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Differential Adaptive Responses to Chronic Stress of Maternally Stressed Male Mice Offspring

Sooyoung Chung, Gi Hoon Son, Sung Ho Park, Eonyoung Park, Kun Ho Lee, Dongho Geum, and Kyungjin Kim

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

It is well established that stress in early life can alter the activity of the hypothalamus-pituitary-adrenal (HPA) axis, but most studies to date have focused on HPA reactivity in response to a single acute stress. The present study addressed whether stress in pregnant mice could influence the adaptive responses of their offspring to chronic stress. Male offspring were exclusively used in this study. Elevated plus maze tests revealed that 14 d of repeated restraint stress (6 h per day; from postnatal d 50–63) significantly increased anxiety-like behavior in maternally stressed mice. NBI 27914, a CRH receptor antagonist, completely eliminated anxiety-related behaviors in a dose-dependent manner, indicating an involvement of a hyperactive CRH system. In accordance with increased anxiety, CRH contents in the hypothalamus and amygdala were significantly higher in these mice. Despite an increased basal activity of the CRH-ACTH system, the combination of chronic prenatal and postnatal stress resulted in a significant reduction of basal plasma corticosterone level, presumably because of a defect in adrenal function. Along with alterations in hypothalamic and hippocampal corticosteroid receptors, it was also demonstrated that a dysfunction in negative feedback inhibition of the HPA axis could be deteriorated by chronic stress in maternally stressed male mice. Taken together, these results indicate that exposure to maternal stress in the womb can affect an animal's coping capacity to chronic postnatal stress. (*Endocrinology* 146: 3202–3210, 2005)

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LIFE EXISTS BY maintaining a complex dynamic equilibrium or homeostasis that is constantly challenged by a variety of intrinsic or extrinsic adverse stressors. Stress is a term that is widely used to describe both the subjective experience induced by a novel, potentially threatening or distressing situation as well as the behavioral and neurochemical reaction to it. The main components of the stress response system are the hypothalamus-pituitary-adrenal (HPA) neuroendocrine axis and locus coeruleus/norephinephrine autonomic system. In the HPA axis, neurochemical signals conveying potential threats reach the hypothalamus, releasing CRH from neurons in the paraventricular nucleus (PVN) to influence rapid secretion of ACTH from the pituitary. ACTH induces the adrenal secretion of glucocorticoids, corticosterone (CORT) in rodents, which interact with specific corticosteroid receptors in the central nervous system to appropriately turn off the hormonal stress response and restore a steady state (1).

The hormones consisting of the HPA axis have protective roles in proper adaptation to stress, but a sustained or dysregulated activation of this axis exerts damaging effects on an organism (2). Chronic, inescapable, or uncontrollable stress may lead to impairment of the normal regulatory mechanism, resulting in various types of pathophysiological states. For example, although glucocorticoids secreted in response to an acute stress enhance the formation of certain types of memories, stress over long periods can inhibit other forms of memories, and even cause memory impairment by inducing hippocampal damage (3–5). Repeated restraint stress for 21 d or longer suppressed neurogenesis in the hippocampal dentate gyrus and even resulted in neuronal atrophy by reduced branching and a shortened dendritic length in hippocampal pyramidal cells (6–9). In addition to memory, excessive stress hormones also have profound influences on a variety of behaviors. Stressed animals exhibited cognitive impairment on spatial recognition memory and increased anxiety in an open field, as well as an enhanced conditioned-fear response (10–13).

The HPA axis is highly susceptible to the effects of environmental factors, including prenatal stress during fetal and neonatal development. There are abundant evidences of hyperactive and/or dysregulated HPA axes in maternally stressed offspring, although there remain some controversies depending on the experimental models. First, several studies reported higher levels of circulating glucocorticoid under basal conditions in adult rodents that were exposed to stress *in utero* (14, 15). More importantly, stressed rats exhibited faster, more robust, and/or more prolonged endocrine responses than control animals in reaction to a variety of stressors (16–19). Maccari and colleagues showed a prolonged CORT secretion by acute restraint stress of maternally stressed adult rats. Such a sensitization of the HPA axis by maternal stress depended on maternal glucocorticoid hypersecretion and could be restored by increasing maternal care (19, 20). Increased HPA axis reactivity in maternally stressed animals is most likely a result of down-regulation of hippocampal corticosteroid receptors, which is crucial for the

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* S.C. and G.H.S. contributed equally to this work.

Abbreviations: CORT, Corticosterone; dpc, days post coitus; EPM, elevated plus-maze; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal; MR, mineralocorticoid receptor; P21, postnatal d 21; PVN, paraventricular nucleus; RM, repeated measures.

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proper activation and inactivation of the endocrine axis (21). Indeed, levels of both glucocorticoid type I and/or type II receptors were reduced in the hippocampus of maternally stressed rats by a maternal glucocorticoid-dependent mechanism (19, 22, 23).

To date, studies investigating the long-lasting influence of maternal stress on the HPA axis of offspring have focused on their reactivity to a single acute stress; thus the coping capacity of prenatally stressed animals to chronic stress is largely unknown. As described above, proper adaptation to chronic stress is integral to the survival of an animal, because repeated stress can induce an overactivity of the HPA axis that can lead to pathophysiology and damage to a variety of physiological systems (3). The present study was undertaken to determine whether maternally stressed animals can adequately adapt to chronic postnatal stress, compared with normal mice.

Materials and Methods

Animals

ICR mice, obtained from the Laboratory Animal Center at Seoul National University, were used in all experiments. Animals were kept in temperature-controlled (22–23 C) quarters under a 12-h light, 12-h dark photoperiod (lights on at 0700 h); standard mouse chow and water were available *ad libitum*, except during the stress period. All animal procedures were approved by the Animal Care and Use Committee at Seoul National University.

Maternal stress

Pregnant mice in the stress group were placed, individually, in a restrainer (a transparent plastic cylinder, 3 cm in diameter and 9 cm long) daily for 6 h (1000–1600 h), a paradigm adopted from a previous report (24). Pregnant mothers were exposed to repeated stress from 8.5 d post coitus (dpc) to parturition (19.5 or 20.5 dpc), because the major organogenesis including critical neural development occurs during this period in the fetal mouse (25). Control pregnant mice remained undisturbed, except for the deprivation of food and water. Pups from the stressed mother were weaned at postnatal d 21 (P21) and reared in an environment identical to that of controls by the age of P49. To exclude possible litter effects, two to three mice from different litters were randomly assigned to a cage.

Chronic postnatal stress

In the present study, male offspring were exclusively used. Each half of the group of male offspring from stressed or control mothers was subjected to repeated immobilization stress for 14 d during late pubertal period to young adulthood (from P50–63). The other halves were used as unstressed groups. The stress scheme for the offspring also involved repeated daily immobilization in a transparent plastic cylinder for 6 h, which was similar to the maternal stress scheme described above. After 14 d of repeated stress, mice were moved to an adjacent, but different animal compartment and habituated there until further examinations on the next day. Body weights were recorded every other day at 0900 h, *i.e.* before the stress session. A schematic diagram for the preparation of stressed animals and the designation of experimental groups (CC, SC, CS, and SS mice, respectively) is summarized in Fig. 1.

Elevated plus-maze (EPM) test

The plus-maze consists of two open arms (5 × 30 cm) and two enclosed arms of the same size with 20-cm-high walls arranged with arms of the same type opposite one another. The apparatus was elevated to a height of 50 cm. Mice were placed individually on the central portion of the apparatus, facing an open arm. The test was carried out for 10 min. Frequency and duration of open and closed arm entries were recorded separately. An entry was defined as movement of all four paws into an arm. The EPM test was performed between 1100 and 1400 h on P64. Mice received vehicle containing dimethylsulfoxide (DMSO) or NBI 27914 (dissolved at 1 or 5 μg/ml in DMSO; Tocris, Bristol, UK), a non-peptide-selective CRH receptor 1 antagonist (26). Vehicle and NBI 27914 were administered sc at a dose of 2 or 10 mg/kg body weight, using a 1-ml tuberculin syringe, 45 min before the EPM test.

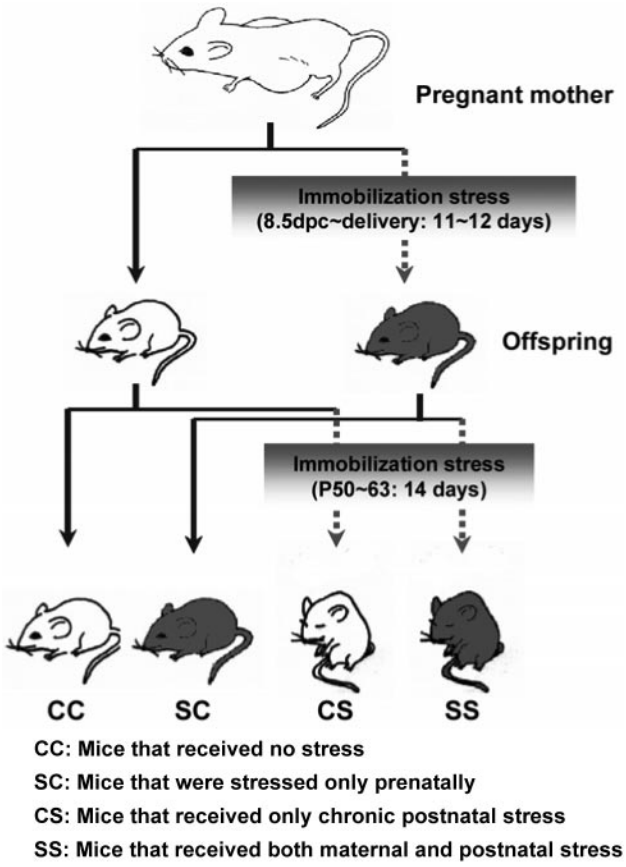

FIG. 1. A schematic diagram for the experimental groups. Pregnant mice in the maternal stress group received daily immobilization stress for 6 h from 8.5 dpc to parturition. Control pregnant mice remained undisturbed. Pups born to the control or stressed mice were weaned at P21 and reared by the age of P49 without any disturbance. Each half of the male offspring from stressed or control mothers, was subjected to chronic restraint stress (14 d) from P50–63. The other halves remained undisturbed. The resulting four experimental groups were denoted as CC, SC, CS, and SS, respectively, as indicated.

Preparation of plasma and tissues

All mice were killed between 1100 and 1400 h with cervical dislocation followed by decapitation for collecting trunk blood on the day after the last stress session (P64). Tissues were isolated on ice and quickly frozen in liquid nitrogen except for the adrenal glands used in static incubation *in vitro*. Brains were rapidly removed and coronally sectioned in a brain matrix. The hypothalamus, hippocampus, and amygdala were bilaterally dissected from 1-mm-thick brain slices on an ice-cold stage according to a brain atlas (27). Frozen tissues and EDTA-plasma were stored at −70 C until assays.

Determination of tissue CRH and CORT content

CRH content in brain tissues and CORT content in adrenal glands were measured by RIA on tissue lysates. For CRH content, indicated brain tissues were homogenized in 600 μl of RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, and 1% Triton X-100], and soluble lysates were obtained by a brief centrifugation. A 100-μl aliquot of each tissue lysate

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was subjected to RIA. Anti-CRH antibody (final dilution of 1:6000) and CRH peptide were purchased from Sigma Chemical Co. (St. Louis, MO; catalog no. C5348 for anti-CRH antibody and C3042 for CRH peptide), and ^{125}I-conjugated CRH was from DuPont NEN Life Science Products (Boston, MA; NEX216). The sensitivity at 80% binding was approximately 1 ng/tube. Intra- and interassay coefficients of variation were 2.7 and 5.1%, respectively, for 10 ng of synthetic CRH. For determining adrenal CORT content, an adrenal gland was lysed in 500 μl of 0.3 N perchloric acid-containing saline. CORT concentration in 50 μl of adrenal lysate was assayed using a commercial CORT RIA kit according to the manufacturer's instructions (Diagnostic Products Corp., Los Angeles, CA).

Measurement of plasma ACTH and CORT

EDTA-plasma was prepared from trunk blood as described elsewhere (15). ACTH concentrations in EDTA-plasma were determined using a double-antibody RIA system kindly provided by the National Hormone and Pituitary Program of National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) according to their instructions with minor modifications. Briefly, 100 μl of plasma was incubated with an anti-ACTH antiserum (AFP6328031) at a final dilution of 1:60,000 and ^{125}I-conjugated ACTH (AFP-2938C; 20,000 cpm per tube) as a tracer at 4 C for 16 h. The intra- and interassay coefficients of variation were 2.4 and 3.7%, respectively, for 100 pg of synthetic ACTH. CORT levels in EDTA-plasma samples were also assayed using the same commercial RIA system described above.

Northern blot hybridization

Total RNA preparation and Northern blot hybridizations were performed as described previously with slight modifications (28). Briefly, total RNA from adrenal glands was isolated by a single-step acid guanidinium thiocyanate-phenol-chloroform method. Fifteen micrograms of each RNA sample were resolved on a 1% formaldehyde agarose gel and transferred for 16 h onto a Nytran filter (pore size, 0.45 μm; Schleicher & Schuell, Inc., Dachen, Germany) by diffusion blotting. A radioactive cDNA probe for each gene was generated by the random priming method in the presence of [α-^{32}P]dCTP using a cDNA fragment of the ACTH receptor (GenBank accession no. NM008560) and β-actin (NM007393). Hybridization was performed at 42 C for 16 h in hybridization solution [50% deionized formamide, 5× sodium chloride-sodium phosphate-EDTA (SSPE; ph 7.4), 5× Denhardt's solution, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA]. After washing off the unbound probe, membranes were exposed to x-ray film at −70 C for 3 d.

In vitro adrenal response to ACTH

Quickly removed adrenal glands were defatted, weighed, and divided into two fragments. Two adrenal fragments from different mice were placed in one well of a 96-well culture plate, rinsed three times with ice-cold Krebs-bicarbonate medium (2.5 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.1 M NaCl, 25 mM $NaHCO_3$, and 11 mM glucose) and preincubated in the same medium at 37 C for 30 min. Adrenal fragments were then incubated at 37 C in 100 μl of fresh Krebs-bicarbonate medium for 2 h in the absence or presence of ACTH (30, 100, or 300 nM at the final concentration; Sigma). CORT in the medium was assayed by RIA as described above.

Western blot analysis

Antibodies to glucocorticoid receptor (GR) (catalog no. Ab2; Calbiochem, Darmstadt, Germany) at a final dilution of 1:10,000 and mineralocorticoid receptor (MR) antibodies (H300; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5000 were commercially available. Whole-cell extracts of hippocampus, hypothalamus, and amygdala were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) in a Bio-Rad (Richmond, CA) Trans-Blot electrophoresis apparatus using Towbin's buffer [25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol]. The blots were blocked in Tris-buffered saline [TBS; 150 mM NaCl, 10 mM Tris (pH 7.6), and 2 mM $MgCl_2$] containing 0.5% Tween 20 and 3% BSA and incubated with a primary antibody at room temperature for 1 h. The blots were then washed four times with TBS/0.5% Tween 20. Primary antibody binding was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were then washed four times as described, and immunoreactive bands were visualized by an exposure to x-ray film for 0.5 or 2 min after application of Amersham enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Response to an acute stress

On the next day after 14 d of postnatal stress sessions (P64), mice from all four groups were exposed to an acute immobilization stress for 30 min at 1100 h with similar, but not same, restrainers used during chronic stress sessions. Mice were killed at 0 (just before an acute restraint stress), 30 (at the end of stress), 60, and 90 min after the beginning of 30 min of restraint stress, and plasma was collected to determine CORT and ACTH concentrations.

Statistical analysis

All data were statistically evaluated by standard two-way or one-way ANOVA, except changes in body weights and weight gains, which were evaluated by two-way ANOVA with repeated measures (RM-ANOVA). Bonferroni multiple comparison test was used for post*hoc* comparison and statistical significance was set at $P < 0.05$.

Results

Changes in body weight during chronic stress period

Body weights of the four groups of mice during the 14 d of stress sessions were measured every other day at 0900 h. Mean body weights of all groups were statistically the same at the beginning of chronic stress; measured body weights

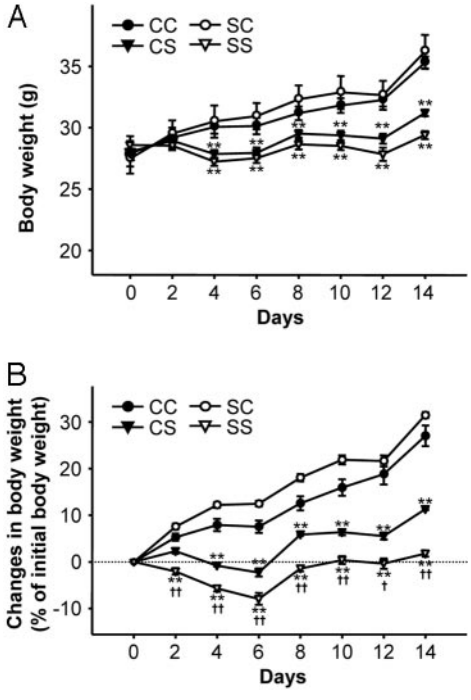

FIG. 2. Changes in body weight during 14 d of repeated stress session. A, Body weight of each mouse was measured every other day at 0900 h and is presented as mean ± SEM; B, changes in body weight were presented as mean percentage ± SEM of initial body weight. **, $P < 0.01$ *vs.* nonstressed CC and SC mice, respectively; †, $P < 0.05$, and ††, $P < 0.01$ *vs.* CS; n = 8 for each group.

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are shown in Fig. 2A. Repeated immobilization stress significantly inhibited increments of body weights in both control and maternally stressed mice during 14 d of period ($F_{(7,196)} = 289.995$, $P < 0.01$ for days; $F_{(3,28)} = 12.847$, $P < 0.01$ among groups by RM-ANOVA). There were also significant interactions between days and groups ($F_{(21,196)} = 30.884$, $P < 0.01$). Weight gain is expressed as percent change from initial body weight in Fig. 2B. Repeated restraint stress also reduced weight gain in both CS and SS mice ($F_{(7,196)} = 313.946$, $P < 0.01$ for days; $F_{(3,28)} = 111.229$, $P < 0.01$ for groups; and $F_{(21,196)} = 39.040$, $P < 0.01$ for interaction by RM-ANOVA). Within 6 d after repeated stress began, body weights of the unstressed groups (CC and SC) constantly increased, whereas weight gain of stressed groups was retarded (CS) or diminished (SS) when compared with their initial body weight. In the second week, the body weight of stressed groups gradually began to recover but did not reach the body weights of unstressed groups until the stress session ended. Interestingly, the maternally stressed mice were more susceptible to immobilization-evoked growth inhibition than the controls. Furthermore, although CS mice began to show a gradual increase in weight gain after 12 d of repeated restraint, SS mice were still retarded in their growth, compared with their initial body weights (Fig. 2B). Based on the features in stress-evoked growth inhibition, we used repeated immobilization stress for 14 d in the remaining part of the present studies.

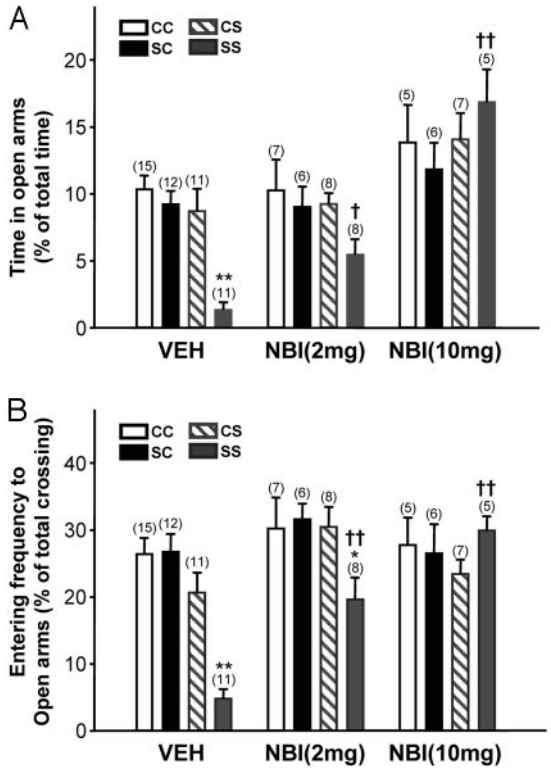

FIG. 3. Increased anxiety-like behavior in the EPM. Time spent in open arms (A) and entering frequency to open arms (B) in the EPM over the 10 min of test time were presented as mean percentage ± SEM. Mice were injected with vehicle [DMSO (VEH)] or NBI 27914 [2 or 10 mg/kg body weight; NBI(2 mg) and NBI(10 mg), respectively] 45 min before test began. *, $P < 0.05$, and **, $P < 0.01$ *vs.* CC and SC; †, $P < 0.05$, and ††, $P < 0.01$ *vs.* vehicle-treated SS mice. *Numerals on the bars* indicate the number of mice used in each group.

CRH receptor-mediated intensive anxiety in SS mice

Stress is reported to be able to increase anxiety-like behaviors in rats (13). The extent of this behavior was assessed using an EPM, which exploits the conflict between an animal's innate tendency to explore novel areas and their aversion for heights and open spaces (29). The relative portions in open arms to the total test time or the total number of crossings in 10 min were used as indices of anxiety. Several lines of evidence have suggested that maternal stress or prenatal exposure to glucocorticoids might cause an increase in anxiety-like behavior, but these notions are still controversial depending on genetic makeup and the duration of treatment (20, 30). In the present study, prolonged maternal stress alone did not increase anxiety-like behavior of the offspring; instead, it significantly raised the risk of the onset of anxiety-like behaviors by chronic stress. Two weeks of repeated stress induced a high-anxiety state in maternally stressed mice, even when tested on the day after a stress session. SS mice showed a significant reduction in both time spent in open arms and the frequency of entry into them (Fig. 3; $P < 0.01$ *vs.* mice in other groups). Interestingly, NBI 27914, a nonpeptidergic CRH receptor antagonist, when administered sc (2 or 10 mg/kg body weight), obliterated the high-anxiety state of SS mice induced by chronic stress in a dose-dependent manner; however, it did not affect basal anxiety

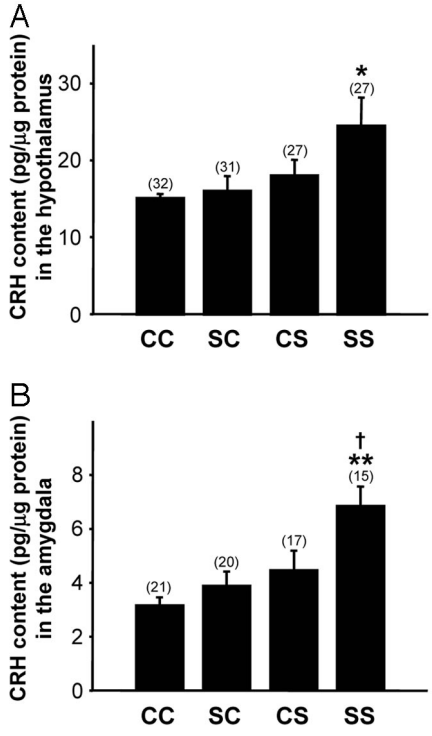

FIG. 4. CRH content in the hypothalamus and amygdala. CRH levels were determined by RIA in tissue lysates from hypothalamus (A) and amygdala (B). Data were normalized to the amounts of soluble protein in the lysates and presented as mean ± SEM. *, $P < 0.05$, and **, $P < 0.01$ *vs.* CC and SC; †, $P < 0.05$ *vs.* CS. *Numerals on the bars* indicate the number of mice in each group.

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levels in other groups of mice at the doses tested (Fig. 3) [for time spent in open arm: $F_{(3,89)} = 2.848$ ($P < 0.05$) for stress scheme, $F_{(2,89)} = 20.194$ ($P < 0.01$) for treatment with NBI 27914, and $F_{(6,89)} = 3.282$ ($P < 0.01$) for interaction; for entering frequency: $F_{(3,89)} = 6.476$ ($P < 0.01$) for stress scheme, $F_{(2,89)} = 10.220$ ($P < 0.01$) for treatment, and $F_{(6,89)} = 3.612$ ($P < 0.01$) for interaction].

Brain CRH system and basal activity of the HPA axis

To investigate a possible correlation of anxiety-like behavior in SS mice with their brain CRH system, CRH content was measured by RIA in hypothalamus and amygdala. Only the combination of both stress paradigms significantly increased CRH in both regions (Fig. 4) (hypothalamus: $F_{(3,113)} = 3.824$, $P < 0.05$; amygdala: $F_{(3,69)} = 8.190$, $P < 0.01$). Then, two other components of the HPA axis, ACTH and CORT levels, were measured in plasma (Fig. 5). Differences between groups were revealed by statistical analysis in both plasma ACTH ($F_{(3,55)} = 15.14$, $P < 0.01$) and CORT ($F_{(3,72)} = 3.836$, $P < 0.05$). Despite markedly elevated ACTH levels (Fig. 5A) ($P < 0.01$ *vs.* CC and SC groups; $P < 0.05$ *vs.* CS group), SS mice exhibited a significant decrease in plasma CORT levels (Fig. 5B) ($P < 0.01$ *vs.* CC, SC, and CS mice).

Effect of prenatal and/or postnatal stress on the adrenal glands

As shown in Fig. 5, the attenuated basal CORT secretion of SS mice did not coincide with an increased ACTH secretion from the pituitary. It is possible that the decrease in CORT secretion was because of an adrenal gland dysfunction. Interestingly, CORT contents in the adrenal glands of the SS group were significantly reduced compared with those of other mice. A decrease in CORT content by chronic stress was not seen in maternally undisturbed CS mice (Fig. 6A) ($F_{(3,28)} = 5.285$, $P < 0.01$). In contrast, normalized adrenal weights relative to body weight were significantly increased in SS mice (Fig. 6B) ($F_{(3,38)} = 5.837$, $P < 0.01$); therefore, it is unlikely that reduced CORT secretion and adrenal content in SS mice result from an adrenal atrophy, which can limit their secretory capacity. To assess whether a similar adrenal response to ACTH was maintained in these mice, mRNA levels of ACTH receptor and the adrenal response to treatment with ACTH *in vitro* were examined. Northern blot analyses revealed that neither prenatal nor postnatal stress influenced the expression of ACTH receptor in the adrenal (Fig. 6C) ($F_{(3,12)} = 1.757$, $P = 0.210$). In addition to the results from Northern blot hybridization, statically incubated adrenal fragments from all groups showed a similar dose-dependent induction of CORT secretion in response to treatment with 30, 100, and 300 nM ACTH ($F_{(3,64)} = 14.779$ for treatment with ACTH, $P < 0.01$; $F_{(3,64)} = 1.419$, $P = 0.245$ for stress scheme), indicating that the decreased plasma CORT was not because of an impairment of adrenal responsiveness to ACTH (Fig. 6D).

Expression of GR and MR

The actions of glucocorticoid are known to be mediated by their nuclear receptors, *i.e.* the high-affinity MR and low-affinity GR. The hippocampus, hypothalamus, and amyg-

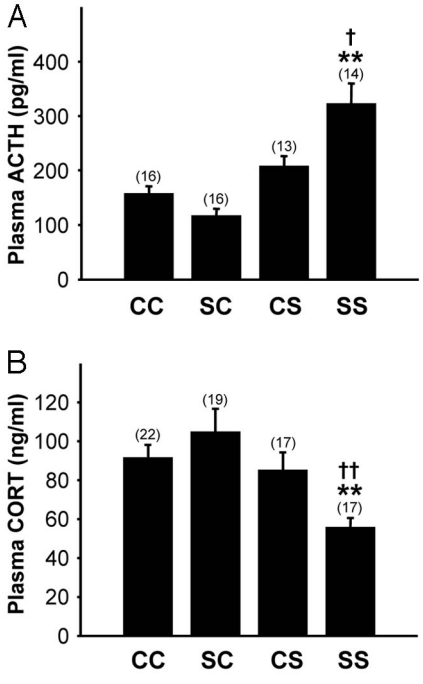

FIG. 5. Plasma ACTH and CORT levels. Plasma ACTH (A) and CORT (B) concentrations were measured by RIA. Plasma samples were immediately prepared from trunk blood and subjected to the assay. **, $P < 0.01$ *vs.* CC and SC; †, $P < 0.05$, and ††, $P < 0.01$ *vs.* CS. The number of mice used in each group is indicated on each *bar*.

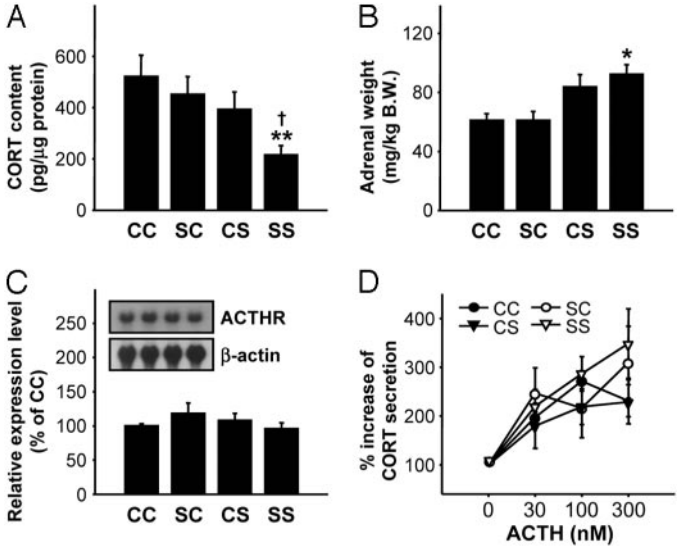

FIG. 6. Adrenal function. A, CORT content was measured by RIA from adrenal lysates and normalized to the soluble protein content. **, $P < 0.01$ *vs.* CC and SC; †, $P < 0.05$ *vs.* CS; n = 8 for each group. B, The weights of adrenal glands relative to their body weights were determined and presented as mean ± SEM. *, $P < 0.05$ *vs.* CC and SC; n = 11 for CC and CS; n = 8 for SC and SS. C, Expression of ACTH receptor (ACTHR) in the adrenal glands was examined by Northern blot hybridization. β-Actin was used as an internal control. D, CORT secretions from statically incubated adrenal fragments in response to treatments with indicated concentrations of ACTH (0, 30, 100, and 300 nM, respectively) was determined by RIA on the incubated media. Data are presented as mean percentage ± SEM (n = 5 for each group).

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dala are primary target tissues for glucocorticoid as a part of the feedback system, and they abundantly express these receptors (21). Therefore, we examined the effects of prenatal and/or postnatal stress on the expression of GR and MR in these brain areas (Fig. 7). Expressions of GR and MR proteins were determined by Western blot analyses. In the hippocampus and hypothalamus, the level of GR showed a significant decrease by both maternal stress and chronic postnatal stress (for hippocampus: $F_{(3,22)} = 8.292$, $P < 0.01$; for hypothalamus: $F_{(3,15)} = 4.369$, $P < 0.05$), but a combination of both stress paradigms did not result in any additive effect. Maternal stress led to a reduced GR level in the amygdala ($F_{(3,40)} = 4.649$, $P < 0.01$), but postnatal stress returned to normal values. Chronic stress in control mice had no effect on GR levels. Maternal stress did not influence MR levels in these tissues. Chronic postnatal stress reduced hippocampal MR proteins only slightly, but both CS and SS mice had significantly lower levels ($F_{(3,27)} = 3.492$, $P < 0.05$).

Effect of prenatal and/or postnatal chronic stress on endocrine responses to an acute stress

To examine the endocrine response to a single stressor in maternally and/or postnatally stressed mice, plasma CORT and ACTH levels were determined before and after 30 min of restraint stress. Two-way ANOVA revealed significant differences in CORT levels by both group ($F_{(3,125)} = 4.032$, $P < 0.01$) and time after stress ($F_{(3,125)} = 250.269$, $P < 0.01$), and there was a significant interaction between these two factors ($F_{(9,125)} = 4.623$, $P < 0.01$). As shown in Fig. 8A, maternally stressed mice (SC and SS mice) exhibited prolonged CORT response to acute stress, compared with the control group, which is consistent with previous studies (16–19). No significant difference was found in CORT levels at 30 min, at the end of an acute stress procedure, between controls and maternally stressed mice. At the 60-min time point, *i.e.* 30 min after acute stress ended, SC and SS showed higher plasma CORT levels, compared with their control group. Interestingly, SS mice retained elevated levels of plasma CORT, compared with their basal levels even by 90 min, when elevated CORT levels returned to basal values in other groups (Fig. 8A). Restraint-evoked increments of plasma ACTH concentrations were apparent in all groups up to approximately 6-fold inductions to their basal values ($F_{(3,83)} = 11.712$, $P < 0.01$). However, prolonged responses in ACTH secretion were not as apparent as CORT in maternally stressed mice (Fig. 8B) ($F_{(3,83)} = 0.220$, $P = 0.882$).

Discussion

The present study clearly demonstrates that maternally stressed male mice exhibit dissimilar responses to repeated postnatal restraint stress, as compared with their controls, indicating different coping capacities to stress. Maternally stressed male mice are more susceptible to the onset of chronic stress-evoked anxiety-like behaviors and show a prolonged CORT secretion in response to acute stress along with a down-regulation of CORT receptor expression in the hypothalamus and hippocampus. Furthermore, 14 d of chronic stress on maternally stressed male mice may lead to adrenal dysfunction, although it also deepens a prolonged endocrine response to an acute stress.

Maternally stressed mice show a more severe retardation of weight gain with repeated immobilization stress than controls. In other studies, overexposure to stress hormones was suggested for stress-induced growth inhibition. Prolonged

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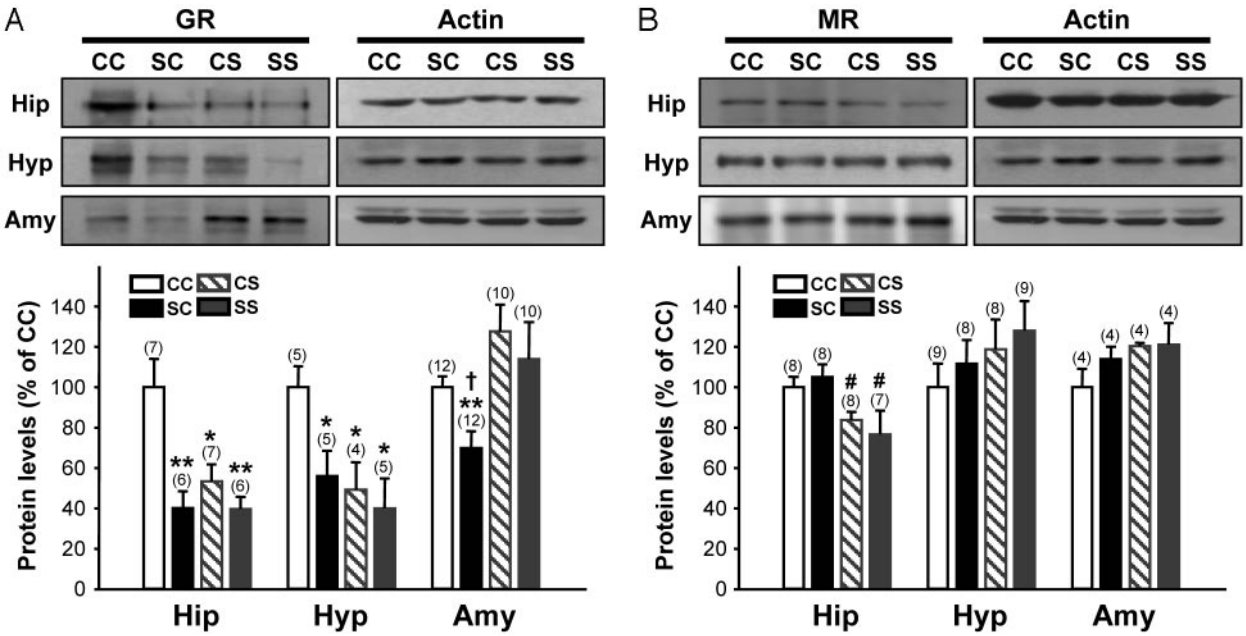

FIG. 7. Expression of GR and MR in the discrete brain regions. To determine the expression levels of GR (A) and MR (B) in hippocampus (Hip), hypothalamus (Hyp), and amygdala (Amy), 40 μg of total protein from each tissue was separated by SDS-PAGE, and Western blotting was performed with anti-GR, -MR, and -β-actin antibodies. Representative blots are shown in *upper panels*. Relative GR and MR immunoreactivities are presented as percentage of mean values from CC mice in *lower panels*. *, $P < 0.05$, and **, $P < 0.01$ *vs.* CC group; †, $P < 0.05$ *vs.* CS and SS group; #, $P < 0.05$ *vs.* CC and SC group. *Digits on the bars* indicate the number of mice used in each group.

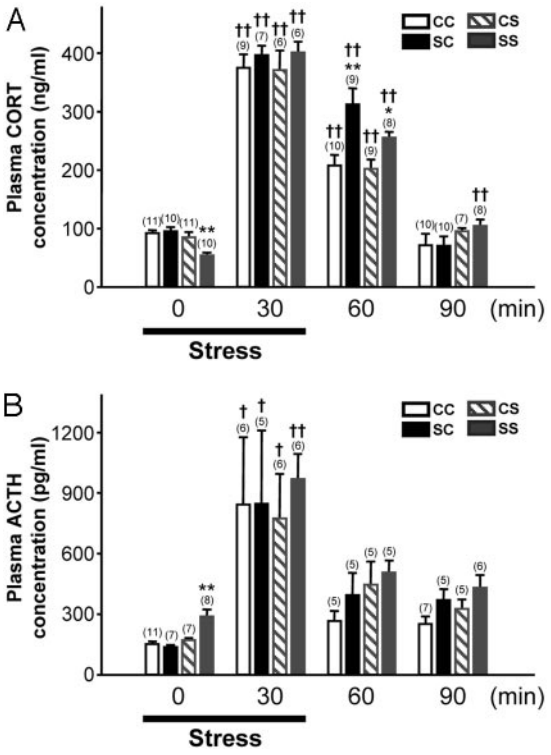

FIG. 8. Prolonged response to an acute stress in SC and SS mice. Plasma CORT (A) and ACTH (B) levels in response to 30 min of immobilization stress were determined by RIA. Mice were killed 0 (just before an acute immobilization stress), 30 (just after stress), 60, and 90 min after the beginning of stress, and plasma samples were prepared. †, $P < 0.05$, and ††, $P < 0.01$ *vs.* basal level of each group; *, $P < 0.05$, and **, $P < 0.01$ *vs.* CC at the same time point. The number of mice used in each group is presented on each *bar*.

activation of the HPA axis with elevations in glucocorticoid levels leads to suppression of GH and inhibition of somatomedin C and other GH effects on their target tissues (31). In addition to the direct effects of glucocorticoid, CRH-induced stimulation in hypothalamic somatostatin secretion may also result in GH suppression (32–34). Thus, severe growth inhibition in SS mice strongly suggests a functional hyperactivity of the HPA axis induced by chronic stress in maternally stressed mice *vs.* their controls.

Interestingly, SS mice apparently had a higher anxiety state, compared with other groups, as revealed by EPM test. Previously, several lines of evidences argued that maternal stress in rats during late gestation could lead to increased anxiety-related behavior in their offspring (20). Moreover, it was also demonstrated that chronic exposure to excess glucocorticoids during the same period mimicked the effect of maternal stress (35, 36). However, rat offspring exposed to glucocorticoid throughout pregnancy showed no increased anxiety, and a similar study using mice failed to induce high anxiety in maternally stressed offspring (30, 36). These controversies strongly suggest that other factors, including genetic background and/or the severity and duration of maternal stress, are critical determinants for the increased anxiety-like behavior of prenatally stressed offspring. In this respect, it should be noted that the maternal stress paradigm in the present study (6 h of immobilization per day for 11–12 d) is quite different in its severity and duration from other studies mentioned above (20, 30). Indeed, by itself, prolonged maternal stress in the present study also failed to induce anxiety-related behavior in adult offspring. However, it is most likely that maternal stress can, at least, potentiate the onset of repeated stress-evoked anxiety, because SS mice did not receive any additional stress on the day of the test. As shown in Fig. 3, inhibition of CRH receptor with a nonpeptide CRH receptor antagonist extinguished the expression of anxiety-like behavior in SS mice. Therefore, it is most likely that intensive anxiety in SS mice was associated with hyperactivity of CRH. In agreement, subsequent results showing that increased CRH contents in the hypothalamus and amygdala (Fig. 4) strongly suggested the importance of CRH in the present experiment. It is well established that an altered functioning of the amygdala by CRH can modulate anxiety-related behaviors. Intraamygdaloid administration of CRH was shown to induce anxiety-related behavior, and inhibition of CRH receptor with locally or systemically injected antagonists blocked this CRH-evoked anxiety thoroughly (26, 37, 38). Hypothalamic CRH can also participate in the expression of anxiety via a subsequent activation of its downstream hormones. For instances, increased GC is also known to be closely related to or facilitate the anxiety-like behaviors (20, 39). CRH receptor antagonist could also block hypothalamic CRH-induced ACTH secretion by inhibition of CRH receptor in anterior pituitary, possibly yielding a reduction in CORT level. However, administration of CRH receptor antagonist at doses used in the present study did not affect the baseline anxiety level of control mice. Thus, considering that plasma CORT levels in SS mice were paradoxically reduced, the increased anxiety of SS mice is most likely a result of the action of increased CRH levels in the amygdala.

Increase in hypothalamic CRH production and subsequent increased plasma ACTH levels indicate the functional hyperactivity of the CRH-ACTH system in SS mice. It is, however, of interest that plasma CORT levels were significantly reduced despite the increased secretion of ACTH in these mice. Expression of ACTH receptor mRNA and the induction of CORT secretion *in vitro* by treatment with ACTH showed no significant differences among the four groups tested; therefore, it is unlikely that reduced adrenal CORT secretion is caused by an impaired adrenal responsivity to ACTH. Rather, reduced adrenal CORT levels suggest a possible dysfunction in CORT synthesis or exhaustion as a result of robust and prolonged secretion by repeated immobilization stress in maternally stressed mice.

Attenuation of CORT-induced feedback via its receptors might contribute to changes of basal HPA axis activity, in particular, increased CRH contents in the amygdala and hypothalamus by prolonged prenatal and postnatal stress. Differential effects of glucocorticoid on CRH systems in the amygdala and hypothalamus were demonstrated elsewhere. For instance, high doses of CORT replacement increased CRH mRNA in the central nucleus of the amygdala, whereas it reduced CRH mRNA expression in the PVN (40). Stereotaxic delivery of CORT to the amygdala increased basal CRH mRNA levels in the central nucleus of the amygdala (41); in contrast, local administration of glucocorticoids suppressed CRH synthesis in the PVN (42, 43). In this study, despite

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reduced amygdala GR levels in maternally stressed mice, a prolonged and repeated CORT response to stress during the stress sessions may have caused an elevation of CORT-mediated positive feedback on the CRH system in the amygdala of SS mice. Maternal stress and repeated postnatal stress reduced GR expression in the hypothalamus, indicating that glucocorticoid feedback inhibition decreased in the PVN. Moreover, GR and MR levels in the hippocampus were influenced by prenatal and postnatal chronic stress. Hippocampal corticosteroid receptors, in particular MR, are thought to play an important role in regulation of basal HPA activity by mediating the inhibitory effect of glucocorticoid (21, 44, 45). Thus, reduced hypothalamic GR and hippocampal MR, in combination with a lower basal secretion of CORT, may likely attenuate feedback inhibition of CRH neurons in SS mice.

The exact molecular mechanism underlying the differential effect of repeated stress on control and maternally stressed mice is still unclear. However, it is evident that SS mice show several paradoxical features in the basal activity of the HPA axis and its reactivity to an acute stress; for example, reduced basal CORT secretion does not correlate only with reduced brain receptors but also with increased hypothalamic CRH content and plasma ACTH, suggesting that the balance of components consisting of the HPA axis, including hormones and brain corticosteroid receptors system, may be severely altered by the combination of chronic prenatal and postnatal stresses. It should be noted that maternally stressed mice (both SC and SS mice) showed prolonged CORT secretion after a single acute stress compared with control mice in the present study, consistent with studies of other researchers (18, 19). Therefore, repeated postnatal stress may activate the stress system and CORT secretion, and maternally stressed mice might have a longer exposure to CORT because of a failure to turn off each stress response properly. Improper termination of stress responses and subsequent overexposure to stress hormones are regarded as a critical determinant for exacerbating a variety of chronic stress-evoked pathophysiological symptoms (2). The accumulative influences of sustained stress system activation are likely to underlie the greater susceptibility of maternally stressed mice to chronic stress.

In conclusion, the present study clearly demonstrates that maternally stressed mice are more susceptible to chronic stress than their controls, because 14 d of repeated restraint stress resulted in neuroendocrine and behavioral alterations only in these animals; control mice maintained normal neurological and endocrine functions after the same stress sessions. There still remain several issues requiring further elucidation. First, a possible effect of the severity and duration of maternal stress on a coping capacity of the offspring should be clarified in future examinations. Moreover, because it has been demonstrated that the age- and gender-dependent differences in HPA axis reactivity might play an important role in response to stress (46, 47), and postnatal stress in this study was administered to only male offspring during the age of late pubertal period to young adulthood, the influences of age and gender on differential adaptive responses of maternally stressed mice also need more consideration. Nevertheless, the present study strongly suggests that stress experience in the womb can result in the increased risk of physiological or neurological abnormalities caused by chronic stress, which maternal stress alone fails to elicit.

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Address all correspondence and requests for reprints to: Kyungjin Kim, Ph.D., School of Biological Sciences, Seoul National University, Seoul 151-742, Korea. E-mail: kyungjin@snu.ac.kr.

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