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Maternal Social Separation of Adolescent Rats Induces Hyperactivity and Anxiolytic Behavior

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Exposure to early stressful adverse life events such as maternal and social separation plays an essential role in the development of the nervous system. Adolescent Sprague-Dawley rats that were separated on postnatal day 14 from their dam and litters (maternal social separation, MSS) showed hyperactivity and anxiolytic behavior in the open field test, elevated plus-maze test, and forced-swim test. Biologically, the number of astrocytes was significantly increased in the prefrontal cortex of MSS adolescent rats. The hyperactive and anxiolytic phenotype and biological alteration produced by this MSS protocol may provide a useful animal model for investigating the neurobiology of psychiatric disorders of childhood-onset diseases, such as attention deficient hyperactive disorder.

Key Words: Maternal social separation, Early stress, Adolescence, ADHD

INTRODUCTION

Abnormal early-life experiences, such as disturbances of the infant-parent relationship, social separation, and abuse have been shown to affect cognitive function as well as emotionality (Pryce et al., 2005; Giachino et al., 2007). Furthermore, those experiences can alter a variety of neurobiological parameters during brain development and lead to adult behavioral alterations (Bremner and Vermetten, 2001; McEwen, 2003).

The maternal deprivation/separation paradigm is a well-established animal model of early-life stress. Also, it has served as a model of psychopathology (Elenbroek and Riva, 2003; Pryce et al., 2005). Depending on a variety of postnatal manipulations, such as a single or repeated separation, the duration of separation, and circadian and thermal conditions (Pryce et al., 2005; Rüedi-Bettschen et al., 2005), however, the regulations of pups' neurobiological systems to guide their development are diverse. Furthermore, different behavioral phenotypes have been reported. Arnold and Siviy (2002) reported hyperactive behavior in a maternal separation group of experimental animals, but not in a deprivation group. However, other investigators reported increased activity in adult rats after 3 min/day of maternal deprivation for 10 days during the first 2 weeks of life (Madruga et al., 2006). Also, different results were obtained regarding fearfulness after different maternal deprivation/separation paradigms (Macri et al., 2004; Madruga et al., 2006).

Previously, Lee et al. (2001) introduced a new animal model of early-life stress, the maternal social separation (MSS) paradigm, i.e., separation of newborn pups from their dam and littermates after postnatal day (PND) 14. They reasoned that separation from their dam and littermates at PND 14 would avoid the stress hyporesponsive period (from PND 2 to PND 14), and that stress-induced hippocampal changes could still occur after PND 14, because maturation and full differentiation of the hippocampal formation, such as synaptogenesis and the establishment of enduring connectivity patterns, do not take place until PND 30 in rodents (Cirulli et al., 2003). Earlier studies found that MSS pups 7 d after PND 14 display decreased cell proliferation and enhanced rate of apoptosis in the granule cell layer, disrupted hippocampal formation, decreased expression of serotonin (Lee et al., 2001), and decreased expression of nitric oxide synthase in hypothalamus (Cho et al., 2002). Recently, we reported that MSS for 3 weeks causes morphological alterations of the apical dendrites of CA3 pyramidal neurons and decreases the number of calretinin-positive dentate pyramidal basket cells which are known to be GABAergic interneurons (Kwak et al., 2008). Compared with studies on neurobiological alterations in MSS rats, however, only a few behavioral studies have so far been performed.

The present study was designed to determine the effects of maternal and social separation on measures of adjustment, innate fear, and social behavior in adolescent rats. We also determined the effect of maternal and social separation on neurobiological systems in the prefrontal cortex.

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ABBREVIATIONS: ADHD, attention deficit hyperactive disorder; MSS, maternal social separation; PND, postnatal day.

METHODS

Animals

Adult Sprague-Dawley male and female rats (Dae han biolink, Umsung, Republic of Korea) were housed in pairs under standard laboratory conditions with artificial 12 h light/dark cycle (lights on at 7:00 a.m) at an ambient temperature of 22°C with free access to food and water. When female of each pair was clearly pregnant, male was removed from the cage. The day of delivery was designated as postnatal day (PND) 0.

All experiments were conducted in accordance with National Institutes of Health guidelines on animal care and experiments. Every effort was made to minimize the number of animals used and to reduce their suffering.

Maternal social separation

On PND 14, ten male pups were separated from their dam and siblings and housed singly in a new cage with free access to food and water. Control pups were left undisturbed with their mothers. All pups were weaned on PND 21, and male offsprings (n=10) were kept in five per cage with food and water *ad libitum*.

Open-field test

Locomotor activity was measured by placing the rats individually in activity cages (90×90×30 cm), with a floor divided into 81 squares. Rats (PND 28) were allowed to acclimatize to the environment of the room for at least 3 h before starting the behavioral test. The test was conducted under dim light conditions. Each rat was placed in the center of the open-field arena before the locomotor activity was monitored for 20 min via CCD camera positioned above the apparatus. Locomotor activity was measured by counting the number of squares crossed.

Elevated plus-maze test

At PND 35, the rats were tested on the elevated-plus maze (open arm: 50×10 cm; closed arm 50×10×40 cm; height 40 cm). Each rat was placed on the center square platform and was allowed to explore the maze for 5 min. The ratio of open arm entries and the time spent on the open arms were calculated.

Forced swim test (FST)

The test was conducted using a modification of the method of Porsolt (Porsolt et al., 1977). Briefly, rats (PND 42) were placed individually in Plexiglas cylinders (height 40 cm; diameter 20 cm) containing water at 25±2°C. Fifteen minutes later, rats were removed and dried before returning to their home cages. Twenty four hours later, the procedure was repeated for 5 min and their activity was videotaped. Immobilized time was determined by 1 or 2 raters who were blinded to the animal group, controls vs. MSS.

Histological procedures

Rats were anesthetized with sodium pentobarbital (50 mg/kg) until a complete lack of response was observed before they were perfused transcardially with 50 mM phosphate-buffered saline (PBS) at pH 7.4, followed by chilled 4% paraformaldehyde (PFA) in 0.1 M PB at pH 7.4. The brains were removed, post-fixed overnight and transferred into 30% sucrose solution for cryoprotection. Frozen coronal sections (40 μm, coordinates 2.7 to 2.2 mm from bregma) were prepared with a cryostat (Leica, Nuβloch, Germany).

Free-floating sections were processed for immunohistochemisry as described (Kwak et al., 2008). Immunohistochemical reactions were carried out by using biotin-avidin system on sections incubated for 16 h with the mouse anti-glial fibrillary acidic protein (GFAP) (1:3000; Dako, Glostrupt, Denmark). The sections were rinsed and stained according to the avidin-biotin horseradish peroxide method (Elite ABC system, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (Sigma, St. Louis, MO, USA) as the chromogen. Sections were washed and mounted onto gelatin-coated slides. Coverslips were mounted using Permount (Fisher, Fair Lawn, NJ, USA).

Quantification

Number of GFAP-positive cells was measured on three 0.1 mm^2 square contour placed over the frontal cortex area 1 and 2 per section using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). All GFAP-positives within the contour were counted under high magnification. The data are presented as means±standard errors of the means (SEM) number of GFAP-positive cells per mm^2.

Statistical analysis

All results were analyzed with the Mann-Whitney's U test using SPSS software 10.0 K (SPSS statistics, Chicago, IL, USA) for independent samples to compare between controls and the MSS group. A value of $p<0.05$ was accepted as statistically significant. The data are presented as means±SEM.

Preparation of Figures

Digital images were processed by using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA). Only general adjustments of contrast and brightness were made. The images were not manipulated otherwise.

RESULTS

Assessment of the effects of maternal social separation on locomotor activity

On PND 28, the total number of square crossings during a 20 min time period was designated as a measure of the locomotor activity. MSS rats appeared to have significantly increased percentage of locomotion behavior throughout the course of the test ($p<0.01$) (Fig. 1).

Assessment of the effects of maternal social separation on anxiety-related behavior

On PND 35, an elevated plus-maze test was performed to assess anxiety-related behavior. Fig. 3 shows the percentages of entries and time spent on the open arms. The percentage of entries into the open arms was significantly increased by maternal and social separation, as compared to controls ($p<0.01$) (Fig. 2A). Furthermore, the percentage of time spent in the open arms was similarly affected by maternal and social separation ($p<0.05$) (Fig. 2B).

Assessment of the effects of maternal social separation on depression-related behavior

The FST is a commonly used paradigm to evaluate despair behavior. Depression-related behavior is inferred from increased latency period in time spent immobile and/or a decreased latency period to become immobile. Interestingly, MSS showed rats significantly decreased immobility time ($p<0.01$) (Fig. 3).

Effect of maternal social separation on GFAP immunoreactivity in the prefrontal cortex

Sirviö and colleagues (2001) demonstrated that metabolic alterations in the prefrontal cortex are related to behavioral deficits in a rodent model of ADHD. Alterations in the number or the functions of astrocytes are important because they undergo a characteristic change in appearance in response to CNS pathology. Therefore, we tested whether the MSS paradigm affects the hypertrophy of astroglial cellular processes in the prefrontal cortex. A well-known feature of reactive astrocytes is the increased expression of GFAP. As shown in Fig. 4A and B, astrocytes containing GFAP immunoreactivity are located in the prefrontal cortex, and we found that the expression level of GFAP in the prefrontal cortex of MSS rats was increased (Fig. 4B). Also, the density of GFAP-positive astrocytes in MSS rats was significantly higher, compared with controls ($p<0.01$) (Fig. 4C).

DISCUSSION

Lee et al. (2001) showed earlier that cell proliferation is decreased and apoptosis is increased in the dentate gyrus of MSS rats. Similar to the MSS model, stress in early life reduces cell proliferation and neurogenesis in the hippocampus of adult rats (Karten et al., 2005). Because decreased neurogenesis is thought to underlie depression-like behaviors (Santarelli et al., 2003) and one of the mood disorder models is an early life stressor (Holsboer, 1999), we expected that MSS rats would display depression and/or

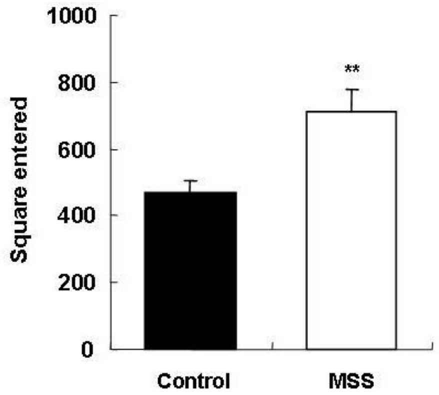

Fig. 1. Comparison between controls and maternal social separation (MSS) group on the open-field test. Data are expressed as mean±SEM. **$p<0.01$, Significant difference from control (Mann-Whitney U-test).

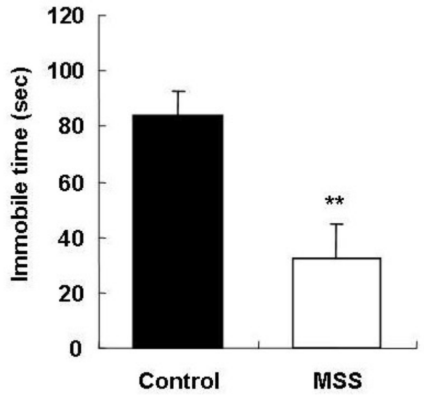

Fig. 3. Effects of maternal and social separation on the forced swim test. Data are expressed as mean±SEM. **$p<0.01$, Significant difference from control (Mann-Whitney U-test).

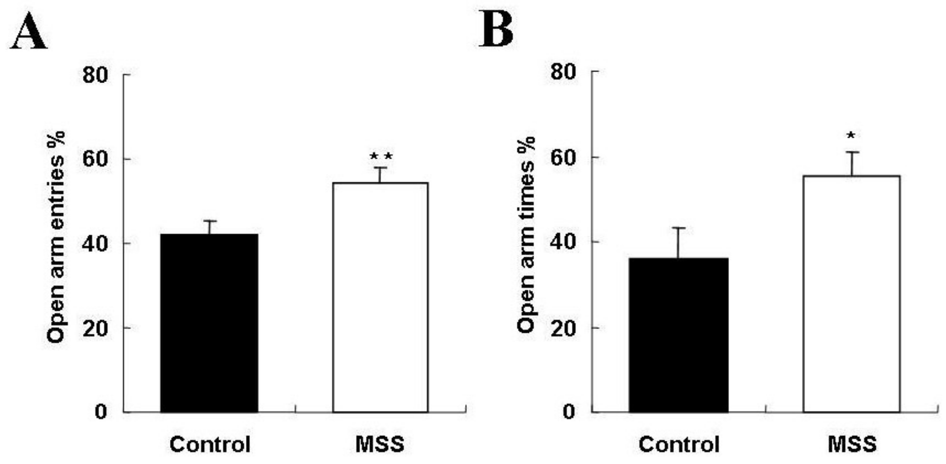

Fig. 2. Effects of maternal and social separation on the elevated plus-maze test. Anxiety-related behavior as reflected by the percentage of entries (A) and time spent (B) on the open arms of the elevated plus-maze by controls and maternal social separation (MSS) rats on PND 35. Data are expressed as mean±SEM. *$p<0.05$, **$p<0.01$, Significant difference from control (Mann- Whitney U-test).

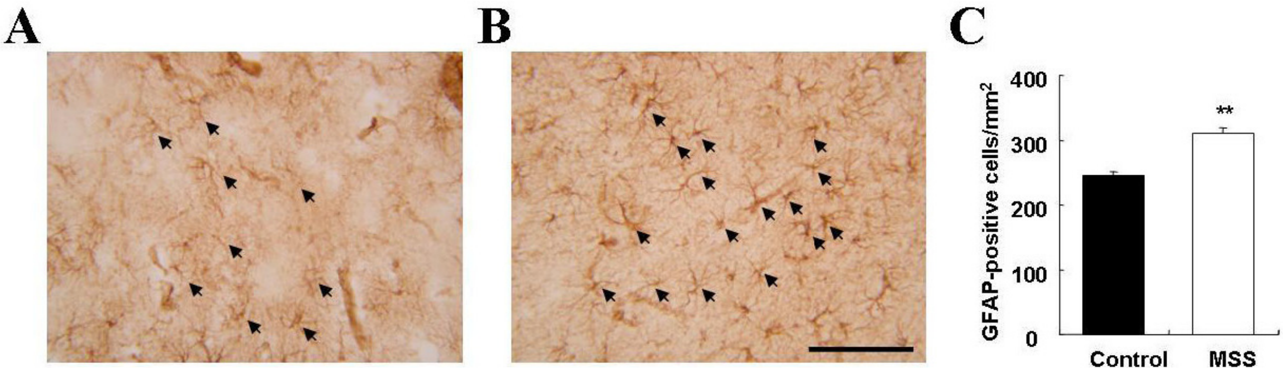

Fig. 4. Representative photomicrographs of GFAP immunoreactivity in the prefrontal cortex of control (A) and MSS (B) rats. Triple images (0.1 mm^2) on the frontal cortex 1 and 2 area were obtained, and density of GFAP-positive cells was calculated (C). Data are expressed as mean±SEM. **$p<0.01$, Significant difference from control (Mann-Whitney U-test).

anxiety-like behaviors.

Deniels et al. (2004) reported that maternally separated animals show a decreased number of entries and time spent in the open arms, suggesting that maternally separated animals display more anxiety-like behaviors than controls. On the contrary, however, our present study revealed that MSS rats show anxiolytic-like behavior in the elevated-plus maze. Fabricius et al. (2008) found that a single 24 h maternal separation on PND 9 resulted in a 20% decrease of neurons in the dentate gyrus in adolescent mice and showed reduced signs of anxiety, similar to our MSS rats. This lack of fear response has been explained by the enriched environment that could reverse the effect from MS (Francis et al., 2002; Fabricius et al., 2008). However, it might not be enough to explain the reason behind an increased fear/anxiety behavioral response.

The forced swimming test has widely been used to assess emotionality, depression and responses to stress in animals. In previous studies, socially separated animals show a significant increase in the time spent immobile in the forced swimming test (Aisa et al., 2008). Interestingly, however, we found that MSS rats showed decreased time spent immobile in the forced swimming test. Even though the latency to immobility has mostly been interpreted to reflect "behavioral despair" (Porsolt et al., 1977), other reports suggested that the behavioral change is related to energy conservation and learning capability (West, 1990), whereas struggling, swimming and diving behaviors are classified as active behaviors. On the other hand, one previous study has suggested that MSS rats display cognitive alterations (Lee et al., 2006). The present finding suggests that the significant decrease of the immobility time in the MSS rats might be due to a memory deficit and/or hyperactivity.

Depending on the maternal separation protocols utilized, several studies reported hyperactive behavior, while others reported a decreased or no difference in activity (Arnold and Siviy, 2002; Colorado et al., 2006). Locomotion in novel environments reflects both motor hyperactivity and sensitivity to environmental stimuli. Our MSS protocol which separates mothers and littermates after PND 14 may cause a hyperactive behavioral profile, indicated by an increased number of movements in the open-field test.

These behavioral profiles, such as an anxiolytic-like activity and hyperactivity, are similar to those found in an animal model of attention-deficit/hyperactivity disorder (ADHD), characterized by dysfunctional levels of poor concentration, increased motor activity, and cognitive and behavioral impulsivity (Paule et al., 2000; Negishi et al., 2005; Sagvolden et al., 2005; Colorado et al., 2006). Those behavioral alterations might occur as a result of structural and/or functional brain changes. A previous study of MSS rats revealed that the volume of the dentate gyrus is decreased in the hippocampus (Lee et al., 2001). We recently reported that the dendrite trees of pyramidal neurons in CA3 shrink, and a number of the basket cells, or interneurons, are decreased in the hippocampus of MSS rats (Kwak et al., 2008). Furthermore, there was a significant difference in the number of astrocytes between prefrontal cortex of controls and MSS rats. Hypofunctionality of catecholaminergic pathways projecting to the prefrontal cortex areas has been proposed as being involved in ADHD pathophysiology (Todd and Botteron, 2001). Structural imaging studies revealed that ADHD subjects have volumetric abnormalities of the frontal and parietal lobes and the basal ganglia (Alward et al., 1996; Filipek et al., 1997). Positron emission tomography studies on ADHD patients showed that cerebral glucose metabolism is reduced in the frontal cortex (Zametkin et al., 1990; Amen and Carmichael et al., 1997). Because cortical astrocytes regulate catecholaminergic neurotransmission and energy homeostasis, it is quite likely that reactive astroglyosis in the prefrontal cortex is associated with ADHD-like behaviors in MSS adolescent rats.

Direct mechanisms underlying the ADHD-like behaviors induced by MSS are not clearly understood. Nevertheless, previous studies indicated that MSS affects many aspects of brain development. At the neurobiological level, neurotransmitter and neuromodulator systems undergo neuronal growth spurts and pruning during the early postnatal period (Cirulli et al., 2003). These growth spurts and pruning are associated with synaptic sensitivity and, therefore, are associated with enhanced vulnerability to negative environments. At the molecular layer of the hippocampal dentate gyrus of rat, the number of synapses increases exponentially until reaching a plateau at the adult value on PND 30 (Cirulli et al., 2003). Thus, it is possible that MSS interferes with the normal process of cell proliferation, cell death and synaptogenesis during neurotransmitter and/or neuromodulator system development, which may ultimately lead to the display of ADHD-like behaviors.

In conclusion, the present study demonstrated that MSS rats have altered astroglial characteristics in the prefrontal cortex that may cause ADHD-like behaviors, such as anxiolytic, hyperactivity and memory deficit. Future studies on the catecholaminergic change in the prefrontal cortex area and psychostimulant effects produced by this MSS protocol may help elucidate the neurological basis for a similar phenotype in ADHD patients.

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