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

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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.



## 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

*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.

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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 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) 15min maternal separation (MS 15min; $N = 16$), or c) 180min 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 (15min) or prolonged period (180min) daily during PND1-6. In contrast to the prolonged MS, early MS for short periods of time (e.g., 15min), an experimental manipulation also known as 'early handling', is associated with increased expressions of maternal care (Macrì et al., 2008), and reduced behavioral 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 to 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).