undergo significant changes, such as increased proliferation, synaptogenesis and myelination (Kosten et al., 2012; Rice and Barone, 2000). Moreover, adverse postnatal

manipulations may interact with neurological insults, thus exacerbating their damaging effects. In fact, early-life stress in the form of MS confers vulnerability to limbic epiliptogenesis, increases susceptibility to seizure-associated microglial activation and neuronal death, and in addition reduces exploratory behavior (Kazl et al., 2009; Salzberg et al., 2007). Furthermore, postnatal stress exacerbates neonatal white matter injury and induces adult hyperglycemia after neonatal cerebral ischemic-hypoxic brain injury, but does not potentiate hippocampal tissue loss (McPherson et al., 2009). The Rice-Vannucci rat model of HI on PND7 is a well-established model of perinatal human encephalopathy, and is associated primarily with cerebral cortex and hippocampal injury (Gill and Perez-Polo, 2008; Rice et al., 1981). Developmental maturity of the rat's brain on PND7 corresponds to that of the late preterm human fetus (Workman et al., 2013).

Individuals with a history of perinatal HI encephalopathy are at high risk of developing long-lasting sensorimotor deficits (Volpe, 2008). Over the last years there has been an increasing interest in exploring the effects of hypoxic-ischemic encephalopathy on cognitive functioning, reporting impairments in attention, executive functioning, visuospatial, ability, and learning and memory (Anderson and Arciniegas, 2010; Rennie et al., 2007; van Handel et al., 2007). Yet less attention has been paid on how this insult may influence emotional behavior.

We recently reported that MS prior to neonatal HI augments the HI-associated spatial learning and reference memory impairments during adulthood. Interestingly, these behavioral effects were not associated with exacerbation of infarct size or hippocampal tissue loss (Tata et al., 2015). However, it could be possible that down-regulation of markers of synaptic plasticity, such as BDNF and synaptophysin, may play some role in these cognitive deficits. BDNF is essential for neurite outgrowth, cell survival and synaptic strengthening (Lu et al., 2005), while synaptophysin, a synaptic vesicle-associated protein commonly used as an

estimate of the number of functional synapses, is involved in neurotransmission (Calhoun et al., 1996; Thiel, 1993; Valtorta et al., 2004). Recent data underline the essential role of hippocampal BDNF and synaptophysin in cognitive functions (Heldt et al., 2007; Liu et al., 2005), as well as in anxiety- and depression-related behaviors (Domingos da Silveira da Luz et al., 2013; Shirayama et al., 2002), and stress the neuroprotective role of neurotrophins against neonatal HI-related brain injury (Almli et al., 2000; Chen et al., 2013; Han et al., 2000). Given the above evidence, in the current paper we extended our study to investigate the hypothesis that MS may interact with neonatal HI, thus exacerbating changes in synaptophysin and BDNF expression in the hippocampus, a structure particularly vulnerable to both experimental conditions. Furthermore, given that perinatal HI encephalopathy has been implicated in emotional dysregulation, we aimed at exploring the effects of neonatal HI on anxiety and depression-like behaviors, as well as whether early stress may have potentiated the effects of neonatal HI on emotionality.

2. Material and Methods

2.1. Animals

Female Wistar rats on the second gestational week were individually housed until delivery. The day of birth was designated as postnatal day 0 (PND0). Sixty infant rats (29 males, 31 females) were included in the experiments and remained with their dams until weaning (PND23). Subsequently, rats of the same gender were housed in groups of 2-3 per cage. All animals were maintained under standard breeding conditions on a 12 h light/dark cycle (8:00 – light on / 20:00 – light off) with food and water available ad libitum. Handling of the pups and behavioral testing was performed by the same personnel based on evidence that factors associated with experimenter may affect behavioral outcomes and familiarity with the experimenter increases consistency in results (Sorge et al., 2014; van Driel and

Talling, 2005). All experimental procedures were conducted in accordance to the Institutional Animal Ethics EL 54 BIO 20.

2.2 Experimental manipulations

2.2.1. Rearing conditions

On PND1 litters comprising both genders were assigned randomly to one of the following conditions: a) no maternal separation (NMS; $N = 25$), b) 15 min maternal separation (MS 15min; $N = 16$), or c) 180 min maternal separation (MS 180min; $N = 19$). The pups of the NMS condition were left undisturbed in their cage with dams, while rats of the MS 15min or MS 180min groups were maternally separated for either a short (15 min) or prolonged period (180 min) daily during PND1-6. In contrast to the prolonged MS, early MS for short periods of time (e.g., 15 min), an experimental manipulation also known as 'early handling', is associated with increased expressions of maternal care (Macrì et al., 2008), and reduced behavioural and endocrine stress reactivity in the offspring (Levine, 2005).

The maternal separation procedures took place between 9:00 and 14:00 h and were performed as previously described (Tata et al., 2015). Heating pads were placed under the containers to compensate for the mother's body heat (PND1-PND6: 32 °C / PND7-PND21: 30 °C) (Arborelius et al., 2004; Huot et al., 2001). During separation periods, dams and pups were kept at different rooms in order to eliminate any potential olfactory, auditory, or visual contact between them. At the end of the separation period, pups were returned to their home cages followed by their dams. No cage cleaning or bedding change took place until PND6. There was no mortality in the MS groups (15min, 180min) compared to the NMS animals. Litter size ranged from 5-9 rats. Given that litter size does not affect maternal behavior in case it ranges between 5 and 18 animals (Champagne et al., 2003), culling was not considered necessary. Furthermore, we have previously shown that there were no significant differences in the amount of maternal care among these three rearing conditions (Kostopoulou, 2012).

2.2.2. Neonatal cerebral Hypoxia-Ischemia

On PND7, pups (N = 24) from each of the three rearing manipulations were exposed to hypoxic-ischemic conditions (HI) as described by Rice and colleagues (Rice et al., 1981; Vannucci and Vannucci, 2005).

Briefly, pups underwent permanent left common carotid artery ligation and following recovery were subsequently exposed to an hypoxic environment (for a detailed description, see Tata et al., 2015). Sham-treated pups from each rearing condition (N = 36) were subjected to the same operation, but without undergoing artery ligation or being exposed to a hypoxic-ischemic environment. After recovery from anesthesia, pups were returned to their home cage where they remained until weaning. No mortality was seen in the sham-treated animals. In the hypoxic-ischemic rats the mortality rate was 12% and prior exposure of these rats to MS did not further increase it. The animals of each experimental group were obtained from 2 to 3 litters.

2.3. Behavioural testing

At approximately 3 months of age (PND 90-100), all young adult animals were individually examined in a battery of behavioral tests in order to assess emotional and exploratory behavior. Specifically, spontaneous locomotor activity and exploration were evaluated with the open field test (OFT) (Denenberg, 1969). Anxiety and depression-like behavior were assessed by the elevated plus maze (EPM) (Walf and Frye, 2007) and the forced swim test (FST) (Duman, 2010), respectively. Behavioral testing took place during the light cycle (between 9:00 – 15:00) and time period between the tests was at least 2 days long. The experimental room was sound attenuated with dim illumination (~ 150 lux). All experimental apparatuses were cleaned thoroughly with a 25% ethanol solution after each trial to eliminate odor cues. Data were collected digitally with a camera placed 160 cm above the experimental arena and evaluated by two researchers blind to the experimental conditions.

2.3.1. Elevated plus maze (EPM)

The EPM is a reliable tool for evaluating anxiety behavior in rodents (Rodgers and Dalvi, 1997; Walf and Frye, 2007). The apparatus was a black wooden apparatus consisted of four arms (50 × 10 cm), two open and two that were arranged to form a "+" and were enclosed by 40 cm high walls. Rats were placed on the junction of the four arms, which was an open central square (10 × 10 cm), facing the open arm opposite to experimenter, and their spontaneous behavior was recorded for 5 min. This task relies upon rodents' unconditioned fear of height/open spaces and their preference towards protected and enclosed areas (e.g., closed arms). Anxiogenic drugs reduce time spent in the open arms, suggesting that less time in the open arms is indicative of higher levels of anxiety.In the current study we calculated a) the percentage time spent in the open arms [open time / (open + closed time) × 100] as an index of anxiety, and b) the number of closed-arms entries, which provides a measure of general activity (Pellow et al., 1985). An entry was recorded when all four paws of the rodent were on the arm, while two paws out defined an exit.

2.3.2. Forced swimming test (FST)

Forced swimming testing was divided into two sessions, a pretest session (15 min) and a test session (5 min), which was administered 24 h later. During testing, each animal was individually placed in a clear Plexiglas cylinder (40 cm height, 18 cm diameter) filled with water (25 ˚C). Normally, during pretest session naïve rodents exhibit a struggling behavior in order to escape. However, because of the inescapable nature of the tank, a naïve animal gradually adopts a passive 'despair' behavior (the so-called 'learned helplessness'), which is characterized by a reduction in vigorous activity and an increased occurrence of immobile posture that allows them to float by performing only the necessary movements. Since antidepressant agents reduce immobility time, this measurement is considered an index of

depression-like behavior (Hédou et al., 2001; Porsolt et al., 1978). In order to assess animals' depressive-like behavior, we analysed the immobility time recorded during the test session.

2.3.3. Open field test (OFT)

The OFT has been widely used to measure locomotor activity and spontaneous exploration of a novel environment (Denenberg, 1969; Walsh and Cummins, 1976). The apparatus consisted of a wooden open square arena (100 × 100 cm) surrounded by 40 cm high walls to prevent escape. The arena's floor was divided into 16 sectors (25 × 25 cm). The four inner sectors marked out the centre, while the twelve outer sectors were defined as the periphery. Rats were placed in the centre of the arena and were allowed to freely explore it for 6 min. The six-minute interval was chosen based on pilot observations in our lab and in order to allow comparison with previous studies of adult stress (Beck and Luine, 1999; Bowman et al., 2001) or maternal separation (Eiland and McEwen, 2012; Farkas et al., 2009; Faure et al., 2007; Jaworska et al., 2008; Knuth and Etgen, 2007).

The behavioral parameters analysed were a) the number of square visits (centre, periphery, total), b) the time spent in the centre, and c) the number of rears. A visit was defined when at least half of the rat's torso was in a sector (Bisagno et al., 2003), while a rear was identified a posture sustained with the hind paws on the floor and the front limbs lifted off it. General motor activity was estimated by the number of square visits (ambulation) and rears in the arena (Walsh and Cummins, 1976). Time spent in the centre was recorded as an index of anxiety (Carola et al., 2002; Prut and Belzung, 2003).

2.4. Tissue preparation and immunohistochemical staining

On PND120-130, animals were euthanized by deep anaesthesia. The brains of 33 animals (N = 5-6 subjects from each experimental group) were immediately removed and post-fixed (4% paraforlmaldehyde in 0.1 M phosphate buffered saline, 3 × 24 h at 4 ˚C). Coronal blocks were taken, gradually hydrated with descending ethanol solutions and

embedded in paraffin. Next, serial coronal sections (5 μm) were taken at the level of dorsal hippocampus (–3.24 mm to –3.36 mm from bregma) (Paxinos and Watson, 2007).

For the detection of BDNF and synaptophysin (SYN), paraffin sections were deparaffinised and hydrated in xylene and graded alcohol solutions. Next, sections were immersed in 3% hydrogen peroxide (H_2O_2)/methanol to block endogenous peroxidase, pretreated with citrate buffer (pH = 6, 1 h) for antigen retrieval, and rinsed with TBS. Following incubation to blocking buffer (10% fetal bovine sodium, 2% normal goat serum in PBS, 30 min), sections were treated (4 ˚C, overnight) with a primary polyclonal antibody against BDNF (Santa Cruz Inc., 1: 600), or monoclonal antibody against SYN (Santa Cruz Inc., 1:20). Subsequently, they were exposed to goat anti-rabbit or goat anti-mouse secondary antibodies (Dako Inc., 1:200, 1 h), for BDNF or SYN, respectively. In order to visualize immunoreactions, an avidin-biotin peroxidase complex (Vecstatin Kit; Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) as the chromogen were used. All sections were finally dehydrated and counterstained with hematoxylin.

2.5. Image analysis

After immunohistochemical processing, tissue images were captured from coronal sections with a 40× objective lens using a digital camera (Nikon DS-5M-L1) connected to a microscope (Nikon Eclipse 50i). Three sections per animal from a total of fifteen sections were chosen with care to allow comparison of similar regions across experimental conditions. This was achieved by their equal distribution (1:5 sections). Immunoreactivity was estimated within the dentate gyrus (DG) molecular layer and CA3 stratum radiatum, and, specifically, from two optical fields per area (Fig. 1). A minimum of 6 microscopic fields were analyzed from each area per animal. Immunoreactivity for BDNF and SYN was measured by quantitative image analysis using Image-Pro Plus software (version 6.3, Media Cybernetics),

with the experimenter blind with respect to condition. In the current study we estimated the percentage of image tissue area stained with BDNF or SYN, as an index of the BDNF or SYN immunoreactivity.

[Insert Figure 1 about here]

2.6. Statistical analyses

All statistical analyses were performed using the SPSS software package (version 22). The effects of the two experimental conditions (rearing, HI) and their possible interaction on behavior (anxiety, depression-like behavior, locomotor activity) as well as on immunohistochemical markers (BDNF, synaptophysin) were analyzed using 3 × 2 ANOVAs with rearing and HI as the between-subjects factors. Because initial analyses revealed no main effect of gender or any interactions of this factor with rearing or HI conditions ($p > 0.05$), findings are not reported in terms of gender. However, it should be stressed that the size of each gender-related subgroup was too small to detect differences. Post-hoc comparisons were conducted, when necessary, using Tukey's test. Furthermore, significant interactions between the two conditions (rearing, HI) were further explored by T-tests. Data are presented as mean values (±SEM). Only *p* values less than 0.05 were considered as statistically significant.

3. Results

3.1. Behavioral evaluation

3.1.1. Elevated plus maze (EPM)

Time spent in open arms: Analyses revealed a significant effect of HI [$F(1, 54) = 5.726$, $p < 0.05$, partial $\eta^2 = 0.096$; Fig. 2]. HI-treated animals spent less time in the open arms [10.33 (2.13)] compared to sham [17.01 (1.79)], suggesting increased levels of anxiety. A

significant effect was also found for rearing [$F(2, 54) = 3.443$, $p < 0.05$, partial $\eta^2 = 0.113$]. Tukey´s post-hoc analyses showed that the percentage of time spent by the MS 180min group in the open arms was significantly smaller ($p < 0.001$) compared to NMS group, while no significant difference was found between NMS and MS15min animals ($p > 0.05$). The two factors did not interact with each other [$F(2, 54) = 1.239$, $p > 0.05$ partial $\eta^2 = 0.04$].

Entries into closed arms: HI condition did not affect general motor activity, as indicated by the number of entries into the closed arms [$F(1, 54) = 3.13$, $p > 0.05$, partial $\eta^2 = 0.055$; Sham: 11.15 (0.64), HI: 12.92 (0.77)]. Similarly, rearing exerted no influence on the same variable [$F(2, 54) = 1.19$, $p > 0.05$, partial $\eta^2 = 0.042$; NMS: 11.194 (0.81), MS 15min: 13.1 (0.93), MS 180min: 11.82 (0.85)] neither interacted with HI [$F(2, 54) = 0.286$, $p > 0.05$, partial $\eta^2 = 0.01$].

[Insert Figure 2 about here]

3.1.2. Forced Swimming Test (FST)

Neonatal HI showed a significant main effect on the immobility time during the FST, as revealed by a two-way ANOVA [$F(1, 54) = 5.826$, $p < 0.05$, partial $\eta^2 = 0.097$]. HI-treated animals exhibited longer floating time [93.63 (9.57)] than sham [57.63 (11.43)] (Fig. 3). On the contrary, rearing did not differentiate significantly the animals [$F(2, 54) = 1.729$, $p > 0.05$, partial $\eta^2 = 0.06$; NMS: 57.778 (12.07), MS 15min: 77.580 (12.59), MS 180min: 91.55 (13.99)]. No statistically significant interaction was found between the two factors [F (2, 54) = 0.178, $p > 0.05$, partial $\eta^2 = 0.007$].

[Insert Figure 3 about here]

3.1.3. Open field test (OFT)

Ambulation and rearing: There were no significant effects for the *central* square crossings measure caused by either the rearing or HI manipulations [rearing: $F(2, 54) =$

1.346, $p > 0.05$, partial $\eta^2 = 0.024$; HI: $F(2, 54) = 2.408$, $p > 0.05$, partial $\eta^2 = 0.082$], and the two factors did not interact with each other [$F(2, 54) = 0.284$, $p > 0.05$, partial $\eta^2 = 0.01$]. However, analysis of entries in the *peripheral* square entries showed that HI animals expressed higher ambulatory activity than sham [$F(1, 56) = 14.341$, $p < 0.001$, partial $\eta^2 = 0.21$], and a similar effect was seen in the *total* number of square visits [$F(1, 56) = 7.076$, $p < 0.01$, partial $\eta^2 = 0.12$; Fig. 4A]. No significant effect of rearing was found for *peripheral* [$F(2, 54) = 1.129$, $p > 0.05$, partial $\eta^2 = 0.04$] or *total* square entries [$F(2, 54) = 1.384$, $p > .05$, partial $\eta^2 = 0.049$]], nor a significant interaction between HI and rearing [peripheral entries: $F(2, 56) = 1.272$, $p > 0.05$, partial $\eta^2 = 0.045$; *total entries:* $F(2, 56) = 0.637$, $p > 0.05$, partial $\eta^2 = 0.023$]. Analysis of the number of *rearings* recorded during the 6-min testing period did not reveal any significant effect of rearing [$F(2, 54) = 0.503$, $p > 0.05$, partial $\eta^2 = 0.018$], HI [$F(1, 54) = 0.691$, $p > 0.013$, partial $\eta^2 = 0.023$]], or interaction between the two factors [$F(2, 56) = 0.453$, $p > 0.05$, partial $\eta^2 = 0.029$].

Time spent in center: HI-treated animals remained in the central squares of the open field for significantly less time compared to sham [$F(1, 56) = 5.205$, $p < 0.05$, partial $\eta^2 = 0.088$; Fig. 4B]. Rearing also affected this variable [$F(2, 54) = 3.309$, $p < 0.05$, partial $\eta^2 = 0.109$]. Tukey post-hoc analyses revealed that the MS 15min group spent less time in the central squares of the arena when compared to the NMS group [$p < 0.05$; NMS: 35.28 (4.46), MS 15min: 22.71 (5.26)], while animals of the MS 180min group did not differ from any of the other two rearing conditions [$p > 0.05$; MS 180min: 31.71 (4.76)]. The effect of HI did not differ as a function of rearing, as indicated by the non-significant interaction [$F(2, 54) = 0.174$, $p > 0.05$, partial $\eta^2 = 0.006$].

[Insert Figures 4A and 4B about here]

3.2. Immunohistochemical evaluation

3.2.1. Brain-derived neurotrophic factor (BDNF)

A significant main effect of HI on the percentage of BDNF-positive area in CA3 region was detected [$F(1, 20) = 11.389$, $p < 0.01$, partial $\eta^2 = 0.363$; Fig. 5). Specifically, BDNF immunoreactivity in CA3 of HI-treated animals was significantly lower than in sham [sham: 18.02 (1.12), HI: 14.93 (0.97)]. Expression of BDNF in the CA3 was also affected by rearing [$F(2, 20) = 10.731$, $p < 0.01$, partial $\eta^2 = 0.518$]. Post-hoc comparisons revealed that the levels of BDNF appeared significantly elevated ($p < 0.05$) in the MS 15min group [19.26 (0.78)] compared to NMS [15.66 (0.90)] and the MS 180min [14.51 (1.46), $p < 0.01$]. No difference between NMS and MS 180min groups was detected ($p > 0.05$). Interestingly, the two factors (HI, rearing) interacted significantly [$F(2, 20) = 3.574$, $p < 0.05$, partial $\eta^2 = 0.263$]. Further t-test comparisons within each rearing condition revealed that HI caused significant reductions in BDNF only in the MS 180min condition [$t(6) = 3.324$, $p < 0.05$]. On the contrary, HI did not have a significant effect in NMS [$t(6) = 1.291$, $p > 0.05$] or MS 15min [$t(8) = 0.508$, $p > 0.05$] conditions.

Regarding BDNF immunoreactivity in DG, statistical analyses showed no significant effect for either HI [$F(1, 20) = 0.101$, $p > 0.05$, partial $\eta^2 = 0.005$] or rearing [$F(2, 20) = 2.454$, $p > 0.05$, partial $\eta^2 = 0.197$], nor an interaction between the two factors [$F(2, 20) = 3.994$, $p > 0.05$, partial $\eta^2 = 0.01$; Fig. 6].

[Insert Figures 5 and 6 about here]

3.2.2. Synaptophysin (SYN)

Similar to BDNF, SYN immunoreactivity was estimated in CA3 and DG hippocampal regions. In CA3, the percentage of SYN-positive area was significantly reduced by HI [F (1, 17) = 60.761, $p < 0.001$, partial $\eta^2 = 0.781$; Sham: 38.2 (0.87), HI: 29.2 (1.7); Fig. 7]. Synaptophysin immunoreactivity was also affected by rearing [$F(2, 17) = 5.482$, $p < 0.05$, partial $\eta^2 = 0.392$]. According to Tukey post-hoc tests, MS 180min animals expressed

significantly lower levels of SYN compared to the NMS ($p < 0.05$) and MS 15min ($p < 0.001$) conditions, while there was no significant difference between the NMS and MS 15min animals [$p > 0.05$; NMS: 35.4 (0.87), MS 15min: 34.8 (1.03), MS 180min: 31(1.96)]. In addition, a significant interaction between the two factors (HI, rearing) was found [$F(2, 17) = 4.915$, $p < 0.05$, partial $\eta^2 = 0.366$]. T-test comparisons in each rearing condition revealed that hypoxic-ischemic rats in both NMS and MS 15min conditions presented significantly reduced percentage of SYN-positive tissue compared to corresponding sham rats [NMS: $t(6) = 8$, $p < 0.001$; MS 15min: $t(6) = 6.606$, $p < 0.01$]. However, in the MS 180min condition no such difference was found between sham- and HI-treated animals. In fact, the loss of SYN due to 3 h of maternal separation was not further exacerbated following HI [$t(6) = 1.420$, $p > 0.05$].

Analysis revealed a significant effect for HI on mean percentage of tissue immunoreactive for SYN in DG [$F(1, 17) = 18.375$, $p < 0.001$, partial $\eta^2 = 0.519$; Fig. 8]. Specifically, the HI-treated rats expressed significantly lower SYN immunoreactivity compared to sham animals [Sham: 19.77 (1.21), HI: 15.77 (0.68)]. Rearing also affected this variable [$F(2, 17) = 3.659$, $p < 0.05$, partial $\eta^2 = 0.301$], with significant reductions in the 3h maternally-separated rats compared to NMS and MS 15min conditions [($p < 0.05$; NMS:18.34 (1.02), MS 15min: 18.963 (0.64), MS 180min: 16 (1.17)]. No differences between NMS and MS 15min groups were found ($p > 0.05$). A significant interaction between the two factors was also revealed [$F(2, 17) = 4.686$, $p < 0.05$, partial $\eta^2 = 0.355$]. Similar to CA3 immunoreactivity, the percentage of DG which was positive to SYN was not different between the sham and HI-treated rats in the MS 180min condition [$t(3.138) = 0.055$, $p > 0.05$]. On the contrary, HI significantly reduced the expression in both NMS [$t(6) = 5.135$, $p < 0.05$] and MS 15min [$t(6) = 5.125$, $p < 0.05$] conditions.

[Insert Figures 7 and 8 about here]

4. Discussion

Cumulative evidence demonstrate that early-life experiences shape brain development and behavior, and may even influence the outcome of neurological insults. In the current study we investigated the possible interaction between early-life stress adversity, due to maternal separation (MS), and neonatal HI on emotional behavior and neuronal plasticity. In particular, we studied whether adult animals that experienced a neurological insult subsequently to MS as neonates exhibit a potentiated emotional response related to anxiety and depression-like behavior. In addition, we investigated the possible effects of these experimental manipulations on synaptophysin and BDNF in the dorsal hippocampus.

4.1. Effects of Maternal Separation and Neonatal Hypoxia-Ischemia on Emotional Behavior

Both human and animal studies suggest that adverse experience during development augments emotionality. Severe stress due to physical, emotional or sexual abuse renders humans more vulnerable to develop depression and anxiety in adulthood (Heim et al., 2010; Springer et al., 2007). Similarly, postnatal stress in rodents caused by neonatal MS increases emotionality, an effect that tends to be associated with HPA axis dysregulation (Heim and Nemeroff, 2001).

In our study, early life stress increased anxiety-like behavior during adulthood. Indeed, three hours of daily MS during the first six postnatal days significantly potentiated the rats' unconditioned fear towards open spaces The reduced open-arms preference was not associated with a general decrease in activity, as implied by the comparable number of closed-arm entries among the three conditions (NMS, MS 15min, MS 180min). In line with this, there was no significant difference among the NMS, MS 15min and MS 180min groups with respect to the horizontal (ambulation) or vertical (rearing) activity in the open field test

(OFT), a finding also reported by others (Jones et al., 2009; Shalev and Kafkafi, 2002; Vivinetto et al., 2013).

Our finding is in accordance with previous studies reporting that 3 h of daily MS over the first 2-3 postnatal weeks may lead to increased anxiety-like behavior during adulthood (Aisa et al., 2007; Daniels et al., 2009; Huot et al., 2002; Kalinichev et al., 2002; Pascual and Zamora-León, 2007; Wigger and Neumann, 1999). This behavioral effect seems to be mediated by alterations in the HPA axis reactivity. Maternally separated animals with higher anxiety levels tend to oversecrete corticosterone when exposed to stressors as adults (Aisa et al., 2007; Eiland and McEwen, 2012; Kalinichev et al., 2002; Wigger and Neumann, 1999), while this is not the case in maternally separated rats that do not express increased anxiety-like behavior (Grace et al., 2009; Hulshof et al., 2011; Roman et al., 2006; Slotten et al., 2006). Interestingly, MS seems to increase the vulnerability of adult rats to anxiety-like behavior when subsequently exposed to chronic stress (Eiland and McEwen, 2012), although other researchers failed to conclude this (Hulshof et al., 2011).

While the aforementioned studies stress the association between early adversity and increased anxiety, it was not known if a similar neonatal manipulation of shorter duration would have the same behavioral effect. To the best of our knowledge, this is the first study to report augmented anxiety in adult rats that experienced three hours of daily MS for a much shorter period, specifically for six days. Increased anxiety manifestations have also been reported in adult rodents that experienced short periods of early adversity, such as 5 days (PND4-PND9) of neonatal isolation (separation from both siblings and mother) (Knuth and Etgen, 2007). However, the different type of adversity model employed in that study does not allow direct comparisons with our findings.

Another aspect of emotionality that is affected by early-life stress is depression-like behavior, which is typically identified either by expression of gustatory anhedonia or

behavioral despair (Porsolt et al., 1978; Willner et al., 1987). While under normal conditions rats show a preference for sweetened fluids over water, two or three weeks of maternal separation significantly reduce the preference for sucrose solution in adult rats (Aisa et al., 2007; Daniels et al., 2009; Hui et al., 2011). Similarly, rats that experience adversity during the first 2-3 postnatal weeks due to MS, isolation or limited nesting exhibit a "despair" behavior which is expressed by increased duration of immobile posture in the forced swimming test (FST) (Aisa et al., 2007; Hui et al., 2011; MacQueen et al., 2003; Sung et al., 2010; Veenema et al., 2006). Nevertheless, other studies failed to report heightened despair as a result of similar paradigms of postnatal manipulation (Greisen et al., 2005; Marais et al., 2008). Despite the increased anxiety, no signs of depression-like behavior were noted in our MS180 group, as implied by no increase in the immobility time estimated based on floating duration during the testing phase. A major difference between the current study and the majority of studies reporting depressive-like behavior during adulthood is the longer duration of the experimental manipulation (2-3 weeks) compared to ours (6 days). This novel finding suggests that the impact of a much shorter in duration paradigm of MS does not entail depression-like behavior during adulthood.

Our EPM and FST findings support that the brief MS condition (15 min), a procedure known as "early handling", does not affect anxiety and depression-related behaviors. Previous findings regarding the effects of neonatal handling on emotional behavior are inconsistent. According to several reports, adult rodents that experienced brief daily MS as neonates tend to be more resilient to stress, given their reduced emotional arousal, attenuated response of the HPA axis to stressors, as well as increased expression of glucocorticoid receptors in the hippocampus and prefrontal cortex (Bilbo et al., 2007; Levine, 2005; Meaney et al., 1988; Vallée et al., 1997). However, it has been suggested that these beneficial effects of neonatal handling are mediated by changes in maternal care expressed towards pups (i.e., high amounts

of liking, grooming and arch-backed nursing) (Liu et al., 1997). In our study, however, no significant increases in maternal care behaviors were detected in the MS 15min group (Tata et al., 2014). Given that the beneficial effects of neonatal handling seem to be exerted by enhanced maternal care, it is possible that the absence of effect may be related to the non-significant changes in this type of maternal behavior.

Another aim of this study was to investigate the impact of neonatal HI on behaviors related to emotionality. Traditionally, research of human encephalopathy has focused on its effects on brain structure and developmental consequences at very young age, while recent findings underscore the negative impact of this neurological insult on both cognitive functioning and emotional behavior (Armstrong-Wells et al., 2010; van Handel et al., 2010, 2007). According to recent findings, children with a history of neonatal encephalopathy experience increased rates of anxiety and depression as indicated by their teacher's evaluations (van Handel et al., 2010). Meanwhile, the vast majority of animal studies have investigated the long-term effects of neonatal HI on cognition (e.g., Arteni et al., 2010; Huang et al., 2009; Karalis et al., 2011) and less attention has been paid to emotional aspects of behavior. It should be noted, however, that recently there has been an interest in exploring emotional behavioral outcome measures in perinatal HI models (Smith et al., 2014).

In the current study, anxiety and depression-like behavior in young adult rats was estimated based on their performance on the EPM and FST, respectively. The evaluation of EPM performance revealed a reduction of proportion of time spent in the open arms with respect to sham animals, a behavioral profile indicative of increased levels of anxiety. Similar to human studies, information regarding alterations in emotional behavior due to neonatal HI is limited in experimental models of neonatal HI. Our EPM finding is in agreement with previous studies that report increased anxiety in neonates (PND 13), young adult or aged rodents following perinatal asphyxia (Hoeger et al., 2000; Morales et al., 2010; Weitzdoerfer et al.,

2004). However, no significant changes in anxiety were found in young adult rats that had been exposed to neonatal HI (Arteni et al., 2010; Schlager et al., 2011). Some possible reasons why this discrepancy in findings might be observed include methodological differences between the studies. Specifically, in Schlager and colleagues (2011) the index of anxiety being used was the percentage of time spent in the border areas of a different maze, namely the open field (Schlager et al., 2011). Regarding the non-significant change found by Arteni et al. (2010), it is possible that the existence of a railing on the open arms of EPM may have interfered with rats' behavior (Arteni et al., 2010). In fact, it has been shown that the presence of railings (ledges) increases open-arms' exploration in the EPM (Fernandes and File, 1996; Treit et al., 1993), thus possibly affecting entries and time spent in those.

Similar to limited animal data regarding long-term effects of neonatal HI on anxiety, a lack of investigations is observed regarding its possible outcomes on depressive-like behavior. Our study is the first to report longer duration of immobility in FST due to neonatal HI, a behavior that reflects a state of despair in the rat (Porsolt et al., 1978), which, however, was not augmented in our maternally separated rats. The paucity of data regarding the outcome on emotional behavior of neonatal HI is surprising given that it causes alterations in brain areas and neurochemical pathways involved in emotionality. Specifically, it is well documented that neonatal HI causes neuronal injury and neurochemical alterations in the hippocampus, amygdala, and prefrontal cortex (Aridas et al., 2014; Nuñez et al., 2007; Vannucci and Vannucci, 2005), areas that play a key role in emotional regulation (Delgado et al., 2008; Sanders et al., 2010). Furthermore, extensive human research has shown a high prevalence of depression and anxiety disorders in stroke survivors (Waje-Andreassen et al., 2013; Zhang et al., 2014), symptoms that hinder recovery or increase the risk of poor long-term functional outcome post-stroke (Sagen et al., 2009; Yuan et al., 2014). In line with this, a meta-analysis of data from adult animal models of ischemic stroke showed that, overall, antidepressant

treatments improve significantly the behavioral score relevant to depression as well as infract volume post-stroke (McCann et al., 2014).

In addition to the indices of increased anxiety and depression-related behaviors, neonatally hypoxic-ischemic rats were significantly differentiated from the sham in specific parameters of the OFT. Specifically, HI animals expressed higher locomotor activity, as estimated by the total number of sector visits, a difference mainly attributed to more squares visits in the periphery. In addition, we found that the time spent in the center of the OFT was significantly lower among the HI-treated rats. This variable is considered to reflect higher anxiety response since, given the natural thigmotactic tendency exhibited by rats, the central area is thought to be an aversive part of the OFT (Carola et al., 2002; Lamprea et al., 2008). Increased locomotion estimated by the number of crossings or total distance may suggest that HI-treated animals spontaneously exhibit hyperactivity, a common adverse outcome of hypoxic-ischemic encephalopathy in humans (De Haan et al., 2006; Herrera-Marschitz et al., 2014; Volpe, 2008). According to existing evidence, increased locomotion has been found in adolescent, young adult or aged rats following neonatal HI or perinatal asphyxia paradigms (Fan et al., 2006; Lubics et al., 2005; McAuliffe et al., 2006; Rojas et al., 2013; Schlager et al., 2011; Venerosi et al., 2006). The horizontal ambulatory activity has been also used as a sign of exploration (Prut and Belzung, 2003). Although a negative correlation between emotionality and ambulation was originally proposed, such that an “emotional” animal has little ambulatory activity, it was later shown that high activity scores on day 1 of OFT represent the dimensions of both exploration and emotional reactivity (Denenberg, 1969). In other words, high ambulation may also indicate high emotionality. The interpretation of increased locomotor activity as enhanced emotionality seems to be in accordance with our finding of reduced time spent by the HI group in the center.

4.2. Effects of Maternal Separation and Neonatal Hypoxia-Ischemia on Synaptophysin and BDNF Expression in the Hippocampus

As mentioned above, our neonatally HI-exposed animals expressed behaviors indicative of increased anxiety and despair. Accumulating evidence suggests that a variety of psychiatric disorders, including depression and anxiety, have been associated with alterations in BDNF expression (Mitchelmore and Gede, 2014). Evidence that antidepressant treatments increase BDNF in the serum of depressed patients (Dwivedi, 2013) and that BDNF replacement therapy is actively being used in human and animal models of diseases, including depression, further support a link between disruption in BDNF signaling and psychopathology (Nagahara and Tuszynski, 2011).

Our analysis revealed a significant decrease in the CA3 percent area positive for BDNF in the HI-treated animals. However, this HI main effect appears to be mainly attributed to the type of rearing,. Specifically, BDNF immunoreactivity levels were significantly lower only in rats that experienced 3 h of MS and subsequently exposed to neonatal HI, while comparisons between sham and HI in NMS and MS 15min conditions did not reveal any significant differences. This effect implies that daily MS for three hours during the first six postnatal days may render the hippocampus more vulnerable to the subsequent insult, thus leading to significant reduction in the expression of BDNF in the specific hippocampal subregion.

Existing data regarding alterations in BDNF expression following neonatal MS are not consistent. While some studies report decreases in hippocampal BDNF based on mRNA or protein expression measurements, others failed to show similar alterations. A determining factor that may explain this discrepancy is the duration of postnatal manipulation. Specifically BDNF downregulation is mainly associated with separation of 2-3 weeks (3hrs/day) (Aisa et al., 2009a; Jaworska et al., 2008; Lippmann et al., 2007; MacQueen et al., 2003), but not with one episode of 24 h maternal deprivation (Choy et al., 2008; Roceri et al., 2002).

In addition to BDNF changes, our experimental conditions significantly altered synaptophysin (SYN) expression in both CA3 and DG. Specifically, rats that experienced prolonged MS expressed significantly less immunoreactivity compared to the other conditions (NMS, MS 15min). In fact, environmental enrichment is linked to increases in SYN immunoreactivity (Hirase and Shinohara, 2014; Koo et al., 2003), while adverse experiences (e.g., early stress) downregulate its levels. Specifically, MS of neonates for approximately 3 weeks (3-4hrs/day) decreases SYN mRNA expression or its immunoreactivity in adult rodents (Aisa et al., 2009a; Andersen and Teicher, 2004). To the best of our knowledge, this is the first study to report decreases in SYN immunoreactivity after a much shorter duration of maternal separation (6 days).

Significant decreases in SYN immunoreactivity in DG and CA3 hippocampal subregions were also found in HI-treated rats approximately 120-130 days after the insult, an effect indicating synaptic loss. According to existing data, SYN immunoreactivity is not altered in CA1 immediately after neonatal HI or in frontal cortex and striatum 22 months following perinatal asphyxia (Van de Berg et al., 2000; Zhao et al., 2012). Decreases in synaptophysin levels have been detected in adult rats that suffered cerebral HI due to middle cerebral artery occlusion, an effect that characterizes mainly tissue surrounding areas of infraction, but not intact areas (Millerot-Serrurot et al., 2007; Tuor et al., 2001). The upregulation of SYN in those areas in contrast to regions of damaged tissue implies that brain ischemia causes not only synaptic loss but may also trigger a compensation mechanism which leads to formation of new synaptic connections. Our measurements were only taken from hippocampal regions that are particularly vulnerable to HI damage. It would be of interest in future studies to investigate the impact on SYN immunoreactivity in brain regions not adjacent to lesioned areas, such as prefrontal cortex or striatum.

In the current study estimation of both BDNF and SYN expression was conducted in the hippocampus, a brain area particularly vulnerable to neonatal HI (Vannucci and Vannucci, 2005). We recently reported severe loss of hippocampal tissue as a result of neonatal HI, as well as greater deficits in spatial reference memory, but not potentiation of hippocampal loss or infarct size due to prolonged MS prior to the neurological insult (Tata et al., 2015). Given the exacerbated cognitive impairment of the stressed HI rats and the essential role of both BDNF and SYN in cognitive functioning, it could be hypothesized that MS may have interacted with HI causing down-regulation of these two markers. Our finding of HI-related decrease in CA3 BDNF levels detected only in the stressed rats is supportive of such an explanation, which implies that the behavioral deficits' exacerbation may be mediated by the observed region-specific BDNF decrease. Regarding SYN, the HI-related downregulation of this marker was similar in all environmental manipulation conditions (NMS, MS 15min, MS 180min), an effect which was also evident to similar extent in the sham MS 180min animals. However, there was no exacerbation of the effect in the MS 180 min / HI rats, maybe due to the major decreases in SYN immunoreactivity already caused by maternal separation condition, as indicated by the immunoreactivity measurements in the MS 180min/sham group.

Neonatal rats had suffered neonatal HI on the 7^{th} day, age that is comparable to that of the late preterm human newborn (Workman et al., 2013). Our prolonged MS experimental condition may correspond to the stress experience of preterm infants due to Neonatal Intensive Care Unit (NICU) adverse environment. Specifically, there is growing concern that environmental parameters of the NICU, such as excessive noise or ambient light, may act as sources of stress, thus disrupting newborns' growth and development (Blackburn, 1998; Lai and Bearer, 2008; McMahon et al., 2012). In turn, this environment-associated stress may increase preterm infants' vulnerability to neurological insults, such as perinatal asphyxia.

Given that prematurity increases the risk for neonatal HI (Volpe, 2009), the importance of exploring the interaction between stress and neonatal HI is further stressed.

Conclusion

To summarize, in the current study we explored the interaction of early-life stress in the form of maternal separation and neonatal HI, a model of hypoxic-ischemic encephalopathy in humans, on emotion-related behaviors and two markers of synaptic plasticity, the neurotrophin BDNF and the vesicle-associated protein, synaptophysin, in dorsal hippocampus. Based on existing evidence, experience of stress during adulthood increases the brain vulnerability to neurological insults during the same period (Caso et al., 2007; DeVries et al., 2001; Madrigal et al., 2003), while we recently reported exacerbation of spatial memory deficits in HI-treated rats that were maternally separated prior to the neurological insult (Tata et al., 2015). Although our current findings do not imply an enhanced emotionality in the stressed HI animals, it was interesting to show, for the first time, that maternal separation during just the first six postnatal days can increase anxiety during adulthood. Given the paucity of data regarding the outcome of neonatal HI on emotion-related behaviors, future studies should attempt the concurrent administration of different emotional behavior batteries in order to contribute to a better understanding of the long-term effects of neonatal HI and its interaction with postnatal adversity. Furthermore, the implication of prefrontal cortex in emotion and the limitation of data regarding the impact of MS on BDNF and/or synaptophysin levels in that area (Andersen and Teicher, 2004; Roceri et al., 2004) emphasize the need to further explore possible long-term effects on the prefrontal cortex.

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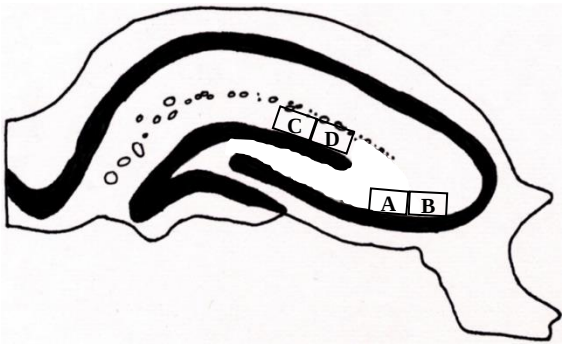

Figure 1. A schematic representation of a coronal section from the rat hippocampus (-2.76 to -2.92 from bregma). Measurements of BDNF and Synaptophysin-positive areas were taken from two adjacent regions of CA3 radiatum (A, B) and dentate gyrus (C, D).

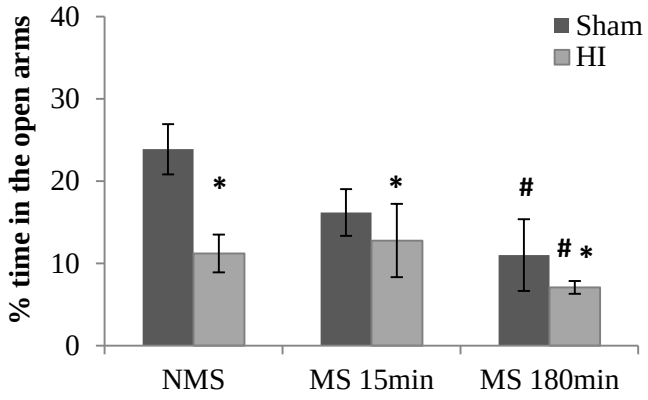

Figure 2. Time spent in the open arms expressed as a percentage of the total time spent in both open and closed arms of the elevated plus maze (EPM). HI-treated animals spent significantly less time in the open arms than the sham group (*$p < 0.05$, HI main effect). Prolonged maternal separation (MS 180min) significantly decreased the time in the open arms compared to the non-maternally separated group (NMS) (#$p < 0.05$, rearing main effect); n = 6-11 per treatment group.

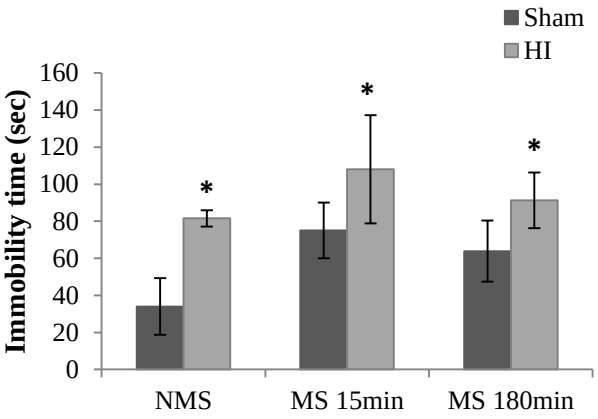

Figure 3. Immobility time in the forced swimming test (FST). HI-treated rats spent more time floating on the water without attempting to escape compared to sham animals (*p < 0.05, HI main effect). Rearing did not influence immobility time (p > 0.05, rearing main effect); n = 6-11 per treatment group.

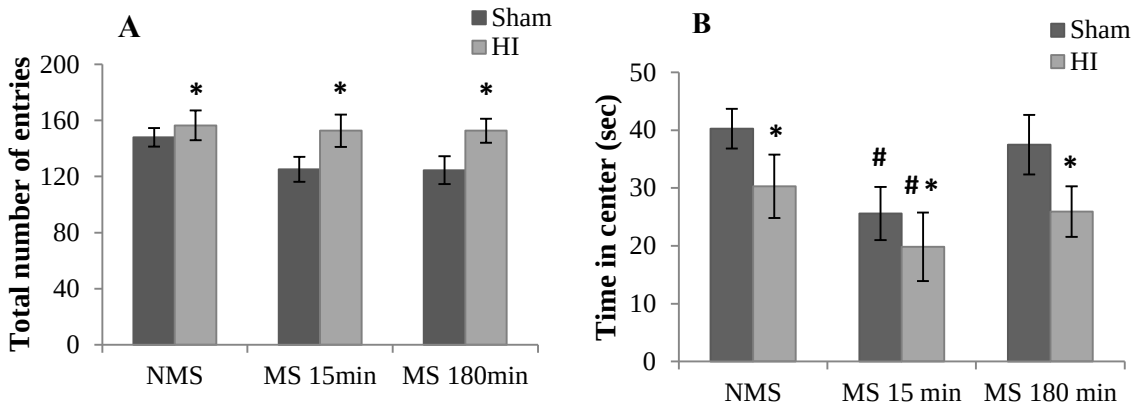

Figure 4. (A) Total number of entries in the open field test (OFT) and (B) time spent in the centre of the arena during a 6-min period. HI-treated animals exhibited increased ambulation since they crossed more squares compared to sham-treated (*$p < 0.001$, HI main effect). No effect of type of rearing was found ($p > 0.05$, rearing main effect). In addition, HI-treated animals decreased the time spent in the central squares of the OFT compared to the sham-treated (*$p < 0.05$, HI main effect), while the MS 15 min group spent less time in the centre of the arena compared to the NMS group (#$p < 0.05$, rearing main effect); n = 6-11 per treatment group.

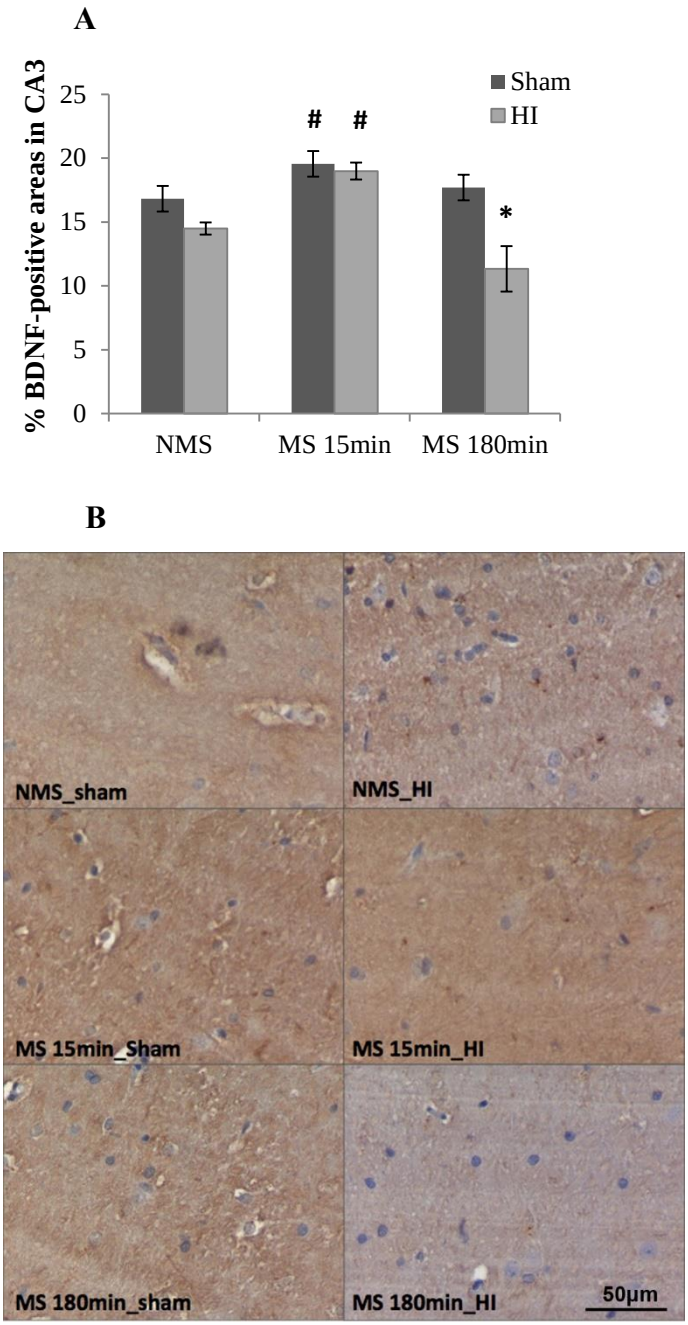

Figure 5. (A) Mean percent BDNF-positive area in CA3. The HI reduced the immunoreactivity only in the MS 180min condition as revealed by the significant decrease compared to the corresponding sham group (*$p < 0.05$). No significant HI-related decrease was found in the other two conditions (NMS, MS 15min). Regarding the effect of rearing, a significant increase in the immunoreactivity was found in animals of the MS 15min condition compared to the

NMS and MS 180min conditions (*#p* < 0.05, rearing main effect). (B) Representative photomicrographs from the CA3 apical neuropil stained with BDNF and visualized with DAB. Images were taken at x40 magnification, scale bar = 50μm; *n* = 4-5 per treatment group.

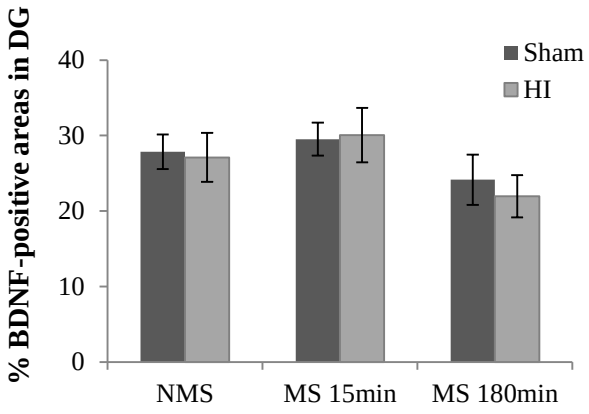

Figure 6. Mean percent BDNF-positive area in DG. Neither the type of rearing nor HI affected BDNF immunoreactivity in DG ($p > 0.05$); n = 4-5 per treatment group.

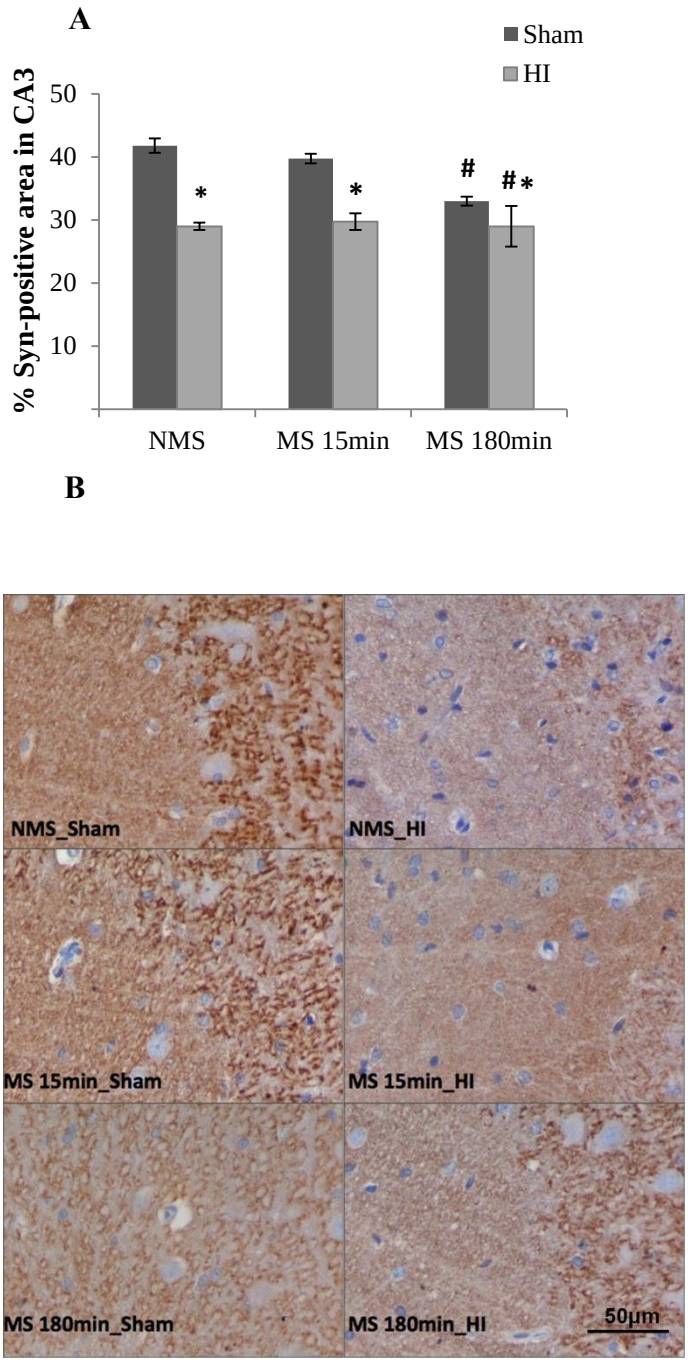

Figure 7. (A) Percent of CA3 area positive for synaptophysin was significantly less in HI-treated rats compared to sham (*$p < 0.001$, HI main effect) as well as in MS 180min condition compared to NMS and MS 15min (#$p < 0.01$, rearing main effect). In addition, a significant interaction between the two factors (HI and rearing) was emerged mainly due to the significant

decrease detected in the sham rats that were 3h maternally (MS 180min/Sham) to the levels of the HI-treated rats. (B) Representative photomicrographs from the CA3 apical neuropil stained with synaptophysin and visualized with DAB. Images were taken at x40 magnification, scale bar = 50μm; *n* = 4-5 per treatment group.

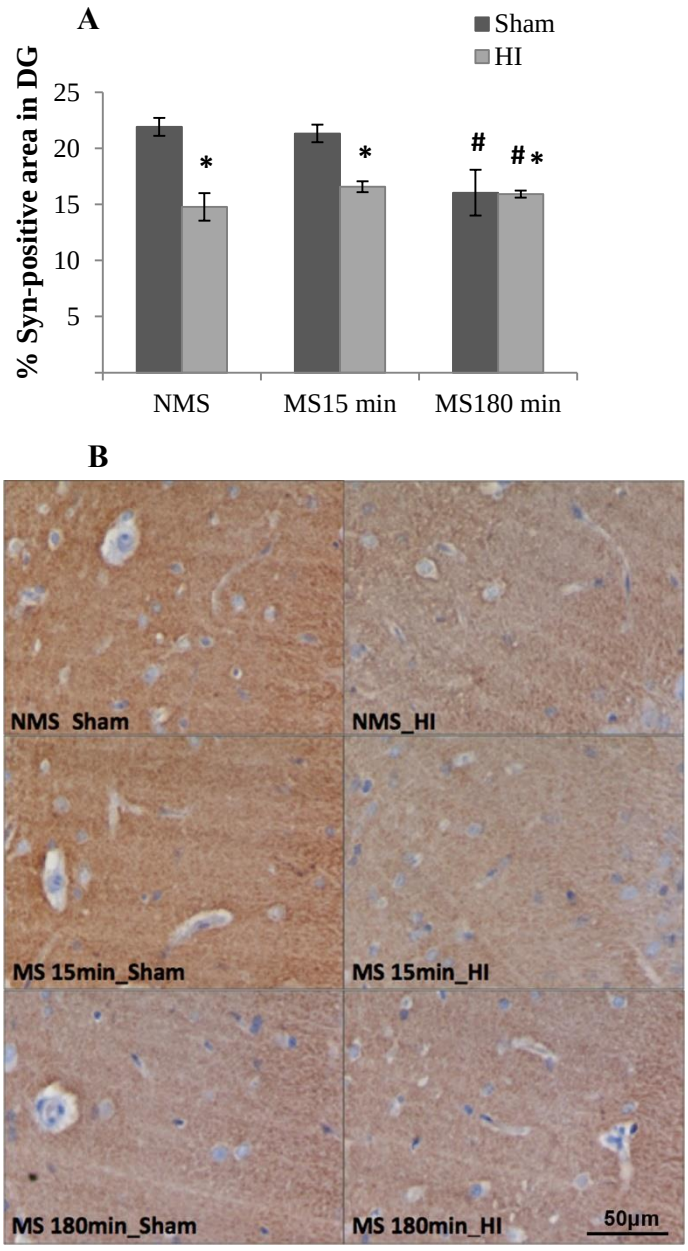

Figure 8. (A) Percent of DG area positive for synaptophysin was significantly less in HI-treated rats compared to sham (*$p < 0.001$, HI main effect). Regarding rearing effect, post-hoc analysis revealed that rats of the MS 180min condition expressed significantly less immunoreactivity

for synaptophysin in DG compared to NMS and MS 15min conditions (#$p < 0.01$, rearing main effect). A significant interaction between rearing and HI was found since the MS 180min/Sham group expressed immunoreactivity levels similar to those of the HI-treated rats. (B) Representative photomicrographs from the dentate gyrus neuropil stained with synaptophysin and visualized with DAB. Images were taken at x40 magnification, scale bar = 50μm; n = 4-5 per treatment group.