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Prolonged maternal separation disturbs the serotonergic system during early brain development

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ABSTRACT

Early life stress interrupts brain development through the disturbance of various neurotransmitter and neurotrophic factor activities, but the details remain unclear. In the current study, we focused on the serotonergic system, which plays a critical role in brain development, and examined the time-dependent influence of prolonged maternal separation on male Sprague-Dawley rats. The rats were separated from their dams for 3 h twice-daily during postnatal days (PDs) 2–20. The influence of prolonged maternal separation was analyzed on PDs 7, 14, 21, and 28 using HPLC to assess concentrations of serotonin and 5-hydroxyindoleacetic acid and using real-time RT-PCR to measure mRNA expression of the serotonin 1A and 2A receptors in various brain regions. HPLC revealed imbalance between serotonin and 5-hydroxyindoleacetic acid in midbrain raphe nuclei, the amygdala, the hippocampus, and the medial prefrontal cortex (mPFC) on PDs 7 and 14. Furthermore, real-time RT-PCR showed attenuation of mRNA expression of the serotonin 1A receptor in the hippocampus and the mPFC and of the serotonin 2A receptor only in the mPFC on PDs 7 and 14. The observed alterations returned to control levels after maternal separation ended. These findings suggest that the early life stress of prolonged maternal separation disturbs the serotonergic system during a crucial period of brain development, which might in part be responsible for emotional abnormalities later in life.

1. Introduction

Early life stress has been reported to be associated with various learning deficits and psychiatric disorders later in life (De Bellis, 2005; Gilmer and McKinney, 2003). Animal experiments have also shown that it can cause various behavioral abnormalities (Cui et al., 2006; Lee et al., 2007; Matsumoto et al., 2009) and learning deficits (Brunson et al., 2005; Oomen et al., 2010). While these findings collectively suggest that early life stress impairs normal brain development, the mechanism by which these abnormalities are caused remains unknown.

Maternal separation, during which offspring are separated from their mothers before weaning, has often been used as an animal model of early life aversive experience (Cirulli et al., 2009). Several studies have reported that maternal separation induces behavioral abnormalities related to the serotonergic (5-HTergic) system, such as anxiety-like behavior, depression, and aggressive behavior (Huot et al., 2001; Kalinichev et al., 2002; Leon Rodriguez and Duenas, 2013; Veenema et al., 2006). In addition, maternal separation during the development stage has been shown to alter the 5-HTergic system in rats from the juvenile through the adult stages of development, but these alterations are not always consistently similar, indicating that influences on the 5-HTergic system remain unclear (Arborelius and Eklund, 2007; Franklin et al., 2011; Lee et al., 2007; Matthews et al., 2001; Xue et al., 2013). Despite much accumulated evidence of the effects of maternal separation on the 5-HTergic system from juvenile to adult animals, information about the effects of maternal separation during early postnatal life remains relatively sparse.

The mammalian brain has a period of rapid growth, which is known as the brain growth spurt (BGS) and is characterized by a dramatic increase in weight (Dobbing and Sands, 1979). The BGS in the rat corresponds to postnatal days (PDs) 5 through 16 (Burns et al., 1984; Micheva and Beaulieu, 1996). Many important ontogenetic events, such as axonal/dendritic arborization and synaptogenesis, occur during this period (Burns et al., 1984; Dobbing and Sands, 1979). Compared to the adult brain, the developing brain during this period has relatively higher neuronal and

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synaptic plasticity (Hensch, 2004, 2005). Therefore, the BGS is considered very important for ontogenesis and acquisition of various higher brain functions. Concurrently, the BGS is also a period during which the brain is particularly susceptible to various insults, including aversive experiences such as maternal separation.

During periods of brain development, including the BGS, various neurotransmitter and neurotrophic factor activities are related to the formation of neuronal networks. Among these, serotonin (5-HT) is known as one of the key molecules involved in neural circuit formation. 5-HTergic neurons in midbrain raphe nuclei, including the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN), project to broad areas of the brain, such as the amygdala (Amy), the hippocampus (Hip), and the medial prefrontal cortex (mPFC) (Vertes, 1991; Vertes and Martin, 1988), which together are profoundly linked to the pathogenesis of many neuropsychiatric disorders (Millan, 2003). Many reports have indicated that 5-HT plays a different role during brain development than it does in the adult brain. For instance, some studies have shown that 5-HT is more strongly associated with synaptogenesis during brain development than during later life (Chen et al., 1994; Yan et al., 1997). In addition, the role of 5-HT receptors in the developing brain is markedly different from that in the adult brain. In particular, findings concerning the 5-HT1A receptor (5-HT1AR) and the 5-HT2A receptor (5-HT2AR) have indicated that temporary expression during brain development greatly influences synaptogenesis and emotional behavior later in life (Beique et al., 2004a, 2004b; Gross et al., 2002; Zhang, 2003).

Despite the 5-HTergic system having an important role in brain development as described above, there is insufficient knowledge about time-dependent changes in the 5-HTergic system due to maternal separation. In order to identify a susceptible period of brain development, we examined time-dependent changes in the 5-HTergic system using high-pressure liquid chromatography (HPLC), and we analyzed the mRNA expression of 5-HT receptors using real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) techniques in the brain of rat offspring during and after maternal separation. In the real-time RT-PCR experiment, we focused on the mRNA expression of 5-HT1AR and 5-HT2AR among many subtypes of the 5-HT receptor for the above-stated reasons.

2. Material and methods

2.1. Animals

Pregnant Sprague-Dawley rats purchased from Japan SLC (Hamamatsu, Japan) were used in the present study. The rats were individually housed in plastic cages with a light/dark cycle of 12 h each (lights on from 0600 to 1800) in a temperature-controlled room ($22 \pm 2\,^{\circ}C$) and given food and water ad libitum. All experiments were conducted in compliance with the guidelines for experimental use and care of laboratory animals set forth by the Kagawa University Animal Ethics Committee.

2.2. Maternal separation parameters

Pregnant rats ($n = 40$) were allowed to give birth and the day of birth was designated as PD 0. Pups that were collected from 40 litters were then randomly redistributed to the dams on PD 2, so that each dam received 8 pups (male/female = 4/4). The random assignment was done in order to exclude biological bias, such as genetic and prenatal factors. Each of these redistributed groups was then randomly assigned to one of the two testing groups: the mother-reared control (MRC) group or the maternal deprivation (MD) group. MD rats were individually separated from their dams for 3 h, two times each day (from 0900 to 1200 and from 1300 to 1600; the pups were returned to their dams from 1200 to 1300 to avoid negatively affecting the pups' nutritional state) between PD 2 and PD 20 using a modified procedure based on previous studies (Eklund and Arborelius, 2006; Leon Rodriguez and Duenas, 2013). Other than during the separation periods, MD pups were returned to their home cage and reunited with their dams. Pups from the MRC group were allowed to remain in their home cage with their dams, and not handled except for changing their bedding on PD 8 and 15. All pups were weaned on PD 21. After weaning, 2 male offspring were housed per plastic cage until use. Some animals were weighed on PDs 2, 5, 9, 13, 17, 21, 25, and 30, but these animals were not used during brain sampling because our handling of them may have adversely influenced the sampling results.

2.3. Brain tissue sampling for each experiment

Brain samples for HPLC were obtained by decapitating the rats with a guillotine on PDs 7, 14, 21, and 28. Sampling on PD 7 and 14 was adjusted immediately after 3 h separation. All samples were obtained between 1200 and 1500 to minimize the effects of circadian change. Brain samples for real-time RT-PCR were obtained using a slightly modified method (Wang et al., 2011). Briefly, the rats from both groups were anesthetized with 7% chloral hydrate in saline (0.7 mL/100 g body weight, i.p.) and were intracardially perfused with medical-grade physiologic saline at time points (age and time) identical to those at which the HPLC samples were obtained. This perfusion is useful for washing the blood from the blood vessels to suppress endogenous RNase activity found within leukocytes (Macfarlane and Dahle, 1993). After the treatment for each experiment (HPLC or real-time RT-PCR), brains were removed from the skulls and sectioned in the coronal plane to yield 1-mm-thick slices using Brain Matrix (Roboz Surgical Instrument Co., Gaithersburg, MD, USA). The Amy, the Hip, the mPFC, the DRN, and the MRN were dissected from each slice under a stereoscopic microscope (Leica Geosystems, Heerbrugg, Switzerland). Samples were stored at $-80\,^{\circ}C$ until required.

2.4. Nutrient assay

Blood samples for nutritional assessment during maternal separation were collected from the left ventricle prior to perfusion on PD 14. After leaving the blood samples at room temperature (RT) for 30 min, samples were centrifuged at 3000 rpm for 15 min at RT. The serum was then transferred into clean tubes and stored at $-80\,^{\circ}C$ until used in the assay. Nutritional assessments, both static (total protein, albumin, and total cholesterol) and dynamic (transferrin, parvalbumin, and retinol binding protein), were outsourced to Oriental Yeast Co. Ltd. (Tokyo, Japan) and SRL Inc. (Tokyo, Japan).

2.5. Serum corticosterone assay

Immediately before perfusion on PDs 7, 14, 21, and 28, blood samples were collected from the left ventricle. After leaving the blood samples at RT for 30 min, they were centrifuged at 3000 rpm for 15 min at RT. The serum was then transferred into clean tubes and stored at $-80\,^{\circ}C$ until used in the assay. Serum corticosterone levels were measured with a specific enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's protocol.

2.6. Monoamine measurement by HPLC

Brain tissue was homogenized in a solution containing 0.2 M perchloric acid, 100 μM disodium ethylenediamine tetraacetate (EDTA/2Na), and 100 ng isoproterenol (internal standard). These solutions were then centrifuged at 13,000 rpm for 20 min at $4\,^{\circ}C$. Deproteinized supernatant was collected into clean tubes and adjusted to pH 3 via the addition of 0.1 M acetic acid. These samples were filtered using a 0.45-μm syringe filter (Millipore (Merck), Billerica, MA, USA). A moving phase buffer (0.1 M sodium acetate, 0.1 M citric acid, 5 mg/L EDTA/2Na, and 190 mg/L octanesulfonic acid [pH 3.5]) with 15% methanol was pumped into a reverse-phase column (3.0 mm diameter × 150 mm length) (Eicompak SC-5ODS; Eicom, Kyoto, Japan) at a rate of 500 μL/min. 5-HT and 5-hydroxyindoleacetic acid (5-HIAA: an intermediate metabolite of 5-HT) were separated on the reverse-phase column and were quantified with an electrochemical detector system (HTEC-500; Eicom). Metabolic efficiency was calculated by dividing metabolite concentrations by neurotransmitter levels and regarded roughly as an index of 5-HT turnover.

2.7. Real-time RT-PCR

Homogenization of brain tissue and extraction of total RNA was performed with QIAshredder and AllPrep DNA/RNA/Protein Mini (Qiagen, Venlo, Netherlands), according to the manufacturer's protocol. The concentration and purity of the extracted total RNA were evaluated by optical density measurements at 260 nm and 280 nm using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Then, a QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA with integrated genomic DNA removal from 0.5 to 1.0 μg of the total sampled RNA. Primer pairs used in the current study were designed in accordance with a previous study (Zhu et al., 2009) and are shown in Table 1. Gene expression was quantified with the LightCycler system (Roche Diagnostics, Basel, Switzerland). Reactions were performed in a 20 μL volume with 2 μL of sample cDNA diluted 10-fold with distilled water, 0.5 μM forward and reverse primers, and the LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche Diagnostics). After the initial denaturation at $95\,^{\circ}C$ for 20 s, amplification was performed under the following conditions: 40 cycles at $95\,^{\circ}C$ for 10 s, $58\,^{\circ}C$ for 5 s, and $72\,^{\circ}C$ for 20 s. Detection of the fluorescent tracers was carried out at the end of the $72\,^{\circ}C$ extension period. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting-curve analysis after amplification. For melting-curve analysis, the PCR products were denatured by gradually increasing the temperature from $65\,^{\circ}C$ in