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Effects of Glucocorticoid on the Amygdala-Dependent Fear Memory and Hippocampal Neurogenesis

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서울대학교 대학원

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ABSTRACT

Glucocorticoid (GC) is a steroid hormone playing diverse roles which enable organisms to respond to and to cope with environmental changes such as stress. The secretion of GC is primarily governed by the hypothalamus-pituitary-adrenal (HPA) axis, a major neuroendocrine circuit in the stress response system. Maternal stress-elicited elevation of GC has programming effects on the long-lasting modification of the HPA axis and brain functions in offspring. However, the prolonged impact of maternal stress on emotional learning remains largely unknown. In addition to stress responsiveness and early life programming, another apparent characteristic of GC is its robust circadian rhythm. However, the significance of the functional GC rhythm in the brain function is not well understood yet. Recently, our group generated transgenic mice with adrenal-specific knockdown of canonical clock protein BMAL1 (A-BMKD), which showed attenuated circadian GC rhythm under constant darkness (Son et al., 2008). In Chapter 1, I intended to investigate how maternal stress affects fear memory in the amygdala, a major target of stress in the brain. In Chapter 2, using A-BMKD transgenic mouse line, I investigated the effect of circadian GC oscillation on hippocampal neurogenesis implicating in cognition deficits and mood disorders in Chapter 2.

1. In Chapter 1, I examined whether maternal stress influences on the amygdala-related learning processes. Maternally stressed mice exhibit normal fear memory acquisition as well as synaptic NMDA receptor

expression in the amygdala; however, fear memory consolidation and the activation of related signaling cascades are significantly attenuated. In accordance with these behavioral aspects, maintenance of long-term potentiation (LTP) evoked in the thalamo-lateral amygdala pathway is significantly attenuated in amygdalar slices from maternally stressed animals, though basal synaptic properties and LTP induction were unaffected in these slices. Interestingly, administration of GC immediately after training or LTP induction restores impaired memory consolidation and LTP maintenance, indicating the weakened aversive memory-enhancing effect of GC in maternally stressed mice. Moreover, the membrane-impermeable form of GC mimics the restorative effect in maternally stressed animals, implying the involvement of a nongenomic mechanism. Taken together, it appears that maternal stress causes dysregulation of amygdala-dependent fear memory in adult offspring by an impairment of amygdalar synaptic plasticity in association with reduced nongenomic action of GC on long-term memory formation.

2. In Chapter 2, I focused on hippocampal neurogenesis in A-BMKD transgenic mice under constant darkness for 7 days. The number of newborn neurons in the hippocampus was significantly declined in the transgenic mice. These mice showed depressive mood states, impaired safety memory, and rapidly shutting off stress reactivity, which are known to be regulated by hippocampal neurogenesis. Moreover, enhanced rhythmic translocation of activated glucocorticoid receptor (GR) into hippocampal nucleus by binding to GC was observed against hypo-GC secretion with

attenuation rhythm in TG mice. In accordance with this finding, transcript levels of brain-derived neurotrophin factor (BDNF) and its receptor TrkB, which are known to be essential for adult neurogenesis and regulated by GC negatively, were reduced in the hippocampus of A-BMKD transgenic mice. Interestingly, a daily oscillation of plasma GC restored by rhythmic intake of drinking water containing GC recovered not only neurogenesis but also gene expression of BDNF and TrkB in the hippocampus, suggesting that BDNF-related signal cascade is one of candidates responsible for the impaired hippocampal neurogenesis in the transgenic mice. Taken together, GC levels as well as its rhythmic secretion are important for maintaining normal neurogenesis associated with gene expression of BDNF and TrkB in the hippocampus.

In conclusion, GC has a critical role in fear memory formation via nongenomic action in the amygdala and the functional circadian rhythm of GC is required for maintaining adult neurogenesis in the hippocampus.

Key words: Glucocorticoid (GC), Maternal stress, Amygdala, Fear memory, Circadian rhythm, Hippocampal neurogenesis, Nongenomic action, Glucocorticoid receptor (GR), Brain-derived neurotrophin factor (BDNF)

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Background and Purpose

BACKGROUND

1. Regulation of glucocorticoid (GC) actions

1.1. Physiological importance of GC

Glucocorticoid (GC; corticosterone in rodents and cortisol in primates) is an adrenal steroid hormone with various effects which enable organism to respond to and to cope with environmental changes such as stress and time (Chrousos and Kino, 2007; Sapolsky et al., 2000). GC exerts wide spread actions in the body which are essential for the maintenance of homeostasis and enable the organism to prepare for physical and emotional stress (Sapolsky et al., 2000). It promotes the breakdown of carbohydrate and protein, and exerts lipid metabolism so as to increase the immediately available energy. GC is also an important regulator in numerous immune and inflammatory responses. It raises blood pressure, has complex effects on bone, exerts both positive and negative effects on cell growth, and is proapoptotic on certain cell types including neuronal populations. Within the central nervous system (CNS), GC mediates organizational events in the developing brain as well as neuronal plasticity and neurodegeneration in adulthood. Animals recall stressful stimuli more vividly than neutral one, since acute GC signaling, for instance, enhances aversive memory formation and evokes heightened alertness and anxiety. Other effects in the CNS include the regulation of food intake, body temperature, pain perception, and neuroendocrine function (Buckingham, 2006; Herbert et al., 2006).

Due to profound influences of GC on numerous biological processes, it is implicated in the pathogenesis of a number of common diseases including hypertension and other cardiovascular disease, insulin resistance, obesity and type II diabetes, autoimmune inflammatory disease, and reproductive dysfunction. In conditions in which sustained and pronounced elevations in circulating GC occur, due to stressful circumstance, hypersecretion of endogenous GC or prolonged administration of exogenous agonists, a plethora of unwanted pathologies mentioned above emerges and becomes exaggerated. Major depression and cognitive impairment are representative conditions of chronically excess GC-induced disorders of the brain. Frequently, altered emotionality such as depressed mood is accompanied with impaired cognition, indicating that excess GC impacts on multiple and inter-related neural circuits. Those adverse effects involve structural changes and synaptic loss in the limbic system that has the function for regulating GC secretion (De Kolet et al., 2005).

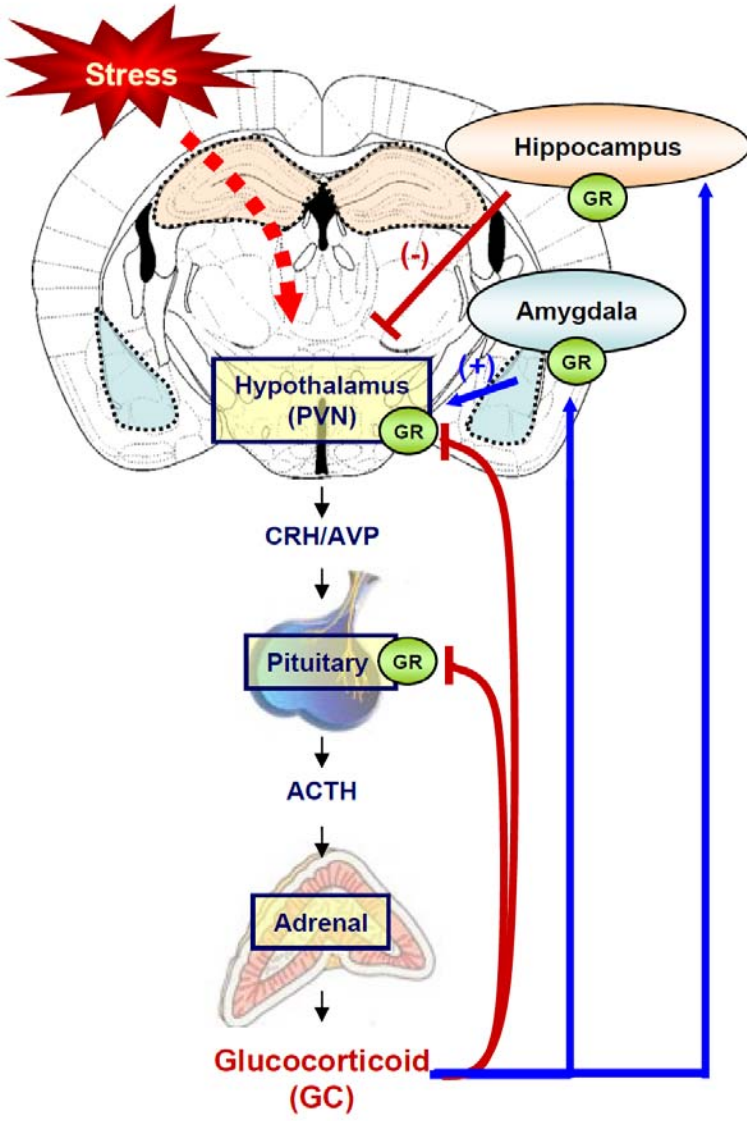
1.2. Stress-responsive GC secretion

The secretion of GC is primarily governed by the hypothalamus-pituitary-adrenal (HPA) axis, a major neuroendocrine circuit in stress response system (Fig. 1). The hypothalamus receives and integrates stress-sensitive neural and humoral information from many sources in discrete brain regions such as the brainstem catecholaminergic pathway and limbic forebrain. It thus acts as a sensor of changes in the external and internal environment. Using this information, the hypothalamus responds to adverse circumstances by releasing of two neurohormones, corticotrophin-

releasing hormone (CRH) and arginine vasopressin (AVP) from neurosecretory parvocellular neurons in the paraventricular nucleus (PVN) that project axon to median eminence. CRH and AVP travel via the hypothalamo hypophyseal-portal blood vessels to the anterior pituitary gland where they act synergistically through their receptors to trigger the release of the adrenocorticotrophic hormone (ACTH) from corticotrophs into the systemic circulation. ACTH, in turn, acts on the adrenal cortex by binding to type 2 melanocortin receptors (MC2R) to initiate the synthesis of GC, which interacts with specific receptors in various target tissues in the brain and periphery. Circulating GC also provides feedback to its upstream regulators to turn off the HPA neuroendocrine activity and restore a steady state (Buckingham, 2006).

In most cases, GC fulfills its central functions through the mediation of two types of nuclear receptors; the type I mineralocorticoid receptor (MR) and the type II glucocorticoid receptor (GR). The MR has a very high affinity for endogenous GC and maintains cellular responses to GC. Stress levels of GC extensively bind to GR, which is necessary for the inhibition of stress responses in HPA axis. The GR is densely expressed in the PVN as well as limbic brain structures including the hippocampus and the amygdala, which are critical for emotional responses and memory, contributing feedback integration of GC (Beato et al., 1995; De Kloet et al., 1998). The hippocampus is involved in terminating anticipatory HPA axis responses, consistent with its role in memory and emotion processing. Hippocampal lesions or local GR inactivation caused diminished feedback efficacy (Van Haarst et al., 1997). While hippocampal GR is required for inhibition of the HPA axis, amygdaloid GR can stimulate HPA responses. For example,

Figure 1. Neuroendocrine regulation of adrenal glucocorticoid (GC). GC is primarily regulated by the hypothalamus–pituitary–adrenal gland (HPA) axis, a major neuroendocrine circuit of the stress response system. When certain neurochemical signals related to stress are received by the hypothalamus, a subset of neurosecretory cells in the paraventricular nucleus (PVN) releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) to stimulate adrenocorticotropic hormone (ACTH) synthesis and secretion from the pituitary. ACTH then induces adrenocortical cells to produce and secrete GC in circulation. GC turns off the HPA axis activity by negative feedback mechanisms at the pituitary and at many sites in the brain, including the hippocampus. Conversely, inputs that arise from the amygdala elicit activation of the HPA axis. Stress levels of GC extensively bind to GC receptor (GR), which is necessary for the inhibition of stress responses in HPA axis. The GR is densely expressed in the PVN, pituitary, hippocampus and the amygdala, contributing feedback integration of GC.


Stress
Hippocampus
GR
(-)
Amygdala
GR
(+)
Hypothalamus
(PVN)
GR
CRH/AVP
Pituitary
GR
ACTH
Adrenal
Glucocorticoid
(GC)

systemic GC increases CRH mRNA expression in the central nucleus of the amygdala (CeA) and stress-induced CRH release is blocked by pretreatment with a GR antagonist (Cook, 2002). Thus, GC may have feed-forward effects in the amygdala that can be linked to enhanced stress excitability.

1.3. Genomic and nongenomic actions of GC

Circulating GC diffuses through cell membranes and binds to intracellular MR and GR that belong to transcription factors. Upon binding of GC, the receptors bind to a multiprotein complex of heat shock proteins and form activated receptor complexes, allowing dissociation from the heat shock proteins and homodimerization with other activated receptors (Kino et al., 2005). The dimerized MR or GR translocate into the cell nucleus, where they bind to the mineralocorticoid response element (MRE) or glucocorticoid response element (GRE). The GRE or MRE modulates gene transcription by transactivation or transrepression (Beato et al., 1996). If transactivation occurs, usually at promoter regions or introns, the receptor complex at the MRE or GRE recruits cofactors and histone-modifying elements, inducing transcription and subsequent protein translation (Datson et al., 2008).

However, recent evidence indicates that GC receptors also act directly and indirectly through nongenomic actions involved with signal transduction initiated from plasma membrane. The mediator of nongenomic GC effects may share similar mechanisms with the estrogen receptor (ER) which is a well-characterized membrane steroid receptor (Riedemann et al., 2010).

Membrane-bound ER coupled with G proteins such as GPR30 has been identified as a potential transducer of estrogen signals that originate at the cell membrane (Qiu et al., 2006). Similarly, a downstream complex that appears to mediate such nongenomic processes is the MAPK found that a short application (15 min) of GC rapidly increased MAPK phosphorylation mediated by G proteins (Li et al., 2001). In this study, a membrane receptor–mediated effect was suggested by the short time frame of action, coupled with the ability of GC conjugated to the membrane-impermeant bovine serum albumin (BSA) to replicate the effect of unconjugated GC. While some authors describe G protein-coupled receptor as a mechanism to explain nongenomic signaling of estrogen, others propose that both membrane and genomic ERs are derived from the same gene transcript, which suggests that the membrane version of the ER is posttranslationally modified by palmitoylation (Hart et al., 2007; Kalita et al., 2005; Milner et al., 2005), a covalent attachment of long-chain fatty acids that increases protein hydrophobicity and membrane association of proteins. While it remains to be shown that classical GC receptors can be palmitoylated and trafficked to the plasma membrane, recent studies have identified a highly conserved motif in the estrogen, progesterone, androgen and GC receptors (Pedram et al., 2007), suggesting that palmitoylation may be a general mechanism that allows nuclear receptors to be inserted to membrane receptors. In addition, MR and GR may also act at the membrane, where they can rapidly modulate synaptic transmission and neuron excitability by affecting the conductance of specific ion channels. These receptors may regulate synaptic plasticity by altering the amount of calcium entering the synapse, which would affect whether synaptic plasticity occurs and synaptic

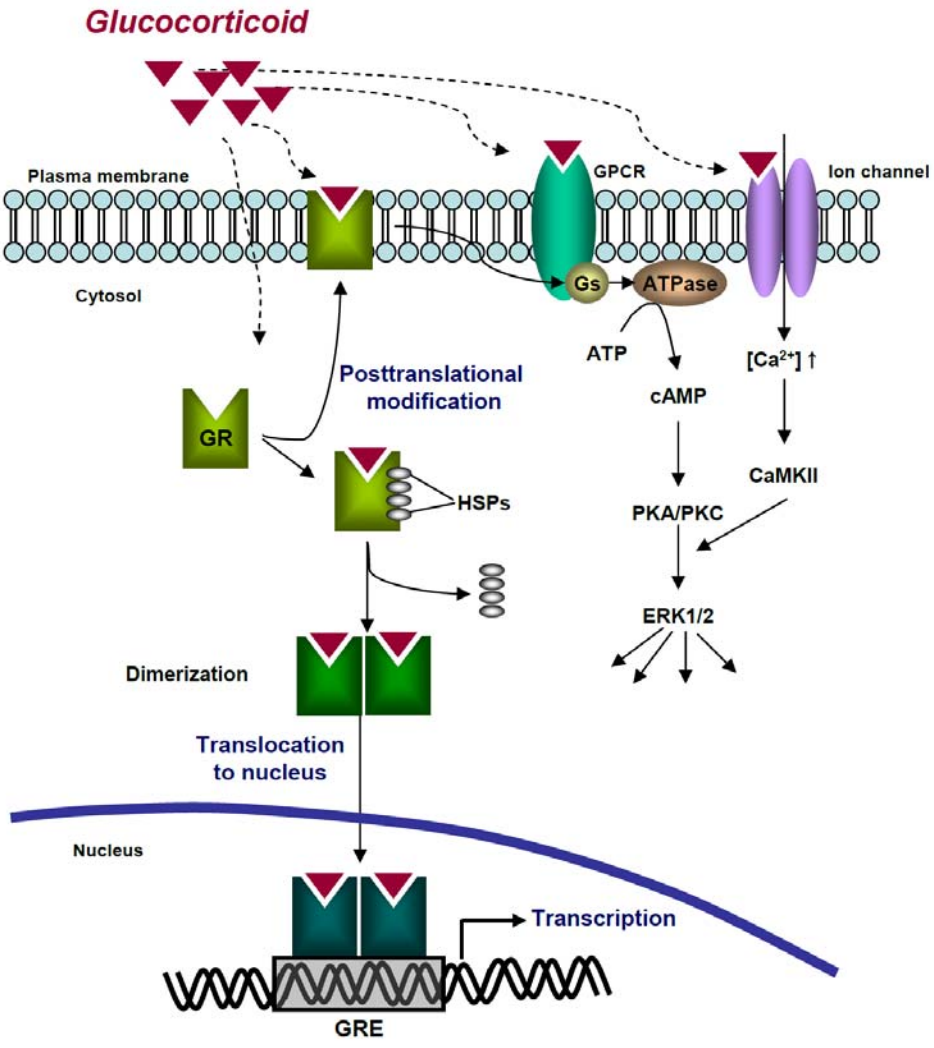
transmission increases between neurons. In this context, Joëls and colleagues demonstrated presynaptic and postsynaptic regulation of glutamatergic neurotransmission by memebrane MR (Karst et al., 2005; De Kloet et al., 2008; Olijslagers et al., 2008). Moreover, this evidence has also included direct anatomical localization of membrane GR to the postsynaptic density (Johnson et al., 2005; Fig. 2).

1.4. Circadian rhythm of GC

In addition to stress responsiveness, another apparent characteristic of GC is its robust daily rhythm. It corresponds to the daily activity-rest cycle, so levels are maximal at the beginning of the dark phase in nocturnal rats, but in the light phase in diurnal humans. Rhythmic releases of ACTH and GC are completely blocked after ablation of the SCN, suggesting that the circadian rhythm of GC has been generally attributed to rhythmic HPA activity regulated by the suprachiasmatic nucleus (SCN) in the ventral hypothalamus as a central pacemaker in mammals (Buijs and Kalsbeek, 2001). Anatomically, there exist both direct and indirect neural connections from SCN to PVN where CRH- and AVP-producing cells reside, and central pacemaker appears to control the HPA axis by modulating the PVN or its output pathways (Dickmeis, 2009)

However, the daily oscillation of GC does not appear to require the periodicity of its upstream hormonal regulators, hypothalamic CRH and ACTH, because GC rhythm was still present when ACTH was constitutively administered to hypophysectomized rats (Meier, 1976). Recently, the influence of the central clock via the splanchnic nerve innervation from the

Figure 2. Diagram of the action of GC via genomic and nongenomic mechanisms. GC is represented by red triangle. GC diffuses through the cell membrane, binds to cytosolic GC receptor (GR), which then bind to heat shock proteins (Hsps) and homodimerize. The Hsps then dissociate and the GR-GR homodimer translocates into the nucleus and binds to GC response element (GRE). Also, GR can be located in plasma membrane via posttranslational modification. Alternatively, GC can trigger signaling cascades, binding to proteins in the plasma membrane, e.g. G-protein-coupled receptors GPCR or ion channels.



(Modified from Riedemann et al., 2010)

SCN to the adrenal gland has been implicated. Denervation of splanchnic nerve blocks the light-induced GC changes (Ishida et al., 2005) and reduces the amplitude of GC rhythm by modulating adrenal sensitivity to ACTH (Ulich-Lai et al., 2006). Though a strong inhibition to the basal GC release during the rest period by the SCN seems evident, both SCN-lesion and splanchnic denervation studies also suggested that the SCN-derived inhibitory mechanisms are insufficient to fully account for the circadian peak of GC rhythm (Buijs et al., 1993; Kalsbeek et al., 1996; Ulrich-Lai et al., 2006), implying the presence of the adrenal intrinsic mechanisms.

1.5. The local adrenal clock in generating the circadian rhythm of GC

Autonomous and self-sustainable natures of the circadian timing system primarily depend on the presence of a genetic mechanism called the molecular circadian clockwork. Core clock components consist of a subset of genes whose protein products are required for the generation and maintenance of circadian rhythm in an organism and even within individual cells. These molecules promote the rhythmic gene expression of each other by two interlocked positive and negative transcription/translation feedback loops. In the core feedback loop, CLOCK and BMAL1 form heterodimers to induce E-box-mediated transcription of the negative regulators Periods (PERs) and Cryptochromes (CRYs). Accumulated PER and CRY proteins intensively repress E-box-mediated transcription until their levels sufficiently decrease once again. Additionally, CLOCK and BMAL1 also control the transcription of nuclear receptors RORα and REV-ERBα which form stabilizing feedback loop with modulating BMAL1 mRNA levels by

competitive actions on RRE element residing in the Bmal1 promoter. The self-sustaining feedback loops described above cycle with an approximately 24-hour of period and constitute the circadian molecular clock machinery (Ko and Takahashi, 2006).

Clock genes are expressed ubiquitously and virtually all of the peripheral organs and tissues possess their own clock machinery. These 'peripheral' oscillators appear to be coordinated by the 'central' oscillator, the SCN. Adrenal gland is also one of the cases and the rhythmic expression of clock components in adrenal gland was found (Ishida et al., 2005; Lemos et al., 2006; Oster et al., 2006; Son et al., 2008). Global analysis of adrenal transcriptome revealed that many key cellular processes can exhibit a rhythmic fashion implying the roles of the adrenal local clock (Lemos et al., 2006; Oster et al., 2006). In this context, several groups have proposed possible molecular mechanisms related to the adrenal peripheral clock. First, Oster and his colleges provided evidence showing that this local clock machinery can be involved in the daily rhythm in the adrenal sensitivity to ACTH (Oster et al., 2006). In addition, photic activation of splanchnic innervation to the adrenal could subsequently entrain the adrenocortical clockwork and augment the circulating GC levels (Ishida et al., 2005).

Finally, it is revealed that the GC biosynthesis is closely linked with adrenal local oscillator by clock controlled expression of steroidogenic acute regulartory protein (StAR), a rate-limiting step of steroidogenesis (Son et al., 2008). This recent finding provided strong evidence on a direct link between StAR and adrenal local clock and its crucial function in generating the adrenal-autonomous GC rhythm *in vivo*. Mice harboring

adrenal-specific BMAL1 knockdown by antisense cRNA expression in adrenocortical GC-producing cells (designated as A-BMKD transgenic mice) clearly presented flattened StAR expression and adrenal steroidogenesis. Interestingly, a significantly dampened but not completely flattened circulating GC pattern is still observed in these animals, a potentially valuable model for studies about physiological relevance of circadian GC rhythm.

2. Programming effect of GC by maternal stress

2.1. Maternal stress as a potent programming factor in early life

The environment in the womb has long-lasting effects on the later life of offspring, which are known as “programming effects” (Seckl, 2008). For example, the low birth weight of pups from mothers stressed in pregnancy has been correlated with pathophysiological symptoms such as cardiovascular disease and metabolic disorders (Nathanielsz, 1999; Welberg et al., 2001). Low birth weight is also associated with affective and cognitive disorders in adulthood. For instance, low birth weight has been linked to schizophrenia, attention deficit/hyperactivity disorder (ADHD), antisocial behavior, increased vulnerability to post-traumatic stress disorder (PTSD), anxiety disorder, learning difficulties and depression (Thompson et al., 2001; Wiles et al., 2005). Therefore, the brain is vulnerable to environmental challenges in early life that contribute to alterations in the cognition and behaviors of offspring (Welberg et al., 2001; Weinstock, 2008).

Despite the increasing evidence pointing to the importance of early life programming in inducing alterations in physiological functioning and mental health, the mechanisms through which these prenatal influences operate remain largely unknown. The major hypothesis has been advanced to explain the link between events in the womb and the later risk of neuropsychiatric and metabolic disorders: fetal overexposure to GC. Maternal stress or administration of GC during pregnancy reduces birth weight and reliably leads to higher blood pressure, glucose levels, altered behavior and HPA axis function in the adult offspring (Seckl and Holmes, 2007). Physiologically, much higher circulating levels of GC in the maternal than in the fetal blood are ensured by a placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which catalyzes GC to an inactive form, thus forming a physiological “barrier” to maternal GC. Similar with the maternal stress, administration of 11β-HSD2 inhibitor during pregnancy or genetic knock out of the enzyme reduces birth weight and generates adversely programmed CNS and peripheral outcomes in the adult offspring (Holmes et al. 2006). From those observations, it is widely accepted that interaction between maternal stress as an environmental factor and genetic blueprint is a critical determinant for the establishment of adult physiology in the later life.

2.2. Maternal stress and the regulation of the HPA axis in adult offspring

The HPA axis is particularly sensitive to GC levels during development. For instance, high GC exposure during fetal development in rats and primates permanently elevates basal GC levels in offsprings. Importantly,

prenatally stressed rats show faster, more robust and/or more prolonged endocrine responses than control animals in response to various stressors. Prenatal GC treatment in rats also increases circulating ACTH levels in the offspring, which is associated with increased CRH in the PVN, and in the central nucleus of the amygdala, where it is crucial for the expression of fear and anxiety (Welberg et al., 2001). Maternal stress also impacts development of CRH neurons in the PVN (Fujioka et al., 1999; Shoener et al., 2006) with greater vulnerability to PVN cell death (Tobe et al., 2005), which has implications for HPA axis programming.

Programmed HPA axis activation has plausibly been ascribed to relative deficiency of GC feedback sensitivity with reduced GR and MR in the limbic regions as feedback sites. Thus, excess GC in pregnancy permanently attenuates GR and MR mRNA expressions in specific subfields of the rat hippocampus. Similarly, maternal GC exposure in guinea pigs results in region-specific changes in MR and GR mRNA in offspring brains. Offsprings whose mothers received an 11β-HSD2 inhibitor during pregnancy have increased GR mRNA expression in the amygdala. It is plausible that increases in GR levels in amygdala augment positive drive onto the HPA axis (Welberg et al., 2001). Taken together, these previous findings strongly suggest that modulation of brain GC receptors is most probable mechanism for the programming effect of maternal stress on the HPA reactivity of adult offspring.

2.3. Influence of maternal stress on the behavior of adult offspring

The limbic regions play a significant role in learning and memory, and

many studies have revealed the altered those functions following prenatal stress exposure. However, there are some controversies in regard to the influence of GC overexposure by maternal stress on the learning and memory of adult offspring, according to experimental model. Maternal stress administered in the form of restraint or daily foot shocks, slowed the acquisition of spatial learning in some (Ishiwata et al., 2005; Lemaire et al., 2000, Son et al., 2006), but not in other studies (Vallée et al., 1997; Cannizzaro et al., 2006). For example, repeated immobilization stress to pregnant rats produced learning deficits associated with an impairment of neurogenesis in the hippocampus (Lemaire et al., 2000). In mice, it is reported that maternal stress can elicit learning deficits due to a reduction in spine density (Ishiwata et al., 2005) or an impairment of NMDA receptor-mediated synaptic plasticity (Son et al., 2006). By contrast, maternal stress did not cause a memory deficit (Vallée et al., 1997), or resulted in a faster rate of learning (Cannizzaro et al., 2006). However, learning ability can be still regarded to be susceptible to the environmental factors in early life as prenatal exposure to dexamethasone, a synthetic GC impaired spatial learning ability of adult offspring while maternal care enhanced it (Brabham et al., 2000; Kamphuis et al., 2003).

The anxiety-related behavior is one of extensively studied neurobehaviors regarding maternal stress. The maternally stressed adult rats have often been described as showing a higher degree of anxiety. They spend less time than controls in the open arms of an elevated plus maze (Estanislau and Morato, 2005, Murmu et al., 2006; Vallée et al., 1997) and exhibit increased defensive freezing and ultrasonic vocalizations in an open field (Dickerson et al., 2005, Takahashi et al., 1992; Williams et al.,

1998). Maternally stressed mice are also more vulnerable to chronic stress-evoked anxiety-like behaviors (Chung et al., 2005). A plausible explanation for the increased hyper-anxious state of prenatally stressed offspring is altered functioning of the amygdala, which mediates fear and anxiety-related behavior (Davis, 1992), containing CRH receptors and CRH producing cells. Indeed, CRH may be the key neurotransmitter that mediates the effect of prenatal stress on anxiety. Firstly, CRH levels are increased in the central nucleus of the amygdala in prenatally stressed or GC-overexposed rats (Cratty et al., 1995; Welberg et al., 2001). Secondly, CRH injections directly into the amygdala increase anxiety-related behavior in rats (Dunn and Berridge, 1990). By contrast, inhibition of CRH receptors in the central nucleus of the amygdala reduced anxiety-related responses under anxiogenic conditions (Rassnick et al., 1993; Liebsch et al., 1995; Lee and Davis, 1997). In spite of extensive studies on maternal stress-evoked anxiogenic behaviors in offspring, there is as yet little evidence concerning the other function of the amygdala, modulation of fear-associated learning and memory.

3. Amygdala-dependent fear memory

3.1. Povlovian fear conditioning and the amygdala

The term ‘fear’ refers to both a psychological state and a set of bodily responses that occur in response to threat. Much progress has been made in understanding how fear is organized in the brain through studies of Pavlovian fear conditioning (Rodrigues et al., 2009). Pavlovian fear

conditioning is a behavioral procedure in which an emotionally neutral conditioned stimulus (CS), such as an auditory tone, is paired with an aversive conditioned stimulus (US), typically a foot shock. After one or several pairings, the CS comes to elicit defensive behaviors, including freezing behavior, as well as increased arousal in the brain and secretion of norepinephrine and GC peripherally.

The amygdala contains a heterogeneity of distinct nuclei, differing by cell type, density, neurochemical composition, and connectivity (LeDoux, 2007). The lateral nucleus (LA) is typically viewed as the sensory gateway to the amygdala because it receives auditory, visual, gustatory, olfactory, and somatosensory information from the thalamus and the cortex. The LA receives sensory information about the CS from thalamic and cortical projections. The thalamo-amygdala pathway is a shortcut of sorts, transmitting rapid and crude information about the fear-eliciting stimulus without the opportunity for filtering by conscious control. The cortico-amygdala pathway, in contrast, provides slower, but more detailed and sophisticated sensory information. Neurons in the LA respond to both the CS and the US, and damage to, or a disruption of, the LA prevents fear conditioning (Romanski et al., 1993). Indeed, single-unit recordings show that cellular plasticity occurs in the LA during fear conditioning (LeDoux, 2007). The central nucleus (CE), in contrast, is viewed as the major output region of the amygdala. The CE controls the expression of the fear reaction, including behavioral, autonomic, and endocrine responses via projections to downstream areas, including hypothalamus, central gray, and the dorsal motor nucleus of the vagus (LeDoux, 2007; Rodrigues et al., 2004). Other nuclei within the amygdala might be involved in fear conditioning, but the

LA and the CE appear to be crucial (Nader et al., 2001).

3.2. Molecular mechanism of fear conditioned memory

In studies of fear conditioning, responses elicited by the CS are often measured during acquisition and retrieval tests. Acquisition is initial learning of the association between the CS and US during the training portion of fear conditioning, when the animal first learns to pair the two. Specifically, it has been proposed that the CS inputs lead to the release of glutamate, which binds to glutamate receptors, including AMPA receptors (AMPARs), NMDA receptors (NMDARs), and metabotropic glutamate receptors (mGluRs) on LA cells. The US then depolarizes these cells while glutamate is bound to NMDARs, allowing Ca2+ to enter through NMDARs. The elevation of intracellular Ca2+ through NMDARs activates protein kinase second messenger cascades that are also essential for memory formation.

Retrieval involves a test of the CS-US association in which the CS is presented alone. Retrieval tests that occur within a few hours after acquisition measure short-term memory (STM), whereas those that occur later measure long-term memory (LTM). STM and LTM tests are used to study memory consolidation, the process through which an unstable STM is converted into a more stable and enduring LTM trace. The Ca2+ signal provided by the activation of glutamate receptors during acquisition triggers a variety of additional intracellular steps including activations of cAMP-dependent protein kinase (PKA) and extracellular-regulated kinase (ERK). Then activated trascription facotrs such as phosphorylated PKA-dependent cAMP response element binding protein (CREB) consolidate the short-

lasting STM into a persistent LTM by initiating macromolecular synthesis. Therefore, the fear memory consolidation is a RNA and protein synthesis-dependent phase (McGaugh, 2000; Rodrigues et al., 2004).

3.3. Synaptic plasticity in the amygdala and fear conditioning

Synapses that transmit auditory information to the LA are capable of long-term potentiation (LTP) whose basic features make it an attractive cellular model of fear learning and memory (Bliss and Collingridge, 1993). A number of studies have examined whether fear conditioning causes LTP-like synaptic changes at auditory inputs onto LA neurons. Actually, LTP induction at auditory inputs to the LA enhances auditory-evoked responses in the LA in a manner similar to the enhancement of CS-evoked responses observed during auditory fear conditioning. This approach has revealed that LTP induction does indeed enhance auditory-evoked field potentials recorded in the LA (Rogan and LeDoux, 1995). Subsequent experiments showed that fear conditioning caused a similar enhancement in field potential responses to an auditory CS that developed in conjunction with conditioned fear responses (Rogan et al., 1997). These findings therefore suggested that LTP occurred in the LA during fear conditioning and were at the time considered the best evidence available linking LTP to learning and memory (Sigurdsson et al., 2007). Moreover, LTP shares many of the molecular mechanisms of fear conditioning, suggesting that LTP-like changes may be a necessary requirement for fear learning and memory. For example, the activation of NMDA receptors is involved in both the induction of LTP in the LA and the acquisition of fear conditioning. In

addition, a number of intracellular signaling pathways, as well as gene transcription and protein synthesis, have also been implicated specifically in the long-term maintenance of LTP and the consolidation of long-term fear memories (Huang et al., 2000; Schafe and LeDoux, 2000; Rodrigues et al., 2004).

3.4. The effects of GC on the fear-conditioned memory

Pretraining systemic or intra-amygdala manipulation of GC influences the LTM of fear conditioning. Posttraining systemic manipulations of GCs also impact fear memory consolidation. Moreover, post-training GR blockade in the amygdala affects auditory fear LTM but not STM, providing more evidences that GC is most important for consolidation processes. Furthermore, posttraining GC administered immediately, but not 3 hours, after auditory fear conditioning facilitates freezing to a tone previously paired with a US but does not alter responses to unpaired tones or to tone or shock alone, suggesting a selective and time-dependent role for GC-facilitated memory of the tone-shock association (Rodrigues et al., 2009).

These mnemonic effects of GC seem to depend on interactions with noradrenaline (norepinephrine) within the amygdala (Quirarte et al., 1997). For example, GR stimulation in the amygdala enhances memory consolidation via a modulation of α1-adrenoceptor-mediated facilitation of β-adrenoceptor–cAMP activity. Because norepinephrine administration rapidly induces increases in cAMP levels, such a potentiation of this intracellular response seems incompatible with the classic view of GC affecting gene transcription through an activation of nuclear GRs. Rather,

this time frame suggests that GC effects on α1-adrenoceptor systems in influencing β-adrenoceptor–cAMP activity might involve rapid, nongenomic actions via membrane-associated GRs (Johnson et al., 2005).

4. Adult neurogenesis in the hippocampus

4.1. Neurogenesis in the adult brain

A central hypothesis of neuroscience has been that in the mammalian brain, the production of neurons occurs only during development and stops before puberty, and that new neurons cannot be formed in the adult brain. This widely held belief has been challenged in recent years by extensive evidence from many mammalian species including non-human primates as well as humans showing that certain brain areas retain the capability to generate new neurons in the adult brain (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999). Adult neurogenesis is a complex multi-step process that originates from precursor cells in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. The neurons born in the SVZ migrate through the rostral migratory stream into the olfactory bulb and become interneurons, while those born in the SGZ migrate into the granular layer of the DG and eventually become mature granule neurons.

Continual production of new neurons in the adult hippocampus was first reported by Altman and colleagues (Altman and Das, 1965). These new neurons originate from self-renewing and multipotent adult neural stem cells (NSCs) residing in the SGZ of dentate gyrus (DG; Bonaguidi et al.,

2011). Two types of NSCs have been identified based on their specific morphologies, proliferative behaviors and expression of unique sets of molecular markers (Mu et al., 2010). Type 1 neural progenitor cells (NPCs) have a radial process spanning the entire granule cell layer and ramifying in the inner molecular layer of DG. They are generally identified by specific molecular markers such as GFAP, Sox2 and Nestin. These cells have been hypothesized to be the quiescent stem cells that generate the second type of NSC, the actively self-amplifying and nonradial cells. These intermediate cells, expressing Sox2, Nestin but not GFAP, subsequently give rise to doublecortin (DCX)-positive neuroblasts that differentiate into glutamatergic dentate granule cells populating the inner third of the granule cell layer. Young adult rats generate approximately 9,000 new cells in the SGZ each day, with a survival rate of ~50% (Cameron and McKay, 2001). The surviving cells send dendrites to the molecular layer of DG to receive inputs from the entorhinal cortex and send axonal projections to the CA3 subfield of the hippocampus to innervate hilar interneurons, mossy cells and CA3 pyramidal cells, thereby integrating into existing neuronal circuits (Van Praag et al., 2002).

4.2. Functional roles of neurogenesis in the adult hippocampus

Most studies investigating the functional impact of new neurons in the hippocampus have tended to focus on their potential role in learning and memory. The reduce cell proliferation in the hippocampus has been shown to produce changes in cognitive tasks associated with the hippocampus (Shors et al., 2001; Garthe et al., 2009). Decreased neurogenesis in the

DG has no effect on hippocampus-independent cued memory, but impairs hippocampus-dependent contextual memory (Winocur et al., 2006; Saxe et al., 2006; Pollak et al., 2008). In rats, context fear deficits do not appear until at least 4 weeks following neurogenesis ablation (Snyder et al., 2009), suggesting that a certain degree of new neuron maturation is critical for context fear conditioning in rats. In the Morris water maze paradigm, rats show deficits in lasting retention of spatial information at least 4 weeks following ablation of hippocampal neurogenesis, but not before (Snyder et al., 2005). In contrast, studies in mice have shown deficits or no change in context fear conditioning and Morris water maze learning in mice of various strains, gender, and ages, from different time points following ablation (Saxe et al., 2006; Meshi et al., 2006; Garthe et al., 2009; Snyder et al., 2009), suggesting that strain, gender, and age-related differences in mice may exist in the time course for new neuron maturation and integration or reactions to different ablation techniques.

More recent studies have linked adult neurogenesis with anxiety regulation and feedback of the stress response. Experimental manipulations associated with reduced number of new neurons in the DG are associated with increased anxiety-like behavior (Revest et al., 2009). Likewise, reduced adult neurogenesis is associated with impaired modulation of the HPA axis; corticosterone levels show a delayed return to baseline after stress in mice lacking new neurons. Furthermore, reduced neurogenesis is associated with impaired responsiveness of the HPA axis to a dexamethasone suppression test (Snyder et al., 2011). Taken together, these findings suggest that new neurons may play an important role not only in the cognitive functions of the hippocampus, but also in its anxiety

and stress regulatory functions.

4.3. Neurogenesis modulated by GC

Adult neurogenesis in the DG is modulated by a large number of environmental and endogenous factors, but stress is one of the most potent environmental parameters known to suppress adult neurogenesis. Exposure to different forms of chronic stress, including social subordination, immobilization, physical restraint and footshock can suppress adult neurogenesis in multiple species (Gould et al., 1997, 1998; Czéh et al., 2001, 2002). Several lines of evidences support that the most likely mechanism by which stress suppresses adult neurogenesis in the hippocampus is via activation of the HPA axis and subsequent elevation of GC levels. First, both acute and chronic treatment with GC leads to a decrease in neurogenesis (Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1998; Karishma and Herbert, 2002). Second, GC also inhibits the proliferation and differentiation of neural progenitors and the survival of young neurons (Wong and Herbert, 2004). Third, adrenalectomy increases adult neurogenesis in the DG (Cameron and Gould, 1994). Adrenalectomy also leads to an increase in granule cell death, suggesting that while proliferation increases, survival of the cells decreases. The cell death can be rescued by adding corticosterone to the drinking water (Gould et al., 1990).

Given that GC is often elevated in human patients suffering major depression, stress is thought to exacerbate depression and the regulation of SGZ neurogenesis by antidepressants also has been extensively studied

in a variety of species. In contrast to stress, both physical and chronic chemical antidepressant treatments increase cell proliferation in SGZ. For instance, treatment of depression with selective serotonin reuptake inhibitors (SSRIs), the most common class of antidepressants, increases adult DG neurogenesis as measured by uptake of bromodeoxyuridine (BrdU) in the SGZ (Santarelli et al., 2003). Importantly, this increase is seen only in the SGZ and not in the SVZ, suggesting a specificity of the antidepressants to regulate hippocampal neurogenesis. Critically important, the proneurogenic effects of antidepressants are only seen with chronic, and not with acute, treatment, mirroring the time course for therapeutic action of these classes of antidepressants in humans. Furthermore, antidepressants are able to prevent or reverse the stress-induced decrease in neurogenesis (Warner-Schmidt and Duman, 2006).

Brain-derived neurotropic factor (BDNF) has an established role in the regulation of proliferation in the DG. BDNF has high levels of expression in the adult DG (Nibuya et al., 1995; Berchtold et al., 1999). The action of BDNF is mediated by the specific tyrosine kinase receptor TrkB, which is also widely expressed in the DG (Barbacid, 1994). GC and BDNF exert distinct actions on hippocampal neurogenesis with GC reducing new neuron formation (Cameron and Gould, 1994) and BDNF serving to promote neurogenesis (Lee et al., 2002; Scharfman et al., 2005). Given the opposing effects of GC and BDNF on structural plasticity in the DG, it has been hypothesized that specific adverse effects of GC on hippocampal neurogenesis may involve attenuation of BDNF expression or signaling (Smith, 1996). By contrast, BDNF expression is markedly stimulated by many kinds of antidepressant agents (Nibuya et al., 1995; Suri and Vaidya,

2012). Moreover, hippocampal BDNF infusion in animals receiving chronic GC was capable of rescuing the depressive-like behavior evoked by GC exposure on the forced swim test (Gourley et al., 2008), implicating its antidepressants-like role related with the pathophysiology of mood disorders (Duman and Monteggia, 2006).

PURPOSE

As described above, considerable evidence suggests that the brain is under the influence of environmental factors in early life (Weinstock, 2008). Previously, we showed that spatial learning associated with hippocampal NMDA receptor-mediated synaptic plasticity was defective in maternally stressed mice (Son et al., 2006). However, other prenatal stress-induced defects have not been well characterized in these mice. First, Chapter 1 is designed to address following issue: Does chronic maternal stress exert a long-lasting influence on functions of the amygdala which is involved in regulation of emotional memory?

Second, the physiological significance of the GC rhythm in functions of adult brain is not as well understood. Mice in which the adrenal clockwork was disrupted by BMAL1 knockdown in adrenocortical GC-producing cells, designated as the A-BMKD transgenic mice (Son et al., 2008) showed significantly attenuated circadian GC levels in the plasma under constant darkness. In Chapter 2, using A-BMKD mice, I addressed the question of what the functional consequence of attenuated GC rhythmicity in adult hippocampal neurogenesis is.

CHAPTER 1

Impairment of Fear Memory Involved with Nongenomic Glucocorticoid Action on the Amygdala in Maternally Stressed Adult Offspring

ABSTRACT

The environment in early life elicits profound effects on fetal brain development that can extend into adulthood. However, the long-lasting impact of maternal stress on emotional learning remains largely unknown. Here, I focus on amygdala-related learning processes in maternally stressed mice. In these mice, fear memory consolidation and certain related signaling cascades were significantly impaired, though innate fear, fear memory acquisition, and synaptic *N*-methyl-D-aspartate (NMDA) receptor expression in the amygdala were unaltered. In accordance with these findings, maintenance of long-term potentiation (LTP) at amygdala synapses, but not its induction, was significantly impaired in the maternally stressed animals. Interestingly, amygdala glucocorticoid receptor (GR) expression was reduced in the maternally stressed mice, and administration of glucocorticoids (GCs) immediately after fear conditioning and LTP induction restored memory consolidation and LTP maintenance, respectively, suggesting that a weakening of GC signaling was responsible for the observed impairment. Furthermore, microinfusion of a membrane-impermeable form of GC (BSA-conjugated GC) into the amygdala mimicked the restorative effects of GC, indicating that a nongenomic activity of GC mediates the restorative effect. Together, these findings suggest that prenatal stress induces long-term dysregulation of nongenomic GC action in the amygdala of adult offspring, resulting in the impairment of fear memory consolidation. Since modulation of amygdala activity is known to alter the consolidation of emotionally influenced memories allocated in other brain regions, the nongenomic action of GC on

the amygdala shown herein may also participate in the amygdala-dependent modulation of memory consolidation.

Key words: Maternal stress, Amygdala, Fear memory, Long-term potentiation (LTP), Glucocorticoid (GC), Nongenomic action

INTRODUCTION

The environment in the womb has long-lasting effects on the later life of offspring which are known as “programming effects” (Seckl, 2008). For example, the low birth weight of pups from mothers stressed in pregnancy has been correlated with pathophysiological symptoms such as cardiovascular disease and metabolic disorders (Nathanielsz, 1999; Welberg et al., 2001). Moreover, the brain is also vulnerable to environmental challenges in early life that contribute to alterations in the cognition and behaviors of offspring (Welberg et al., 2001; Weinstock, 2008). Previously, our group showed that spatial learning associated with hippocampal NMDA receptor-mediated synaptic plasticity was defective in maternally stressed mice (Son et al., 2006). However, other prenatal stress-induced defects have not been well characterized in these mice. In the present study, I have questioned whether and how maternal stress affects the amygdala, a major target of stress in the brain that is also known as a key limbic brain region involved in the regulation of emotion, particularly fear-related processes.

The amygdala is an important locus for integrating modulatory influences on emotion through numerous neural and hormonal systems that are affected by stress and that control stress-evoked neurobehavioral alterations (LeDoux, 2007; Joëls and Baram, 2009). Considering such interactions with stress-responsive neuroendocrine systems, it is plausible that the amygdala is a primary target of the programming effects of maternal stress. Indeed, several lines of evidence have suggested that either maternal stress or prenatal GC exposure increases amygdala-

dependent anxiety-like behaviors in offspring (Cratty et al., 1995; Welberg et al., 2001; Fan et al., 2009). In this regard, our group also has reported that maternally stressed mice were more vulnerable to chronic stress-evoked anxiety-like behaviors associated with hyperactivity of the corticotrophin-releasing hormone (CRH) system in the amygdala (Chung et al., 2005). In spite of extensive studies on maternal stress-evoked innate fear and anxiogenic behaviors in offspring, there is as yet little evidence concerning one of the most important amygdala functions, i.e. the processing of fear-associated learning and memory.

The basolateral complex of the amygdala (the BLA complex), which is comprised of the lateral, basal, and accessory basal nuclei, is believed to be responsible for the consolidation of emotionally influenced memory (McGaugh, 2000). It is important to note that activation of the stress-responsive hypothalamus-pituitary-adrenal (HPA) neuroendocrine axis (Fig. 1) and the subsequent secretion of adrenal glucocorticoid (GC) are reported to be heavily involved in aversive memory formation, presumably by exerting an effect on the BLA (Roozendaal et al., 2002; De Quervain et al., 2009; Rodrigues et al., 2009). Therefore, in the present study, I investigated alterations in the GC effects on the BLA in adult male offspring of mothers exposed to prolonged stress during pregnancy. For this purpose, I examined amygdala-based fear memory, maintenance of amygdala LTP and related signaling events. Lastly, I examined the possible involvement of nongenomic GC actions (Fig. 2) on the amygdala of maternally stressed mice.

MATERIALS AND METHODS

Animals and the maternal stress procedure. ICR mice, obtained from the Laboratory Animal Center at Seoul National University, were used in all of the experiments and kept in temperature-controlled (22-23°C) quarters under a 12-h light/dark photoperiod (lights on at 8:00 A.M.). Standard mouse chow and water were available *ad libitum*. The maternal stress procedure was performed as described previously (Chung et al., 2005; Son et al., 2006, 2007). Briefly, pregnant ICR mice were prepared by mating with adult males. Pregnant mice of the stressed group were placed individually in a restrainer (a transparent plastic cylinder, 3 cm in diameter and 9 cm long) daily for 6 h (10:00 A.M.-4:00 P.M.) from 8.5 day postcoitum (dpc) to 18.5 or 19.5 dpc (the day before parturition). Control pregnant mice remained undisturbed. After weaning on postnatal day 21, the pups born from a stressed mother (STR) were reared in an environment identical to that of the controls (CTL). The STR and CTL groups were separately housed, but three to five mice from different litters were randomly assigned to a cage to exclude possible litter effects. Male offspring at 8-12 weeks of age were used in all experiments. All behavioral tests were performed during the light period (between 11:00 A.M. and 3:00 P.M.). All animal procedures are approved by the Animal Care and Use Committee of Seoul National University.

Passive avoidance test. Adult male mice were trained in an apparatus (PACS-30, Columbus Instruments Int'l Co., Columbus, OH) in which two compartments were divided by a guillotine door. The larger compartment

was made of black Plexiglas and maintained in darkness, while a lamp (60 W) illuminated the compartment made of white Plexiglas. On the training day, each mouse was placed in the illuminated compartment, facing away from the dark compartment. When the mouse turned around, the door leading to the dark compartment opened. When a mouse entered the dark compartment to the extent that all four paws were on the dark side, the door closed, and a foot shock (1 mA, 3 s) was delivered automatically. Mice were then removed from the apparatus and returned to their home cage. Retention was examined 24 h later using a procedure similar to that of the training session, except that no foot shock was administered. A maximum step-through latency of 3 min was allowed per test session.

Pavlovian fear conditioning. Contextual fear conditioning was carried out in the conditioning chamber (13 × 13 × 25 cm) with 5 repetitions of foot shocks (1 mA, 1 s) at 90-s intervals after habituation for 10 min in the same chamber on the day before training. On the next day, conditioned mice were placed in the same chamber, and the “freezing” time was measured over a period of 5 min. Conditioned freezing was defined as immobility except for respiratory movements. The total freezing time in the test period was represented as a percentage. Auditory fear conditioning was conducted as described previously (Schafe and LeDoux, 2000) with a slight modification. Mice were habituated for 10 min in the conditioning chamber without disturbance on the day before conditioning. Mice were then placed in the conditioning chamber, and 5 conditioning trial repetitions, each consisting of a tone (30 s, 5 kHz, 75 dB) that terminated with a foot shock (1 mA, 1 s), were administered. The intertrial interval was 90 s. Conditioned

fear responses were tested at the indicated time after conditioning. For each test, mice were placed in a distinct context and re-exposed to three tones at 90 s intervals after a 5 min period of exploration. Freezing behaviors were scored during the tone presentation. The total freezing time in the test period was represented as percentage of the average duration to each tone presentation. Metyrapone (dissolved at 10 mg/mL in saline; Tocris Bioscience, Bristol, UK; 100 mg/kg b.w.; i.p.), a GC synthesis inhibitor or saline as a vehicle, was administered 3 h before conditioning. Dexamethasone (DEX) 21-phosphate disodium salt (dissolved at 100 μg/mL 3% ethanol in saline; Sigma, St. Louis, MO; 1 mg/kg b.w.; s.c.), corticosterone (dissolved at 50 μg/mL in 3% ethanol in saline; CORT, Sigma; 0.5 mg/kg b.w.; s.c.) or vehicle (3% ethanol in saline) was injected immediately after training.

Measurement of spontaneous motor activity. Spontaneous motor activity in the fear conditioning chamber was measured under conditions similar to those for fear memory testing. Mice were habituated in the tone-testing chamber (13 × 13 × 25 cm) with a bedded floor for 10 min on the day prior to measuring the activities. On the next day, mice were placed in the same chamber, and then spontaneous motor activities were recorded for 10 min (the time period required for the tone-testing) and analyzed using the EthoVision Pro system (Noldus Information Technology, Wageningen, Netherlands). The distance moved was employed as an index of motor activity.

Unconditioned fear response to predator odor. To examine innate fear, mice were placed in the chamber with a beaker containing 30 μl of a synthetic fox feces odor, timethylthiazoline (TMT; PheroTech, Delta, British Columbia, Canada), which was reported previously to elicit freezing in rodents (Wallace and Rosen, 2001). TMT-evoked freezing was recorded for 10 min after TMT exposure and scored as a percentage of the time spent in the freezing behavior at 2-min-intervals. Freezing behavior was defined the same as in the Pavlovian fear conditioning case.

Tissue preparation and immunoblot analysis. For tissue preparation, mice were sacrificed between 11:00 A.M. and 2:00 P.M., and the brain was quickly removed and placed on ice. The amygdala, hippocampus, and hypothalamus were dissected from 1-mm-thick brain slices prepared with a brain matrix and quick frozen in liquid nitrogen. Whole cell lysates and postsynaptic density proteins were prepared as previously described (Son et al., 2006). Protein samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) in a Bio-Rad (Richmond, CA) Trans-Blot electrophoresis apparatus was employed 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 5% bovine serum albumin (BSA), and incubated with primary antibodies at room temperature for 1 h. The blots were then washed four times with TBS/0.5% Tween-20. The bound primary antibody was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson

ImmunoResearch, West Grove, PA). The blots were then washed further four times. Immunoreative bands were visualized with Amersham ECL reagents according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden). The blots were analyzed with Bio1D image analysis software (Vilber-Lourmat, Marne-la-Vallée, France) and were expressed as relative optical densities (R.O.D.). The antibodies used in the immunoblotting were as follows: Anti-GluN1 (BD Bioscience, San Jose, CA), GluN2A, GluN2B, GluA1 (Millipore), actin (Sigma), PSD-95, extracellular-regulated kinase 1 and 2 (ERK1/2), phosphorylated ERK1/2, cAMP response element binding protein (CREB), phosphorylated CREB (Cell Signaling Technology, Danvers, MA), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies are all commercially available.

Immunohistochemistry. Mice were sacrificed 15 min after fear conditioning and DEX treatment. Brains were removed and cut into 3-mm-thick sections with a brain matrix, including the amygdala. The sectioned brain tissues were postfixed in phosphate-buffered saline (PBS, pH7.6) containing 4% paraformaldehyde overnight at 4°C and then cryoprotected in 30% sucrose for 24 h at 4°C. Frozen coronal sections were cut at a 20 μm thickness on a cryostat. After blocking with PBS containing 10% normal horse serum and 0.3% Triton X-100, slices were incubated at room temperature for 1 h in anti-phosphorylated ERK1/2 (at 1:200 dilutions; Cell Signaling Technology) in PBS-3% horse serum-0.1% Triton X-100. After several washes with PBS, Cy3-conjugated anti-rabbit IgG antibody (1:500)

was applied for 30 min. Subsequently, sections were washed, mounted, and observed under fluorescence microscopy (Axiovert 200M, Carl Zeiss, Göttingen, Germany). Sections from comparable anteroposterior levels (1.3-1.9 mm posterior to the bregma) containing the BLA regions were selected for quantifying nuclear pERK1/2-positive cells. Cell counts were taken from at least three sections per mouse and scored using a defined boundary approximately equivalent to the size of the BLA.

Determination of serum GC levels. Mice were sacrificed between 1:00 P.M. and 2:00 P.M.. Trunk blood was collected and centrifuged at 10,000 ×*g* for 20 min to obtain serum. Serum samples were quick frozen and stored at -70°C until use. GC levels were assayed using a commercial radioimmunoassay kit, according to manufacturer's instructions (Diagnostic Products Co., Los Angeles, CA).

Electrophysiology. Electrophysiological experiments were performed as described in a previous report (Kim et al., 2007). Control mice and maternally stressed mice were anesthetized with halothane and sacrificed. The isolated brains were placed in an ice-cold modified artificial cerebrospinal fluid (aCSF) solution containing the following: 175 mM sucrose, 20 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, and 11 mM D-(+)-glucose, and aerated with 95% O_2/5% CO_2. Coronal slices (300 μm for whole cell recordings and 400 μm for field recordings), including the LA, were cut and incubated in normal aCSF containing the following: 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26

mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, and 11 mM D-(+)-glucose, then continuously bubbled at room temperature with 95% O_2/5% CO_2. A submersion-type recording chamber was continuously superfused with aCSF (31.0-33.0°C for whole cell recordings and 33.0-34.5°C for field recordings) containing picrotoxin (100 μM for whole-cell recordings and 10 μM for field-potential recordings). I included picrotoxin in the recording solution to isolate excitatory synaptic transmission and to block feed-forward GABAergic inputs to principal neurons in the LA. For field potential recordings, where afferents were stimulated at a stronger intensity, a lower concentration of picrotoxin was used to preserve the inhibitory tone and thus prevent multi-synaptic firing. I chose brain slices containing a well-isolated, sharply defined trunk (containing thalamic afferents) crossing the dorsolateral division of the LA, which is a site of convergence for somatosensory and auditory inputs (Pitkanen et al., 1997). The sizes of the LA and central amygdala were relatively constant in these slices, and the trunk closest to the central nucleus of the amygdala was used when multiple trunks were observed. Thalamic afferents were stimulated using a concentric bipolar electrode (MCE-100, Rhodes Medical Instruments, CA) placed on the midpoint of the trunk between the internal capsule and medial boundary of the LA. Regions and cells of interest for all recordings were located beneath the midpoint of the trunk horizontally spanning the LA. Whole-cell currents were filtered at 1 kHz, then digitized at 20 kHz using a MultiClamp 700A digitizer (Molecular Devices, Sunnyvale, CA). Recordings were obtained by using pipettes with series resistances of 2.5-3.5 Mohm when filled with the following solution: 100 mM Cs-gluconate, 0.6 mM EGTA, 10 mM HEPES, 5 mM NaCl, 20 mM tetraethylammonium, 4 mM Mg-ATP,

0.3 mM Na-GTP, and 3 mM QX314, with the pH adjusted to 7.2. While voltage-clamped, a minor proportion (< 5%) of recorded neurons exhibited spontaneous excitatory postsynaptic currents (EPSCs) with faster decay times and larger amplitude (> 100 pA). As these are characteristic of inhibitory neurons, they were excluded from the analysis (Kim et al., 2007). Extracellular field potentials were recorded by using a parylene-insulated microelectrode (573210; A-M Systems, Carlsborg, WA) in 400-μm-thick slices. As shown in previous studies (Kim et al., 2007; Kim et al., 2009; Huang et al.. 2000), field potentials (population spikes) at thalamo-LA synapses exhibited a constant and short latency of approximately 4 ms, reliably followed by high-frequency stimulation without failure, and could be blocked by kynurenic acid. This indicates that the field potentials measured in the present study reflect glutamatergic and monosynaptic responses at thalamo-LA synapses. LTP was induced by six trains of high-frequency stimuli (HFS; 100 Hz, 1 s duration, 1 min interval) with the same intensity and pulse duration as the baseline stimuli. Vehicle, DEX (dissolved in aCSF at a final concentration of 20 nM), or BSA-conjugated DEX (DEX-BSA; dissolved in aCSF at a final concentration of 200 nM) was applied immediately after six repetitions of HFS for 30 min. To obtain stable, long-term recordings, I began field recordings at least 3 h after preparation of the slices. To improve the signal-to-noise ratio, data were averaged using a three-point running average in time-lapse experiments.

Cannula implantation and infusion of DEX-BSA. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and bilaterally implanted

with 26-gauge stainless steel cannulas (model C315G; Plastic Products, Roanoke, VA) into the BLA complex (AP -1.3 mm, ML ±3.6 mm, DV -3.8 mm). A 32-gauge dummy cannula was inserted into each guide cannula to prevent clogging. Two jewelry screws were implanted over the skull to serve as anchors, and the whole assembly was affixed on the skull with dental cement. Mice were given at least 1 week to recover before experimentation. After completion of the experiments, correct placement of the injection cannula tips was verified in all animals. For this purpose, brains were removed from animals and fixed overnight in PBS/4% paraformaldehyde. The fixed brains were coronally sectioned into 70-μm thicknesses using a vibratome (World Precision Instruments, Sarasota, FL). Sections were then stained with cresyl violet and examined under light microscopy. DEX-BSA dissolved in saline (2 μg/μl) was administrated bilaterally into the BLA complex via a 33-gauge injector cannula (C315I; Plastic Products) attached to a 10-μl Hamilton syringe. A solution containing 0.4 μg of DEX-BSA in 0.2 μl was administered immediately after auditory fear conditioning at a rate of 0.2 μl/min. After infusion, injector cannulas were left in place for an additional minute to allow the solution to diffuse away from the tip.

Data analysis. The results from behavioral studies, quantitative immunoblot analyses and serum GC levels were statistically evaluated using Student's *t*-test and/or analysis of variance (ANOVA) followed by Neuman-Keuls test as a *post-hoc* comparison. Two-way repeated measure (RM)-ANOVA followed by student's t-test for each time point or one-way

ANOVA was used for statistical evaluation of the average of population spike amplitudes. A probability level of $p < 0.05$ was chosen to denote statistical significance.

RESULTS

Impaired contextual fear memory in maternally stressed mice

To determine whether chronic maternal stress has a long-lasting effect on emotional learning processes, male adult offspring were subjected to a passive avoidance test and contextual fear conditioning, which are well established behavioral paradigms for investigating fear-related memory associated with contextual information.

In the passive avoidance test as described in Fig. 3A, both the control and maternally stressed mice displayed similarly short step-through latencies of less than 20 s prior to receiving a foot shock (training). Control mice exhibited significantly increased latency to enter into the dark compartment where they had previously received a foot shock in the test sessions 24 h after training. However, the maternally stressed mice entered the dark compartment with much shorter latency than controls ($F_{(1, 56)}$ = 30.31, $p < 0.01$ for group differences; $F_{(1, 56)}$ = 39.22, $p < 0.01$ for training; $F_{(1,56)}$ = 28.07, $p < 0.01$ for interaction; Fig. 3B). Impaired fear-associated learning in maternally stressed mice was also revealed by a contextual fear conditioning paradigm (Fig. 4A). Control and maternally stressed mice freely explored a fear conditioning chamber without any significant difference in freezing behaviors before they were exposed to foot shock (training) in the course of contextual fear conditioning (Fig. 4B). However, on the next day after conditioning, the maternally stressed mice displayed fewer freezing behaviors in the same conditioning chamber than the control mice ($F_{(1, 36)}$ = 20.36, $p < 0.01$ for group differences; $F_{(1, 36)}$ = 208.2, $p < 0.01$

for training; $F_{(1,36)}$ = 21.8, p < 0.01 for interaction; Fig. 4B). Together, these results show that maternally stressed adult mice have a defect in fear memory which is related to contextual information. Since maternal stress affects NMDA receptor-mediated synaptic functions in the hippocampus (Son et al., 2006), it is likely that the memory impairments shown in the two tasks are due to, at least in part, a malfunctioning of the hippocampus, which is critical for the acquisition of contextual information.

Impaired auditory fear memory in maternally stressed mice

Unlike contextual fear conditioning, auditory-cued Pavlovian fear conditioning (Fig. 5A) is known to be dependent on the amygdala, not the hippocampus (Fanselow and LeDoux, 1999). To test the possibility that amygdala functions are altered in maternally stressed mice, I examined whether these mice normally acquire conditioned fear responses after auditory-cued fear conditioning. In the auditory fear conditioning paradigm in rodents, the convergence of a neutral-conditioned stimulus (CS) and an aversive unconditioned stimulus (US) leads to fear responses, such as freezing behavior. I tested the fear memory in the animals by measuring the freezing time in two different phases: the acquisition, i.e., initial learning of the association of CS with US during the training of fear conditioning, and the retrieval of conditioned fear, a test of the CS-US association in which the CS is presented alone. In the acquisition session, an auditory tone serving as the CS was paired with a foot shock in five successive trains. The freezing behavior of both the control and maternally stressed mice increased over the number of training sessions without any significant

difference in the acquisition phase, indicating that maternal stress does not significantly alter auditory fear memory acquisition in adult offspring ($F_{(1,56)}$ = 0.054, p = 0.82 for group differences by RM-ANOVA; Fig. 5B). However, freezing was found to be much weaker in the maternally stressed mice compared with control mice when tested 24 hr after conditioning (controls, 53.36 ± 9.26 vs. maternally stressed mice, 12.87 ± 7.31% of freezing; p < 0.01; n = 8 for each group; Fig. 5C), indicating that the acquired fear memory may not be effectively maintained in maternally stressed mice.

Although both groups showed similar freezing responses during the acquisition phase, it cannot be ruled out that the impairment in fear memory retention originated from a decrease in freezing behavior due to unknown reasons (e.g. differences in the expression of fear and/or motor behavior), rather than the fear-associated learning process. I first hypothesized that changes in the unlearned fear response may alter freezing in maternally stressed mice. To test this, I compared innate fear elicited by a synthetic fox feces odor (timethylthiazoline, TMT), which is known to elicit freezing behaviors in rodents as a predator odor stimulus (Wallace and Rosen, 2001), between the maternally stressed and control mice. Mice in the two groups exhibited similar freezing responses over time when exposed to TMT ($F_{(1,40)}$ = 0.039, p = 0.847 for group differences by RM-ANOVA; Fig. 6A), showing that not only unlearned fear, but also fear expression (freezing), were unaltered in maternally stressed mice. Then I tested the possibility that hyperlocomotion alters freezing in maternally stressed mice, since the maternally stressed mice exhibited impaired habituation to a novelty with hyperlocomotion (Son et al., 2007). After 10 min habituation (see the Materials and methods section for additional details), the two

groups of mice were placed in the tone-testing chamber for 10 min (the time period required for the tone-testing) to estimate spontaneous locomotive activity. I, however, failed to find any significant difference in spontaneous motor activity between the two groups of mice under similar conditions of auditory fear testing (controls, 18.13 ± 0.59 vs. maternally stressed mice, 24.59 ± 0.89 m of the distance traveled; $p = 0.19$; $n = 6$ for each group; Fig. 6B), ruling out the possibility that freezing changes are due to hyperlocomotive activity in maternally stressed mice. Taken together, these findings suggest that freezing is a reliable measure of learned fear in maternally stressed mice, and that learned fear, but not unlearned fear, is specifically impaired in these mice.

Considering the essential roles of the glutamate receptors in the amydala in the acquisition of fear memory (Goosens and Maren, 2004; Rodrigues et al., 2004), I examined the synaptic association of the glutamate receptor subunits, GluN1 and GluN2A/B (subunits of NMDA receptor) as well as GluA1 (a subunit of AMPA receptor) in the amygdala extracts. There were no obvious differences between the two groups in terms of protein expression levels or synaptic association of these molecules, as revealed by immunoblot analyses of both the whole cell lysate and postsynaptic density (PSD) protein-enriched fraction prepared from the amygdala (Fig. 6C). These findings are in contrast with our previous study, in which synaptic protein composition in the hippocampus was found to be altered in maternally stressed mice (Son et al., 2006).

Effect of maternal stress on consolidation of auditory fear memory

Memory consolidation is a process of converting short-term memory (STM) into stable long-term memory (LTM) over a period of time after the acquisition of information (McGaugh, 2000). Auditory fear memory encoded in the amygdala has been shown to be consolidated within 24 h and short-term fear memory is known to be maintained for up to 4 h (Schafe and LeDoux, 2000). Therefore, if there is a defect in the consolidation of auditory fear memory, memory retention assessed 24 h after conditioning would be impaired, while memory retention assessed within 4 h after conditioning would be intact. To determine whether the consolidation of auditory fear memory is defective in maternally stressed mice, I compared freezing behaviors in response to CS between maternally stressed and control mice at different time points after auditory fear conditioning. Maternally stressed mice exhibited reduced freezing at time points greater than 4 h after conditioning compared with control mice, while they exhibited unaltered freezing at time points within 2 h after conditioning ($F_{(1,76)} = 21.04$, $p < 0.01$ for group differences; $F_{(4,76)} = 15.81$, $p < 0.01$ for time differences, and $F_{(4,76)} = 4.593$, $p < 0.01$ for interaction; $p < 0.01$ between control and maternally stressed mice at 4 and 24 h after auditory fear conditioning; Fig. 7A). These findings suggest that prenatal stress impairs the consolidation of auditory fear memory in adult offspring.

Consolidation of auditory fear memory requires the activation of MAPKs (mitogen-activated protein kinases), particularly ERK1/2, and active ERK1/2 stimulates a subset of transcription factors, including CREB proteins in the amygdala, which in turn promotes the gene expression required for maintaining long-term fear memory (Schafe et al., 2000; Stanciu et al., 2001; Rodrigues et al., 2004). To determine whether the activation of

ERK1/2 and CREB is also impaired in maternally stressed mice, phosphorylation of ERK1/2 and CREB in the amygdala was examined by immunoblotting at 15, 60, and 240 min after auditory fear conditioning (Fig. 7B). Control mice exhibited rapid and transient increases in phosphorylation of both ERK1/2 and CREB after fear conditioning, whereas maternally stressed mice exhibited impairment in the conditioning-induced phosphorylation of both of them. Thus, these findings strengthen the notion that fear memory consolidation processes are impaired in maternally stressed mice.

Effects of glucocorticoid (GC) on auditory fear memory consolidation and ERK1/2 phosphorylation in maternally stressed mice

Adrenal GC hormone is known to be involved in the consolidation of aversive memory (Rodrigues et al., 2009). Importantly, it was reported that prenatal stress alters the HPA neuroendocrine axis reactivity which controls adrenal GC secretion in adult offspring (Chung et al., 2005). It is therefore conceivable that alterations in the GC signaling cascade may account for the observed defects in fear memory consolidation in maternally stressed mice. To test this, I examined the effects of metyrapone (MET: a GC synthesis inhibitor), corticosterone (CORT: an endogenous rodent GC) and dexamethasone (DEX: a synthetic GC) on fear memory retention assessed 24 h after auditory fear conditioning (Fig. 8A). MET-injected control mice (a systemic injection 3 h prior to fear conditioning) exhibited reduced freezing compared with vehicle-injected control mice. In contrast, CORT-injected maternally stressed mice (a systemic injection immediately after

conditioning) exhibited increased freezing comparable to that observed in control mice injected with either vehicle or CORT. DEX (a systemic injection immediately after conditioning), which has a higher affinity for the glucocorticoid receptor (GR) than mineralocorticoid receptor (MR), mimicked the effect of CORT (for controls, $F_{(3,23)} = 134.3$, $p < 0.01$; for maternally stressed mice, $F_{(3,27)} = 248.4$, $p < 0.01$ by one-way ANOVA; Fig 8B.). Thus, these findings suggest that pharmacological activation of GRs immediately after fear conditioning restores fear memory consolidation in maternally stressed mice.

Since pharmacological activation of GRs apparently restores fear memory consolidation in maternally stressed mice, I reasoned that DEX injection after conditioning also restores conditioning-induced phosphorylation of ERK1/2 in these mice. To test this, I examined expression levels of phosphorylated ERK1/2 in the whole cell extracts of the amygdala by immunoblotting at 15 min after auditory fear conditioning (for controls, $F_{(3,14)} = 18.86$, $p < 0.01$; for maternally stressed mice, $F_{(3,14)} = 36.51$, $p < 0.01$ by one-way ANOVA; Figs. 9A and B). Prenatal stress impaired the conditioning-induced phosphorylation of ERK1/2 in adult offspring, whereas DEX injection after conditioning apparently restored it in these mice. In contrast to the post-conditioning administration, DEX injection without conditioning did not significantly affect ERK1/2 phosphorylation levels in either group, indicating that the enhancing effect of DEX on ERK1/2 phosphorylation requires fear conditioning.

To more precisely examine whether restoration of ERK1/2 phosphorylation in stressed animals takes place in the BLA complex, I compared the number of phosphorylated ERK1/2 immunoreactive (pERK-ir)

cells in this region by the same condition used in the immunoblot analyses (for controls, $F_{(3,20)} = 4.754$, $p < 0.05$; for maternally stressed mice, $F_{(3,20)} = 12.05$, $p < 0.01$ by one-way ANOVA; Figs. 10A and B). As in the results obtained by immunoblotting, fear conditioning significantly increased the number of pERK-ir cells in control mice, but not in maternally stressed mice. Also, in maternally stressed mice, post-conditioning injection of DEX increased the number of pERK-ir cells to levels comparable with those in the fear-conditioned control mice.

Collectively, our findings show that exogenously injected GC restores both ERK1/2 phosphorylation as well as fear memory consolidation in maternally stressed mice. The restored phosphorylation of ERK1/2 after conditioning appears to mediate the restorative effect of GC on fear memory consolidation.

Serum GC profiles after auditory fear conditioning and the basal GC receptor level

In the next set of experiments, I intended to further address the molecular and cellular changes underlying the impaired memory consolidation observed in maternally stressed mice. I first examined changes in serum GC profiles after auditory fear conditioning. Both control and maternally stressed mice exhibited a significant elevation in serum GC levels 30 min after conditioning. The increased GC levels returned to baseline in the control mice within 180 min, whereas the increased GC levels in the maternally stressed mice were maintained at significantly higher levels than in the control mice ($F_{(1,22)} = 4.390$, $p < 0.05$ for group

differences; $F_{(2,22)} = 32.39$, $p < 0.01$ for time differences; $F_{(2,22)} = 8.816$, $p < 0.05$ for interaction; Fig. 11A). Thus, fear conditioning appears to evoke a prolonged incremental increase in the circulating GC levels in maternally stressed mice. This may result from impaired GC signaling resulting in a reduction in the feedback inhibition of GC secretion**.**

The actions of circulating GC are mediated by binding either to the high-affinity MR or to the low-affinity GR (De Kloet, 2004). The results shown in Figs. 9, 10, and 11A demonstrate that maternally stressed mice are much less sensitive to elevated serum GC after auditory fear conditioning than control mice. One possible explanation is that a lower receptor expression level accounts for the lower sensitivity to GC in maternally stressed mice. To test this idea, I examined the GR and MR expression levels in the amygdala, hippocampus and hypothalamus, all of which are major target sites of GC in the brain (Fig. 11B). The GR protein expression was significantly reduced in all tested tissues in the maternally stressed mice compared with the control mice (amygdala: 58.73 ± 6.86% of controls in normalized R.O.D., $p < 0.01$; hippocampus: 39.81 ± 7.28%, $p < 0.01$; hypothalamus: 63.11 ± 8.74%, $p < 0.05$; n = 6 for each group). In contrast to GR, the basal expression of MR was not significantly altered in any of the tissues examined. Therefore, it is suggested that the reduced GR expression is responsible for the impairment in memory consolidation and conditioning-evoked ERK1/2 phosphorylation in the amygdala of maternally stressed mice.

Basal synaptic transmission in the thalamo-lateral nucleus in the amygdala (LA) pathway

It is reasonable that altered basal transmission in the thalamo-LA pathway might be responsible for the observed impairment in maternally stressed mice, since this pathway is one of the major excitatory inputs to the BLA complex, particularly during auditory-cued fear conditioning (Pitkanen et al., 1997; LeDoux, 2007). To test this possibility, I examined excitatory synaptic transmission in the thalamo-LA pathway by measuring both the input-output relation and paired-pulse facilitation (PPF) ratio of excitatory postsynaptic currents (EPSCs) using a whole cell patch-clamp method (Fig. 12A). EPSCs recorded with different stimulus intensities were not significantly different between the two groups, indicating that basal synaptic function in the amygdala of maternally stressed mice is unaltered ($F_{(1,132)}$ = 0.776, p = 0.396 for group differences by RM-ANOVA; Fig. 12B). I next examined the PPF ratio, an index of presynaptic release probability, using several interpulse intervals (35, 50, 100 ms, respectively) in the two groups. As shown in Fig. 12C, no significant difference in PPF ratio was observed between the two groups ($F_{(1,34)}$ = 0.05, p = 0.826 for group differences by RM-ANOVA), suggesting that presynaptic release is normal in maternally stressed mice. Together, these findings suggest that basal synaptic transmission in the thalamo-LA pathway is not significantly changed in maternally stressed mice.

Synaptic plasticity in the thalamo-LA pathway

I next measured both the induction and maintenance of LTP, a cellular substrate for memory (Bliss and Collingridge, 1993), in the thalamo-LA

pathway of maternally stressed and control mice. It is widely held that LTP in this pathway is tightly correlated with auditory fear memory (Rodrigues et al., 2004; Rumpel et al., 2005). I recorded population spikes (PSs) in the thalamo-LA pathway using extracellular field recordings, and induced LTP by applying high-frequency stimulation (HFS; six trains of 1 sec, 100 Hz stimulation; 1 min intertrain intervals). Initial potentiation was not significantly different between the maternally stressed and control mice, whereas the maintenance phase of LTP was significantly impaired in maternally stressed mice ($F_{(1,741)} = 7.214$, $p < 0.05$ for group differences by RM-ANOVA; Fig. 13A). This particular result correlates with the behavioral results in which LTM, but not STM, was impaired in the maternally stressed mice.

Based on the behavioral results shown in Fig. 8 and a previous report showing GC-enhanced excitability of the amygdala (Duvarci and Paré, 2007), I questioned whether an application of GC immediately after HFS would restore stable LTP in maternally stressed mice. Therefore, I explored the effects of DEX on LTP maintenance in both maternally stressed and control mice by applying DEX for 30 min after the last train of HFS to mimic the post-conditioning injection of DEX in the behavioral experiments. As shown in Fig. 13B, the application of DEX (20 nM in aCSF) after HFS evidently restored stable LTP in maternally stressed mice compared with DEX-treated control mice ($F_{(1,456)} = 0.023$, $p = 0.883$ for group differences by RM-ANOVA).

Considering the rapid time course of DEX treatment (i.e., for 30 min immediately after LTP induction), the restorative effect of DEX on LTP might be mediated by nongenomic effects of GC rather than by the well-known

classical genomic mechanism. To test this possibility, I used a membrane-impermeable form of DEX, BSA-conjugated DEX (DEX-BSA). DEX-BSA (200 nM in aCSF) dramatically restored stable LTP in the maternally stressed mice compared with the DEX-BSA-treated control mice ($F_{(1,627)}$ = 0.002, p = 0.97 for group differences by RM-ANOVA; Fig. 13C), suggesting a nongenomic action of GC on LTP maintenance.

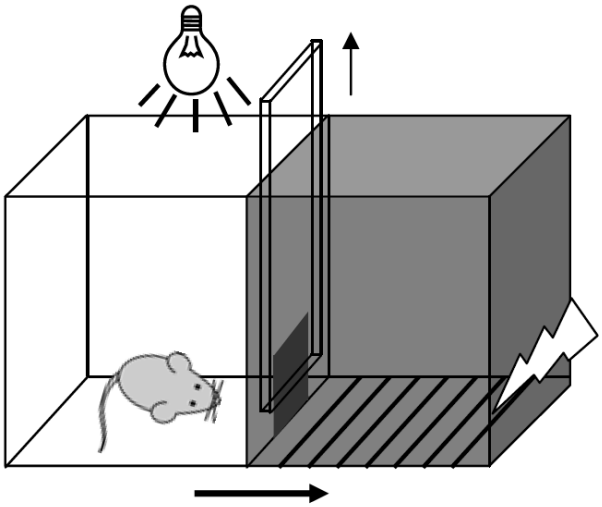
Based on 3 h of field recordings in the three treatment groups (vehicle, DEX, and DEX-BSA groups) of maternally stressed and control mice, I summarized the average of the PS amplitude during the first 30 min and last 30 min after the sixth HFS. As shown in Fig. 13D, during the first 30 min, the PS amplitude in all of the tested groups was statistically the same (for controls, $F_{(2,15)}$ = 0.081, p = 0.923; for maternally stressed mice, $F_{(2,17)}$ = 2.99, p = 0.077 by one-way ANOVA). However, post-HFS application of DEX or DEX-BSA restored impaired LTP maintenance in the stressed mice to levels comparable with those seen in the vehicle-treated slices prepared from the control mice (for maternally stressed mice, $F_{(2,17)}$ = 5.77, $p < 0.05$ by one-way ANOVA; Fig. 13E), while the same applications did not induce any significant PS amplitude change in the control slices within this LTP protocol, presumably due to the robust synaptic plasticity-evoking condition (for controls, $F_{(2,15)}$ = 0.67, p = 0.53 by one-way ANOVA; Fig. 13E). Collectively, the restoration of LTP by the post-HFS application of either DEX or DEX-BSA suggests that membrane-associated GRs play a critical role in LTP maintenance in the amygdala of maternally stressed mice.

Nongenomic action of DEX-BSA on auditory fear memory formation

Given that I had observed the restorative effect of DEX-BSA on LTP in the amygdala of maternally stressed mice, I further examined the effects of DEX-BSA on amygdala-related fear memory formation in both the maternally stressed and control mice. I microinjected DEX-BSA (0.4 μg in 0.2 μl) or BSA into the BLA through preinstalled cannulas, since DEX-BSA does not penetrate the blood-brain barrier. DEX-BSA was infused immediately after auditory fear conditioning and freezing was monitored 24 h after conditioning. As shown in Fig. 14A, microinjection of DEX-BSA significantly increased the freezing levels in the maternally stressed mice, but not in the control animals ($F_{(1,20)} = 22.89$, $p < 0.01$ for group differences; $F_{(1,20)} = 10.68$, $p < 0.01$ for treatment; $F_{(1,20)} = 9.565$, $p < 0.01$ for interaction). The freezing levels of the maternally stressed mice that received DEX-BSA were comparable with those seen in the control mice. These results suggest that a nongenomic action of GC restores fear memory consolidation in maternally stressed mice, and that GC signaling cascades, presumably initiated from the amygdaloid membrane, are impaired in these mice.

Figure 3. Fear memory of maternally stressed mice in a passive avoidance test. (A) A schematic diagram for passive avoidance test. (B) Step-through latencies into the dark compartment before (training) and after a foot shock (test) expressed as means ± SEM (**: $p < 0.01$ vs. control; $n = 15$ for each group). CTL and STR indicate control and maternally stressed mice, respectively.

A.



B.

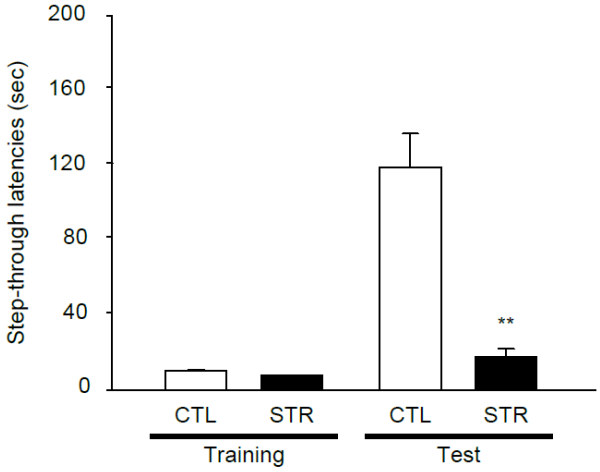
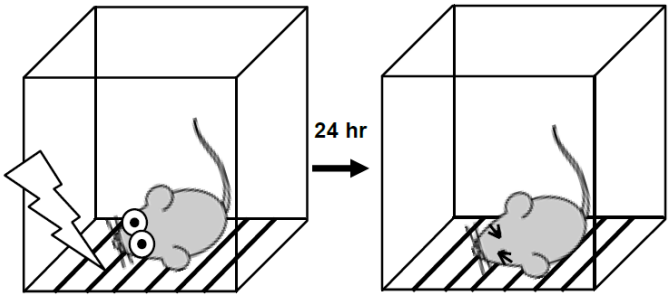

Step-through latencies (sec)
200
160
120
80
40
0
**
CTL
STR
CTL
STR
Training
Test

Figure 4. Contextual fear conditioning in maternally stressed mice. (A) A schematic diagram for contextual fear conditioning paradigm. (B) Freezing percent before (training) and after pairing of the context and foot-shock (test) (**: $p < 0.01$ vs. CTL; n = 10 for CTL and n = 11 for STR).

A.


24 hr

B.

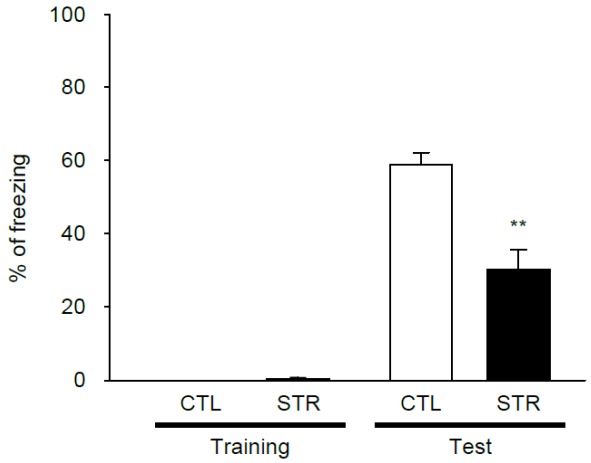

100
80
60
40
20
0
% of freezing
**
CTL
STR
CTL
STR
Training
Test

Figure 5. Impaired retrieval of auditory fear memory, but not fear memory acquisition in maternally stressed mice. (A) A schematic diagram for auditory fear conditioning paradigm (CS; conditioned stimulus, US; unconditioned stimulus). (B) Fear memory acquisition curves represented as freezing percent during five repetitions of tone and foot-shock pairing. (C) Freezing percent during tone exposure without foot shock at 24 h after training presented as means ± SEM (**: $p < 0.01$ vs. CTL; n = 8 for each group).

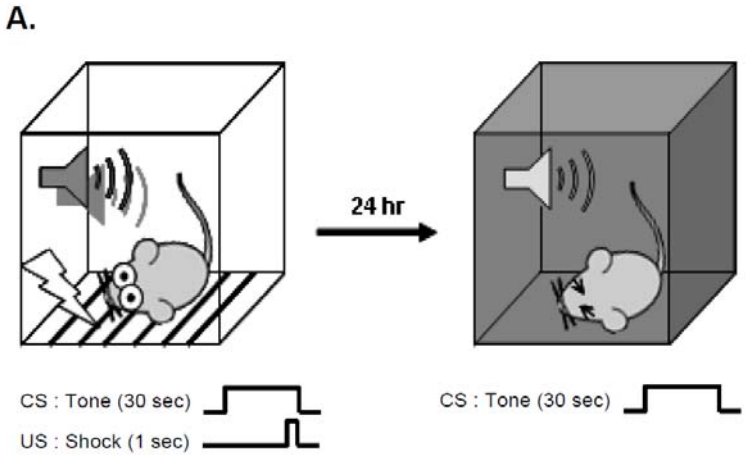

A.
24 hr
CS : Tone (30 sec)
US : Shock (1 sec)
CS : Tone (30 sec)
B.
CTL
STR
% of freezing
Trials
C.
% of freezing
**
CTL
STR

Figure 6. Normal innate fear, spontaneous motor activity and expression of amygdaloid glutamate receptors in maternally stressed mice. (A) Freezing behaviors in response to a predator odor during a 10min trial. Freezing was measured by at 2 min intervals (n = 6 for each group). (B) Spontaneous motor activities for 10 min in the test chamber are expressed as the means ± SEM of the distance traveled ($p < 0.185$ between two groups by Student's t test; n = 6 for each group). (C) Immunoblot analysis of synaptic molecules in whole-cell lysates (WL) and PSD-enriched fractions (PSD) from dissected amygdala of the control and maternally stressed mice.

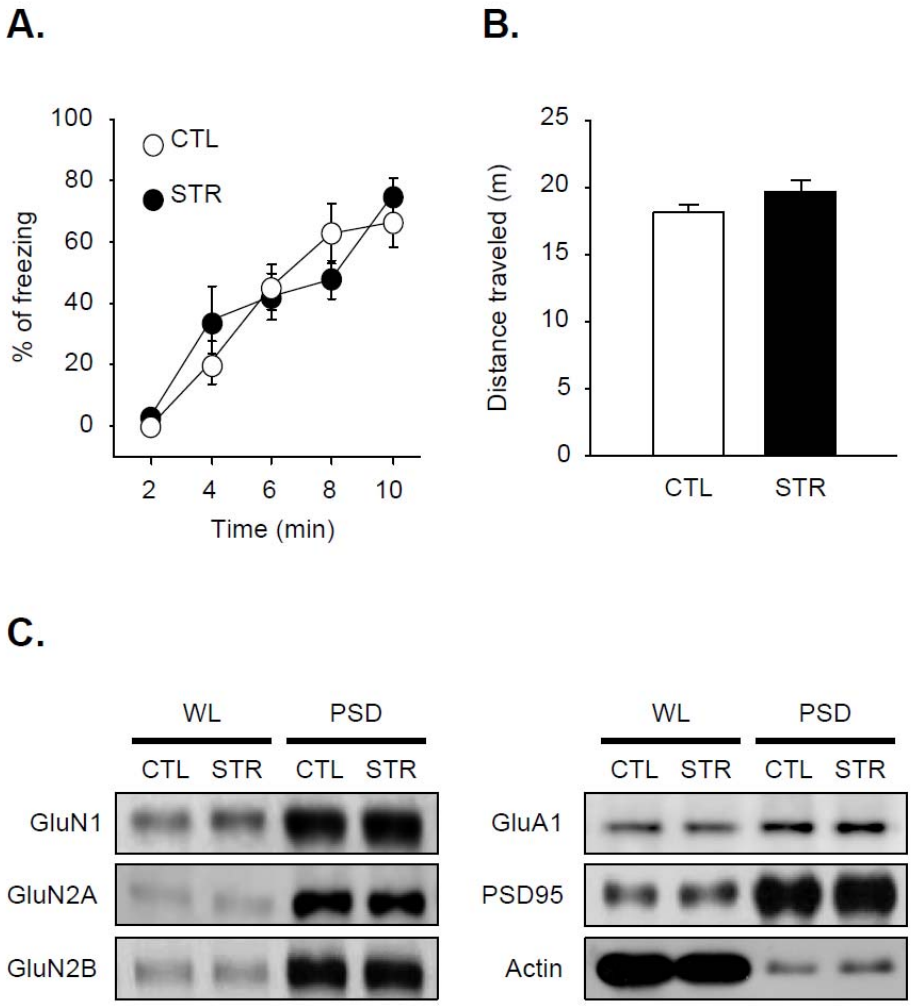
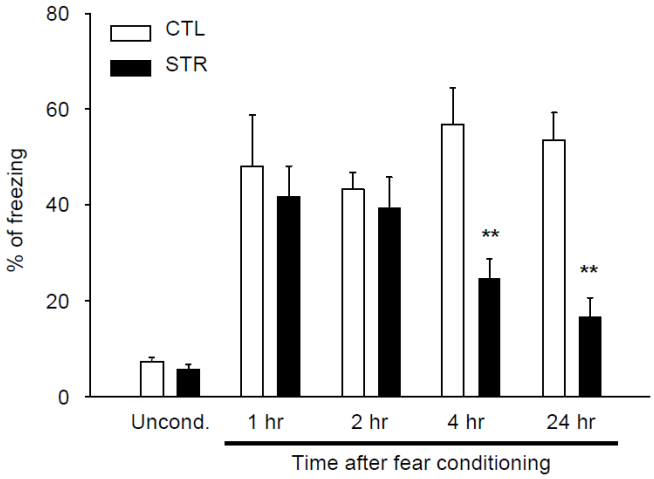

A.
B.
100
80
60
40
20
0
% of freezing
CTL
STR
2
4
6
8
10
Time (min)
25
20
15
10
5
0
Distance traveled (m)
CTL
STR
C.
WL
PSD
CTL
STR
CTL
STR
GluN1
GluN2A
GluN2B
WL
PSD
CTL
STR
CTL
STR
GluA1
PSD95
Actin

Figure 7. Impairment of fear memory consolidation. (A) Fear responses to the auditory cue in unconditioned mice (Uncond.) or mice at 1, 2, 4, or 24 h after training (**: $p < 0.01$ vs. CTL; $n = 6$-12 for each group). (B) Immunoblot analyses of ERK1/2 and CREB phosphorylation on the amygdala whole cell lysates prepared at 15, 60, or 240 min after fear conditioning. Actin was used as an internal control to ensure a similar loading amount.

A.


80
60
40
20
0
% of freezing
CTL
STR
**
**
Uncond.
1 hr
2 hr
4 hr
24 hr
Time after fear conditioning

B.

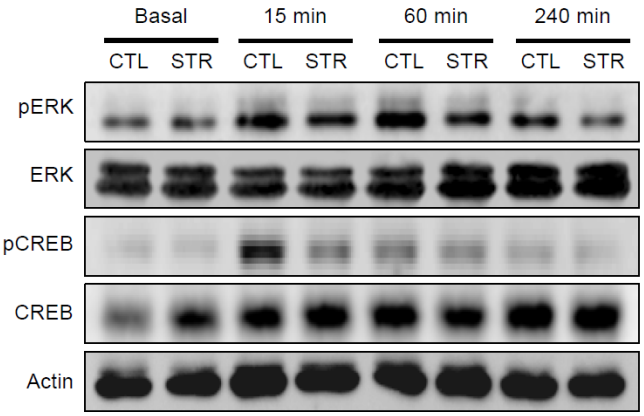
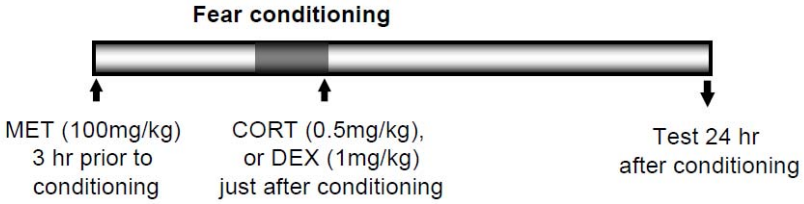

Basal
15 min
60 min
240 min
CTL
STR
CTL
STR
CTL
STR
CTL
STR
pERK
ERK
pCREB
CREB
Actin

Figure 8. Involvement of GC in the impairment of auditory fear memory in maternally stressed mice. (A) Experimental scheme. (B) Effect on fear memory formation of MET, a GC synthesis inhibitor, administered 3 h before conditioning, or GCs such as CORT or DEX administered immediately after conditioning (**: $p < 0.01$ vs CTL receiving the same treatment; ††: $p < 0.01$ vs naive group with the same prenatal manipulation; n = 6–9 for each group).

A.


Fear conditioning
MET (100mg/kg)
3 hr prior to
conditioning
CORT (0.5mg/kg),
or DEX (1mg/kg)
just after conditioning
Test 24 hr
after conditioning

B.

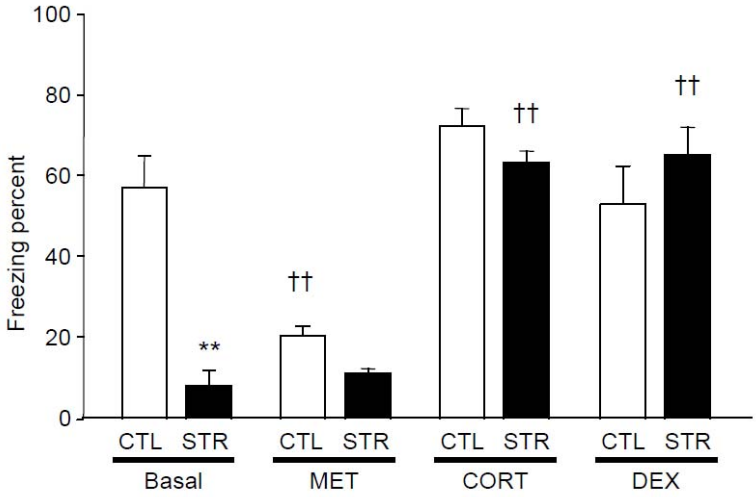

Freezing percent
100
80
60
40
20
0
**
††
††
††
CTL STR
CTL STR
CTL STR
CTL STR
Basal
MET
CORT
DEX

Figure 9. Phosphorylated ERK1/2-immunoreactive (pERK-ir) cells in the BLA complex. (A) Representative images for pERK1/2-ir nuclei (red) counterstained with DAPI (blue). (B) The numbers of pERK1/2-ir cells per amygdala section are represented as a bar chart (**: $p < 0.01$ vs CTL having received the same treatment; ††: $p < 0.01$ vs naive group with the same prenatal manipulation; $n = 6$ for each group).

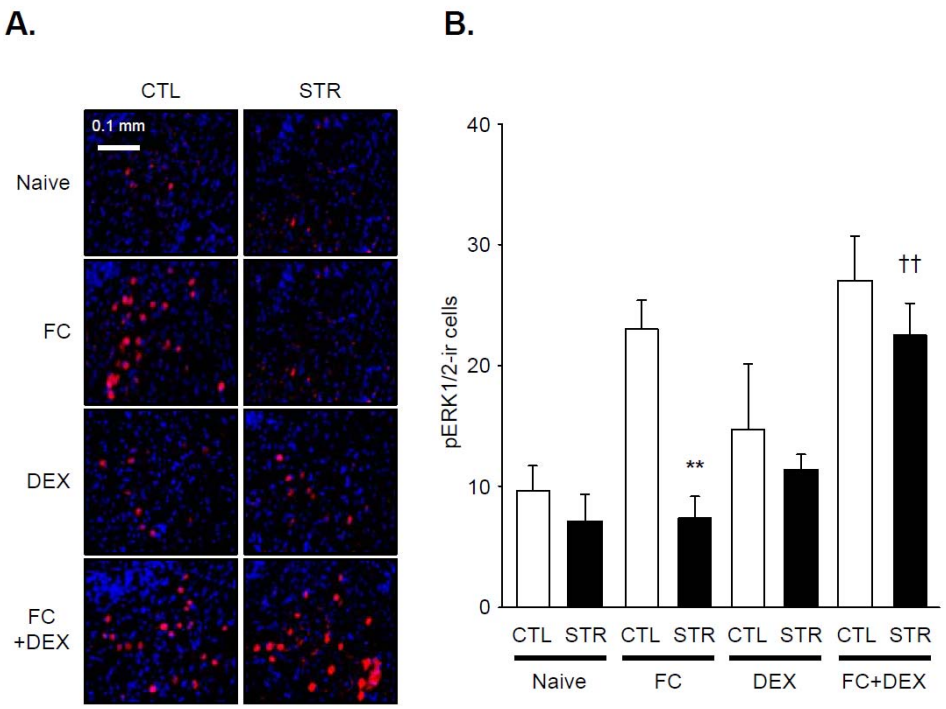

A.
CTL
STR
0.1 mm
Naive
FC
DEX
FC
+DEX
B.
pERK1/2-ir cells
40
30
20
10
0
**
††
CTL STR CTL STR CTL STR CTL STR
Naive
FC
DEX
FC+DEX

Figure 10. Fear conditioning-induced phosphorylation of ERK1/2 levels in the amygdala. (A) Immunoblot analyses of ERK1/2 phosphorylation (pERK1/2) in the amygdala whole-cell lysates prepared from naive mice (Naive), fear-conditioned mice (FC), DEX-injected naive mice (DEX), and DEX-injected mice after fear conditioning (FC+DEX). Each sample was prepared by pooling 3-4 animals. Actin was used as an internal control to ensure a similar loading amount. (B) Relative pERK1/2 levels are presented as the means ± SEM of R.O.D., where the mean value of naive CTL is set as 1 (*: $p < 0.05$ between CTL-FC and STR-FC; ††: $p < 0.01$ vs naive group with the same prenatal manipulation; n = 5 for Naive and FC and n = 4 for DEX and FC+DEX). The R.O.D. for each pERK1/2 band was normalized to the corresponding pan-ERK1/2 immunoreactive band and reference samples.

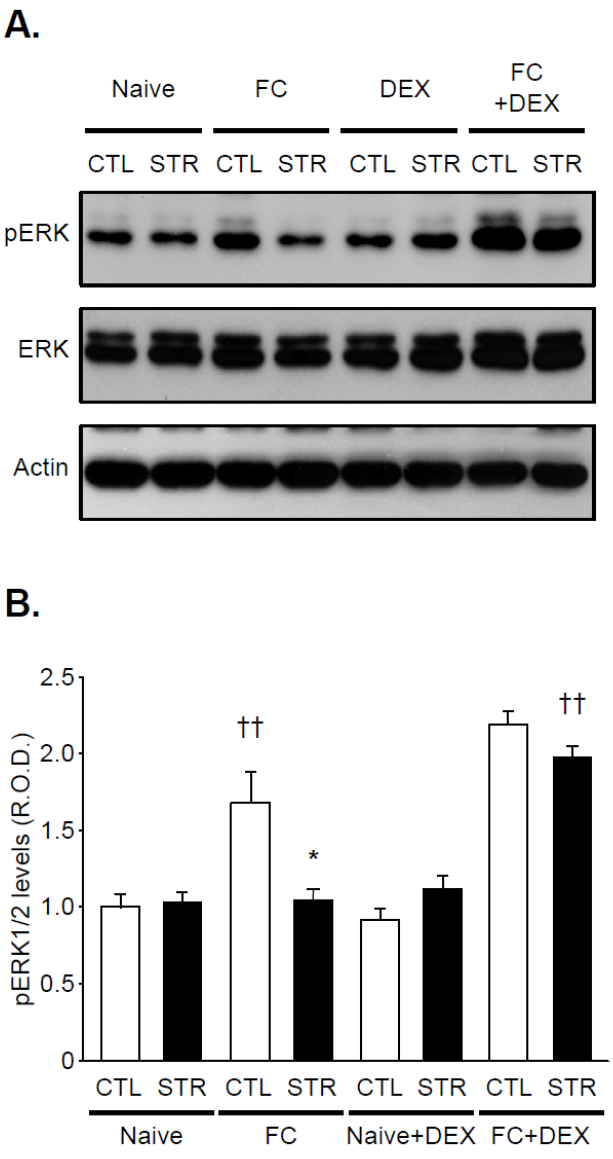

A.
Naive
FC
DEX
FC +DEX
CTL STR CTL STR CTL STR CTL STR
pERK
ERK
Actin
B.
pERK1/2 levels (R.O.D.)
2.5
2.0
1.5
1.0
0.5
0
††
*
††
CTL STR CTL STR CTL STR CTL STR
Naive
FC
Naive+DEX
FC+DEX

Figure 11. Serum GC levels in response to auditory fear conditioning and expression of GC receptors in various brain regions. (A) Radioimmunoassay of serum GC levels from sacrificed mice without training (basal) or 30 and 180 min after fear conditioning (*: $p < 0.05$ vs CTL at the same time point; †: $p < 0.05$ and ††: $p < 0.01$ vs basal serum GC levels; n = 4–6 for each group). (B) Protein levels of the GR and MR in the amygdala (AMY), hippocampus (HIP), and hypothalamus (HYP), with actin as an internal control. Relative GR levels are presented in the lower panel as the means ± SEM of R.O.D., where the mean value from each CTL tissue is set as 1 (*: $p < 0.05$ and **: $p < 0.01$ vs CTL; n = 6).

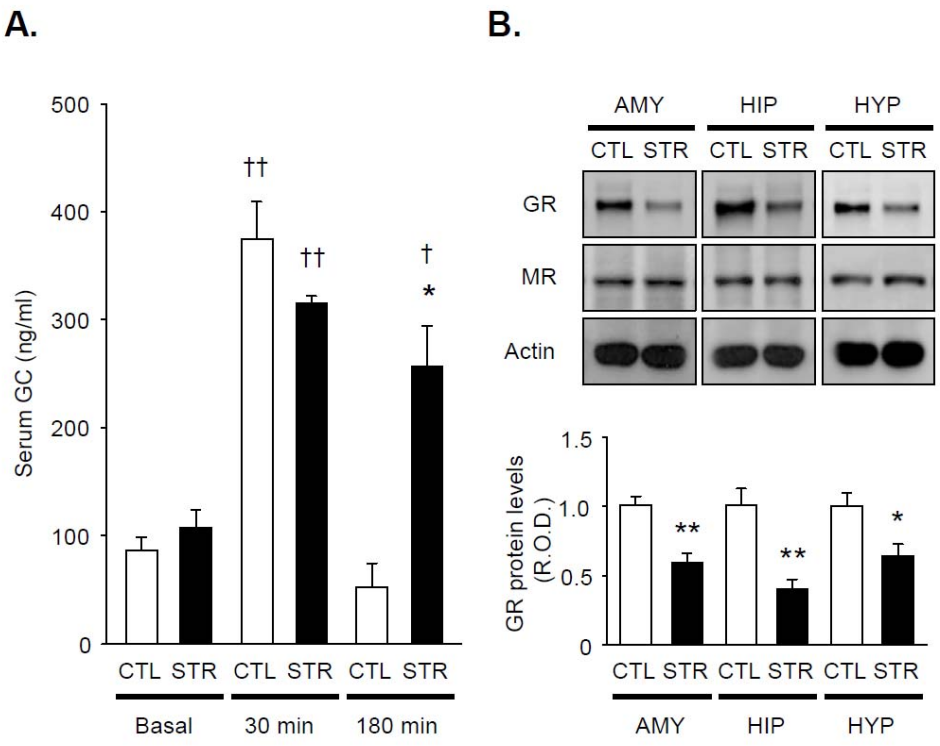

A.
B.
Serum GC (ng/ml)
500
400
300
200
100
0
††
††
†
*
CTL STR CTL STR CTL STR
Basal
30 min
180 min
AMY
HIP
HYP
CTL STR CTL STR CTL STR
GR
MR
Actin
GR protein levels (R.O.D.)
1.5
1.0
0.5
0
**
**
*
CTL STR CTL STR CTL STR
AMY
HIP
HYP

Figure 12. Basal synaptic transmission in the thalamo-LA pathway of mouse brain slices. (A) Schematic representation of a brain slice containing the amygdala showing the position of the recording and stimulation pipettes. (B) Input-output curves for evoked excitatory postsynaptic currents (EPSCs) in CTL and STR groups (n = 7 for each group). (C) Paired-pulse facilitation in amygdaloid neurons with 35-, 50-, or 100-ms interpulse intervals (n = 8 for CTL and n = 11 for STR).

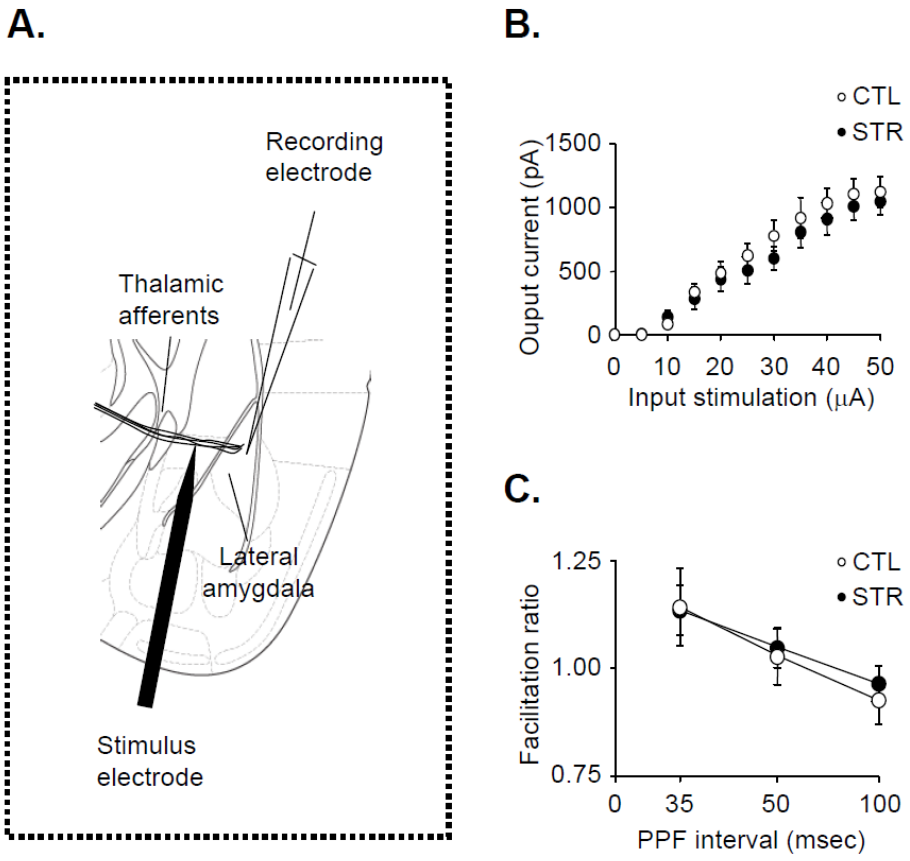

A.
Recording electrode
Thalamic afferents
Lateral amygdala
Stimulus electrode
B.
CTL
STR
Ouput current (pA)
1500
1000
500
0
0 10 20 30 40 50
Input stimulation (μA)
C.
CTL
STR
Facilitation ratio
1.25
1.00
0.75
0 35 50 100
PPF interval (msec)

Figure 13. Synaptic plasticity of the thalamo-LA synapses. (A) High frequency stimulus (HFS)-induced PS amplitude (*: $p < 0.05$ vs CTL; n = 7 for CTL and n = 8 for STR). (B) Effect of DEX treatment immediately after six times of HFS (n = 5 for each group). (C) Population spikes (PS) amplitudes with membrane-impermeable DEX-BSA conjugate (DEX-BSA) treatment after HFS (n = 6 for CTL and n = 7 for STR). Calibration: 2 ms, 0.2 mV. (D) Early-phase of LTP expressed as the means ± SEM of the PS amplitude for the first 30 min after HFS in each group from the results in A (Vehicle), B (DEX), and C (DEX-BSA). (E) The means ± SEM of PS amplitudes for the last 30 min (from 2.5 to 3 h0 after HFS application (*: $p < 0.05$ vs CTL by Student's t test and †: $p < 0.05$ vs vehicle-treated STR).

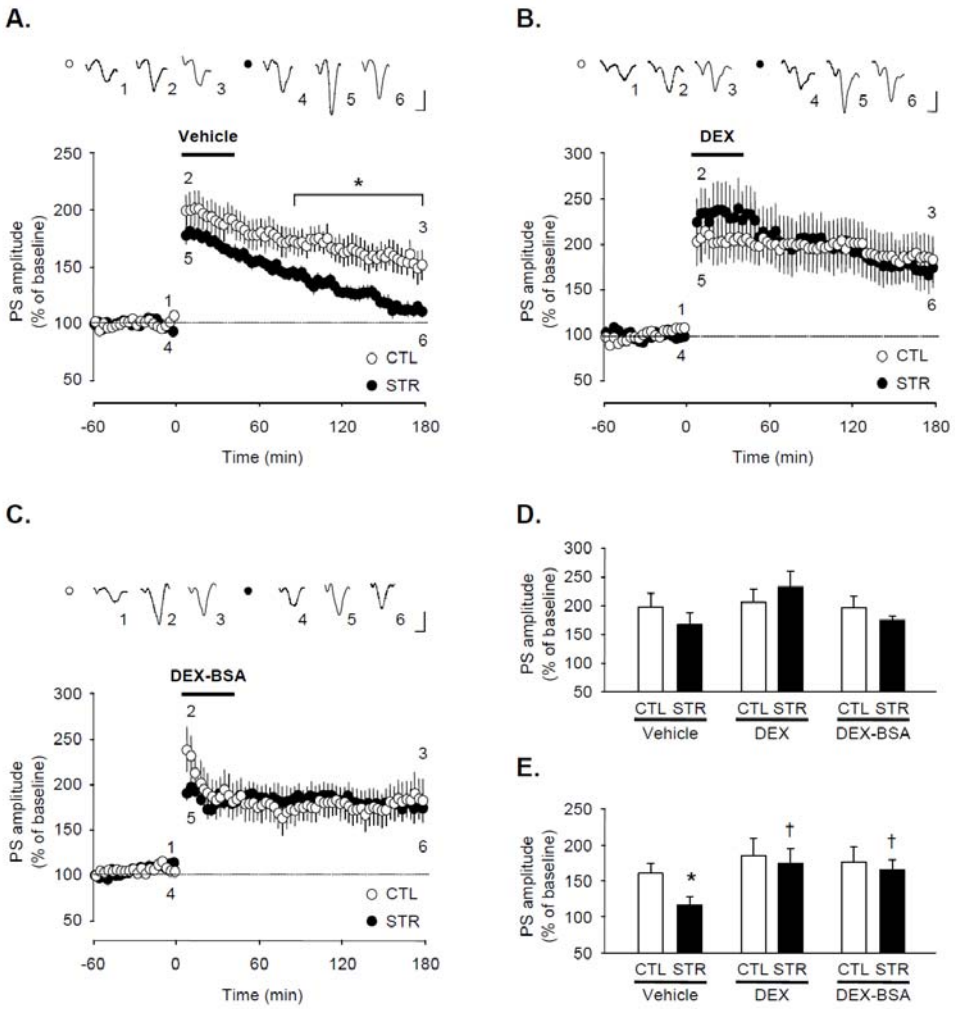

A.
Vehicle
PS amplitude (% of baseline)
Time (min)
CTL
STR
B.
DEX
C.
DEX-BSA
D.
CTL STR
Vehicle
DEX
DEX-BSA
E.

Figure 14. Nongenomic action of GC in fear memory formation elucidated by using DEX-BSA. (A) Effects on fear memory formation of microinjection of DEX-BSA immediately after fear conditioning (**: $p < 0.01$ vs. CTL and ††: $p < 0.01$ vs. vehicle treated STR; $n = 5$ for each vehicle (BSA)-treated group, $n = 7$ for each DEX-BSA-treated group). (B) A representative photograph and illustration of the position of an implanted infusion needle. The position is represented by the symbol on three different rostrocaudal planes, with coordinates relative to the mouse bregma (○, vehicle injected control mice; ●, vehicle injected maternally stressed mice, □, DEX-BSA injected control mice; ■, DEX-BSA injected maternally stressed mice).

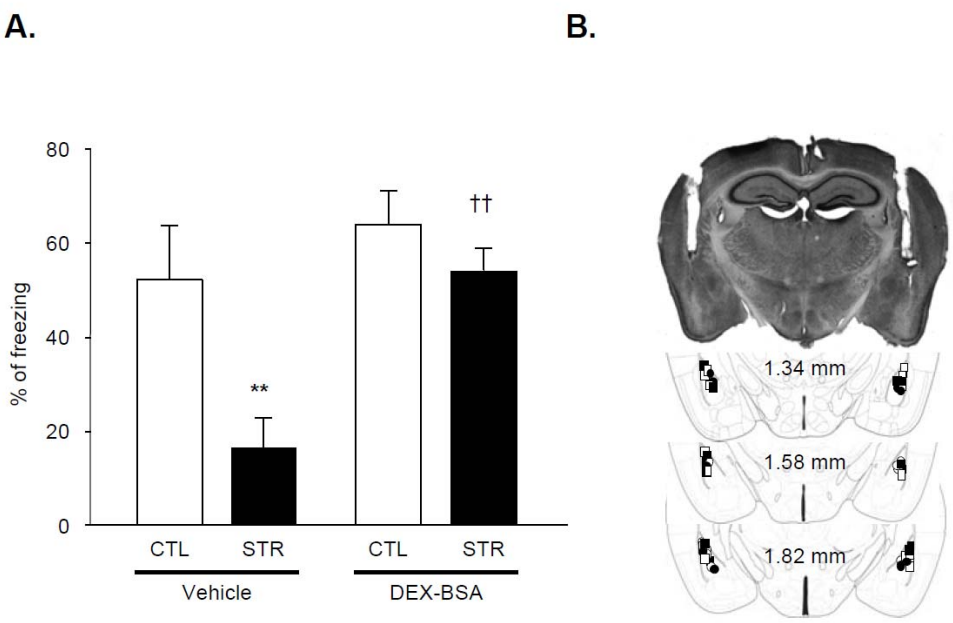
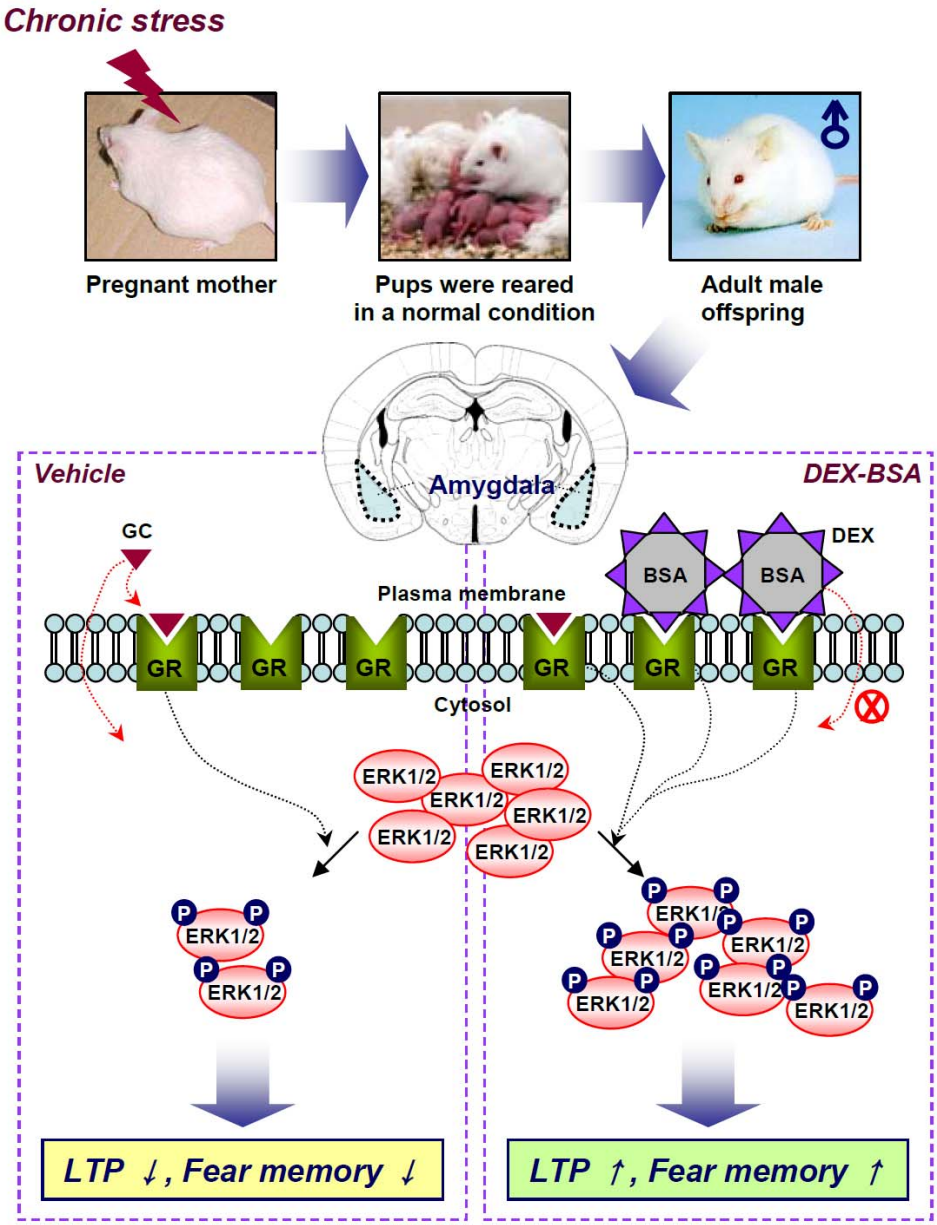

A.
B.
80
60
40
20
0
% of freezing
**
††
CTL
STR
CTL
STR
Vehicle
DEX-BSA
1.34 mm
1.58 mm
1.82 mm

Figure 15. Impairment of fear memory consolidation in maternally stressed male mouse offspring invovled with nongenomic GC action on the amygdala. Impaired LTP in the thalamo-LA amygdala pathway and conditioned fear memory of stressed offspring were recovered by DEX-BSA treatment. These findings provide strong evidence that GC-induced ERK1/2 signaling pathway initiated from plasma membranes in the amygdala, which is attenuated in maternally stressed mice, plays an important role in the regulation of fear memory and synaptic plasticity, independent of GR-mediated transcription.


Chronic stress
Pregnant mother
Pups were reared in a normal condition
Adult male offspring
Amygdala
Vehicle
DEX-BSA
GC
DEX
BSA
BSA
Plasma membrane
GR
Cytosol
ERK1/2
P
LTP ↓, Fear memory ↓
LTP ↑, Fear memory ↑

DISCUSSION

The present study was designed to elucidate whether maternal stress causes long-lasting alterations of amygdala-dependent emotional learning capabilities in adult rodent offspring. Indeed, male offspring from stressed mothers exhibit impairments in fear memory consolidation and LTP in the thalamo-LA pathway. Interestingly, these abnormalities are rescued by membrane-impermeable DEX-BSA, which is known to activate membrane-initiated GC signaling, suggesting that maternal stress impairs emotional learning, at least in part, by weakening the nongenomic action of GC on the amygdala. Physiologically, the adverse effects of prenatal stress on emotional memories, especially fear, suggest that psychiatric disorders related to aversive experiences can be programmed by a stressful environment *in utero*.

In the auditory fear conditioning paradigm, maternally stressed mice displayed problems in the memory consolidation process, resulting in an LTM defect, in contrast to the normal fear responses during the training period (normal fear memory acquisition) and the retention tests within 2 h after fear conditioning (STM formation). It is also important to note that the abnormal motor activity and/or innate fear level in maternally stressed mice may compromise the measurement of fear memory, since I used freezing behavior as measure of fear expression. However, it was found in the present study that after habituation, spontaneous motor activity in the testing chamber was not altered within the time period required for testing conditioned fear. It was also demonstrated that prenatal stress does not alter the unlearned fear response to predator odor in adult offspring.

Therefore, a neural mechanism responsible for amygdala-dependent memory consolidation is evidently an important substrate of the organizing effects of prenatal stress, although the precise molecular targets require further investigation.

The most striking finding in the present study is the nongenomic effect of GC on the amygdala of maternally stressed mice in the amelioration of fear memory impairment. As shown in Fig. 13C, impaired LTP in the thalamo-LA amygdala pathway of stressed offspring was recovered by DEX-BSA treatment. In accord with this finding, bilateral posttraining infusions of DEX-BSA into the amygdala enhanced the conditioned fear response in stressed animals to a level comparable with that of their controls (Fig. 14). These findings provide strong evidence that a membrane-initiated GC signaling in the amygdala, which is attenuated in maternally stressed mice, plays an important role in the regulation of fear memory and synaptic plasticity, dependent of ERK phosphorylation, as illustrated in Fig. 15.

A recent study (Roozendaal et al., 2010) also has suggested a critical role for the nongenomic action of GC on the insular cortex in object recognition learning. In support of these findings, the presence of membrane GR has been demonstrated in an amphibian species (Orchinik et al., 1991). Although membrane GR in mammalian neurons has not been fully characterized, evidence for membrane-associated GR has recently been reported (Riedemann et al., 2010). In this context, it should be noted that Johnson et al. (2005) found GR (classical GR) immunoreactive sites on the postsynaptic membranes of amygdala neurons. It is therefore plausible that downregulation of putative membrane GR in the amygdala of

maternally stressed mice underlies the observed impairments in both fear memory consolidation and LTP. However, whether this putative membrane GR is a membrane-associated form of classical GR, or an as yet unidentified isotype, remains to be determined.

Although the present study is focused on local storage processes in the amygdala, this regional brain tissue, particularly the BLA complex, has been proposed to interact with other brain regions in order to modulate the memory consolidation related to a variety of emotionally arousing experiences (McGaugh, 2004). For example, Malin and McGaugh (2006) demonstrated an involvement of the amygdala, hippocampus and anterior cingulate cortex. More recently, critical interactions between the BLA and medial prefrontal cortex (mPFC) were also demonstrated (Roozendaal et al., 2004; Roozendaal et al., 2009). According to this model, amygdala activation during the consolidation phase modulates the activities in other brain regions so as to consolidate acquired memory. Thus, this model may explain why so many different learning tasks are affected by modulation of the amygdala. In fact, in addition to fear-related tasks, object recognition learning has been shown to be affected by modulation of the amygdala (Roozendaal et al., 2008). Therefore, the reduced nongenomic effect of GC on the amygdala of maternally stressed mice may not be limited to the impairment in the consolidation of auditory fear memory investigated here, it may also modulate the consolidation of emotionally influenced memory, possibly by affecting neural activity in other brain regions.

The modulatory effect of GC on memory consolidation appears not to be mediated solely by the amygdala. For example, microinjection of a GR agonist into the mPFC after passive avoidance training rapidly increases

ERK1/2 phosphorylation in the BLA within 15 min, and *vice versa* (Roozendaal et al., 2009). This rapid effect of GR agonists on ERK1/2 phosphorylation, which is also observed in the present study, supports the nongenomic action of GC on memory consolidation processes. Based on these findings, it is tempting to speculate that downregulation of putative membrane GR in the amygdala and perhaps also other interconnected brain regions, is responsible for the impairments in both memory consolidation and conditioning-enhanced ERK1/2 and CREB phosphorylation in maternally stressed mice. Consistently, reduced GR expression in stressed animals was found in various brain regions, including the hippocampus, hypothalamus and amygdala (see Fig. 10B). Therefore, the reduced expression of GR, possibly membrane-associated GR, in the various interconnected brain regions also may influence neuronal activities reciprocally, thereby dampening the activity of the neural network required for memory consolidation.

In conclusion, the present study clearly demonstrates, for the first time, that prenatal stress produces long-term impairment in both fear memory consolidation and amygdala LTP maintenance in the adult offspring mice. The observed impairments in memory consolidation and LTP appear to involve a reduced nongenomic action of GC on the amygdala of maternally stressed mice. Therefore, our study suggests that the consolidation of emotionally influenced memory is a critical target of the programming effects of maternal stress, resulting in defective behaviors and psychiatric dysfunctions in the offspring.

CHAPTER 2

Adult Neurogenesis in the Hippocampus Affected by Attenuated Glucocorticoid Rhythmicity

ABSTRACT

Glucocorticoid (GC) is an adrenal steroid with diverse physiological effects, showing a robust circadian rhythm. However, whether circadian GC oscillation influences on the brain functions remains as a question. Here, I examined the effect of diurnal GC rhythm on the hippocampus, where new neurons are generated in and which involves with cognitive functions and mood regulation, by using adrenal speicifc BMAL1 knockdown (A-BMKD) transgenic mice previously reported to show apparently reduced daily oscillation of GC under constant darkness (Son et al., 2008). In these mice, the number of newborn neurons in the hippocampus was significantly decreased when they produced the attenuated circadian GC rhythm. At the same time, transgenic mice showed depressive mood states, impaired safety memory, and rapidly shutting off stress reactivity, which are known to be regulated by hippocampal neurogenesis. Interestingly, enhanced rhythmic translocation of activated glucocorticoid receptor (GR) into hippocampal nucleus by binding to GC was observed against hypo-GC secretion with attenuated rhythm in transgenic mice. In accordance with this finding, transcript levels of brain-derived neurotrophin factor (BDNF) and its receptor TrkB, which are known to be essential for adult neurogenesis and regulated by GC negatively, were reduced in the hippocampus of transgenic mice. Moreover, rhythmic GC supplementation rescued neurogenic activity as well as BDNF and TrkB gene expression in the hippocampus, suggesting that weakening of BDNF cascades is responsible for the impaired hippocampal neurogenesis in transgenic mice. Taken together, not only GC levels but also its rhythmic secretion is

important for maintaining normal neurogenesis associated with BDNF signaling cascades in the hippocampus.

Key words: Glucocorticoid (GC), Circadian rhythm, A-BMKD, Hippocampal neurogenesis, Depression, Memory, Stress, Glucocorticoid receptor (GR), Brain-derived neurotrophin factor (BDNF), Rhythmic GC supplementation

INTRODUCTION

The subgranular zone (SGZ) of the dentate gyrus (DG), a subfield of the hippocampus continues to make new neurons during adulthood in rodents, as in several other species, including humans (Gross, 2000). This phenomenon called as neurogenesis is modulated by many environmental and endogenous factors, but stress is one of the most potent environmental parameters known to suppress adult neurogenesis. Glucocorticoid (GC) is an important mediator of the stress response in hippocampal neurogenesis. Elevated GC by exposure to acute or chronic stress, as well as to chronic GC administration, reduces the numbers of newborn neurons in the DG. Conversely, removal of endogenous GC by adrenalectomy (ADX) stimulates neurogenesis in the hippocampus (Cameron and Gould, 1994; Lucassen et al., 2010; Oomen et al., 2007).

Given that GC is often elevated in human patients suffering major depression, stress is thought to exacerbate depression, and the regulation of hippocampal neurogenesis by antidepressants also has been studied. In contrast to stress, antidepressants stimulate the proliferation of the progenitor cells in the SGZ, and the response to antidepressant takes around 21~28 days. This period corresponds to the development of a neuronal phenotype in newly formed cells in the DG. Furthermore, the inhibitory effects of GC on neurogenesis can be reversed by chronic treatment with a variety of antidepressants (Warner-Schmidt and Duman, 2006). Therefore, altered neurogenesis in the hippocampus plays a central role either in the development of depression or the therapeutic response to antidepressants (Duman, 2004).

GC undergoes a robust daily oscillation, which is driven by the master circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus *via* the hypothalamus-pituitary-adrenal (HPA) axis. Moreover, our group showed that the adrenal gland oscillator tightly linked to steroidogenesis by the steroidogenic acute regulatory protein (StAR) is also required for circadian GC production by using transgenic (TG) mice in which a canonical clock protein BMAL1 is knockdowned by an antisense RNA expression under adrenal-specific ACTH receptor (MC2R) promoter (Son et al., 2008). Some studies suggested that such diurnal rhythm of GC is involved in the neurogenesis interacting with antidepressants, given that flattened profiles of GC rhythm were observed in depressed patients (Deuschle et al., 1997). For example, artificially dampening the circadian GC rhythm prevents antidepressants from stimulating neurogenesis, and restoring the GC rhythm also regains the action of those drugs in rats (Huang and Herbert, 2006; Pinnock et al., 2007; Pinnock and Herbert, 2008). However, the precise significance of the GC rhythm in the adult neurogenesis is not as well understood as the consequences of general experimental models using acute and chronic GC administration or artificial removal of adrenals.

In this chapter, I intended to investigate the direct effect of circadian GC oscillation on hippocampal neurogenesis *in vivo* using trangenic system instead of surgical methods. For this purpose, our transgenic mice were subjected, which show the attenuation in circadian GC rhythm under constant darkness due to adrenal-specific abrogation of clock machinery (Son et al., 2008). In contrast to ADX model lacking whole adrenal glands, these mice have not only intact rhythm of GC release during ordinary light-

dark schedule but also normal secretion of catecholamines from the adrenal medulla even under dark-dark condition. In this regard, our transgenic mouse line can serve as a valuable animal model for solving a precise link between hippocampal neurogenesis and basal GC rhythm.

MATERIALS AND METHODS

Genotyping of A-BMKD transgenic mice. Transgenic animals were identified by PCR amplification of tail genomic DNA. PCR genotyping was carried out with the following three primers: MC2R promoter up for genotyping, 5'- ATA TGT TCC GGC CTT TCC TG-3'; AS-BMAL1 dn for genotyping, 5'-TTG GCG TAT CTA CCA CAG GA-3'; endogenous MC2R dn for genotyping, 5'-TGG GAT AGG GAG TTT GTG GA-3'.

Animal care and handling. Wild-type and A-BMKD transgenic male C57BL/6J mice at 9-12 weeks of age were kept in temperature-controlled (22~23℃) quarters under a 12-hr light and 12-hr dark (LD) photoperiod (light-on at 8:00 a.m.), with standard mouse chow and water available *ad libitum*. For dark-dark (DD) conditions, mice were kept in constant darkness for the indicated duration from the light-off time after entrainment for more than 10-days under LD conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Measurement of plasma GC levels. GC levels in plasma samples were assayed using a commercial CS RIA kit (DPC, Los Angeles, CA) as described previously (Chung et al., 2005). Mice were sacrificed at ZT/CT 00 or 12 in Fig. 17. In Fig. 22, stressed mice were sacrificed 0, 60, or 150 min after restraint stressor given at CT6 under 1-week-DD condition. Trunk blood was collected in EDTA-containing tubes and centrifuged at 10,000 ×*g*

for 5 min to obtain plama. Plasma samples were quick frozen and stored at -70°C until use. GC levels were assayed using a commercial radioimmunoassay kit, according to manufacturer's instructions (Diagnostic Products Co., Los Angeles, CA).

Analysis of neurogenesis. Mice were administered BrdU (100 mg/kg i.p.) [(+)-5´ bromo-2´-deoxyuridine; 97%; Sigma, St. Louis, MO] twice per day (8 hr interval) for 2 days prior to sacrifice. After anesthesia with sodium pentobarbital (50 mg/kg, i.p.), mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Brains were collected and postfixed overnight in 4% PFA at 4°C. The following day, serial coronal sections (40 μm) along the entire rostrocaudal extension of the hippocampus were cut on a cryostat and stored in a cryoprotective solution (50% glycerol in 0.1 M PBS) at −20°C until further processed. BrdU immunohistochemistry was performed on every sixth free-floating section (n = 4 - 8 per group) and subsequent immunostaining was performed by the free-floating method as described previously (Sun et al., 2004) with modifications. For BrdU staining, sections were incubated with 1N HCl for 30 min at 37°C, washed with PBS, and blocked with 3% BSA and 0.3% Triton X-100 in PBS for 45 min. The following primary antibodies were applied overnight: anti-BrdU, 1:500 and Doublecortin (DCX), 1:500 (Abcam, Cambridge, MA). After several washes with PBS, Alexa 594-conjugated anti-rat IgG antibody (1:500) and Alexa 488-conjugated anti-rabbot IgG antibody (1:500) were applied for 30 min. Subsequently sections were washed, mounted, and observed with a fluorescence microscopy

(Axiovert 200M, Carl Zeiss, Göttingen, Germany). BrdU/DCX double positive cell counts were done exhaustively throughout the dentate gyrus (DG) or subventricular zone (SVZ) of both sides.

Forced swim test. All behavior tests of A-BMKD transgenic mice were performed between ZT/CT 4 and 8 under dim red light to exclude the influence of photic stimulus on behaviors. Mice were placed in a transparent beaker (10-cm diameter) containing water (21-25°C) with a depth of 15 cm. They remained in the water for 6 min and were then removed and allowed to dry in a clean cage before returning to their home cage. The water was changed for each subject. During the test time, mice were monitored from the side by a video camera, and data were stored for later analysis. Only the last 4 min of the test were scored for to assess the time of immobility (Roybal et al., 2007). Immobility was defined as no volitional body or limb movement, and is presented as the mean ± SE % of the immobile time per total test time.

Elevated plus maze (EPM) test. The forced-swim test was carried out as described previously (Chung et al., 2005). The plus-maze consists of two open arms (5 X 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. I analyzed the duration of open arm entries using the EthoVision Pro system (Noldus Information Technology, Wageningen, Netherlands). An entry was defined as movement of all four

paws into an arm.

Pavlovian fear conditioning. Contextual fear conditioning was carried out in the conditioning chamber (13 × 13 × 25 cm) with 3 repetitions of foot shocks (0.5 mA, 1 sec) at 90-sec intervals after habituation for 10 min in the same chamber on the day before training. Conditioned mice were placed in the same chamber, and the “freezing” time was measured over a period of 5 min at 2 hr or 24 hr after conditioning for measuring short-term memory (STM) or long-term memory (LTM) each. Conditioned freezing was defined as immobility except for respiratory movements. The total freezing time in the test period was represented as a percentage. Auditory fear conditioning was conducted as described previously (Schafe and LeDoux, 2000) with a slight modification. Mice were habituated for 10 min in the conditioning chamber without disturbance on the day before conditioning. Mice were then placed in the conditioning chamber, and 3 conditioning trial repetitions, each consisting of a tone (30 sec, 5 kHz, 75 dB) that terminated with a foot shock (0.5 mA, 1 sec), were administered. The intertrial interval was 90 sec. Conditioned fear responses were tested at 2 hr or 24 hr after conditioning. For each test, mice were placed in a distinct context and re-exposed to three tones at 90 sec intervals after a 2 min period of exploration. Freezing behaviors were scored during the tone presentation. The total freezing time in the test period was represented as percentage of the average duration to each tone presentation.

Safety conditioning paradigm. The forced-swim test was carried out as described elsewhere (Pollak et al., 2008). Animals were handled daily for 3

days prior to the safety-training procedure. Safety conditioning protocol was carried out over 3 days, followed by a test day 24 hr after the last training day. Safety conditioning consisted of four explicitly unpaired unconditioned stimulus (US; electric shock, 0.5 mA, 1 sec) and conditioned stimulus (CS; tone, 30 sec, 350 Hz, 75 dB) presentations (one session per day for 3 days). The fear conditioning protocol was matched to the number of auditory CS and US presentations of the safety conditioning paradigm and thus constituted four paired CS-US presentations per day (see Fig. 21A for details). The precise timing of stimuli varied within session and across days. A memory recall test, consisting of the sole presentation of one CS (30 sec), was carried out 24 hr after the last training day. In any instance, the behavior during the CS period (30 sec) was compared to the corresponding length of time (30 sec) prior to the onset of the CS (pre-CS period).

Extraction of nuclear proteins and immunoblot analysis. All procedures were performed at 4 °C on ice. Frozen tissues were homogenized in proteinase inhibitor containing lysis buffer (300 mM sucrose, 10 mM Tris (pH 7.8), 1.5 mM $MgCl_2$, 0.25% NaDOC and 0.5% NP-40). Lysates (400 µL) were over-layered onto 350 µL of 400 mM sucrose cushion buffer without detergent and centrifuged at 3000 rpm for 10 min. The pellet was then resuspended for SDS-PAGE gel running. Protein samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) in a Bio-Rad (Richmond, CA) Trans-Blot electrophoresis apparatus was employed 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 5% bovine serum albumin (BSA), and incubated with primary antibodies at room temperature for 1 h. The blots were then washed four times with TBS/0.5% Tween-20. The bound primary antibody was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). The blots were then washed further four times. Immunoreative bands were visualized with Amersham ECL reagents according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden). The blots were analyzed with Bio1D image analysis software (Vilber-Lourmat, Marne-la-Vallée, France) and were expressed as relative optical densities (R.O.D.). The antibodies used in the immunoblotting were as follows: Anti-glucocorticoid receptor (GR), lamin B (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Sigma). These antibodies are all commercially available.

Corticosterone supplementation. To experimentally reinstate the robust rhythm of corticosterone in A-BMKD mice, drinking water was replaced by a corticosterone-containing solution (3 mg/L of corticosterone in water) under DD conditions. Corticosterone was first dissolved in 100% ethanol and then diluted in drinking water to reach its appropriate concentration. The final concentration of ethanol was 1%. The vehicle solution contained 1% ethanol in water only.

RNA Isolation and RT-PCR. RNA analyses were performed as described previously with modifications (Son et al., 2008). Mouse tissues were rapidly

removed, frozen in liquid nitrogen, and stored at -70 °C until use. Total RNA was isolated by the single-step acid guanidinium thiocyanate–phenol–chloroform method. For RT-PCR, 500 ng of each RNA sample was reverse-transcribed with MMLV reverse transcriptase (Promega). Then, aliquots of the cDNA were subjected to quantitative real-time PCR in the presence of SYBR Green I (Sigma). Gene expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences used for real-time RT-PCR were as follows: BDNF up, 5'-GAA CAT AGC CGA ACT ACC CAA TCG-3'; BDNF dn, 5'-CCT TAT GAA TCG CCA GCC AAT-3'; TrkB up: 5'-GTT CCG GAG AAC ATC AC-3'; TrkB dn: 5'-GCC GGA ATC CAC AAT TGT AAG G -3'; CBP up: 5'-TTA GCC AGC AAA CAG AGC AT-3'; CBP dn, 5'-CAA TTC CCA CTG ATG TTT GC-3'; AK-2 up: 5'-GGA AAC TGG TGA GTG ACG AA-3'; AK-2 dn, 5'-TCC ATG AGG TCA TCA AGC AT-3'; CHN-1 up: 5'-CGC ATC TCA AGA GAG TGA CC-3'; CHN-1 dn, 5'-CCA CCA CCA GTC TCT GAT AGC-3'; Dexras-1 up: 5'-TGC TGT CTG TAC CCA AGA GC-3'; Dexras-1 dn, 5'- GTG GCC GAG AGA AGG TAG AG-3'; Egr-1 up: 5'-CGA GTC GTT TGG CTG GGA TA-3'; Egr-1 dn: 5'-GAA CAA CCC TAT GAG CAC CTG AC-3'; SGK1 up: 5'-TGT GAA GTC CCT TCT GTG GA-3'; SGK1 dn, 5'-CCA TCT TCG TAC CCG TTT CT-3'; GAPDH up: 5'-CAT CCA CTG GTG CTG CCA AGG CTG T-3'; and GPADH dn: 5'-ACA ACC TGG TCC TCA GTG TAG CCC A -3'. GAPDH up: 5'-CAT CCA CTG GTG CTG CCA AGG CTG T-3'; and GPADH dn: 5'-ACA ACC TGG TCC TCA GTG TAG CCC A -3'.

Data analysis. The results from behavioral studies, quantitative immunohistochemical analyses and plasma GC levels were statistically

evaluated using Student's *t*-test and/or analysis of variance (ANOVA) followed by Neuman-Keuls test as a *post-hoc* comparison. A probability level of $p < 0.05$ was chosen to denote statistical significance.

RESULTS

Attenuated circadian GC rhythm in A-BMKD transgenic mice

Previously, our group revealed that cyclic expression of steroidogenic acute regulatory protein (StAR), a rate-limiting enzyme in steroidogenesis (Stocco, 1999) is directly controlled by heterodimer of CLOCK and BMAL1, the core clock genes composing molecular clock machinery (Ko and Takahashi, 2006). The resulting daily oscillation in steroidogenesis contributes to the generation of a robust GC rhythm (Son et al., 2008). Moreover, our group examined the role of the adrenal peripheral clock in producing the daily GC rhythm *in vivo* by generation of transgenic (TG) mice, in which the molecular clockwork is specifically ablated in the adrenal GC-producing cells (Son et al., 2008). Under 1-week-dark/dark (DD) condition, the adrenal StAR rhythm is completely abolished and the plasma GC oscillation also is strongly attenuated in adrenal-specific BMAL1 knockdown (A-BMKD) transgenic mice in which a part of the BMAL1 coding region was expressed in an antisense orientation under tissue-specific control by using ACTH receptor (MC2R) promoter (Fig. 16). In these mice, the 1.3 kb MC2R promoter and mouse Bmal1 coding sequence spanning +27 to +1268 region with strong knocking-down activity were used in producing the transgene construct (Fig. 17A).

At first, I confirmed the circadian GC profiles in WT and A-BMKD transgenic mice after entrainment under 12-hour light/dark (LD) condition at least for 10 days followed by transferring to LD or DD cycles as illustrated in Fig. 17B. In the presence of light cue, trangenic mice showed normal

daily variations in plasma GC levels ($F_{(1,13)}$ = 0.055, p = 0.82 for genotype; $F_{(1,13)}$ = 79.31, p < 0.01 for time; $F_{(1,13)}$ = 0.074, p = 0.79 for interaction; Fig. 17C). However, the plasma GC oscillations were strongly attenuated, although some daily variation was retained in trangenic mice under DD conditions ($F_{(1,29)}$ = 10.24, p < 0.01 for genotype; $F_{(1,29)}$ = 67.89, p < 0.01 for time; $F_{(1,29)}$ = 16.98, p < 0.01 for interaction; Fig. 17D) as previously reported (Son et al., 2008).

Reduced neurogenesis in the hippocampus of A-BMKD transgenic mice

The dentate gyrus (DG) of the hippocampus (Fig. 18A) is particularly sensitive to GC levels by responding with altered adult neurogenesis. To explore the possible role of the circadian changes of GC levels in hippocampal neurogenesis, I examined whether attenuated GC rhythm is associated with alterations in structural plasticity of the DG by using A-BMKD transgenic mice. The neurogenesis in the DG was analyzed 2 days after administration of BrdU (100 mg/kg i.p.) twice per day (8 hr interval). In this injection paradigm, the colocalization of BrdU with immature neuronal marker, doublecortin (DCX), reflects the initial proliferation of cells to become neurons later. Hippocampal BrdU/DCX-positive cell counts were significantly reduced in A-BMKD trangenic mice compared to WT mice under DD condition ($F_{(1,136)}$ = 329.6, p < 0.01 for genotype; $F_{(1,136)}$ = 6.972, p < 0.01 for photoperiod; $F_{(1,136)}$ = 76.5, p < 0.01 for interaction), in contrast to similar neurogenic activity in the DG of both group under LD cycles (Figs. 18B and C). These results suggest that reduced proliferation in the DG is

an effector of reduced daily variation of GC, implicating that the circadian changes of GC levels play a critical role in hippocampal neurogenesis.

Depression-like behavior and anxiety in transgenic mice

Multiple studies have confirmed that GC is invovled in neurgoenesis-related anxiety and depression-like states in rodents (Stone and Lin, 2008; Gourley et al., 2008). To examine the influence of weakening GC circadian oscillation in depressive mood, A-BMKD trangenic mice were subjected to forced swim test (FST), a task used for detecting depression-like behavior in rodents (Fig. 19A). These mice exhibited more immobility time only under DD conditions (WT, 34.53 ± 2.56 vs. TG mice, 47.56 ± 3.91% of immobility; $p < 0.01$; n = 10 for WT and n = 9 for TG mice; Fig. 19C), but not under normal LD cycles (WT, 45.00 ± 2.45 vs. TG mice, 40.44 ± 3.04% of immobility; $p = 0.26$; n = 10 for each group; Fig. 19B), suggesting that attenuated GC rhythm can develop a depression-like phenotype in accordance with reduced hippocampal neurogenesis which is involved with the etiology of major depression in humans. However, I found that transgenic mice did not show significant changes in anxiety revealed by the elevated plus maze test (EPM; Fig. 19D) regardless of photoperiod, although there was a tendency to stay a little bit more in open arm (for LD conditions, $F_{(1,42)} = 3.337$, $p = 0.075$ for genotype; $F_{(1,42)} = 0.141$, $p = 0.71$ for time; Fig. 19E; for DD conditions, $F_{(1,30)} = 0.535$, $p = 0.075$ for genotype; $F_{(1,30)} = 2.921$, $p = 0.098$ for time; Fig. 19F). Even though many previous reports suggest a relationship between anxiety and depressive behavior, I did not detect this correlation in the two most frequently used animal

models, the FST and the EPM in the present study. This is an interesting outcome, revealing that reduced GC daily rhythm does not necessarily trigger a generalized anxious state and can induce depression-like phenotypes without exacerbating anxiety.

Normal fear conditioned memory of transgenic mice under 1-week-DD cycles

The observation of adult neurogenesis in the DG has led to a number of interesting functional hypotheses including learning and memory based on the consideration about hippocampal functions in the acquisition and consolidation of declarative memories (Snyder et al., 2005). Actually, experiments using pharmacological, physical or genetic ablation strategies have implicated adult neurogenesis in hippocampus-dependent cognitive functions (Deng et al., 2010). Therefore, I decided to use the trangenic model to examine whether deficient neurogenesis elicited by attenuated GC oscillation would have any appreciable effect on learning behaviors using fear conditioning paradigm. In the hippocampus-dependent contextual fear conditioning test, the mice learned to fear the environment associated with an aversive stimulus, such as a foot shock (Phillips and LeDoux, 1992). I measured the contextual fear memories 24 hr after training and found a similar level of freezing responses in both groups regardless of light-dark schedule (WT-LD, 66.23 ± 6.79 vs. TG-LD, 65.12 ± 6.59% of freezing; p = 0.91; n = 9 for each group; Fig. 20B; WT-DD, 65.94 ± 2.78 vs. TG-DD, 57.26 ± 4.04% of freezing; p = 0.098; n = 21 for WT and n = 17 for TG mice; Fig. 20E), in addition to normal learning curves on

training day ($F_{(1,32)}$ = 0.0062, p = 0.938 for genotype by RM-ANOVA for LD condition; Fig. 20A; $F_{(1,52)}$ = 0.643, p = 0.430 for genotype by RM-ANOVA for DD condition; Fig. 20D). I also examined the performance of these mice in another type of conditioned memory, cued fear conditioning in both normal LD and 1-week-DD conditions. In this task, mice learned to fear a neutral stimulus, such as a tone, by pairing it with an electrical foot shock (Phillips and LeDoux, 1992). Similar to the results in the contextual fear conditioning, I found no significant difference in the freezing response measured 24 hr after training between wildtype and transgenic groups in all photoperiod (WT-LD, 63.83 ± 10.46 vs. TG-LD, 57.16 ± 11.29% of freezing; p = 0.67; n = 9 for each group; Fig. 20C; WT-DD, 59.74 ± 5.02 vs. TG-DD, 62.15 ± 6.05% of freezing; p = 0.77; n = 21 for WT and n = 17 for TG mice; Fig. 20F). These results indicate that the declined neurogenesis in the hippocampal regions observed in the A-BMKD transgenic mice had no significant effect on the conditioned fear memory.

Long-term effects of attenuation of GC rhythm on anxiety and fear memory

Although there is no significant effect of attenuated GC oscillation for a week in anxiety and fear conditioned memory, the possibility that 1-week-DD cycle is not sufficient for alternation in those behaviors can not be excluded. Therefore, I tried to reveal sustained effects of disrupted circadian variation of GC in those behaviors using long-term DD condition for a month (Fig. 21A). A-BMKD transgenic mice maintained a weak rhythm of plasma GC, showing lower peak level at CT12 than in control animals

until one month after the beginning of a DD cycle (WT, 124.81 ± 21.07 vs. TG, 63.07 ± 12.77 ng/mL at CT12; $p < 0.05$; n = 4 for each group; Fig. 21B). Also, numbers of new neurons co-immunostained with BrdU and DCX in the DG were still reduced in transgenic mice (WT, 13.69 ± 0.94 vs. TG, 9.63 ± 1.17 of cell numbers; $p < 0.01$; n = 4 for each group; Fig. 21C). There was no evidently accelerated reduction in neurogenesis by long-term DD exposure compared to its levels under 1-week-DD condition in both groups (Fig. 18). Instead, transgenic mice showed significantly increased anxiety levels in the testing of EPM after long-term exposure to attenuated circadian GC oscillation (WT, 9.83 ± 3.03 vs. TG, 22.79 ± 4.63% in open arms during last 5 min; $p < 0.05$; n = 12 for WT and n = 14 for TG mice; Fig. 21D). Also, sustained hypo-GC production evoked the significantly impaired contextual fear memory (WT, 67.27 ± 4.79 vs. TG, 42.17 ± 5.22% of freezing; $p < 0.01$; n = 11 for WT and n = 10 for TG mice; Fig. 21E) and slightly but not significantly increased auditory fear memory (WT, 60.30 ± 6.41 vs. TG, 75.48 ± 7.34% of freezing; $p = 0.13$; n = 11 for WT and n = 10 for TG mice; Fig. 21F). These results suggest that anxiety and fear-conditioned memory are less-sensitive but still reactive to attenuated GC rhythm with extended exposure duration.

Impaired safety memory of transgenic mice after 1-week-DD exposure

As shown above, fear conditioning results from a positive correlation (pairing) of a previously neutral conditioned stimulus (CS; context, tone) and an aversive unconditioned stimulus (US; electric foot shock). During safety conditioning, by contrast, a CS that is negatively correlated (explicitly

unpaired) with an aversive US becomes a positive signal (predictor) for safety and reduces the expression of conditioned fear (Cándido et al., 2004). Recently, it is revealed that safety memory promotes the cell proliferation in the DG, while such effect of safety memory is abolished in mice with ablated hippocampal neurogenesis (Pollak et al., 2008). Considering such dependency of safety memory on neurogenesis, I observed safety learning in A-BMKD transgenic mice which showed the attenuation in hippocampal neurogenesis under DD cycles for a week compared to. Safety conditioning is carried out over 3 days, one session per day, and comprises a simple conditioned inhibition of fear paradigm consisting of several explicitly unpaired presentations of the aversive US and the tone CS (Fig. 22A). After safety training, freezing response to the experimental context in the presence of the CS was significantly reduced in safety-trained mice regardless of the genotype ($F_{(1,62)} = 0.78$, $p = 0.38$ for genotype; $F_{(1,62)} = 47.72$, $p < 0.01$ for CS; $F_{(1,62)} = 0.011$, $p = 0.92$ for interaction; Fig. 22B) and the degree of changed freezing levels of both groups were almost same in normal LD cycles (WT, -19.43 ± 3.22 vs. TG, -20.98 ± 2.50 % of freezing; $p = 0.70$; n = 16-17 for each group; Fig. 21C). However, freezing behaviors during CS presentation were less reduced in transgenic mice compared to wildtype mice under DD condition ($F_{(1,62)} = 0.84$, $p = 0.36$ for genotype; $F_{(1,62)} = 22.72$, $p < 0.01$ for CS; $F_{(1,62)} = 3.16$, $p = 0.08$ for interaction; Fig. 22D; WT, -28.24 ± 2.41 vs. TG, -12.89 ± 2.65% of freezing; $p < 0.01$; n = 16-17 for each group; Fig. 22E), indicating that safety memory was impaired in transgenic mice only when their circadian amplitude of GC was reduced. Normal contextual and auditory fear conditioned memory of transgenic mice under 1-week-DD condition shown

in Fig. 20 can exclude the possibilities of abnormal encoding of contextual information or hearing problems in these mice, respectively. Moreover, safety memory seems to be more sensitive to decline in hippocampal neurogenesis evoked by attenuated circadian GC rhythm under 1-week-DD condition than fear conditioned memory, which was impaired only after exposure to long-term DD cycles for a month.

Stress reactivity of A-BMKD transgenic mice

The hippocampus provides negative control of the HPA axis with highly expressed GR. Recently, it has been reported that a small subset of newborn neurons within the DG are critical for hippocampal negative control of the HPA axis (Snyder et al., 2011). I therefore examined plasma levels of GC after stress in conditions that activate the HPA axis in transgenic mice bearing reduced neurogenesis in the DG. To test the response to, and recovery from, a stressor, I subjected mice to 30 min of restraint stress at CT6 after DD cycles for a week and measured plasma GC immediately (CT6.5), 60 (CT7.5) or 150 min (CT9) later (Fig. 23A). To exclude masking effects of diurnal GC decrease on stress-evoked plasma GC levels, mice were given a restraint stressor at CT6, a rising period from circadian GC rhythm. I also examined plasma GC levels in non-stressed animals at CT9, the same time point with 150 min after stress in stressed mice, considering circadian elevation of GC independent from stress-induced HPA axis activation. Similar with our previous report (Son et al., 2008), plasma GC levels evoked by a 30-min immobilization were not significantly changed in transgenic mice despite impaired circadian profiles

in these mice. While wildtype mice showing prolonged GC elevation until 150 min after stressor, plasma GC almost reached to basal levels 60 min after stress, suggesting faster terminated action of HPA axis by stress in TG animals ($F_{(1,37)} = 10.76$, $p < 0.01$ for genotype; $F_{(1,37)} = 25.10$, $p < 0.01$ for time after stress; Fig. 23B). Non-stressed mice of both genotypes did not show significant GC elevation at CT9 compared to that of stressed WT mice, ruling out the effect of circadian fluctuation of GC secretion on the accelerated termination of stress-elicited HPA axis action in TG mice.

Translocation of GR into the nucleus of hippocampal cells

The faster shutting off stress reactivity in transgenic mice is contrast to prolonged elevated GC levels after stress in mice with complete loss of GR in the forebrain (Furay et al., 2008). Therefore, I hypothesized that the expression or dynamics of GR in the hippocampus might be altered, which accelerates negative feedback of GC in A-BMKD transgenic mice. To verify the hypothesis, I examined both GR expression in whole cell lysates and its nuclear translocation occurring by receptor activation after binding to GC from hippocampal tissues. In Fig. 24, the result shows that the nuclear translocation of GR is tightly regulated by GC in the hippocampus of WT mice. Thus, levels of nuclear GR were low at CT6 when GC levels are low. By contrast, at CT12 associated with increased GC levels, GR largely translocated into the nuclei. Also, this phenomenon is reversible and nuclear GR was promptly decreased following the decrease in GC levels. These observations support previous studies that GR in the hippocampus also follow a rhythm with increased GR occupancy during periods of

elevated GC levels as GC secretion follows a circadian rhythm (Reul and de Kloet, 1985; Spencer et al., 1993; Kitchener et al., 2004). Although there is no significant change of GR levels in whole cell lysates, nuclear GR in the hippocampus of transgenic mice were higher than those of wildtype mice especially at CT 12, showing more robust oscillation pattern of GR translocation (WT, 3.17 ± 0.53 vs. TG, 5.50 ± 0.56 of A.U.; $p < 0.05$; n = 3 for each group; Fig. 24). From the result, it is likely that a specific mechanism exists in the hippocampus compensated for weakening rhythms of GC in circulation, eliciting hyperactive states of GR.

Rescue of impaired neurogenesis in the DG by rhythmic GC supply

It was reported that nocturnal corticosterone (CORT; an endogenous rodent GC) intake by drinking water supplemented with CORT restored a daily pattern of plasma CORT concentration in ADX rats close to the sham group (Fig. 25A; Malek et al., 2007). By using the CORT supply in drinking water, I tried to recover daily profile of GC concentrations in A-BMKD transgenic mice with increasing levels at CT12 close to the wildtype group. For the proper concentration of CORT that increases plasma GC in transgenic mice enough but maintains GC levels in wildtype mice, I used the dose with 3 mg/L of CORT in drinking water (Fig. 25A). Mice were fed CORT containing water under DD cycles for 7 days after a day in LD for adaptation to CORT-containing water. These mice were injected with BrdU and sacrificed to detect the effect of CORT on neurogenesis, which was quantified in the hippocampus with immunohistochemistry. Compared with vehicle group, newborn neurons in the hippocampus were increased in

transgenic mice fed with CORT containing water ($F_{(1,362)} = 1813$, $p < 0.01$ for genotype; $F_{(1,362)} = 736.9$, $p < 0.01$ for CORT treatment; $F_{(1,362)} = 415.8$, $p < 0.01$ for interaction; Fig. 25B), suggesting that attenuated daily variation of GC is the direct cause for reduced neurogenic activity in the DG.

Neurogenesis levels in the subventricular zone (SVZ) of transgenic mice

Adult mammalian brain creates new neurons not only in the DG of the hippocampus, but also in the SVZ (Fig. 26A). Suppression of GC secretion after ADX increases births of neurons in the DG (Cameron and Gould, 1994; Gould et al., 1992), whereas mitotic activity in the SVZ remains unchanged (Rodriguez et al., 1998), suggesting a site-specific inhibitory influence of GC. In accordance, neurogenesis levels in the SVZ were not affected not only by genotype, but also by rhythmic CORT supplement ($F_{(1,44)} = 0.62$, $p = 0.44$ for genotype; $F_{(1,44)} = 0.074$, $p = 0.79$ for photoperiod; Fig. 26B). From these results, it seems that neurogenesis in the hippocampus, not in the SVZ, is sensitive to GC rhythms as well as its levels.

Expression profiles of GC-responsive genes in the hippocampus

Given that I had observed the restorative effect of rhythmic CORT uptake on neurogenesis in the hippocampus of A-BMKD transgenic mice (Fig. 25B), I next wanted to further explore the mechanism responsible for the change in neurogenic action regulated by circadian GC rhythm. To this

end, I used a candidate-based approach to assess whether there were changes in the expression of genes previously linked to GC signaling in the hippocampus (Schaaf et al., 1997; Datson et al., 2001, 2012; Revest et al., 2005; Sarabdjitsingh et al., 2009). First of all, I selected BDNF and its receptor TrkB as target genes responsible for the observed impairment in hippocampal neurogenesis, considering that they regulate the proliferation in the DG and that their expression is modulated by GC. To measure gene expression of those genes in the hippocampus, I conducted real-time PCR with cDNA samples from hippocampal tissues of vehicle or CORT-fed mice sacrificed at CT12 after 1-week-DD exposure, when the peak level of GC is attenuated in the transgenic mice. As revealed in Fig. 27, exposure to reduced diurnal GC rhythm decreased gene expressions of BDNF and TrkB in the hippocampus of vehicle-treated TG mice. Interestingly, rhythmic CORT supplement significantly increased the mRNA levels of those genes in the hippocampus of transgenic mice, but not in that of control animals. The hippocampal BDNF and TrkB mRNA levels of the transgenic mice that received CORT-containing water were comparable with those seen in the control mice (BDNF: WT-VEH, 1.012 ± 0.044 vs. TG-VEH, 0.78 ± 0.041; $p < 0.01$ between two groups; WT-CORT, 0.94 ± 0.084 vs. TG-CORT, 1.013 ± 0.093 of A.U.; $p < 0.05$ vs. vehicle-treated group; TrkB: WT-VEH, 0.98 ± 0.065 vs. TG-VEH, 0.79 ± 0.025 ; $p < 0.01$ between two groups; WT-CORT, 0.90 ± 0.052 vs. TG-CORT, 1.029 ± 0.094 of A.U.; $p < 0.01$ vs. vehicle-treated group; n = 6-16 for each group). These results suggest that BDNF and its action via TrkB can be suggested as mediators for neurogenic activity regulated by diurnal GC oscillation, which was impaired under DD condition but restored by rhythm GC supply in transgenic mice.

I also measure mRNA levels of other genes which are known to be responsive to GC and function as transcription factor (CBP, CREB-binding protein; Egr1, Early growth response protein 1), protein kinase (AK2, Adenylate kinase 2; SGK1, serum-and glucocorticoid-induced protein kinase 1) or signaling molecules (CHN1, N-Chimerin 1; Dexras1, dexamethasone-induced Ras-related protein 1) (Schaaf et al., 1997; Datson et al., 2001, 2012; Revest et al., 2005; Sarabdjitsingh et al., 2009). Rhythmic GC supplementation elicited the restoration of reduced expression of CBP and AK2 genes in hippocampus of transgenic mice showing attenuated GC rhythm. These similar gene expression patterns with those seen in BDNF and TrkB genes suggest that CBP and AK2 are also involved with the effect of circadian GC rhythm in hippocampal neurogenesis (CBP: WT-VEH, 1.00 ± 0.050 vs. TG-VEH, 0.67 ± 0.059 ; p < 0.01 between two groups; WT-CORT, 0.85 ± 0.12 vs. TG-CORT, 1.089 ± 0.16 of A.U.; p < 0.05 vs. vehicle-treated group; AK2: WT-VEH, 1.035 ± 0.073 vs. TG-VEH, 0.68± 0.033; p < 0.01 between two groups; WT-CORT, 0.84 ± 0.11 vs. TG-CORT, 0.98 ± 0.15 of A.U.; p < 0.05 vs. vehicle-treated group; n = 6-16 for each group). Hippocampal mRNA expression of other genes does not seem to be affected by altered circadian GC oscillation, except SGK1 whose expression in the hippocampus was downregulated at CT12, but not recovered by rhythmic GC intake in transgenic mice (SGK1: WT-VEH, 1.05 ± 0.071 vs. TG-VEH, 0.78 ± 0.038; p < 0.01 between two groups; n = 6-16 for each group). These results implicate that unknown factor is required to recover SGK1 expression levels in the hippocampus of A-BMKD transgenic mice. Therefore, further studies are required to solve the precise link between circadian GC oscillation and hippocampal gene

expression for regulating neurogenesis in the DG.

Figure 16. A schematic diagram of the A-BMKD transgenic mouse. Cyclic expression of steroidogenic acute regulatory protein (StAR) is directly controlled by CLOCK:BMAL1 heterodimer, and the resulting daily oscillation in steroidogenesis contributes to the generation of a robust GC rhythm. A part of the BMAL1 coding region was expressed in an antisense orientation under adrenal-specific control by using ACTH receptor (MC2R) promoter in adrenal-specific BMAL1 knockdown (A-BMKD) transgenic mouse. The adrenal StAR rhythm is completely abolished and the plasma GC oscillation also is strongly attenuated in transngenic mice under dark/dark (DD) condition.

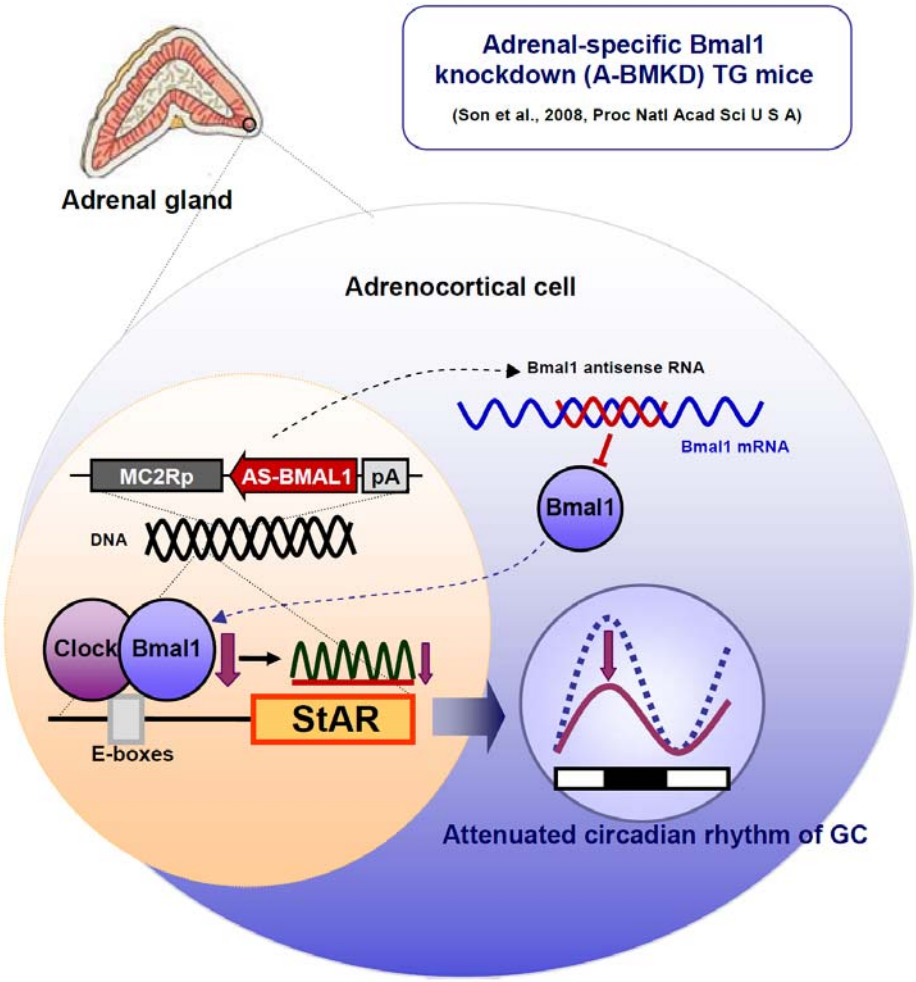
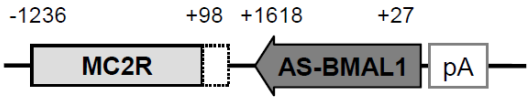
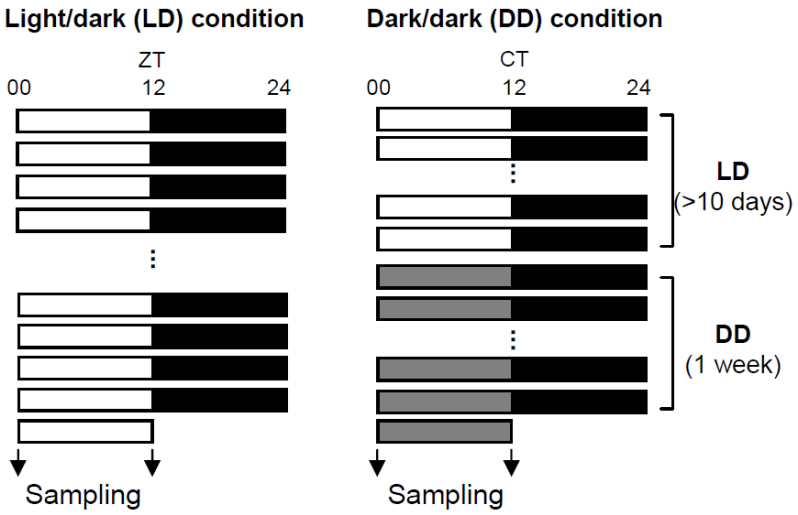

Adrenal-specific Bmal1 knockdown (A-BMKD) TG mice
(Son et al., 2008, Proc Natl Acad Sci U S A)
Adrenal gland
Adrenocortical cell
Bmal1 antisense RNA
Bmal1 mRNA
MC2Rp
AS-BMAL1
pA
DNA
Bmal1
Clock
Bmal1
StAR
E-boxes
Attenuated circadian rhythm of GC

Figure 17. Attenuated circadian GC rhythm in A-BMKD transgenic mice. (A) A schematic diagram of the transgene for the A-BMKD mice. (B) Experimental scheme for the preparation of samples. Wild type (WT) and transgenic (TG) mice housed under 12-hr of LD photoperiod (B) or DD condition (C; 6-7 days after light-off) were sacrificed at ZT or CT 00 or 12 hrs. Plasma GC levels were measured by RIA and expressed as means ± SEM (n = 4-5 for LD and 5-9 for DD; **: $p < 0.01$ between WT and TG).

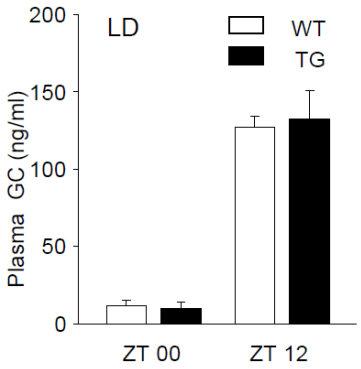
A.


-1236
+98
+1618
+27
MC2R
AS-BMAL1
pA

B.


Light/dark (LD) condition
ZT
00
12
24
Sampling
Dark/dark (DD) condition
CT
00
12
24
LD
(>10 days)
DD
(1 week)
Sampling

C.


LD
WT
TG
Plasma GC (ng/ml)
200
150
100
50
0
ZT 00
ZT 12

D.

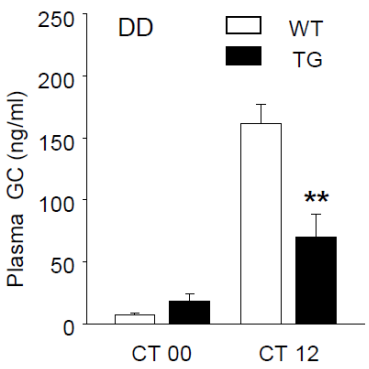

DD
WT
TG
Plasma GC (ng/ml)
250
200
150
100
50
0
**
CT 00
CT 12

Figure 18. The number of newborn neurons in the DG of hippocampus. (A) The diagram shows subregions such as DG (dentate gyrus), CA1 and CA3 of the hippocampus. (B) Immunofluorescence for the doublecortin (DCX) (green) and BrdU (red) was stained in the DG of wildtype and transgenic mice. Nuclei were counterstained with DAPI (blue). (B) Quantification of BrdU+/DCX+ cells expressed as means ± SEM (n = 5 for each group; **: $p < 0.01$ between WT and TG).

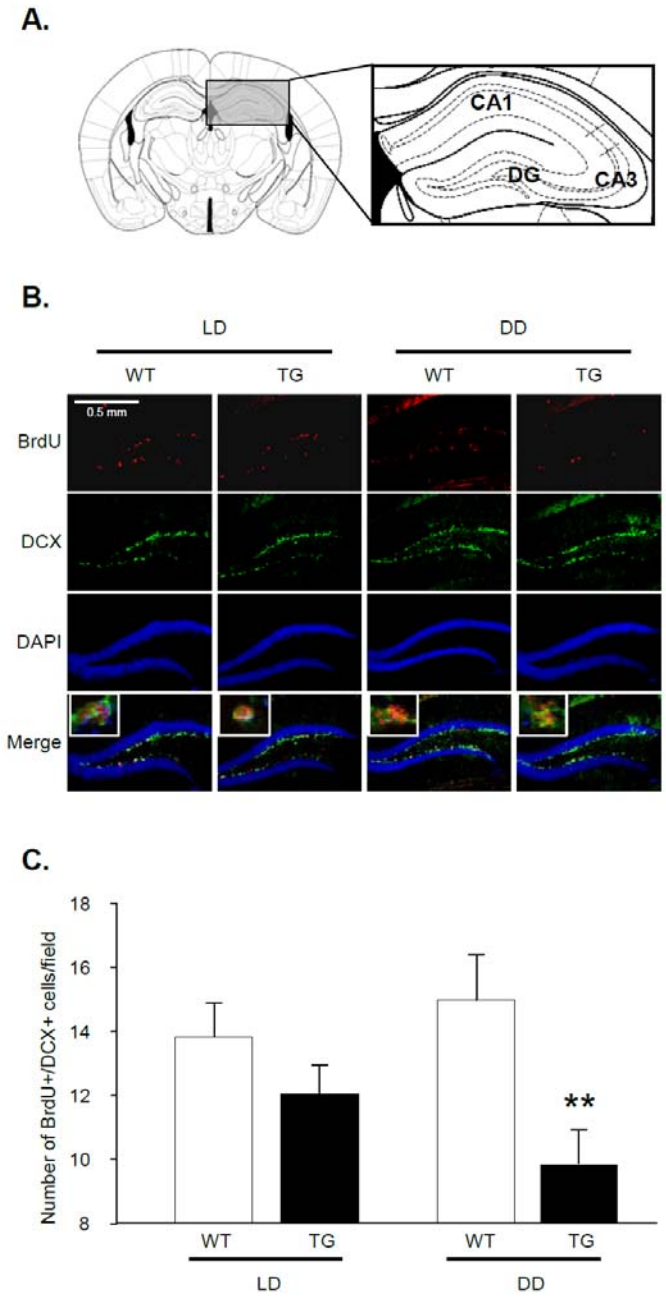

A.
CA1
DG
CA3
B.
LD
DD
WT
TG
WT
TG
0.5 mm
BrdU
DCX
DAPI
Merge
C.
Number of BrdU+/DCX+ cells/field
18
16
14
12
10
8
**
WT
TG
WT
TG
LD
DD

Figure 19. Depression-like and anxiety behaviors. Depression-like behavior was measured through forced swim test (A) in normal LD (B) or DD (C) conditions. The percentage of immobility time was measured and expressed as means ± SEM (n = 9-10 for each group; **: $p < 0.01$ between WT and TG). With elevated plus maze test (EPM; D), anxiety levels were expressed as the percentage of time spent in open arms during 10 min-testing under LD (E) and DD (F) conditions (n = 11 for WT and n = 13 for TG under LD condition; n = 9 for WT and n = 8 under DD condition).

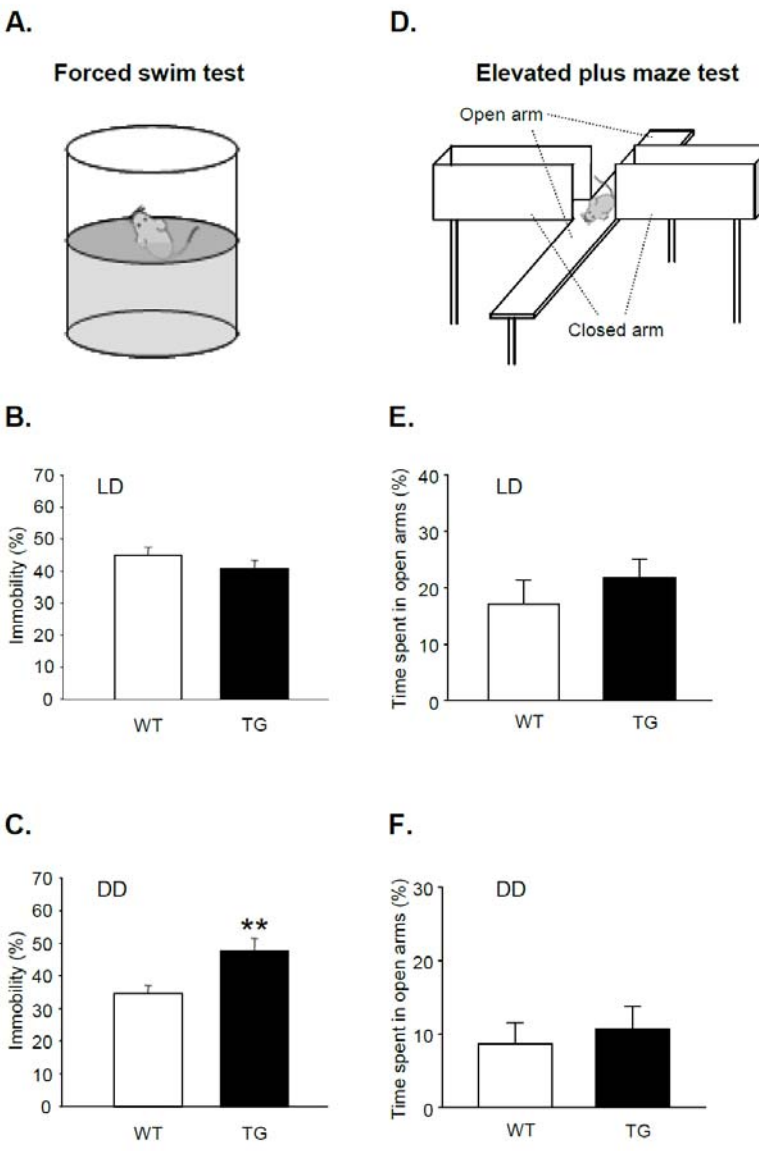

A.
Forced swim test
D.
Elevated plus maze test
Open arm
Closed arm
B.
LD
Immobility (%)
70
60
50
40
30
20
10
0
WT
TG
E.
LD
Time spent in open arms (%)
40
30
20
10
0
WT
TG
C.
DD
**
Immobility (%)
70
60
50
40
30
20
10
0
WT
TG
F.
DD
Time spent in open arms (%)
30
20
10
0
WT
TG

Figure 20. Fear memory in A-BMKD transgenic mice. Fear memory acquisition curves represented as freezing percent during three repetitions of tone and foot-shock pairing (A, D). Fear memory was measured using both contextual (B, E) and auditory fear (C, F) conditioning paradigm. (n = 9 for each group under LD condition; n =10 for each group under DD condition).

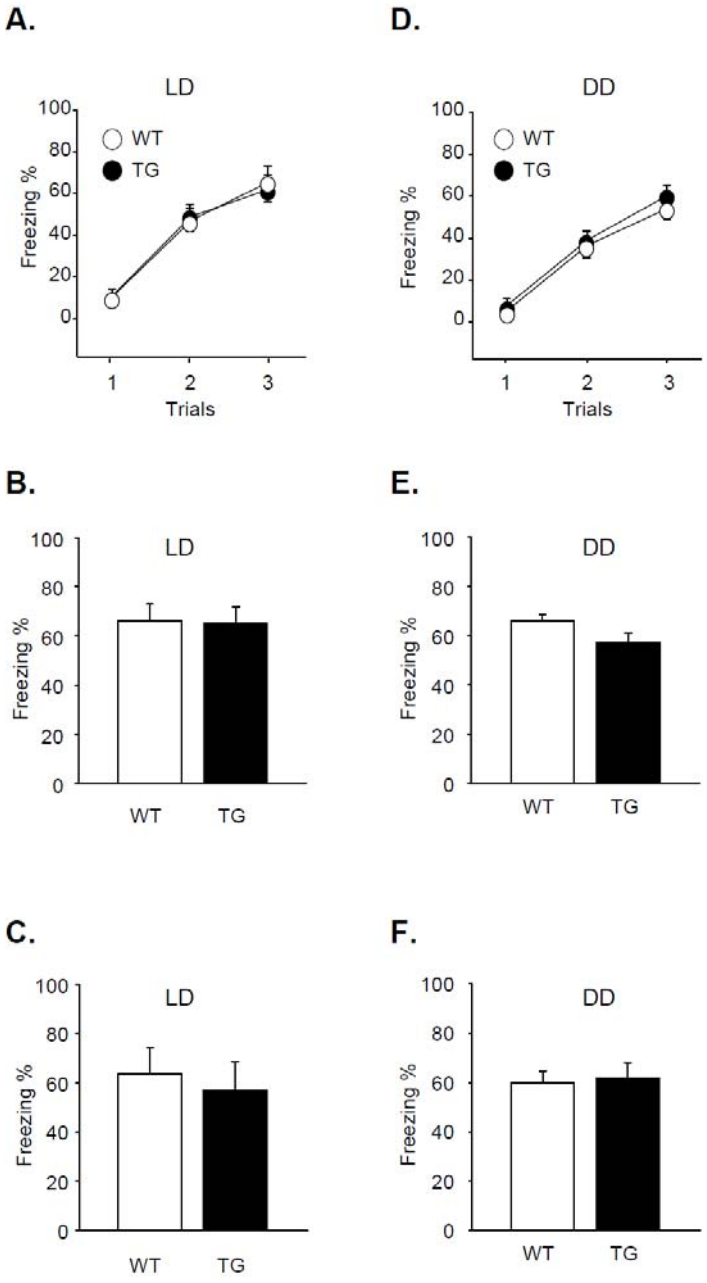

A.
LD
WT
TG
Freezing %
Trials
D.
DD
B.
E.
C.
F.

Figure 21. Impairments both in anxiety and fear-conditioned memory under long-term DD condition for a month. (A) Experimental scheme for the extended DD condition until one month. Wildtype and transgenic mice housed under long-term DD condition were sacrificed at CT 00 or 12 hrs for plasma GC measurement. (B) Plasma GC levels were measured by RIA and expressed as means ± SEM (n = 4 for each group; *: $p < 0.05$ between WT and TG). (C) Quantification of BrdU+/DCX+ cells expressed in the DG as means ± SEM (n = 4 for each group; **: $p < 0.01$ between WT and TG). Anxious behavior and conditioned fear memory were measured through elevated plus maze (D; n = 12-14 for each group; *: $p < 0.05$ between WT and TG), contextual fear conditioning paradigm (E; n = 10-11 for each group; *: $p < 0.01$ between WT and TG) and auditory fear conditioning paradigm (F; n = 10-11 for each group), respectively.

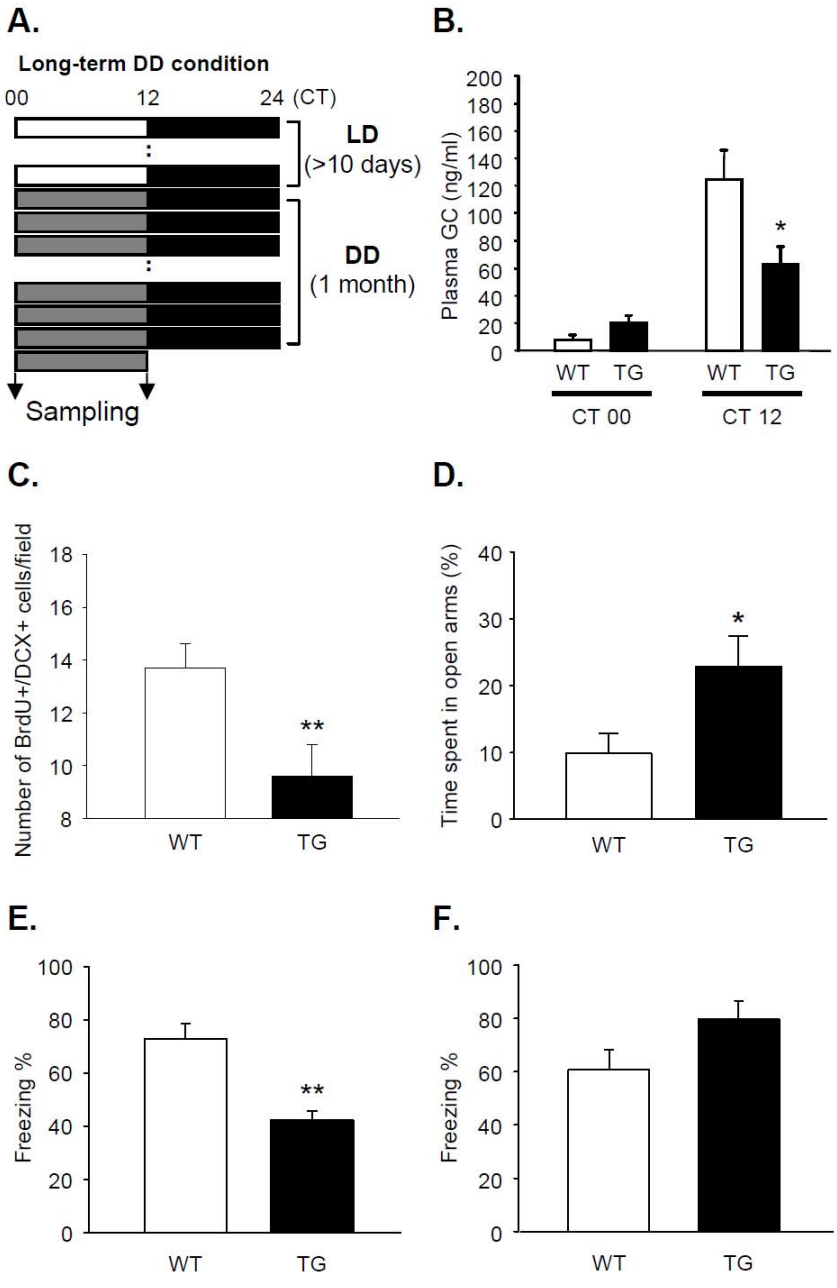

A.
Long-term DD condition
00
12
24 (CT)
LD
(>10 days)
DD
(1 month)
Sampling
B.
Plasma GC (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
*
WT
TG
WT
TG
CT 00
CT 12
C.
Number of BrdU+/DCX+ cells/field
18
16
14
12
10
8
**
WT
TG
D.
Time spent in open arms (%)
40
30
20
10
0
*
WT
TG
E.
Freezing %
100
80
60
40
20
0
**
WT
TG
F.
Freezing %
100
80
60
40
20
0
WT
TG

Figure 22. Impaired safety memory in A-BMKD transgenic mice. Hippocampal neurogenesis-related memory was measured through safety learning paradigm (A) in normal photoperiod (B, C) or constant dark (D, E) conditions. The percentage of freezing was measured before (preCS) and during conditioned stimulus (CS; safety tone) exposure. The percentage changes of freezing levels are expressed as means ± SEM (n = 16-17 for each group; *: $p < 0.05$ and **: $p < 0.01$ between WT and TG).

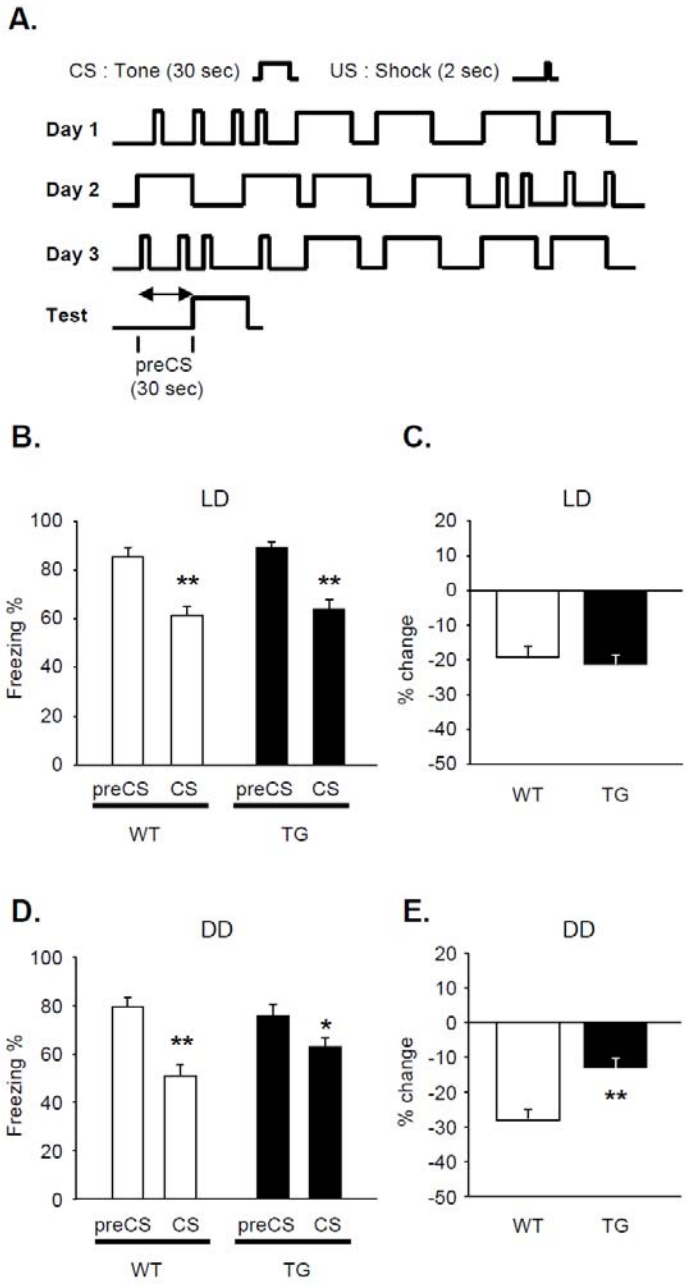
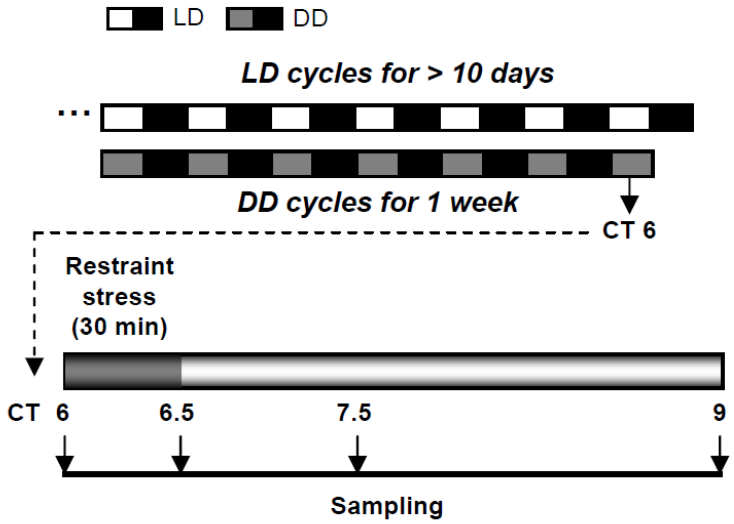

A.
CS : Tone (30 sec)
US : Shock (2 sec)
Day 1
Day 2
Day 3
Test
preCS
(30 sec)
B.
LD
Freezing %
100
80
60
40
20
0
**
**
preCS
CS
preCS
CS
WT
TG
C.
LD
% change
20
10
0
-10
-20
-30
-40
-50
WT
TG
D.
DD
Freezing %
100
80
60
40
20
0
**
*
preCS
CS
preCS
CS
WT
TG
E.
DD
% change
20
10
0
-10
-20
-30
-40
-50
**
WT
TG

Figure 23. Stress reactivity of A-BMKD transgenic mice. (A) An experimental scheme for stress reactivity using restraint stress during 30 min. Wildtype and transgenic mice housed under DD conditions for 7 days were given a stressor at CT 6 and killed according to indicated time points. (B) Elevated plasma GC response to restraint stress was rapidly recovered to basal levels in transgenic mice compared to WT mice (n = 4-7 for each group; *: $p < 0.05$ between WT and TG at the same time point).

A.


LD
DD
LD cycles for > 10 days
DD cycles for 1 week
CT 6
Restraint
stress
(30 min)
CT 6
6.5
7.5
9
Sampling

B.

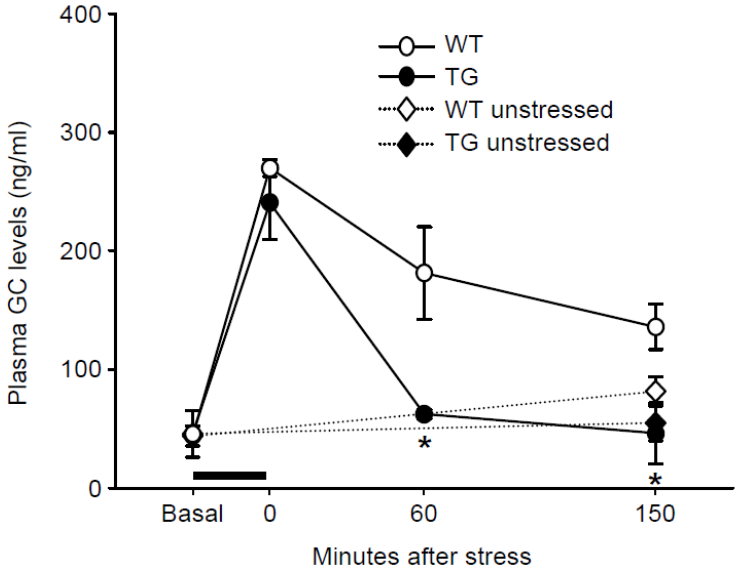

400
300
200
100
0
Plasma GC levels (ng/ml)
WT
TG
WT unstressed
TG unstressed
*
*
Basal
0
60
150
Minutes after stress

Figure 24. GR nuclear translocation states in hippocampus of A-BMKD transgenic mice. (A) Whole cells and nuclear extracts from hippocampus were analyzed by western blotting at CT 6, 12, 18, and 24 h after DD conditions for 7 days. Each sample was prepared by pooling 3-4 animals. Actin and lamin B were used as an internal control to ensure a similar loading amount. (B) Protein bands on the corresponding X-ray films were quantified by densitometry. Relative GR levels are presented as the means ± SEM of R.O.D., where the mean value of WT at CT24 is set as 1 (n = 3 for each group; *: $p < 0.05$ between WT and TG). The R.O.D. for each GR band was normalized to the corresponding actin or lamin B band.

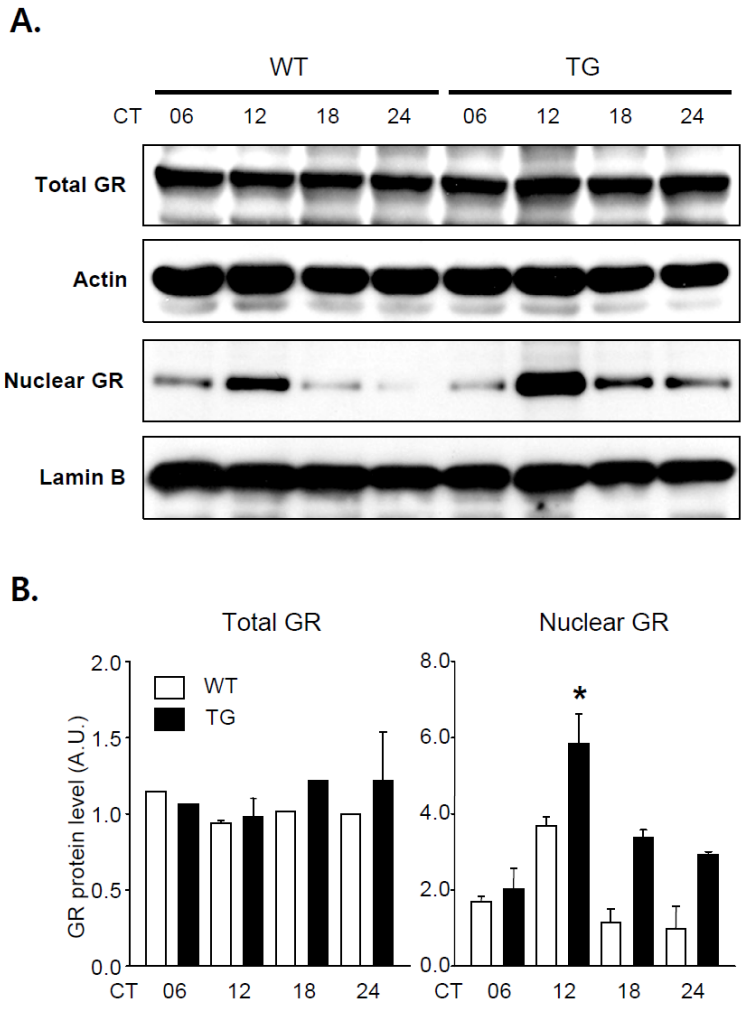
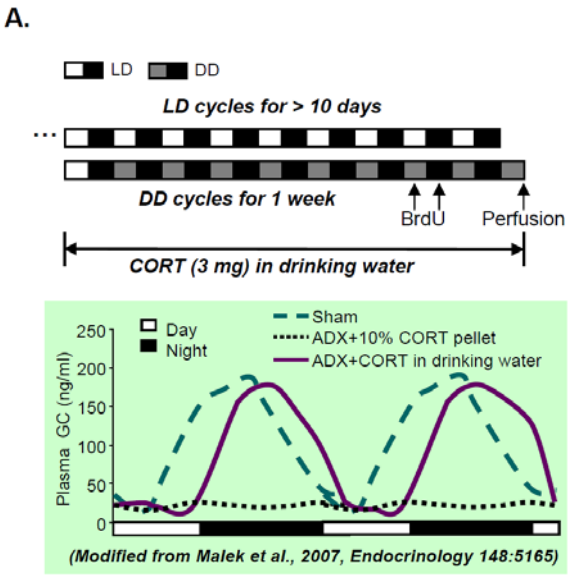

A.
WT
TG
CT 06 12 18 24 06 12 18 24
Total GR
Actin
Nuclear GR
Lamin B
B.
Total GR
Nuclear GR
WT
TG
GR protein level (A.U.)
2.0
1.5
1.0
0.5
0.0
8.0
6.0
4.0
2.0
0.0
*
CT 06 12 18 24
CT 06 12 18 24

Figure 25. Hippocampal neurogenesis with corticosterone (CORT) supply in drinking water. (A) Experimental scheme for CORT (3 mg/L) supply in drinking water under DD condition. (B) Numbers of newborn neurons in the hippocampus with CORT supply (n = 5-7 for each group; **: $p < 0.01$ between WT and TG, ††: $p < 0.01$ vs. vehicle-treated group).


A.
LD
DD
LD cycles for > 10 days
DD cycles for 1 week
BrdU
Perfusion
CORT (3 mg) in drinking water
Day
Night
Sham
ADX+10% CORT pellet
ADX+CORT in drinking water
Plasma GC (ng/ml)
250
200
150
100
50
0
(Modified from Malek et al., 2007, Endocrinology 148:5165)

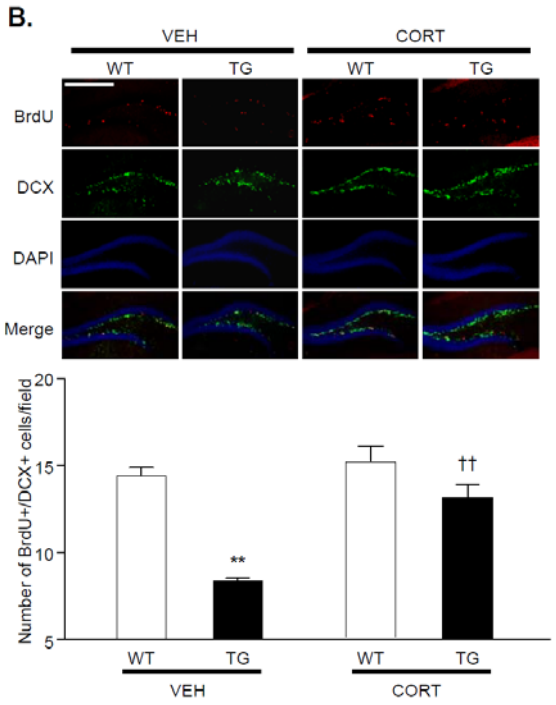
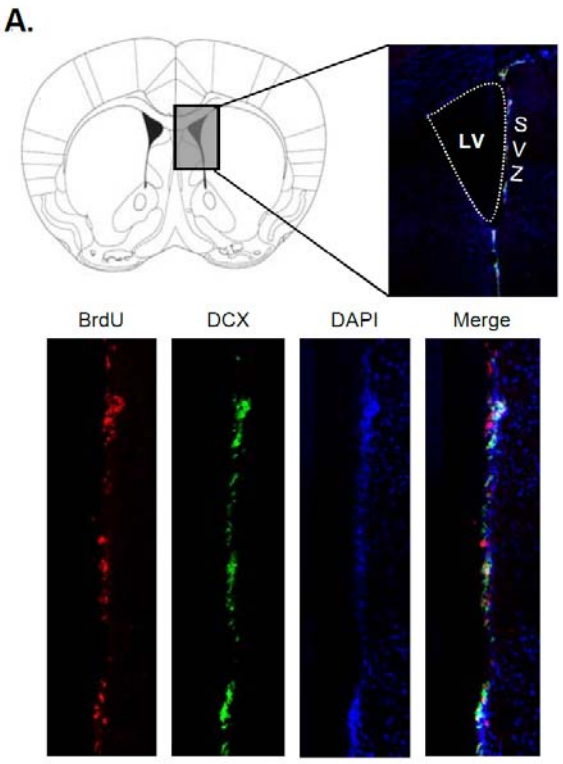

B.
VEH
CORT
WT
TG
WT
TG
BrdU
DCX
DAPI
Merge
Number of BrdU+/DCX+ cells/field
20
15
10
5
**
††
WT
TG
WT
TG
VEH
CORT

Figure 26. Neurogenesis levels in the subventricular zone (SVZ). (A) The diagram shows the subventricular zone (SVZ) and laternal ventricle (LV). Immunofluorescence for the doublecortin (DCX) (green) and BrdU (red) was stained in the SVZ of wildtype and transgenic mice. Nuclei were counterstained with DAPI (blue). (B) Numbers of newborn neurons in the SVZ with CORT supply.


A.
LV
S
V
Z
BrdU
DCX
DAPI
Merge

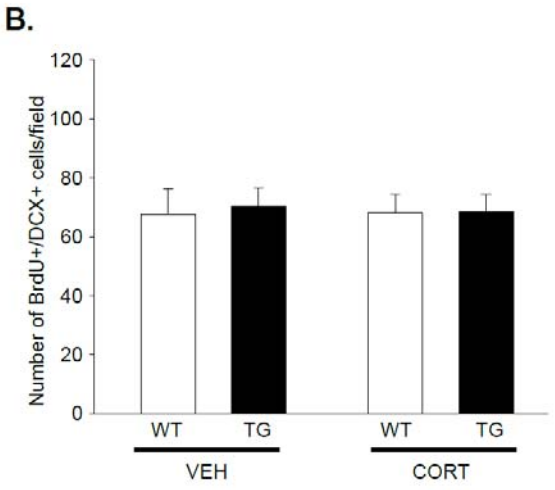

B.
Number of BrdU+/DCX+ cells/field
120
100
80
60
40
20
0
WT
TG
WT
TG
VEH
CORT

Figure 27. Gene expression profiles of GC-responsive genes in the hippocampus of A-BMKD transgenic mice. Wildtype and transgenic mice housed under DD conditions supplied with CORT-containing water for a week were killed at CT12. mRNA expression profiles were obtained by real-time RT-PCR in the hippocampus (CBP, CREB-binding protein; Egr1, Early growth response protein 1; AK2, Adenylate kinase 2; SGK1, serum- and glucocorticoid-induced protein kinase; CHN1, N-Chimerin 1; Dexras1, dexamethasone-induced Ras-related protein 1). Data are normalized with GAPDH and expressed as means ± SE of A.U., where the mean vehicle-treated WT value is defined as 1 (n = 6-16 for each group, **: $p < 0.01$ between WT and TG, †: $p < 0.05$ and ††: $p < 0.01$ vs. vehicle-treated group).

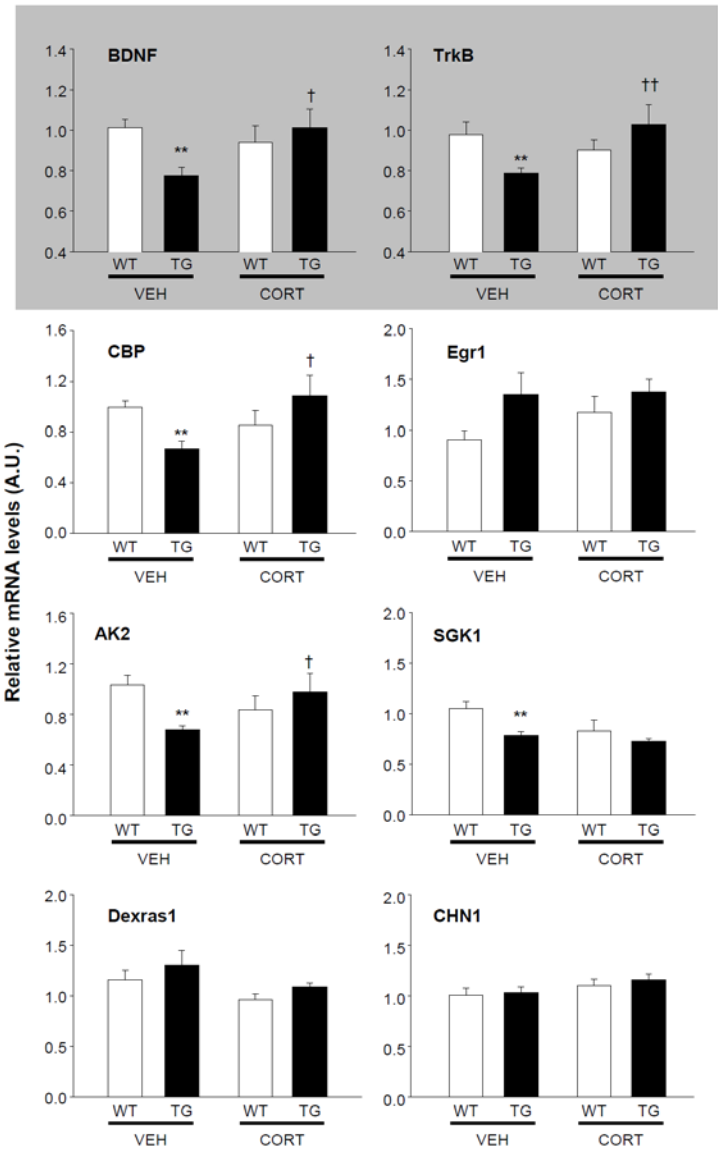
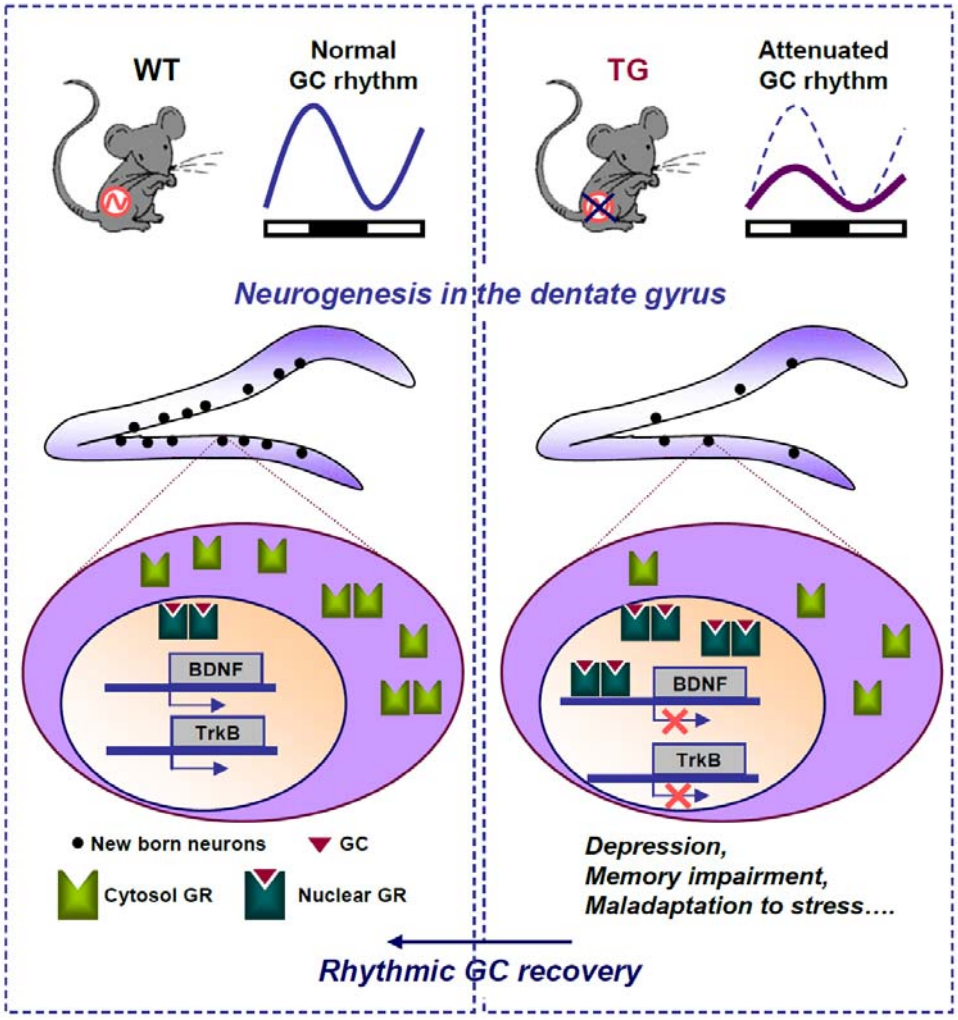

BDNF
TrkB
CBP
Egr1
AK2
SGK1
Dexras1
CHN1
Relative mRNA levels (A.U.)
WT
TG
VEH
CORT
**
†
††

Figure 28. A schematic diagram of hippocampal neurogenesis regulated by circadian GC rhythm via BDNF-related signaling cascades. As revealed in the A-BMKD transgenic mice, attenuated GC rhythm produces impairment in hippocampal neurogenesis, probably due to reduced BDNF and TrkB expression by hyperactive GR in the hippocampus. A restored GC rhythm rescued neurogenic activity as well as both gene expressions in the hippocampus. These results suggest that the daily oscillation of GC plays significant role in pathophysiology of neurogenesis-related diseases such as depression.


WT
Normal
GC rhythm
TG
Attenuated
GC rhythm
Neurogenesis in the dentate gyrus
BDNF
TrkB
BDNF
TrkB
New born neurons
GC
Cytosol GR
Nuclear GR
Depression,
Memory impairment,
Maladaptation to stress….
Rhythmic GC recovery

DISCUSSION

In this chapter, I tried to elucidate the effect of circadian GC release on the adult neurogenesis in the DG. For this purpose, I used A-BMKD transgenic mouse showing attenuated diurnal rhythm of GC under dark/dark (DD) condition for a week (Son et al., 2008). Indeed, transgenic mice exposed to 1-week-DD cycles exhibited impairments in hippocampal neurogenesis accompanied with depressive behaviors and altered gene expression of BDNF and TrkB in the hippocampus. Moreover, reduced numbers of newborn neurons as well as BDNF transcripts in the hippocampus were rescued by rhythmic GC supplementation, indicating that attenuated daily variation of GC itself impairs hippocampal neurogenesis via BDNF-related cascades. Physiologically, these results suggest that hippocampal neurogenesis implicating mood disorders with regard to the pathogenesis of major depression can be regulated by not only the GC levels but also its circadian profiles, presenting a new therapeutic perspective.

The number of new neurons co-labeled with BrdU and DCX in the DG is reduced in A-BMKD transgenic mice only when they show a reduced daily variation of GC under DD cycles (Figs. 18 and 21C). The decreased cell proliferation in the DG has been shown to produce changes in mood regulation and cognitive tasks (Shors et al., 2001; Garthe et al., 2009). In accordance with this finding, transgenic mice display depressive states under unescapable situations, although they exhibit normal anxiety levels (Fig. 19). Likewise, safety memory, which requires newborn neurons generated in the hippocampus (Pollak et al., 2008), is impaired in

transgenic mice under DD condition (Fig. 22). By contrast, these mice show normal contextual fear memory (Fig. 20), which also has been reported to be decreased by impaired neurogenesis in the DG (Winocur et al., 2006; Saxe et al., 2006). These findings raise the possibility that the hippocampus containing declined numbers of newborn neurons by attenuated circadian GC secretion can maintain the ability to encode contextual information, but can not separate contextual information involved with emotional cues such as safety. Therefore, depression-like behavior or safety memory is thought to be more sensitive to neurogenesis levels in the hippocampus regulated by GC rhythm, compared to anxiety or contextual fear memory respectively, suggesting that newborn neurons permit selected changes in brain functions. However, contextual fear memory and anxiety eventually become vulnerable to long-term exposure to hypo-rhythmicity of GC with extended DD cycles up to one month (Figs. 22D and E). From the results, it is possible that certain degree of new neuron maturation rather than neuronal generation is critical for those behaviors. Moreover, involvement of other factors more than hippocampal neurogenesis in those time-dependent impairments should not be excluded. One of the probable candidates responsible for the observation is the amygdala implicated both in fear conditioned memory and in anxious behavior. Therefore, prolonged effects of attenuated circadian GC rhythm not only on hippocampal neurogenesis but also on other discrete brain regions should be scrutinized in the future.

Recently, new neurons in the hippocampus also play an important role in stress regulatory functions associated with negative feedback of the HPA axis (Snyder et al., 2011). Elevated GC by stress is recovered to basal condition rapidly in A-BMKD transgenic mice subjected to 1-week-DD

cycles, suggesting the effect of reduced hippocampal neurognesis on inappropriate stress reactivity. The accelerated shut-off stress responsiveness can also be explained by the hyperactive GR states in the hippocampus of transgenic mice (Fig. 23), regarding the role of hippocampal GR for negative feedback of HPA axis. Moreover, increased nuclear GR in the hippocampus of neurogenesis-impaired transgenic mice is notable in that GR is important for GC-exerted neurogenesis regulation. For example, treatments with the GR antagonist and agonist normalize the GC-induced reductions and ADX-induced increments in hippocampal neurogenesis, respectively (Montaron et al., 2003; Crochemore et al., 2005; Oomen et al., 2007). Considering such a negative control of GR in neurogenesis, enhanced GR activity in the hippocampus for producing hypo-GC with attenuated rhythm can be one of the underlying mechanisms for the reduced neurogenesis in transgenic mice. However, a target molecule responsible for such opposite phenomenon with increased GR activity against decreased ligand secretion at its circadian peak time is still elusive.

GC exerts its effects classically on the transcription of various genes via the glucocorticoid response element (GRE) located at the promoters by binding to GR (McEwen, 2007; Fig. 2). BDNF, a important regulator for proliferation in the DG, is one of such GC-responsive genes based on several studies reported that excess GC decreased the expression of BDNF in the brain (Suri and Vaidya, 2012) in addition to the putative GRE sites residing on BDNF promoter (Fuchikami et al., 2008). Implying the role of GR for such GC-regulated BDNF gene expression, GR agonist has been show to suppress BDNF mRNA in neuronal cells, whereas MR agonist

stimulated it (Kino et al., 2010). Therefore, down-regulated gene expressions of BDNF at CT12, when reduced peak level of GC followed by enhanced translocation of GR in hippocampal cells appears, suggest that BDNF is a mediator for decreased hippocampal neurogenic activity in A-BMKD transgenic mice. Moreover, not only neurogenesis levels but also BDNF expression in the hippocampus rescued by rhythmic GC supply also supports that BDNF is the molecular target for adult neurogenesis regulated by circadian rhythm of GC. In addition to the regulation of BDNF mRNA levels, GC impinges on BDNF signaling through modulation of its TrkB receptor. In contrast to the emerging consensus from reports that GC reduces BDNF mRNA, studies about the influence of GC on the TrkB mRNA levels have been far less consistent; excess GC exhibits either no change, or an enhancement in TrkB mRNA, whereas ADX does not appear to change it in the hippocampus (Barbany and Persson, 1993; Schaaf et al., 1997; Vellucci et al., 2001, 2002). In the present study, however, hippocampal TrkB mRNA levels are apparently affected by circadian GC rhythm, showing that the restorative effect by enhanced rhythmicity of GC is robust (Fig. 27). Therefore, BDNF cascades can be suggested as a target of circadian GC variation for regulating neurogenic activity in the hippocampus.

Moreover, I found that rhythmic GC supplementation elicited the restoration of reduced expression of CBP and AK2 genes in hippocampus of transgenic mice. These similar gene expression patterns with those seen in BDNF and TrkB suggest that CBP and AK2 are also involved with the effect of circadian GC rhythm in hippocampal neurogenesis. Actually, binding of BDNF to TrkB receptors initiates signaling pathways including

MAPK that induce phosphorylation of CBP and CREB. Then, phosphorylated CBP and transcription factors such as CREB lead to transcriptional activation (Riccio, 2010). Also, BDNF itself is also known to be one of target genes regulated by CBP (Suri and Vaidya, 2012). The function of CBP is also reported to be increased by the cAMP-PKA cascade initiated from adenylyl cyclase using ATP as substrate. Therefore, adenylate kinase which regulates ATP concentration can contribute CBP-dependent transcription (Koos, 2011). Therefore, downregulation of CBP and AK2 could contribute to the effects of BDNF regulating neurogenesis, which is reduced in the transgenic mice.

Previously, it appears that the circadian rhythm of GC interacts with other signaling pathways in regulating neurogenesis. For example, artificially dampening the circadian GC rhythm prevents a serotonin uptake inhibitor, fluoxetine, from stimulating neurogenesis; however, restoring the GC rhythm also restores the action of fluoxetine used as an antidepressant (Huang and Herbert, 2006). Similarly, another antidepressant, L-NAME, a nitric oxide synthase inhibitor also stimulates hippocampal neurogenesis depending on rhythmic changes of GC (Pinnock et al., 2007). Increasing the possible crosstalk between BDNF and circadian GC variation, stimulating action of L-NAME on BDNF as well as on neurogenesis is blocked in GC rhythm-flattening ADX rat with CORT pellet (Pinnock and Herbert, 2008). However, those findings still have questions whether diurnal GC rhythm itself modulates the neurogenesis in the hippocampus since they used ADX model with rhythmic recovery of GC by daily injection. Here, I provide strong evidence for the direct effect of diurnal GC rhythm on hippocampal neurogenesis in terms of that the reduced number of newborn

neurons in the DG of animals bearing attenuated circadian GC oscillation were fully recovered by rhythmic GC supplementation. Further, one major goal for future research with A-BMKD transgenic mice will be to better understand the reciprocal crosstalk between circadian rhythmicity of GC and antidepressant-related signaling pathways.

In conclusion, the present study clearly demonstrates that weakening of GC rhythm itself produces impairment in hippocampal neurogenesis by using the A-BMKD transgenic mice, which overcome limitations in ADX models in regard to intact adrenal medulla secreting catecholamines and low possibility of developmental problems thanks to normal GC secretion under general light/dark schedules. These mice also showed pathological symptoms such as depressive mood, reduced ability of discriminating safety from danger, and altered coping ability to stress. The observed impairments appear to be involved with altered BDNF cascades accompanied with enhanced rhythmic GR translocation into the hippocampal nucleus in contrast to the attenuated GC rhythm in the transgenic mice (Fig. 28). Therefore, this study suggests the significant role of daily oscillation of GC in the hippocampal neurogenesis implicating cognition and mood regulation.

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국문 초록

글루코코르티코이드 (Glucocorticoid; GC)는 스트레스와 같은 환경적 변화에 대해 개체가 반응하고 대응할 수 있도록 도와줌으로써 다양한 기능을 수행하는 스테로이드 호르몬 중 하나이다. GC의 분비는 주로 스트레스 반응계의 주된 내분비축으로 알려져 있는 시상하부-뇌하수체-부신피질축 (Hypothalamus-Pituitary-Adrenal axis; HPA axis)에 의해 주로 지배된다. 모체를 통한 스트레스에 의해 상승된 GC는 자손의 HPA axis와 뇌기능의 변화를 야기하고 이를 지속적으로 유지하게 하는 프로그래밍 효과를 지닌다. 그러나 모체를 통한 스트레스의 감정 학습에 대한 장기적인 영향에 대한 연구는 아직 미진한 실정이다. 이와 같은 스트레스에 대한 반응성 및 프로그래밍 효과에 더하여, GC의 또다른 특징은 뚜렷한 일주기 리듬을 지닌다는 것이다. 그러나 뇌기능에 대한 정상적인 GC 리듬의 중요성에 대한 연구는 아직 미진하다. 본 연구실에서는 부신 피질 특이적으로 생체시계의 핵심 인자인 Bmal1 유전자를 억제한 형질전환 생쥐 (A-BMKD transgenic mice)를 제작하였고, 이 생쥐가 지속적인 암주기에 노출되었을 때 GC의 일주기 리듬이 감소되어 있음을 밝혔다 (Son et al., 2008). 본 연구의 제 1장에서는 스트레스의 주된 대상인 편도체 관련 공포 기억이 모체를 통한 스트레스에 의하여 어떠한 영향을 받는지에 대한 연구를 시도하였다. 제 2장에서는 A-BMKD 형질전환 생쥐를 이용하여 인지 능력 감퇴 및 우울증과 같은 감정 질환에 관계가 깊은 해마 신경 세포

생성에 대한 GC 일주기 리듬의 영향을 알아보고자 하였다.

제 1장에서는 모체를 통한 스트레스가 편도체 관련 학습 과정에 어떠한 영향을 미치는지에 대하여 연구하고자 하였다. 모체로부터 스트레스를 받은 생쥐는 정상적인 공포 기억 습득뿐만 아니라 편도체 시냅스 상의 NMDA 수용체 발현에도 변화가 없음에도 불구하고 공포 기억의 저장과 이와 관련된 신호 전달 체계의 활성은 현저히 줄어든 양상을 보였다. 이러한 행동학적 결과와 부합하게도 모체로부터 스트레스를 받은 생쥐의 편도체 뇌 절편에서 측정한 기저 수준의 시냅스 활성과 장기강화 (Long-term potentiation; LTP) 유도는 정상적이나, LTP의 지속성이 현저히 떨어져 있었다. 흥미롭게도 학습 혹은 LTP 유도 직후에 GC를 처리해주면 감소되었던 기억의 저장과 LTP의 지속성이 회복됨을 보임으로써, GC의 공포 기억 강화 효과가 모체로부터 스트레스를 받은 생쥐에서 약화되어 있다는 것을 밝혔다. 더욱이 세포막을 통과하지 못하는 유형의 GC가 모체 스트레스를 받은 생쥐에서 관찰된 회복 효과를 그대로 재현한 결과를 통하여 비게놈적(nongenomic) 기작의 연관성이 시사되었다. 이러한 결과들을 종합해 볼 때, 모체를 통한 스트레스는 편도체 장기 기억 형성에 중요한 GC의 nongenomic 작용을 감소시킴으로서 시냅스 가소성 저해를 수반하는 편도체 관련 공포 기억 장애를 초래한다는 사실이 입증되었다.

제 2장에서는 일주일 동안의 지속적인 암주기 하에서 A-BMKD 형질전환 생쥐의 해마 신경 세포 생성에 대해 초점을 맞추어 연구를 진행하였다. 이 생쥐의 해마에서 새롭게 생성된 신경 세포의 수는 유의하게 감소되어 있었고, 신경 세포 생성과 연관된 것으로 알려진

우울 행동, 안전 기억 상실, 비정상적인 스트레스 반응성 등의 증상이 나타났다. 또한 약한 일주기 리듬으로 인한 GC 분비의 감소에 반하여, 해마에서는 GC에 의해 활성화되어 핵으로 이동한 GC 수용체가 증가된 양상이 나타났다. 이 결과와 부합하여, 성체 신경 세포 생성에 필수적임과 동시에 GC에 의해 감소되는 것으로 알려진 뇌유래 신경영양인자(Brain-derived neurotrophin factor; BDNF)와 그 수용체인 TrkB의 전사체 양이 형질전환 생쥐의 해마에서 감소되어 있는 것으로 나타났다. 흥미롭게도 GC가 포함되어 있는 식수의 일주기적 섭취에 의한 GC의 일주기 리듬 회복이 해마에서의 신경 세포 생성뿐만 아니라 BDNF와 TrkB 유전자 발현을 정상적인 수준으로 되돌린 결과로 미루어 볼 때, BDNF 관련 신호 기작이 A-BMKD 형질전환 생쥐에서 관찰된 신경 세포 생성의 감소를 야기하는 후보 인자 중 하나일 것으로 생각된다. 즉, GC의 양 뿐만 아니라 그 일주기 리듬이 해마에서의 정상적인 신경 세포 생성을 유지하는데 매우 중요하다고 할 수 있겠다.

결론적으로, GC는 편도체에서의 nongenomic 작용을 통한 공포 기억 형성에 중요한 역할을 수행하며, 그 일주기적 분비 또한 해마의 성체 신경 세포 생성에 필수적이라 할 수 있겠다.

Key words: Glucocorticoid (GC), Maternal stress, Amygdala, Fear memory, Circadian rhythm, Hippocampal neurogenesis, Nongenomic action, Glucocorticoid receptor (GR), Brain-derived neurotrophin factor (BDNF)