Experimental Neurobiology
Vol. 18, pages 88～96, December 2009

Experience of Neonatal Maternal Separation May Lead to a Long-term Modulation in the Neuronal Activity of Nucleus Accumbens in the Offspring

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ABSTRACT

Dysfunction of the nucleus accumbens (NAcb) is implicated in the development of anhedonia, a core symptom of major depressive disorder. In order to define the neural basis of depression-like behaviors induced by experience of neonatal maternal separation (MS), both basal and stress-induced neuronal activations in the NAcb of adolescent rats with MS experience were examined parallel with palatable food intake. Rat pups were separated from dam daily for 180 min during the first two weeks of age (MS), and non-handled control (NH) pups were left undisturbed. After weaning on postnatal day (PND) 22, a half of NH or MS pups were subjected to 1 h of restraint stress every even day during PND 28～40 (NH/R or MS/R), and then had free choices of chow and chocolate cookie for 1 h immediately after returned to home cage. The rest half of NH and MS pups (NH/C or MS/C) received free choices of chow and cookie in the same time schedule with stress group, just omitting restraint stress. Cookie intake was significantly decreased in MS/C, whereas c-Fos expression in the NAcb and plasma corticosterone increased, compared to NH/C. Restraint stress suppressed cookie intake and increased the NAcb c-Fos expression in NH/R, but not in MS/R. The plasma corticosterone of NH/R, but not of MS/R, increased following repeated restraint stress. These results suggest that the increased neuronal activation in the NAcb of MS/C may be implicated in the development of anhedonia by MS experience, perhaps, in relation with a blunted responsivity of the hypothalamic-pituitary-adrenal axis to stress.

Key words: c-Fos, depression, maternal separation, nucleus accumbens, palatable food intake, stress early in life

INTRODUCTION

A number of studies have indicated a strong correlation between traumatic events during early life and the development of behavioral abnormalities

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Received November 13, 2009
Accepted for publication December 21, 2009

later in life, such as anxiety disorders (Kendler et al., 1992; Furukawa et al., 1999) and depression (Heim et al., 2000; Heim et al., 2001). Neonatal maternal separation is considered as an animal model of stressful experience early in life. Many of studies have demonstrated its impact both on the activity of the hypothalamic-pituitary-adrenal gland (HPA) axis; i.e., permanent alterations in the characteristics of the HPA response to stress (Ladd et al., 1996; Van Oers et al., 1998; Vazquez et al., 2000) and on the development of depression- (Ladd et al., 2000; Khoury et al., 2006) and anxiety-like behaviors (Kalinichev et al., 2002; Daniels et al., 2004) later in life. We have previously reported that rats experienced maternal separation repeatedly during the first two weeks of life exhibit depression- and anxiety-like behaviors (Lee et al., 2007) with altered response of the HPA axis to stress challenges later in life (Kim et al., 2005a; Kim et al., 2005b; Ryu et al., 2008).

Anhedonia (diminished interest or pleasure) is a core symptom of major depressive disorder. Development of anhedonia has been ascribed to dysfunction of the reward pathway, in which the nucleus accumbens (NAcb) plays a pivotal role (Di Chiara et al., 1999; Yadid et al., 2001). Palatability and hedonic value of food play central roles in nutrient intake, and recent studies have demonstrated that the NAcb is strongly implicated in the motivational mechanisms for feeding (Bassareo et al., 2002; Kirkham et al., 2002; Kelley et al., 2005) and the hedonic property of palatable food ingestion (Di Chiara and Bassareo, 2007; Sahr et al., 2008; Yamamoto, 2008). It is well known that stress affects food intake, and chronic stress has been reported to induce dramatic neurochemical alterations in the NAcb, leading to depressive phenotypes (Moreau et al., 1995; Di Chiara et al., 1999). The striatal dopaminergic activity was suggested to be associated with the severity of anhedonia in depressed patients (Gorwood, 2008), and it has been reported that acute and repeated immobilization stresses differentially affect dopaminergic activities in sub-regions of the striatum including NAcb (Lucasa et al., 2007). These reports together led us to hypothesize that palatable food intake may be suppressed in relation with a dysfunction of the reward pathway in our animal model of maternal separation (MS) that showed depression-like behaviors in our previous study (Lee et al., 2007).

We have examined both basal and stress-induced neuronal activation, referred by c-Fos expression, in the NAcb of our MS model, parallel with palatable food intake, in order to define the neural basis of depression-like behaviors induced by MS experience. Also, c-Fos expression in the central nucleus of the amygdala (CeA) was examined, since the CeA has been implicated in anxiety disorders (Pugh et al., 1997; Hui et al., 2004) and our MS animal model shows anxiety-like behaviors as well (Lee et al., 2007; Ryu et al., 2009).

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were purchased (Samtako Bio, Osan, Korea), and cared in a specific-pathogen-free barrier area with constant control of temperature (22±1°C), humidity (55%), and a 12/12 hr light/dark cycle (lights-on at 0700 h). Standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and membrane filtered purified water were available *ad libitum*. Animals were cared according to the Guideline for Animal Experiments, 2000, edited by the Korean Academy of Medical Sciences, which is consistent with the NIH Guidelines for the Care and Use of Laboratory Animals, revised 1996. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Seoul National University.

Experimental protocol

Nulliparous females and proven breeder males were used for breeding in the laboratory of the animal facility, and the pups were reared in a controlled manner to minimize and standardize unwanted environmental stimulation from *in utero* life. Maternal separation (MS) was performed daily for 180 min from postnatal day (PND) 1 through 14, following the procedure that we previously described (Kim et al., 2005a; Lee et al., 2007; Ryu et al., 2008), and then the pups were left with their dam undisturbed until weaning on PND 22. The non-handled (NH) group remained undisturbed until weaning except for routine cage cleaning. Weanling

male pups were singly caged with *ad libitum* access to standard chow and water.

NH and MS pups were either subjected to 1 h of restraint stress every other day during PND 28～40 (NH/R or MS/R), or left undisturbed in home cage (NH/C or MS/C). Restraint stress was performed with placing each rat in a restraint cage (a plastic box with openings for aeration, which is adjustable to the size of each rat allowing to move four limbs but not to turn around) for 1 h between 0800 h and 1000 h daily, and the rats had free choices of chow and chocolate cookie (Kraft Foods Global, Inc., East Hanover, NJ, USA) for 1 h immediately after returned to home cage. Rats in the control groups (NH/C or MS/C) received free choices of chow and cookie with the same time schedule, just omitting restraint stress. Otherwise all rats had *ad libitum* access to chow and water during the whole experimental period, and the amounts of cookie or chow consumed during 1 h of the test session were recorded each day. All rats were sacrificed on PND 41; i.e., 24 h after the last feeding test session, and processed for c-Fos immunohistochemistry and corticosterone assay. The NH/R and MS/R groups received 1 h of restraint stress before the sacrifice on PND 41.

Immunohistochemistry

All rats were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.), and transcardially perfused with heparinized isotonic saline followed by ice cold 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in 0.1 M sodium phosphate buffer. The brains were immediately dissected out, blocked, post-fixed for 2 h, and transferred into 30% sucrose (Sigma, St. Louis, MO, USA) for cryoprotection. Forty-micron coronal sections were cut on a freezing, sliding microtome (HM440E, Microm Co., Germany) and alternate sections were collected throughout the rostro-caudal extent of the nucleus accumbens of striatum (NAcb; between bregma 0.84 mm and 1.56 mm; Paxinos and Watson, 2005), and the central nucleus of amygdala (CeA; between bregma −2.04 mm and −2.64 mm; Paxinos and Watson, 2005). c-Fos immunohistochemistry was performed with standard DAB reaction using commercial ABC kit (Vectastain Elite Kit, Vector Laboratories, CA, USA) as previously described (Jahng et al., 2004). Polyclonal rabbit anti-c-Fos antibodies (1 : 20,000 dilution, Oncogene Research Products, San Diego, CA) were used as primary antibody and biotinylated anti-rabbit IgG (1 : 200 dilution, Vector Laboratories, CA, USA) as secondary. Immunostained sections were mounted onto gelatin-coated slides, air dried overnight, dehydrated through graded ethanol to xylene, and then coverslipped with Permount.

The number of stained cells in the NAcb and the CeA sections was blind-counted by hand from the consecutively digitized images (1,280×1,024 micron) of each brain area using Olympus BX-50 microscope (Olympus Co., Tokyo, Japan). Cell counts from three NAcb (closest sections to bregma 1.08 mm) or three CeA (closest sections to bregma −2.28 mm) sections in each rat were averaged per section, and the individual mean counts were averaged across rats within experimental groups.

Plasma corticosterone assay

Cardiac blood was collected into 1.5-ml microcentrifuge tubes containing 5 μl heparin, and centrifuged at 3,000×g for 10 min at 4°C. The plasma was transferred into new tubes, frozen in liquid nitrogen and stored at −80°C until used for the assay. Plasma corticosterone levels were determined by radioimmunoassay using ^{125}I-labelled Coat-A-Count kit (DPC, CA, USA). The sensitivity of the assay was 5.7 ng/ml. The intra-assay coefficient of variation was 4～12.2%. All rats were sacrificed between 0800 h and 1100 h to minimize diurnal variation in the plasma corticosterone level.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and preplanned comparisons between groups performed by *post hoc* Fisher's PLSD test, using StatView software (Abacus, Berkeley, CA, USA). Cookie and chow intake data were further analyzed by repeated measures ANOVA. Significance was set at $p<0.05$, and all values were presented as means±SEM.

RESULTS

One hour cookie intake was gradually increased during the experimental period in each group, con-

Table 1. Cookie or chow intake during 1 h period following restraint stress

PND	Cookie (g)				Chow (g)			
	NH/C	NH/R	MS/C	MS/R	NH/C	NH/R	MS/C	MS/R
28	0.90±0.17	0.24±0.12†	0.67±0.13	0.46±0.18	0.12±0.06	0.02±0.02	0.06±0.03	0.35±0.19‡
30	1.97±0.32	0.82±0.28*	1.20±0.21*	1.05±0.22	0.17±0.07	0.17±0.08	0.10±0.05	0.08±0.02
32	2.42±0.43	1.19±0.35*	1.88±0.23	1.69±0.25	0.08±0.04	0.15±0.05	0.07±0.03	0.12±0.06
34	3.22±0.46	2.01±0.59	2.71±0.17	2.58±0.54	0.12±0.02	0.14±0.07	0.13±0.05	0.13±0.07
36	3.98±0.61	3.23±0.72	3.04±0.32	3.37±0.77	0.10±0.02	0.13±0.07	0.13±0.03	0.08±0.04
38	4.96±0.68	3.61±0.82	3.24±0.24*	3.64±0.83	0.13±0.07	0.27±0.20	0.22±0.12	0.16±0.11
40	6.88±0.80	3.99±1.22*	4.12±0.59*	5.55±0.63	0.23±0.16	0.16±0.08	0.33±0.20	0.14±0.07

Pups in NH/R and MS/R groups were subjected to 1 h of restraint stress every even day during PND 28～40 and then had free choices of chow and chocolate cookie for 1 h immediately after returned to home cage. NH/C or MS/C pups received free choices of chow and cookie in the same time schedule with stress group, just omitting restraint stress. NH/C: non-handled control, MS/C: maternal separation control, NH/R: non-handled & repeated restraint, MS/R: maternal separation & repeated restraint, PND: postnatal day, *$p<0.05$, †$p<0.01$ vs. NH/C on each day, ‡$p<0.05$ vs. MS/C, Data are presented by means±SEM.

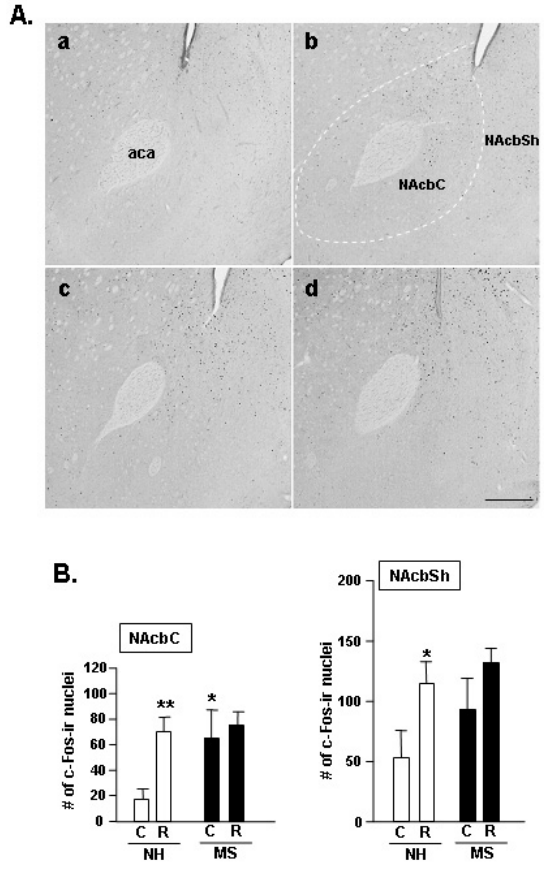

Fig. 1. Representative photographs of c-Fos immunohistochemistry (A) in the nucleus accumbens of NH/C (a), MS/C (b), NH/R (c) and MS/R (d), and quantificational analyses of c-Fos immunoreactive neurons (B). Pups in NH/R and MS/R groups were sacrificed immediately after the last stress session. NH/C: non-handled control, MS/C: maternal separation control, NH/R: non-handled & repeated restraint, MS/R: maternal separation & repeated restraint, aca: anterior commissure, NAcbC: nucleus accumbens core, NAcbSh: nucleus accumbens shell, *$p<0.05$, **$p<0.01$ vs. NH/C, n=6 each group, Scale bar; 500 μm.

trary to one hour chow intake showing no differences (Table 1). Interestingly, cookie intake of MS/C (maternal separation and no restraint) decreased significantly ($p<0.05$) on PND 30, 38, and 40 compared with NH/C (non-handled and no restraint) on each day, but chow intake did not differ between the groups. Repeated measures ANOVA revealed main effect of maternal separation on cookie intake [$F_{(1,6)}=3.499$, $p=0.0068$] and no effect on chow, supporting the idea that MS experience may affect palatable food intake of the offspring later in life.

Repeated exposure to restraint stress appeared to suppress cookie intake, but not chow, in NH pups. That is, cookie consumption was decreased significantly in NH/R (non-handled and restraint stress) on PND 28 ($p<0.01$), 30, 32 and 40 ($p<0.05$, respectively) compared with NH/C on each day, but chow intake did not differ between the groups (Table 1). Repeated measures ANOVA revealed main effect of stress on cookie intake of NH pups [$F_{(1,6)}=2.610$, $p=0.0372$] and no effect on chow. Restraint stress did not further affect cookie intake of MS pups; i.e., cookie intake of MS/R (maternal separation and restraint stress) did not differ from MS/C on each day observed. Repeated measures ANOVA revealed no effect of stress either on cookie or chow intake of MS pups over the whole experimental period, although a significant increase by restraint was observed in chow intake on PND 28 ($p<0.05$, MS/C vs. MS/R) (Table 1). Neither maternal separation nor restraint stress

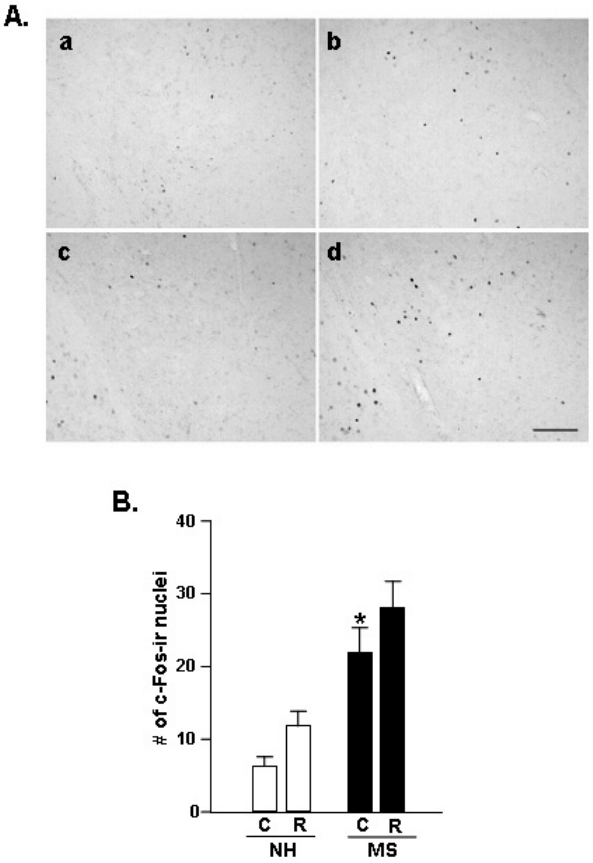

Fig. 2. Representative photographs of c-Fos immunohistochemistry (A) in the central amygdala of NH/C (a), MS/C (b), NH/R (c) and MS/R (d), and quantificational analysis of c-Fos immunoreactive neurons (B). Pups in NH/R and MS/R groups were sacrificed immediately after the last stress session. NH/C: non-handled control, MS/C: maternal separation control, NH/R: non-handled & repeated restraint, MS/R: maternal separation & repeated restraint, *p<0.05 vs. NH/C, n=6 each group, Scale bar; 500 μm.

produced significant effects on body weight gain of NH or MS pups during the experimental period (Data not shown).

c-Fos immunoreactivity in the nucleus accumbens (NAcb), the reward center, of pups in MS/C group appeared to be increased, compared to NH/C pups (Fig. 1A (a, b)). Quantificational analysis showed that number of c-Fos-ir nuclei in the NAcb core is increased significantly by MS experience ($p<0.05$, MS/C vs. NH/C) (Fig. 1B). c-Fos-ir nuclei in the NAcb of NH pups increased significantly both in the core ($p<0.01$, NH/C vs. NH/R) and the shell ($p<0.05$, NH/C vs. NH/R) following repeated exposure to restraint stress; however, neither the NAcb core nor the shell of MS pups showed an increase

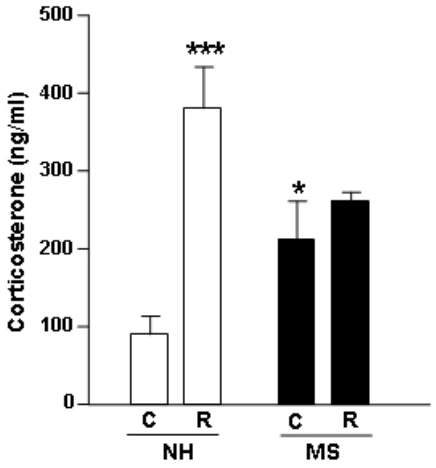

Fig. 3. Plasma corticosterone levels analyzed by radioimmunoassay. Pups in NH/R and MS/R groups were sacrificed immediately after the last stress session. NH/C: non-handled control, MS/C: maternal separation control, NH/R: non-handled & repeated restraint, MS/R: maternal separation & repeated restraint, *p<0.05, ***p<0.001 vs. NH/C, n=6 each group.

in c-Fos-ir nuclei following repeated restraint (Fig. 1).

Interestingly, c-Fos expression in the central amygdala (CeA) appeared to be increased by MS experience; i.e., number of c-Fos-ir nuclei in the CeA of MS/C pups increased significantly ($p<0.05$) compared with NH/C pups per se (Fig. 2). Number of c-Fos-ir nuclei in the CeA of NH/R or MS/R pups tended to be increased following repeated exposure to restraint stress, compared to NH/C or MS/C, respectively; however, statistical significances were not found. Basal plasma level of corticosterone was elevated in MS/C pups ($p<0.05$) compared with NH/C pups (Fig. 3). Plasma corticosterone levels were significantly increased in NH pups ($p<0.001$, NH/C vs. NH/R), but not in MS pups, following repeated restraint stress (Fig. 3).

DISCUSSION

We have demonstrated that consumption of chocolate cookie, but not standard chow, is suppressed in adolescent rats that experienced neonatal maternal separation (MS). Anhedonia (diminished interest or pleasure) is a core symptom of major depressive disorder, and it has been reported that consumption of palatable food is decreased in an animal model of depression (Tacchi et al., 2008). The MS animal model used in this study showed

depression-like behaviors with decreased ambulatory activity and increased immobility during forced swim test (Lee et al., 2007). Thus, it is likely that decreased intake of chocolate cookie, highly palatable food, in the MS pups may reveal anhedonia, further supporting the development of depression in our animal model of maternal separation. Development of anhedonia has been ascribed to dysfunction of the reward pathway, in which the nucleus accumbens (NAcb) plays a pivotal role (Di Chiara et al., 1999). In this study, c-Fos expression, a conventional marker for neuronal activation, was increased in the NAcb of MS pups as compared with NH pups, and the increase was statistically significant in the NAcb core. Previous studies have reported that the NAcb core is required for normal preference for a large reward (Cardinal and Cheung, 2005) and handles generic motivational value of food whereas the NAcb shell integrates the motivational valence and novelty (Bassareo et al., 2002). Taken together, it is suggested that increased neuronal activities in the NAcb core of MS pups may be implicated in the decreased cookie intake; i.e., MS experience may lead to increased activity of the NAcb neurons, perhaps, suppressing motivational and/or reward values of palatable food in the offspring later in life. Further studies to identify the neurons expressing c-Fos in the NAcb of MS pups are currently under our consideration.

Several human studies have demonstrated that acute stress increases not only the frequency and amount of food intake but also intake of highly palatable food (Oliver et al., 2001; Zellner et al., 2006). Whereas, some other studies have reported that stress may result in a decreased energy intake in human (Pollard et al., 1995; Adam and Epel, 2007). Stress, in fact, can lead to both under- and over-eating, and little is known about what determines direction of eating (Adam and Epel, 2007; see for review). In contrast to humans, rats and mice consistently lose weight in response to stress, and it has been suggested that decreased food intake and weight loss serve as the most reliable marker of stress severity (Armario, 2006). It has been reported that adult rats exposed to restraint stress for 3 h daily for three consecutive days showed decreases in food intake and body weight on the days of stress (Harris et al., 1998; Miragaya and Harris, 2008). In the present study, 1 h of restraint stress given every other day at adolescence (PND 28～40) did not affect chow intake and weight gain of both NH and MS pups (data not shown); however, it suppressed cookie intake in NH group, but not in MS, on the days of stress. Previous report suggested that restraint stress differentially activates brain neuronal populations depending on the developmental age of rats and time of restraint (Kellogg et al., 1998). Thus, it is plausible that the restraint dose used in this study may not be so severe to affect weight gain or daily food intake of adolescent rats that are in a great demand for growth; however, it may still be enough to affect the brain reward system involved in pleasure seeking behavior, such as caving for palatable food. Also, it should be noticed that although restraint stress suppressed cookie intake of NH pups, it did not further suppress cookie intake of MS pups. These results led us to assume that neuronal activation in the NAcb of MS pups by restraint stress may differ from NH pups. Indeed, number of c-Fos expressing neurons was significantly increased in the NAcb, both core and shell, of NH pups following repeated restraint; whereas, this increase was not observed in MS pups. Previous reports have suggested that increased Fos expression in the NAcb may be related with decreased food intake (Konsman and Blomqvist, 2005) and aversive response to palatable food (Yamamoto, 2007). Therefore, it is concluded that c-Fos expression in the NAcb neurons may play a role in the regulatory mechanism underlying restraint-induced suppression of palatable food intake, and the experience of neonatal maternal separation may lead to blunted activation of the NAcb neurons responding to restraint stress later in life.

The amygdala is a limbic brain region that is involved in the control of emotional behavior, such as anxiety or depression (Davis, 1997), and especially, the central nucleus of the amygdala (CeA) has been shown to respond to hormonal changes associated with stress and fear, the core symptoms of anxiety disorders (Pugh et al., 1997; Hui et al., 2004). In the present study, c-Fos expression in the CeA of MS pups was significantly increased as compared to NH pups, and our MS animal model shows anxiety-like behaviors (Lee et al., 2007; Ryu

et al., 2009). Thus, it is suggested that increased neuronal activity in the CeA of MS pups, referred by c-Fos expression, may be implicated in the development of anxiety-like behaviors by MS experience. This is supported by a previous report that gene expression of corticotropin-releasing hormone (CRH) is increased in the CeA of adult male rats that experienced MS (a 24 h MS at the age of 9 days) and this may be related with its anxiety-like behavior (Barna et al., 2003). Since CRH upstream promoter carries a functional AP-1 (c-Fos/c-Jun) site (Malkoski and Dorin, 1999), it is plausible that the neurons expressing c-Fos in the CeA of MS pups may comprise CRH containing neurons being implicated in anxiety-like behaviors. In this study, resting levels of plasma corticosterone were significantly increased in MS pups as compared to NH pups, in accordance with our previous report (Ryu et al., 2008). It has been reported that corticosterone application to the amygdala increases not only CRH expression in the CeA but also anxiety-like behavior (Shepard et al., 2000). Taken all together, it is concluded that increased plasma corticosterone in MS pups may contribute, at least partly, to the development of anxiety-like behaviors, perhaps, in mediation of the increased activity of the CeA neurons.

In this study, repeated exposure to restraint stress markedly increased the plasma corticosterone levels of NH pups, concurring with previous reports that repeated exposure to stressors induces a long-term increase of corticosterone levels (Marti et al., 1993; Ottenweller et al., 1994). Interestingly, c-Fos expression in the NAcb (both core and shell) of NH pups was also increased following repeated exposure to restraint stress, as mentioned above. Human studies have demonstrated that the secretion of dopamine over the NAcb responding to stressors is proportional to cortisol responses (Oswald et al., 2005; Wand et al., 2007). Together, it is suggested that the increased c-Fos expression in the NAcb of NH pups may be related with increased plasma corticosterone as a result of the HPA axis activation responding to repeated restraints. Both the plasma corticosterone increase and the NAcb c-Fos expression by repeated restraints were not observed in MS pups. The interaction between the responsivity of the HPA axis to stress and the development of anhedonia has been suggested to be mediated by dopamine neurotransmission in the reward pathway responding to stress. That is, dopamine neurotransmission in the NAcb responding to food is blunted by chronic mild stress, an animal model of depression (Di Chiara et al., 1999), and the striatal dopaminergic activity was associated with the severity of anhedonia in depressed patients (Gorwood, 2008). Collectively, it is concluded that MS experience may lead to the development of anhedonia in relation with a decreased responsivity of the HPA axis to stress, perhaps, accompanied by a blunted dopaminergic activity in the brain reward system. We have observed that gene expression of tyrosine hydroxylase, the rate limiting enzyme of dopamine synthesis, responding to restraint stress is blunted in the ventral tegmental area of MS pups, where the NAcb dopaminergic inputs are originated from (Yoo et al., 2009).

The NAcb has been viewed as a transition area between the ventral striatum and the extended amygdala including the CeA (Gray, 1999; Cardinal et al., 2002), and the CeA has been reported to interact with the HPA axis (Shepard et al., 2000; Shepard et al., 2003). However, in this study, c-Fos expression in the CeA of NH pups was not increased following repeated restraints, despite the increases of plasma corticosterone and the NAcb Fos expression. A single exposure of NH pups to restraint stress on PND 40, not like repeated exposure, increased not only the plasma corticosterone and the NAcb Fos expression but also the CeA Fos expression (Noh et al., 2008). Thus, it is likely that c-Fos expression in the CeA of NH pups may be habituated to the repeated exposure to restraint stress, concurring with previous report (Melia et al., 1994), but neither the NAcb Fos expression nor the plasma corticosterone does. It has been suggested that repeated exposure to restraint stress desensitizes c-Fos response in many, but not all, of the brain regions (Melia et al., 1994; Umemoto et al., 1997; Stamp and Herbert, 1999). Currently, we do not know the regulatory mechanism underlying the differential habituation of c-Fos response in brain regions to repeated restraint.

ACKNOWLEDGMENTS

This study was supported by a grant from the Brain Research Center of the 21st Century Frontier Research Program (2009K001269) funded by the Korea Government (Ministry of Education, Science and Technology), and the R&D Grant funded by KHIDI (A080918).

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