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Neonatal Maternal Separation Alters the Capacity of Adult Neural Precursor Cells to Differentiate into Neurons Via Methylation of Retinoic Acid Receptor Gene Promoter

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ABSTRACT

BACKGROUND: Early life stress is thought to contribute to psychiatric disorders, but the precise mechanisms underlying this link are poorly understood. As neonatal stress decreases adult hippocampal neurogenesis, which, in turn, functionally contributes to many behavioral phenotypes relevant to psychiatric disorders, we examined how in vivo neonatal maternal separation (NMS) impacts the capacity of adult hippocampal neural precursor cells via epigenetic alterations in vitro.

METHODS: Rat pups were separated from their dams for 3 hours daily from postnatal day (PND) 2 to PND 14 or were never separated from the dam (as control animals). We isolated adult neural precursor cells from the hippocampal dentate gyrus at PND 56 and assessed rates of proliferation, apoptosis, and differentiation in cell culture. We also evaluated the effect of DNA methylation at the retinoic acid receptor (RAR) promoter stemming from NMS on adult neural precursor cells.

RESULTS: NMS attenuated neural differentiation of adult neural precursor cells but had no detectible effect on proliferation, apoptosis, or astroglial differentiation. The DNA methyltransferase (DNMT) inhibitor, 5-aza-dC, reversed a reduction by NMS of neural differentiation of adult neural precursor cells. NMS increased DNMT1 expression and decreased expression of RARα. An RARα agonist increased neural differentiation and an antagonist reduced retinoic acid-induced neural differentiation. NMS increased the methylated portion of RARα promoter, and the DNMT inhibitor reversed a reduction by NMS of RARα messenger RNA expression.

CONCLUSIONS: NMS attenuates the capacity of adult hippocampal neural precursor cells to differentiate into neurons by decreasing expression of RARα through DNMT1-mediated methylation of its promoter.

Keywords: Adult neurogenesis, Dentate gyrus, DNA methylation, DNA methyltransferase, Maternal separation, Retinoic acid receptor

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One of the fundamental issues in neurobiology is how environmental factors alter molecular states in the brain, ultimately leading to behavioral phenotypes. Neonatal and postnatal stress are thought to have long-lasting effects on individuals, resulting in heightened risk for many psychiatric disorders, including schizophrenia, substance abuse disorders, personality disorders, and mood and anxiety disorders (1). The precise mechanisms of this process are still poorly understood in humans.

In rodents, neonatal maternal separation (NMS) alters behavioral phenotypes related to neuropsychiatric disorders later in life. Defective prepulse inhibition (PPI) is nonselectively associated with many neuropsychiatric disorders, including schizophrenia, bipolar disorder, schizotypal personality disorder, obsessive-compulsive disorder, and panic disorder in humans (2). NMS reduces PPI from adolescence to adulthood but not before puberty in rats (3–7). Moreover, NMS exacerbates stress responses and anxiety-like behaviors (8–10), heightens preference for ethanol (8,11), and induces cognitive impairments (5,12) in rats by the time they reach adulthood.

NMS induces a host of neuronal phenotypes in many rodent brain regions (13), but neuronal alterations in the hippocampus are likely to mediate some of the long-lasting effects of NMS on behaviors (14). Indirect evidence suggests that adult neurogenesis in the hippocampus contributes to the behavioral effects of NMS. First, NMS reduces adult neurogenesis in the rat hippocampal dentate gyrus in vivo (15). Second, direct alterations in adult neurogenesis in the hippocampus affect PPI (16), mood-related behaviors (17), and fear-related memory (18–20).

Epigenetic alterations in hippocampal neural precursor cells are increasingly appreciated as contributors to many aspects of adult neurogenesis (21,22). Methyl-CpG binding domain

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protein 1, a member of the methylated DNA-binding protein family, binds methylated gene promoters and facilitates transcriptional repression. Loss of this gene reduces neural differentiation in vivo and in vitro (23) through a basic fibroblast growth factor 2 promoter in vitro (24) and induces PPI deficits and defective fear conditioning and heightens anxiety- and depression-related behaviors in vivo (25).

We hypothesized that NMS alters the rate of adult neurogenesis in the hippocampal dentate gyrus via methylation of a neurogenesis-related gene. Because adult neural precursor cells represent a small fraction of the total hippocampal cell population, in vivo analysis cannot identify an epigenetic modification for this specific cell population. To circumvent this technical obstacle, we evaluated the impact of in vivo environmental stress on adult neural precursor cells in the hippocampal dentate gyrus, using our in vitro cell culture system. Our cell culture system uses adult dentate gyrus-derived neural precursor cells (ADP) and does not include ependymal cells (26). Pups were separated from their dams on postnatal days (PNDs) 2 to 14, and we evaluated how this environmental stress altered the capacity of in vitro adult neural precursor cells and DNA methylation at PND 56. Rats become sexually mature by 6 weeks of age (i.e., enter adolescence). They are considered to be young adult from PND 63, reaching socially mature adulthood around 6 months of age (27). We focused on young adulthood, because onset of many neuropsychiatric disorders occurs during the period from late adolescence through young adulthood.

METHODS AND MATERIALS

Animals

Pregnant Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were delivered on gestation day 14 and singly housed. All rats were housed in standard animal cages with ad libitum access to food and water in a temperature-controlled environment (22°C ± 1°C) on a 12-hour light/dark cycle (light phase: 6:00 AM–6:00 PM). All procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

Neonatal Maternal Separation

We used a brief maternal separation procedure previously reported by Plotsky and Meaney (28). Pups were cross-fostered on PND2 to minimize litter differences; eight male and two female pups were placed in each litter. Ten pups per litter were assigned to neonatal maternal separation or typical animal facility rearing (AFR) groups. Because cross-fostering could have long-lasting effects on emotional behaviors (29), this factor was held constant for both groups. Under the cross-fostering condition, NMS, but not AFR, results in reduced adult neurogenesis (15). Maternal separation took place for 3 hours per day (9:30 AM to 12:30 PM each day) from PND 2 to PND 14. Dams were removed from the cage and placed in a separate cage; pups were also removed from the cage, placed in a clean plastic cage with wood-chip bedding in an incubator to maintain an ambient temperature at 27°C to 30°C in another room, and returned 3 hours later to the original cage with the dams. Pups in the NMS group were permitted to position themselves, which included huddling with littermates, during the separation period. Pups in the AFR group were not disturbed and were maintained with dams. Bedding for both AFR and NMS groups was changed once a week by an animal care technician.

The same pool of animals that simultaneously underwent NMS was randomly divided into two subgroups. One subgroup was tested for fear conditioning and the other for the present cell culture analysis. The efficacy of our NMS procedure was validated as NMS-treated rats showed fear-related phenotypes (30). We removed all pups from the dam for 3 hours each day. Other published procedures keep two to three pups with the dam to minimize her stress and subsequent maternal abuse. The precise environmental factor in the NMS procedure that causes behavioral phenotypes cannot be easily or definitively isolated. Nonetheless, the version we employed has been demonstrated to cause robust behavioral phenotypes (30) and alteration in adult neurogenesis (15). In the literature, control for NMS is our AFR, brief handling, or both. A brief-handing group is handled for 30 seconds to 15 minutes; however, this also inevitably results in maternal separation during handling. Thus, this control does not isolate the impact of handling alone. As pointed out by Matthews and Robbins (31), it is not realistic to apply a pure experimental condition that would permit definitive descriptions of the effects of handling or maternal separation. In reality, the AFR and brief isolation with handing do not result in consistently different behavioral phenotypes (8,31–35). We conducted a pilot study to compare the impact of the AFR and 15-minute handling but did not find phenotypic differences in anxiety-related behaviors between these two groups and thus did not include the handling control.

Isolation and Culture of ADP Cells

At weaning, male and female rats were separated and group-housed. At PND 56, all eight male rats from each of the NMS groups and AFR groups were used to dissect the dentate gyrus. We used four NMS groups and four AFR groups as one set. Tissues from 32 rats of each treatment group (NMS or AFR) were pooled and digested using proteases and DNase (Worthington Biochemical Corp., Lakewood, New Jersey). ADP cells were isolated using Percoll-gradient centrifugation and then prepared in monolayer culture in nonserum medium with basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, California), using our standard procedure (26). Each cell culture was derived from 32 rats (8 rats per foster mother and 4 foster mother lines per treatment group). As each assay was repeated in three to six cell cultures, the sample size ranged from three to six.

Drugs

We used retinoic acid (Invitrogen), Ro 41-5213 (Enzo Life Sciences, Farmingdale, New York), and 5-aza-dC (Sigma, St. Louis, Missouri). Staurosporine was kindly donated by Asahi-Kasei Corporation (Tokyo, Japan) and CD1556 was kindly donated by Garderma (Sophia-Antipolis, France).