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Research report

Effects of maternal separation on behavior and brain damage in adult rats exposed to neonatal hypoxia–ischemia

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HIGHLIGHTS

- We examined if maternal separation (MS) interacts with the effects of neonatal hypoxia–ischemia (NHI).
- Both MS and NHI impaired spatial learning and memory.
- Maternal separation augmented spatial memory impairments only in hypoxic–ischemic rats.
- There was no exacerbation in infarct size, hippocampal damage or corpus callosum thickness in MS rats.

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ABSTRACT

Animal studies suggest that maternal separation, a widely used paradigm to study the effects of early life adversity, exerts a profound and life-long impact on both brain and behavior. The aim of the current study was to investigate whether adverse early life experiences interact with neonatal hypoxia–ischemia, affecting the outcome of this neurological insult at both functional and structural levels during adulthood. Rat pups were separated from their mothers during postnatal days 1–6, for either a short (15 min) or prolonged (180 min) period, while another group was left undisturbed. On postnatal day 7, a subgroup from each of the three postnatal manipulations was exposed to a hypoxic–ischemic episode. Behavioral examination took place approximately at three months of age and included tests of learning and memory (Morris water maze, novel object and novel place recognition), as well as motor coordination (rota-rod). We found that both prolonged maternal separation and neonatal hypoxia–ischemia impaired the animals' spatial learning and reference memory. Deficits in spatial but not visual recognition memory were detected only in hypoxic–ischemic rats. Interestingly, prolonged maternal separation prior to neonatal hypoxia–ischemia augmented the reference memory impairments. Histological analysis of infarct size, hippocampal area and thickness of corpus callosum did not reveal any exacerbation of damage in hypoxic–ischemic rats that were maternally separated for a prolonged period. These are the first data suggesting that an adverse postnatal environmental manipulation of just 6 days causes long-term effects on spatial learning and memory and may render the organism more vulnerable to a subsequent insult.

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1. Introduction

Hypoxic–ischemic brain injury in human perinatal period is a major contributor to mortality and chronic disability [1]. According to existing evidence, infants who suffered hypoxia–ischemia are at high risk of experiencing sensorimotor deficits as well as impairments in learning and memory throughout development and during adulthood [2,3]. A well-established animal model of perinatal hypoxia–ischemia, based on the original work of Levine, has been developed by Rice and his colleagues, and consists of the permanent unilateral ligation of carotid artery followed by

Abbreviations: ARRT, accelerating rota-rod test; MS, maternal separation; CC, corpus callosum; MWM, morris water maze; HE, hematoxylin–eosin; NORT, novel object recognition test; LFB, luxol fast blue; NPRT, novel place recognition test; NHI, neonatal hypoxia–ischemia; PND, postnatal day; NMS, no-maternal separation.

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exposure to hypoxia in 7-days old rats [4,5]. This animal model of neonatal hypoxia–ischemia (HI) induces damage restricted to the hemisphere ipsilateral to the carotid artery occlusion. The injury is mainly observed in the subcortical and periventricular white matter, cerebral cortex, hippocampus and striatum and is largely characterized by the occurrence of neurodegeneration, infarction and white matter loss [5–8]. Although human hypoxic–ischemic encephalopathy has been also associated with cognitive dysfunction, the vast majority of animal studies using the HI model have focused on the mechanisms of brain injury and strategies of neuroprotection, and less attention has been paid to long-term impairment in learning and memory.

Neonatal HI in rats occurs in early life, a period of critical importance to normal development. According to a growing body of evidence, early-life events can affect the central nervous system, at both the structural and functional levels, thus having a profound and life-long impact on nervous system and behavior. For example, early stress in rats as a result of separation from their dam during the first weeks of life causes neuroendocrine and behavioral changes that persist into adulthood. Specifically, maternal separation enhances anxiety and depression-like behaviors [9,10] and predisposes to cognitive dysfunction [11–14]. Furthermore, it increases reactivity of the hypothalamic–pituitary–adrenal (HPA) axis to stress [15] and down-regulates markers of synaptic plasticity [11,16,17].

Because of the importance of early events in neural development and function, there has been an interest in exploring how environmental manipulation may interact with the outcome of HI. Exposure of adult rodents to psychological, restraint, or social stress increases infarct size and neuronal damage in CA1, and further worsens cognitive function and behavioral scores after cerebral ischemia [18–22]. Similarly, stress subsequent to cerebral ischemia during adulthood reduces cell proliferation and survival and induces depressive behavior in ischemic rats [23]. Despite the consistency among studies regarding the detrimental effects of adult stress on the outcome of cerebral ischemia, little attention has been paid to the influence of early life stress on the outcome of HI. Moreover, existing findings are contradictory, indicating either reduction in brain damage in neonatal rats exposed to 3-day restraint stress *after* a hypoxia–ischemia [24], or exacerbation of injury in internal capsule and thalamus at 10-days old rats which were maternally-separated *prior* to the neonatal hypoxic–ischemic insult (PND7) [8]. To the best of our knowledge, no study has explored the long term effect of maternal separation prior to HI on HI-related brain damage and behavioral deficits in adult rats.

The aim of the current study was to investigate whether early stress interacts with HI potentiating HI-related brain damage and behavioral deficits. To this end, we examined if prolonged maternal separation (180 min) potentiates infarct size, hippocampal tissue loss and thickness of corpus callosum, which are considered markers of brain damage associated with HI insult. In addition, we explored the interaction between maternal separation and HI in spatial and non-spatial forms of learning and memory during adulthood.

2. Materials and methods

2.1. Animals

Pregnant Wistar female rats on the second gestational week were individually housed and maintained on a 12:12 light/dark cycle (8:00—light/20:00—dark) with food and water available *ad libitum*. The day of birth was designated as postnatal day 0 (PND0). Totally, 60 neonates of either sex were included in the experiment. All animal experiments were conducted in accordance to the Institutional Animal Ethics EL 54 BIO 20.

2.2. Rearing conditions

On PND1 litters were assigned randomly to one of three rearing conditions: (a) no-maternal separation (NMS; $N = 25$), (b) 15 min maternal separation (MS 15 min; $N = 16$), and (c) 180 min maternal separation (MS 180 min; $N = 19$). In NMS condition, pups and mother remained undisturbed in their cage for the first 6 postnatal days. Maternal separation conditions involved daily separation of the pups from their dams during the first 6 postnatal days for a short (15 min) or prolonged (180 min) period of time. The process of separation was implemented as described previously [9,25]. Briefly, mothers were first removed from their cages and placed in an adjacent cage. Next, the pups were placed as a litter in a plastic container with nesting material. Heating pads were placed under the containers to compensate for the mother's body heat. The experimental manipulations occurred between 09:00 and 14:00 h for a period of 15 min or 180 min depending on the experimental group. The MS 15 min experimental condition, a manipulation also known as "early handling", is considered to render the rodents more resilient to stress [26]. On the contrary, daily MS for at least 1 h increases basal levels of the major stress hormone, corticosterone [27,28], a finding supporting the characterization of this experimental manipulation as a model of neonatal stress. In our study, dam and pups were placed at different rooms during the separation period to prevent any potential contact (auditory, olfactory, or visual) between them. At the end of the separation period, pups were returned to their home cage followed by their dam. No cage cleaning or bedding change took place until PND6.

2.2.1. Neonatal hypoxia–ischemia

On PND7 a subgroup of pups from each of the three rearing conditions was exposed to hypoxic–ischemic conditions as described by Rice and his colleagues [5]. Shortly, 7-day-old Wistar rats ($N = 24$) of either sex, weighing 11–17 g, were anesthetized with diethyl ether. Their left common carotid artery was exposed through a midline neck incision, and permanently ligated with 5–0 silk. Following 1 to 1.5 h recovery [29], hypoxia was induced by placing the animals in a plexiglass cage ($25 \times 20 \times 22$ cm), submerged in a 37 °C water bath to maintain a stable temperature, and exposing them to a gas mixture of 8% oxygen–92% nitrogen for 1 h. The gas composition was monitored continuously by an oximeter to maintain constant hypoxic environment. Sham-operated pups ($N = 36$) underwent the same operation but they had neither undergone artery ligation nor exposed to a hypoxic environment. Upon completion of hypoxic exposure, the pups were returned to their home cage. They were weaned on PND23 and, subsequently, housed 2–3 per cage by sex until the end of the experiment. All animals were housed under standard breeding conditions and were allowed free access to food and water. A 12-h light/dark cycle (8:00—light/20:00—dark) was maintained. The animals of each experimental group were obtained from 2 to 3 litters.

2.3. Behavioural testing

Behavioural assessment took place approximately at 3 months of age in order to examine aspects of learning and memory as well as motor coordination. Reference and working memory were evaluated by the Morris water maze (MWM) Test [30]. Spatial and non-spatial (visual) episodic memory were assessed by the novel place recognition test (NPRT) and the novel object recognition test (NORT) [31,32], respectively, while the accelerating rota-rod test (ARRT) examined motor coordination and learning [33,34]. The time period between the tests was at least 2 days long.

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