Role of hippocampal metabotropic glutamate receptor 5 (mGluR5) in stress-induced response: physiological and behavioral analyses

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ABSTRACT

Role of hippocampal metabotropic glutamate receptor 5 (mGluR5) in stress-induced response: physiological and behavioral analyses

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An individual's behavior is generally based on genetic blueprint and previous experiences. A coping strategy, affected by personal interpretation of past events, can be determined by behavioral controllability of stress. In this study, I investigated the role of mGluR5 in stress-induced physiological and behavioral responses by using two different behavioral models; the helplessness paradigm and the restraint stress model.

An increased or decreased mGluR5 expression was found in the hippocampus of the repetitive exposure to the uncontrollable stressor. An increased mGluR5 protein expression level is accompanied by the helplessness

(HL) (+) behavior, a decreased EEG theta power and an increased blood corticosterone concentration, suggesting the negative adaptation to the stressor. In contrast, a decreased mGluR5 protein expression level is accompanied by the HL (-) behavior, no changed EEG and no changed corticosterone level, suggesting the positive adaptation to the stressor.

Moreover, the increased mGluR5 protein expression level is positively related to the increased mGluR5 mRNA level, no changed DNA methyltransferase (DNMT) 3a mRNA level, and a decreased methylation site in the CpG island of the mGluR5 gene, while decreased mGluR5 protein expression level is positively related to the decreased mGluR5 mRNA level, the increased DNMT 3a mRNA level, and increased DNA methylation sites of the mGluR5 gene.

Thus these results suggest that mGluR5, one of the candidate molecules modulating specific forms of memory, played a critical role in the development of controllability-dependent stress coping strategies, and epigenetic modulation of mGluR5 gene is involved in the adaptation mechanism.

Keywords: mGluR5, Stress, DNA methylation, Coping strategy, Hippocampus

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I. INTRODUCTION

When organisms are under stress, they develop a coping strategy, either controllable or uncontrollable, which is manifested via their behaviors. Eventually, those who are able to control stress specialize their behaviors to escape or prevent stressful situations and those who are unable to control stress reorganize the associative part of the brain for the acquisition of new behavioral strategies to adapt stressful situations[1].

Once an individual pattern to cope with a stressor is established, it can maintain for a long time, sometimes throughout one's whole life. It is assumed

that a genetic change is involved in this long-term biological change. Among many possible genetic changes, epigenetic changes are focused, because epigenetic regulation allows for an integration of intrinsic and environmental signals in the genome that are not attributable to changes in the primary DNA sequence[2-4]. A greater emphasis has been placed on the role of epigenetic mechanisms in facilitating the adaptation of an organism to changing environments through regulation of gene expression level. It seems that epigenetic modulation is deeply involved in establishing coping strategy to repetitive stress when memory function is needed to establish a new strategy.

When an organism is exposed to the same stressor repeatedly, its coping strategy to the facing stressor depends on previous experience of the stress episode. The memory of the previous stress episode will be the key determinant of the coping strategy, such as memory of resisting stress or exhaustion by the stress. Metabotropic glutamate receptor 5 (mGluR5) is a candidate molecule for the establishment of coping strategy because mGluR5 is crucially related with the acquisition of new memory, especially when confronted with the previous memory[5].

In this study, I hypothesized that epigenetic change of mGluR5 induced by the stress episode is crucial for the establishment and maintenance of the coping strategy to the similar subsequent stress episode.

1. Stress and stress response

1) General Adaptation Syndrome and Central Adaptation Syndrome

The General Adaptation Syndrome (GAS) was developed by Hans Selye[6]. The GAS is divided into three phases; alarm, resistance, and exhaustion phases. During the alarm reaction phase, a stressor disturbs homeostasis and the brain subconsciously perceives the stressor and prepares the body either to fight or to run away. Then, the reaction moves to the resistance phase where it attempts to cope by using mechanisms of protection and defend the stress. In the 'exhaustion' phase, the organism's resources are eventually depleted and the organism is unable to maintain normal function. The initial autonomic nervous system symptoms, such as sweating and raised heart rate, may reappear. Selye also distinguished two types of stress, 'eustress' and 'distress'. Eustresses enhance function, for example through challenging work, whereas distresses refer to persistent stresses that are not resolved through coping or adaptation and may lead to illnesses, for example anxiety or depression.

The Central Adaptation Syndrome (CAS), developed by Huether[7], described the differential effects of controllable and uncontrollable stress on neural structures. According to this theory, stress affects the brain and changes

brain structures. The strength of the stress reaction and of its short-term and long-term effects depend on the controllability of the stressor. Controllable stress triggers the facilitation of neuronal networks involved in the generation of appropriate patterns of coping, whereas uncontrollable stress favors the extinction of inappropriate patterns and the reorganization of neuronal connections underlying certain inappropriate behaviors. Therefore, both controllable and uncontrollable stress response processes are inherent challenges to the development of an individual's behavior.

2) Stress response

The stress response is mediated by the hypothalamic-pituitary-adreno cortical axis that regulates the production and release of corticosterones in the adrenal cortex. And, the stressor evokes cellular mediators that can promote adaptation when the response is efficiently turned on and shut off, but which can also promote pathophysiological processes when the response is overused or dysregulated[8]. Not only the brain is central in the adaptation to stress, as it perceives and determines what is threatening, and orchestrates the behavioral and physiological responses to the stressor, but also the brain is a target of stress. Animal studies have shown stress-induced remodeling of brain architecture, such as dendritic atrophy and loss of dendritic spines in neuronal populations[8-10].

Also, depending on the age of the animal at the time of exposure and the duration and type of stressor experienced, stressor has often divergent effects on stress responses. In relation to these effects, stress is known to influence several distinct cognitive processes, including spatial, declarative, and emotional memories. However, the underlying mechanism of these effects is not clear.

3) Animal model

Early life and repeated chronic stressors have been linked to chronically high levels of corticotropin-releasing-hormone in human and animal studies. And finally these are associated with long-lasting interrelated genetic changes in the central nervous system[11,12]. In this study, the HL paradigm and the repetitive restraint stress model were used.

A HL paradigm was developed from the learned helplessness theory created by Seligman and Maier, which provided a methodological means to study biochemical and physiological correlates of depressive-like behavior of animals. Animals showed HL behavior after repetitive exposure to uncontrollable and unpedictable electric footshocks[13]. Similar to the learned helplessness theory, the HL paradigm provided an easy classification of the responses into 2 large groups in which animals show HL behavior or not, when footshock stress is applied. HL (-) behavior is considered as the positive coping

response and HL (+) behavior as the negative coping response. In particular, HL (+) behavior was regarded as a manifestation of depressive-like behavior similar to the helplessness theory in which freezing was interpreted as the behavioral manifestation to the uncontrollable stressor in the fear conditioning paradigm[14]. The advantage of the HL model is able to observe the behavioral response of animals during exposure to the stress by the researcher. However, this paradigm during stress experiences is inadequate to measure the electrical activity of the brain using EEG, because EEG is interfered by the electric shock stimulation. Therefore, to monitor the real-time electrical activity of the brain, the restraint stress model was used in this study.

2. Epigenetics

The epigenetics refers to mechanisms altering gene expression that are not attributable to changes in the primary DNA sequence. Epigenetic regulation allows for an integration of intrinsic and environmental signals in the genome. Greater emphasis has recently been placed on the role of epigenetic mechanisms for facilitating the adaptation of organisms to changing environments through the regulation of gene expression level[2,4,15,16]. Mechanisms of epigenetic alterations include changes in DNA methylation and associated histone

modifications that influence the chromatin states and impact gene expression patterns[17].

1) DNA methylation

DNA methylation involves the addition of a methyl group to the 5'-position of the cytosine within "CpG" sites. CpG sites are often found in clusters within DNA regions and referred to as CpG islands. It is composed of at least 200 base pairs, of which greater than 50% are CpG sites[18]. CpG islands are present at several gene promoters or coding region, and are often the focus of active DNA methylation and demethylation. In addition, CpG islands occur within intragenic DNA regions and subsequently affect transcriptional activity[19]. In principle, a DNA methylation state is associated with gene silencing[20].

DNA methylation is mediated by DNA methyltransferases (DNMT), and S-adenosyl methionine (SAM) serves as the methyl donor. Three active DNMTs have been identified in mammals; DNMT 1, DNMT3a, and DNMT3b. DNMT1 is the most abundant DNMT, and preferential for maintenance methyltransferase. DNMT3a and DNMT3b are responsible for de novo methylation[21].

DNA methylation has been studied extensively in the area of development because it is a static process following cell differentiation. Because the mammalian brain primarily consists of post-mitotic neurons and glial cells, most

neuroscience researches have not focused on brain epigenetics; however, high levels of DNMT mRNA have recently been reported in adult mammalian brains[22]. This finding raised the possibility that certain brain functions could be regulated by DNA methylation catalyzed by DNMT enzymatic activity. In fact, it has been recently reported that brain DNMT activity or DNA methylation could influence memory formation.

DNA methylation is also a two-faced modification resulting in either gene repression or activation. Generally, a hypermethylated DNA state is normally associated with gene silencing, whereas a hypomethylated DNA state is associated with active gene expression[23].

2) Histone modification

Eukaryotic genomes are organized into chromatins, in which DNA sequences wrap around histone octamers to form nucleosomes. The H3 and H4 histones have long tails protruding from the nucleosome, which can be covalently modified at several places. Modifications of the histone tail include methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation. The core of the histones H2A, H2B, and H3 can also be modified. Modification of histone proteins is another widely studied epigenetic mechanism that regulates the interconversion between the silenced heterochromatin state and the

actively transcribed euchromatin state.

3) Epigenetics and stress

Initial studies investigating learning-induced histone alterations within the adult brain have demonstrated a direct relationship between histone acetylation and the expression of several memory-related genes. Further analysis of the histone microenvironment has also revealed a role for histone phosphorylation occurring in concert with histone acetylation to regulate gene expression changes during fear memory consolidation. Abnormal regulation of histone methylation can result in cognitive decline that in associated with neuropsychiatric disorders, and hence the chromatin-modifying enzymes may also serve as potential therapeutic targets[24].

In addition, fear conditioning is associated with the DNA methylation of the memory suppressor genes, protein phosphatase and calcineurin, and the demethylation of the synaptic plasticity gene, reelin[21,25]. After contextual fear conditioning, DNMT expression in the hippocampus increased, and the DNMT inhibitors, 5-azadeoxycytidine or zebularine, blocked fear memory formation[25].

DNA methylation is more suitable than histone modification to investigate the epigenetic changes on local specific DNA. Therefore, the DNA methylation was used to observe the effect of environmental stimuli on specific gene.

3. Glutamate receptor

Glutamate, the amino acid, is known as the main excitatory neurotransmitter in the mammalian brain. It is released from synapses, and binds to neurons, and thereby activates cell surface receptors. These receptors are characterized as either ionotropic or metabotropic.

1) Ionotropic glutamate receptor

Ionotropic glutamate receptors (iGluRs) are the principal mediators of excitatory neurotransmission. The iGluR is divided into three subtypes, named according to their selective agonists: a-amino-3-hydroxyl-5-methylisoxazol-4-proprionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate (KA). All these receptors pass ions that depolarize neuronal plasma membranes. However, they perform very distinct functions at the synapse and in neural processing. AMPA receptors are found in most excitatory synapses and mediate fast excitatory neurotransmission. Activation of NMDA receptors depends on the coincidence of presynaptic activity (release of glutamate, presence of the co-agonist glycine) and postsynaptic activity (membrane depolarization by

AMPA/KA or other excitatory inputs). Overstimulation of these receptors by glutamate is thought to be a major mechanism for Ca^{2+} overload in neurons, which mediates neuronal injury and death[26,27].

2) Metabotropic glutamate receptor

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCR), which activate intracellular secondary messenger systems when bound by the physiological ligand, glutamate. Activation of mGluRs results in modulating and fine-tuning effects on ion channel function changed via synaptic plasticity and excitotoxicity[28,29].

The mGluRs consist of eight receptor subtypes, which are divided into three groups, based on sequence homology, signal transduction pathways and pharmacological properties. Group I mGluRs (mGluR1 and mGluR5) are coupled to phospholipase C, resulting in an intracellular Ca^{2+} signalling. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs inhibit adenylate cyclase.

The mGluRs have three distinctly separated topological domains: an extracellular hydrophilic N-terminal domain, transmembrane domain, and a variable intracellular C-terminal domain. The extracellular domain contributes to glutamate binding, agonist activation of the receptor and subtype specificity

for group selective agonists. Extracellular and intracellular domains are connected by seven transmembrane domains located hydrophobic segments. The second intracellular loop and the amino portion of the C-terminal tail contribute to G-protein coupling. However, the first and the third intracellular loop seem to play an important role in G-protein activation[30]. The presence of numerous phosphorylation sites at the C-terminus suggests it has targets for several types of kinases that regulate receptor activity. All mGluR subtypes show amino acid sequence similarity and conservation of the 19 cysteine residues[31].

The mGluRs are widely expressed throughout the central nervous system (CNS). Each subtype is specifically distributed in certain brain regions[29]. mGluRl is highly expressed in the cerebellum, the ventral pallidum and the substantia nigra, and mGluR5 is expressed in the striatum, the hippocampus and the cortex. Group II mGluRs are primarily distributed in the forebrain region and group III mGluRs are widely expressed throughout the CNS, except mGluR6, which is essentially expressed in the retina.

3) Glutamate receptor and stress

Corticosterones, released from the adrenal glands, secreted during the diurnal rhythm and during stress. Corticosterones can bind, with different

affinities, to the glucocorticoid receptor and the mineralocorticoid receptor. When released to blood, corticosterones can result in non-genomic effects (mediated by membrane receptors), indirect genomic effects (mediated by membrane receptors and second messengers), and genomic effects (mediated by cytoplasmic receptors that move to the nucleus and act as transcription factors)[32,33]. Corticosterones rapidly induce glutamate release in the hippocampus through a mechanism that may involve a membrane associated form of the mineralocorticoid receptor[34,35]. An indirect way by which corticosterones can influence neurotransmission (glutamatergic, GABAergic, cholinergic, noradrenergic and serotonergic) is through crosstalk with the endocannabinoid system[36].

Acute exposure to the restraint stress rapidly increases glutamate release in the hippocampus[37]. And, using patch-clamp recordings, application of 100 nM corticosterone to hippocampal slices rapidly enhanced the frequency of miniature excitatory postsynaptic potentials in CA1 pyramidal neurons, suggesting that corticosterone increases glutamate release probability in the hippocampus[38]. As discussed above, stress acutely enhances glutamate release in the hippocampus. However, the effects of chronic stress on glutamate release are still mostly unknown.

Not only glutamate release was affected by the stressor, but also total expression level or synaptic expression level of the glutamate receptors

influenced by the stressor. Acute stress has a delayed and sustained impact on postsynaptic glutamate receptor, causing a rapid and transient increase in presynaptic glutamate release [40,41]. Acute stress or corticosterone treatment increases AMPAR and NMDAR responses, but more selectively enhances AMPAR-mediated currents in CA1 neurons than NMDAR mediated currents[41,42]. Glutamate, released during stress, also primes hippocampal excitability by activating mGluR1 and mGluR5, and thus causes a sustained reduction of the activation threshold for forthcoming hippocampal inputs[7]. After exposure to the chronic mild stress during 9 days, the level of mGluR5 receptor protein was increased in CA1 and decreased in CA3 region of the hippocampus[43]. However, the reason for the changes of the mGluR5 expression level is not yet known.

4. Aim of this study

When an organism is exposed to a stressor repetitively, it develops individually different stress response depending on whether the stressor can be controlled or not. The purpose of the study was to investigate the mechanism of individual differences in the coping strategy to the same environmental stressful stimulus. Two behavioral models were used in this study. The modified HL

paradigm was used for studying behavioral changes according to the differential expression of mGluR5 protein, and repetitive restraint stress model was used for investigating underlying mechanism.

II MATERIALS AND METHODS

Experiment 1. Hippocampal mGluR5 in the helplessness behavior after repetitive exposure to uncontrollable stressor

1. Animals and housing

The Sprague-Dawley(SD) rats used for breeding were supplied by the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University (Project license number: #00062). Rats were cared for in accordance with the Guide for Animal Experiments as well as with the NIH Guide for the Care and Use of Laboratory Animals. All animals were raised in a Specific Pathogen Free (SPF) environment, with maintained temperature (22 ± 1℃), controlled humidity (55%), and a 12:12 hour light/dark cycle (with light starting at 7:00 am). The day pups were found in the cage was referred to as postnatal day (PND) 0. On PND 2, litters were culled to eight with an equal sex ratio based on Latin square method from different 12 females, if possible. On PND 12, animals were weaned, and pair-housed throughout the experiment. And, a litter

was comprised 6 male rats.

2. Exposure to electric footshock

Male SD rats were divided into two groups: the shock-exposed group and the control group. A rat of the shock-exposed group received inescapable and unpredictable footshocks twice (on PND 14 and PND 90) in an electric footshock chamber (20.5 × 17 × 20 cm). The shock chamber was equipped with metal rod (stainless steel) flooring connected to a shock generator and a grid scrambler (ENV-412, Med Associates, VT, USA). The control group was neither put into the chamber nor exposed to electric footshock. Rats were handled and weighed every ten days. And prior to the footshock exposure, they were handled every day. On both footshock exposure days, electric footshocks were randomly administered 40 times by as a 4 sec scrambled alternating current (0.8 mA), each of which was separated by an average of 60 sec interval (randomly ranging from 30 to 90 sec and averaging 60 sec).

3. Helplessness behavior

Helplessness behavior was measured by observation. An animals' helplessness behaviors were defined by the following criteria:

- There was no movement (such as lifting feet to avoid a shock, climbing a wall, shrinking back as if startled, etc.) at all for 3 consecutive 4 sec shocks.
- The tail was hanging down when footshock was present.
- Once 1 and 2 are fulfilled, these conditions usually continue to be met until the last footshock.

4. Exploratory activity

Rats, from both the control and shock-exposed groups, were exposed to a new environment (50 × 50 cm) three times on PND 91 for 5 min each. They were given a 5 min break between the trials. The ambulatory activity was measured during the 5 min period in each session.

5. Immunoblotting for hippocampal mGluR5

On PND 92, rats were sacrificed and their hippocampal tissues were quickly dissected. The hippocampus was weighed and homogenized using a glass homogenizer in a volume of homogenizing buffer (0.32 M

sucrose, 100 mM HEPES, pH 7.4) that was five times the tissue weight. Homogenated tissue lysates were then centrifuged. The resulting supernatants were resuspended in a lysis buffer (1% triton X-100, 100 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% Na-deoxycholate), incubated for 30 min in a 4℃ rotator, and then centrifuged. Protein concentrations of the samples were determined by a protein assay that used bicinchoninic acid (BCA; Pierce, CA, USA). Fifty micrograms of protein were run on a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and blotted onto polyvinylidene fluoride (PVDF) membranes in a methanol-based tris-glycine buffer. Membranes were then blocked with 5% non-fat milk (Bio-Rad). Each of the primary antibodies (anti-mGluR5 against mGluR5 C-terminus peptide; anti-beta actin, Santa Cruz, USA) was suspended in 1% non-fat milk and incubated over the membrane with continuous mixing for 2 hrs at room temperature or overnight at 4℃. Membranes were rinsed at least three times with tris-buffered saline (TBS) and tween 20 for 5 min each and were then incubated in 1% non-fat milk which contained a horseradish peroxidase (HRP) conjugated secondary antibody for 1 hr at room temperature. After the final TBS-T rinse, the membranes were suspended in an enhanced chemiluminescence solution (Pierce, CA, USA) and were exposed on Kodak film. Then, western blots were

scanned and quantified by densitometry using Scion Image Analysis Software (National Institutes of Health, Bethesda, MD, USA).

6. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). From the data, overall differences in mGluR5 expression levels were analyzed with one-way analysis of variance followed by pre-planned multiple comparison tests (Tukey-HSD). Cumulative exploratory activity was analyzed with repeated measures ANOVA. And, correlation coefficient between the mGluR5 protein expression level and exploratory activity was analyzed with Pearson's correlation coefficient. All statistical analyses were performed with the SPSS v.18 software. Statistical significance was defined as $p<0.05$.

Experiment 2. Epigenetic modulation of hippocampal mGluR5 in establishing coping strategies after repetitive exposure to uncontrollable stressor

1. Animals and housing

Adult male Sprague-Dawley (SD) rats (9-12 wks) were used. The rats were cared for in accordance with the Guide for Animal Experiments and the NIH Guide for the Care and Use of Laboratory Animals. They were raised in a Specific Pathogen Free environment with temperatures maintained at 22 ± 1℃, controlled humidity at 55%, and a 12:12-hr light/dark cycle (with light starting at 7:00 a.m.). The protocol was approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University (Project license number: #00062).

2. Schedule for exposure to restraint stress

Rats were subjected to 1 hr restraint session per day for 6 consecutive days inside cylindrical acrylic restraint tubes (8 cm diameter × 20 cm height). Control rats were left undisturbed in their home cages. For the antagonist study, a separate group of rats were injected with 0.36 μg/μl

MPEP into the lateral ventricle 30 min prior to stress exposure from Days 2 to 6.

3. Measurement of blood corticosterone

Blood corticosterone concentrations were measured with a radioimmunoassay kit obtained commercially (Siemens Medical Solutions Diagnostics, CA, USA). On Day 6, all rats were sacrificed between 3-4 p.m., 1 hr after the stress exposure was completed, to ensure that circadian rhythms were uninterrupted for stable basal corticosterone levels. Trunk blood was collected immediately after decapitation and was centrifuged at 1500 × g for 20 min. The serum was then removed and stored at -70℃ until assay. Serum samples and ^{131}I radiotracers were mixed in tubes containing antibody-bound corticosterone and incubated for 2 hrs at room temperature. Each of the tubes was then counted for 1 min in a Wallac 1480 Wizard 3 automatic gamma counter (Perkin-Elmer, Waltham, MA, USA).

4. Implantation surgery of EEG telemetry device and cannulae

To measure EEG signals, the telemetry device (Model ETA-F20, Data

Sciences, St. Paul, MN, USA) was implanted in animals anesthetized with chloropent. The device was composed of two double-wound lead wires that were attached to the transmitter body. A 3-4 cm incision was made on the dorsal midline of the head to insert the body of the electrode into the subcutaneous cavity and to place it in the flank near the midaxillary line. The two wires (silicon-insulated except the top) were threaded through 1 mm holes, respectively, which were located 2 mm away from the sagittal line on either side and 2 mm anterior to the lambda suture. The exposed tips of the lead wires were placed between the dura mater and the skull base and then secured to the skull with dental acrylic. After the surgery, animals were given a single dose of antibiotics (ampicillin, 40 mg/kg). They recovered from surgery at least 2 weeks prior to data recording.

To inject the antagonist of mGluR5, the cannulae (22G, 11mm length) was positioned for injection into the intracerebralventricular space (ICV) at 5.2 mm anteroposterior, -1.0 mm in the mediolateral, and -9.1 mm dorsoventrical from Bregma.

5. EEG recording and data analysis

The EEG signals recorded by the telemetry device were sent by a

carrier frequency of 455 KHz to the receiver plate (Model RPC-1, Data Sciences, USA), which was placed under the cage. This radio transmission was read digitally and analyzed with a data acquisition program (Data Quest A.R.T. 4.1, Data Sciences, USA). The EEG signals were recorded for 65 min (pre-exposure period: 5 min, exposure period: 60 min) each day for 6 consecutive days. The EEG acquisition and analyzing system was able to calculate the EEG variances without any data loss. The EEG signals were band-pass filtered with a low cutoff frequency of 0.5 Hz and a high cutoff frequency above 50 Hz. And then the mean value of the EEG variances for each day was calculated.

6. Immunoblotting

On Day 6, rats were sacrificed, and their hippocampal tissues were quickly dissected. The hippocampus was weighed and homogenized using a glass homogenizer in a volume of homogenizing buffer (0.32 M sucrose, 100 mM HEPES, pH 7.4) that was five times the tissue weight. Homogenate tissue lysates were then centrifuged. The resulting supernatants were resuspended in lysis buffer (1% triton X-100, 100 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate), incubated for 30 min in a 4℃ rotator, and then centrifuged. The total protein concentration in each

sample was determined by a BCA assay (Pierce, IL, USA). Fifty micrograms of protein were run on a SDS-PAGE gel and blotted onto PVDF membranes in a methanol-based tris-glycine buffer. The membranes were then blocked with 5% non-fat milk (Bio-Rad, CA, USA). Each of the primary antibodies (anti-mGluR5 against mGluR5 C-terminus and anti-beta actin; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were suspended in 1% non-fat milk and incubated over the membrane with continuous mixing overnight at 4℃. The membranes were rinsed at least three times with TBS and tween 20 for 5 min each and then incubated in 1% non-fat milk containing a HRP conjugated secondary antibody for 1 hr at room temperature. After the final TBS-T rinse, the membranes were suspended in an enhanced chemiluminescence solution (Pierce, IL, USA) and exposed to Kodak film. The western blots were scanned and quantified by densitometry using Scion Image Analysis Software (National Institutes of Health, Bethesda, MD, USA).

7. RNA preparation and cDNA conversion

For RNA preparation (RNeasy mini kit, Qiagen, Germany), 30 mg of hippocampal tissue sample was disrupted and homogenized in 600 μl of RLT buffer. The lysate was then centrifuged for 3 min, and the

supernatant was saved. After adding one volume of 70% ethanol to the cleared supernatant, the sample was transferred to the RNeasy spin column and centrifuged at 8000 × *g* for 15 sec. The RNeasy spin column was centrifuged two more times at 8000 × *g* for 15 sec: once after adding 700 µl of RW1 buffer and again after adding 500 µl of RPE buffer. Finally, 30 µl of RNase-free water was added directly to the spin column membrane for RNA elution. I then used 200 ng RNA as the template for cDNA synthesis using Superscript III (Invitrogen, CA, USA).

8. Real-time PCR

To measure levels of mGluR5 and DNMT mRNA, quantitative real-time PCR was used. The expression of GAPDH mRNA served as an internal control. The real-time PCR reactions were performed with a 7500 Real-time PCR System (Applied Biosystems, CA, USA) using fluorescent SYBR Green technology (Applied Biosystems, CA, USA). Unique 18-25 bp primer pairs from coding sequences were specifically identified using Primer Express (Applied Biosystems, CA, USA). Real-time PCR was performed on 2 µl of cDNA synthesized from 200 ng of total RNA. As an endogenous control, primers were constructed to detect the ubiquitously expressed GAPDH message.

9. gDNA purification, sodium bisulfite treatment, and methylation analysis

The hippocampal tissue was incubated overnight with 300 μl of lysis buffer containing 30 μg of Proteinase K at 55℃. Next, 100 μl of protein precipitation solution was added to the tissue, which was then vortexed for 1 min and centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a clean tube containing 300 μl of 100% isopropanol, was mixed by gentle inversion (about 50 times), and centrifuged at 13,000 rpm for 1 min. The genomic DNA, now visible as a pellet, was washed with 70% ethanol, centrifuged at 13,000 rpm twice, resuspended in 50 μl of DNA hydration solution (containing 1.5 μl of RNase A), and incubated at 37℃ for 60 min. For DNA hydration, the sample was incubated again at 65℃ for 1 hr, and 1 μg of the resulting genomic DNA was then saved for the bisulfite conversion with a commercial kit (EpiTect bisulfite kit, Qiagen, Germany). Bisulfite reactions were prepared and thoroughly mixed in PCR tubes so the bisulfite DNA conversion could be performed using a thermal cycler. For cleanup of bisulfite converted DNA, the PCR tubes containing the bisulfite reactions were briefly centrifuged, and solutions were transferred to clean 1.5 ㎖ microcentrifuge tubes. After

adding 560 µl of lysis buffer, the mixture was vortexed, briefly centrifuged, transferred to the corresponding EpiTect spin columns, and centrifuged at 13,000 rpm for 1 min. Then, 500 µl of wash buffer was added, and the spin column was centrifuged. Desulfonation buffer (500 µl) was also added to the spin column, incubated for 15 min at room temperature, and centrifuged. After adding 500 µl of wash buffer and centrifuging, the spin columns were then placed into clean 1.5 ㎖ microcentrifuge tubes, incubated for 5 min at 56℃, and eluted with 20 µl of elution buffer. The direct sequencing method was used for the methylation analysis on bisulfite-treated gDNA-utilized PCR. Primers flanking the CpG island on the mGluR5 gene were designed to be bisulfite specific (i.e., primers containing non-CpG cytosines such that they are not complementary to non-bisulfite-treated DNA). The nested PCR method was used to enhance the product for sequencing.

10. Statistical analysis

All data were presented as mean ± SEM. The methylation pattern of each CpG site in the matching group was tested by Pearson's chi-squared test. Differences in mGluR5 expression levels, DNMT activity, overall methylation, theta power, and antagonist effects were analyzed with one-

way ANOVA followed by pre-planned multiple comparison tests (Tukey-HSD). All statistical analyses were performed with the SPSS v.18 software. Statistical significance was defined as $p<0.05$.

III RESULTS

Experiment 1. Hippocampal mGluR5 in the helplessness behavior after repetitive exposure to uncontrollable stressor

1. The relationship between helplessness behavior and the protein expression of mGluR5 after exposure to electric foot shock stress

Rats were divided into three groups based on their behavioral responses to electric footshocks on PND 14 and PND 90: 1) the control group (no exposure to footshock; n=8); 2) the HL (-) group (exposure to footshock but no signs of HL behavior during both footshock experiences; n=8); and 3) the HL (+) group (exposure to footshock that showed signs of HL behavior during both footshock experiences; n=8). The behavioral response was measured by delivering electric footshocks once on PND 14 and again on PND 90. Those that showed HL on both days and those that didn't were selected to be used in the study.

The two days (PND 14 and PND 90) denoted their preadolescence and adulthood, respectively. Two times exposure was chosen whether the stressful experience during preadolescence would affect their behavioral controllability during adulthood if they were placed in the same situation. For this reason, only those that responded with the same pattern of behavior (i.e. HL vs. no HL) on

both shock days were used in this experiment. Out of all the animals that were given the same stressor on both days, 81.8 % (27 out of 33 rats) of them showed the same behavior during the second footshock as they did during the first. And among these, 66.7 % (18 out of 27 rats) manifested HL (+) behavior and the rest displayed HL (-) behavior during both shock periods (**Table 1**).

Interestingly, the presence or absence of HL behavior was found to be closely related to the expression of hippocampal mGluR5. The Western blot analysis of mGluR5 protein showed two bands, in which the upper one corresponded to mGluR5 receptor dimers and the lower one mGluR5 monomers. Data for mGluR5 dimers were specifically used for density analysis. And the result showed that mGluR5 expression increased (42 ± 9.7 %) in the HL (+) group and decreased (33 ± 8.9 %) in the HL (-) group ($F_{(2,21)}$=22.7, p<0.01), compared to the control group. However, the mGluR1, which belongs to Group I mGluR, protein expression level was not changed in HL (-) and HL (+) groups compared with control group (HL (-) group; 95 ± 5.7 %, HL (+) group; 102 ± 7.3 %) (**Figure 1**).

Table 1. An occurrence of helplessness behavior in the exposure to footshocks in PNDs 14 and 90.

	HL (PND 14/PND 90)			
	HL (−/−)	HL (−/+)	HL (+/−)	HL (+/+)
Number of rats (%)	9 (27.3)	6 (18.2)	0	18 (54.5)

Rats showed helplessness positive (+) and negative (-) behaviors. Total 33 rats enrolled and 81.8 % of them showed the same behavior during the second footshock as they did during the first.

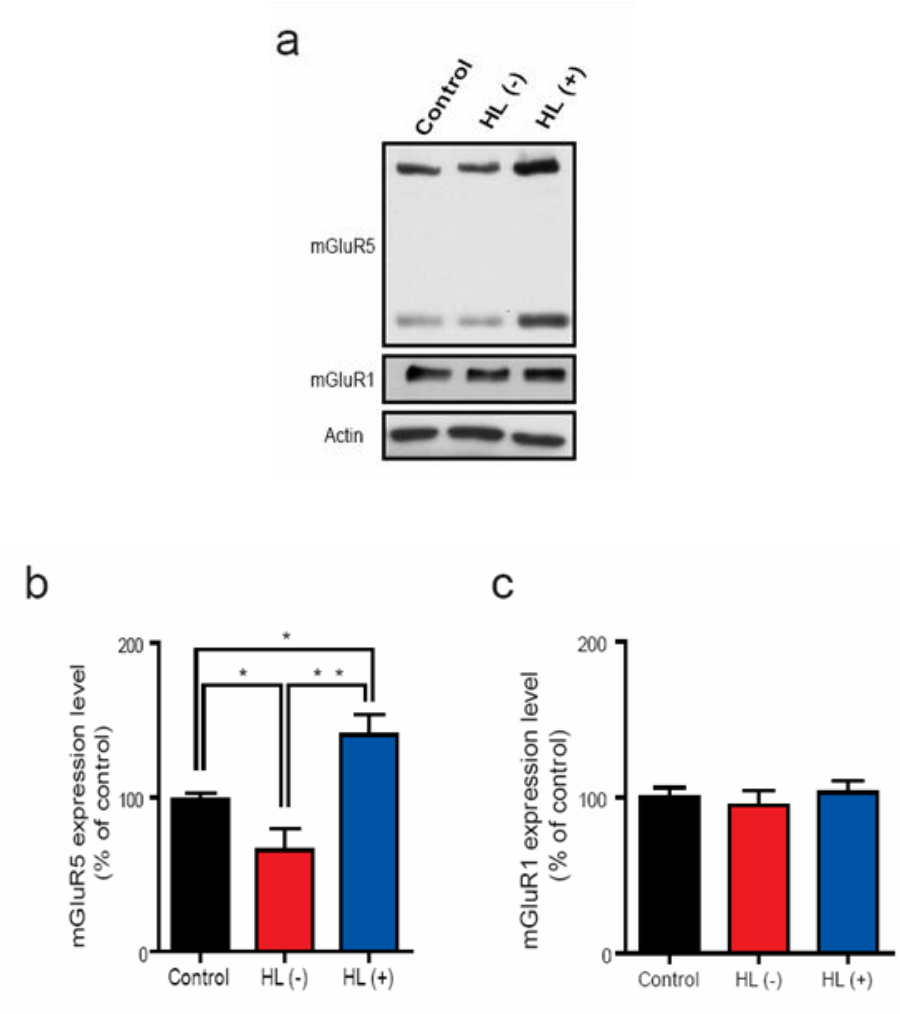


Figure 1. Protein expression of mGluR5 in the hippocampus. (a) mGluR5 protein expression level. Two bands were detected for mGluR5; dimers and monomers. (b) Quantification plot for dimeric mGluR5. Compared to the control group, hippocampal mGluR5 expression level increased in the HL (+)

group and decreased in the HL (-) group. (c) Quantification plot for mGluR1. Compared to the control group, hippocampal mGluR1 expression level was not changed in the HL (+) and HL (-) groups. Data are represented as means + SEM (ANOVA/Tukey-HSD post hoc test *p<0.05, **p<0.01).

2. The exploratory activity after exposure to electric footshock stress

The exploratory activity, which was tested two days after shock deliverance, gradually decreased in the control and HL (-) groups throughout the repeated trials. However, in the HL (+) group, there was no decrease in exploratory activity ($F_{(8,63)}$=4.7, p<0.01, One-way ANOVA) (**Figure 2a**). The high level of exploratory activity of the HL (+) group implied that there was a lack of adaptation to the novel environment. On the third trial, the HL (+) group showed higher exploratory activity than other groups ($F_{(8,63)}$=4.7, p<0.01, vs. the HL(-) group). In the analysis of individual rats, the mGluR5 protein expression levels showed a strong positive correlation with the sum of 3 trials' exploratory activities in each rat (Pearson's correlation coefficient, r=0.832, p<0.01) (**Figure 2b**).

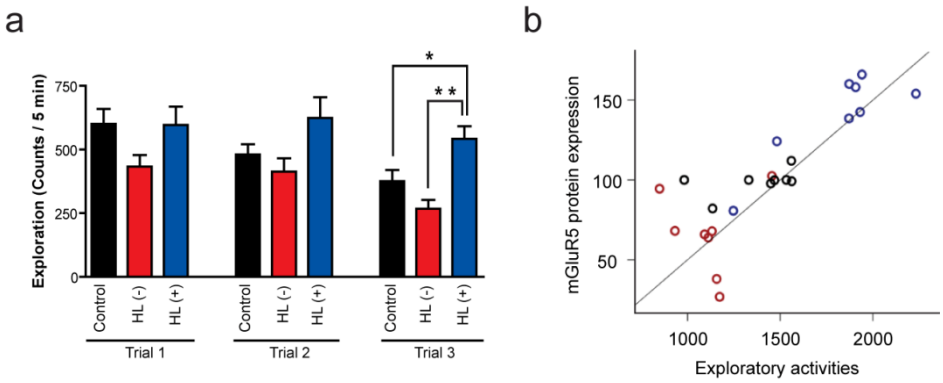


Figure 2. Exploratory activity in a novel environment. (a) Exploration during 5 min in each 3 trials. Rats were exposed to the same environment three times on PND 91. Exploratory activity decreased gradually as the trial was repeated in the control and the HL (-) group, while the HL (+) group did not show any decrease in ambulatory activity during the repeated trials. Data are represented as means ± SEM (ANOVA/Tukey-HSD post hoc test, **P*<0.05, ***P*<0.01). (b) The correlation between the mGluR5 protein expression levels and exploratory activities. The mGluR5 protein expression levels are positively correlated with the cumulative exploratory activities in each rat (Pearson's correlation coefficient, r=0.832, p<0.01).

Experiment 2. Epigenetic modulation of hippocampal mGluR5 in establishing coping strategies after repetitive exposure to uncontrollable stressor

1. Individual differences in mGluR5 protein and mRNA expression after repetitive exposure to restraint stress

To determine whether repetitive exposure to a stressful environment could mediate individual differences in the protein expression of mGluR5, 60 rats were repetitively exposed to restraint stress for 1hr per day for 6 consecutive days. After the final stress exposure, the rats were sacrificed and the hippocampus was prepared for measuring mGluR5 protein expression. The mGluR5 protein level varied from 20.95% to 210.65% (mean 113.78%) of the control group which was not exposed to restraint stress. Two groups were selected according to the mGluR5 protein expression level: the low expression group (LE, 10 rats with the lowest mGluR5 expression level) and the high expression group (HE, 10 rats with the highest mGluR5 expression) (**Figure 3a-c**). The protein expression level in the LE group was 36.34 ± 3.04% (p<0.01) of the control group, and the level in the HE group was 191.53 ± 4.02% of the control group (p<0.01). Real-time PCR analysis revealed that the mGluR5 mRNA levels in the LE group were low (41.41 ± 10.36%, p<0.01) compared to

the control group, while the levels in the HE group were high (174.09 ± 11.38%, $p<0.01$) (**Figure 3d**). Evidently, mGluR5 protein and mRNA expression levels showed parallel patterns in the LE and the HE groups.

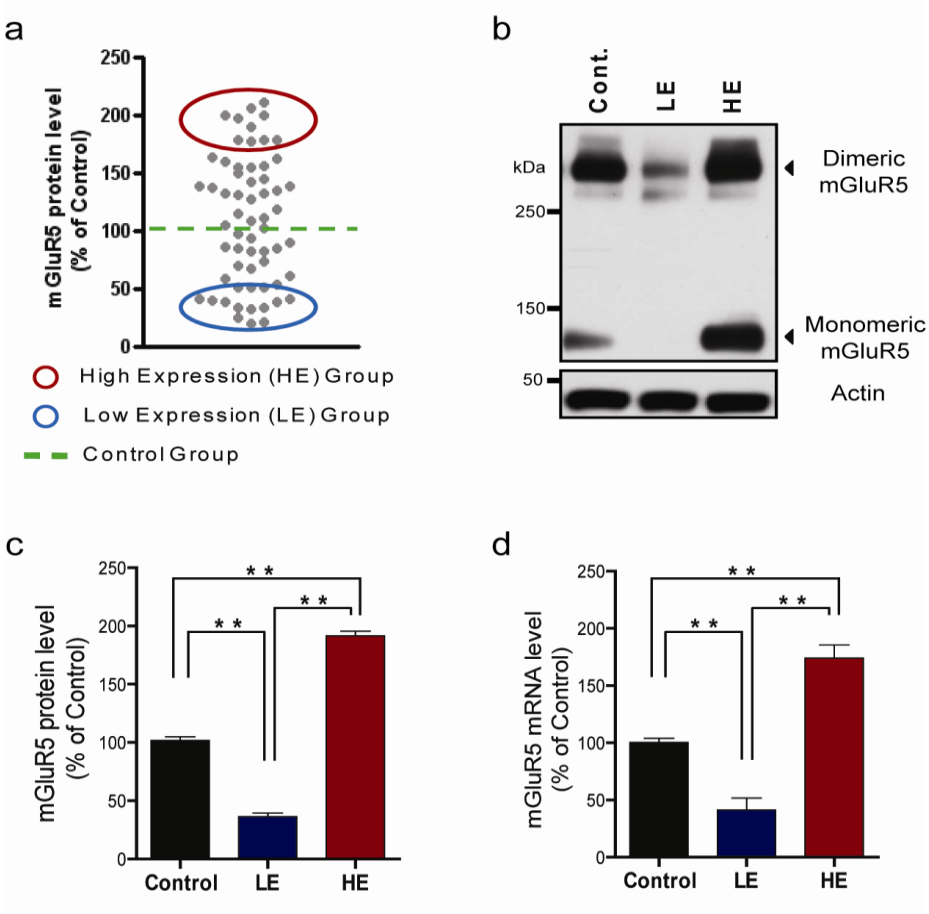


Figure 3. Individual differences in the protein and mRNA expression of mGluR5 after repetitive exposure to restraint stress for 6 days. Rats were sacrificed 1 hr after the last restraint stress exposure on Day 6, and hippocampi were obtained for the analyses. (a) Individual plot of mGluR5 protein expression in the hippocampus. Each gray circle represents one rat. The mGluR5 protein expression was widely distributed compared to the control

group, not exposed to restraint stress. Ten rats with higher mGluR5 expression levels were chosen as the high expression (HE) group, and 10 rats with lower mGluR5 expression levels were chosen as the low expression (LE) group. (b) mGluR5 protein expression levels in the hippocampus. Two bands were detected for mGluR5 by western blot; the upper one corresponds to dimeric mGluR5 and the lower to monomeric mGluR5. (c) Quantification plot for dimeric mGluR5. Hippocampal mGluR5 protein decreased in the LE group and increased in the HE group. (d) mGluR5 mRNA expression in the hippocampus. $^{**}p<0.01$, one way ANOVA followed by Tukey-HSD test.

2. DNA methyltransferase activity after repetitive exposure to restraint stress

To investigate a possible role for mGluR5 gene methylation in the observed individual differences in post-stress mGluR5 expression, I studied whether DNMT mRNA levels in the hippocampus were altered after repetitive exposure to restraint stress. The mRNA levels for two DNMT subtypes, DNMT 1 and DNMT 3a, were measured by real-time PCR. DNMT 1 mRNA levels were increased in both groups after repetitive exposure to restraint stress ($p<0.01$) and showed no group differences (LE; 295.9 ± 58.4%, HE; 290.1 ± 35.0%) (**Figure 4a**). Interestingly, the DNMT 3a mRNA levels increased two fold in the LE group (202.7 ± 20.9%, $p<0.01$), while the levels in the HE group (101.2 ± 10.3%) did not change (**Figure 4b**). These results indicate that an increase in DNMT 3a mRNA is associated with a decrease in mGluR5 expression.

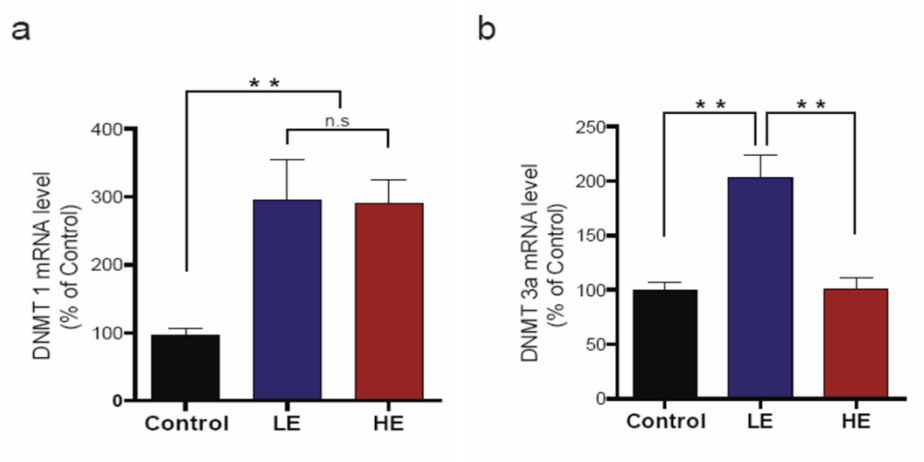


Figure 4. DNA methyltransferase (DNMT) activity after repetitive exposure to restraint stress for 6 days. DNMT 1 and DNMT3a mRNA levels were measured by real-time PCR. (a) Level of DNMT 1 mRNA expression. The DNMT 1 mRNA levels were increased in both groups after exposure to repetitive restraint stress, and no group differences were observed. (b) Level of DNMT3a mRNA expression. The DNMT3a mRNA levels increased in the low expression (LE) group but not in the high expression (HE) group. $^{**}p<0.01$, one way ANOVA followed by Tukey-HSD test.

3. DNA methylation pattern of the mGluR5 gene after repetitive exposure to restraint stress

To elucidate whether an epigenetic mechanism is involved in the regulation of mGluR5 expression after repetitive exposure to stress, the DNA methylation of the CpG island on the mGluR5 gene was analyzed. Computational analysis predicted one CpG island located within the mGluR5 gene. This CpG island contains 31 CpG sites within a stretch of 330 bp in exon IX in the rat mGluR5 gene. To investigate the DNA methylation status, bisulfite conversion and DNA sequencing were used, as they are capable of providing detailed information about methylation status at single CpG site resolution (**Figure 5**).

In the control group, about 20% of all 31 CpG sites were methylated (20.64 ± 4.5%, n=10), suggesting the existence of natural DNA methylation. This epigenetic modulation appears to be necessary to manage ordinary life in rats. Notably, a remarkable increase in CpG methylation sites (58.38 ± 3.7%, $p<0.01$) was found in the LE group, whereas those in the HE group showed similar levels to the control group (16.13 ± 2.94%) (**Figure 6a**). Therefore, the results showed that the overall increase in the methylation rate of the CpG island was associated with a decrease in mGluR5 protein and mRNA levels in the LE group.

Next, to identify which CpG sites are directly responsible for mGluR5 expression, the methylation status of each CpG site within a CpG island was analyzed. I analyzed each methylation site as a decreased, unchanged, or increased methylation site compared with the degree of methylation in the matching CpG site in the comparison group. Three comparisons with Pearson's Chi-square test was performed: control vs. LE, control vs. HE, and LE vs. HE. Twelve (CpGs 3, 5, 9, 10, 11, 12, 14, 16, 18, 19, 22, and 30) out of the 31CpG sites in the LE group showed increased methylation compared with the control group ($p<0.05$), and no decreased sites were observed. Interestingly, the HE group showed decreased methylation at only one site (CpG 24) compared with the controls; although, the overall methylation rates in the HE group were not different from the controls. When comparing the HE and LE groups, 16 CpG sites (CpGs 5, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 22, 24, 25, 29, and 30) in the HE group showed a decreased degree of methylation ($p<0.05$) (**Figure 6b,c**). Therefore, I speculated that the increased methylation of 11 CpG sites in the LE group (CpGs 5, 9, 10, 11, 12, 14, 16, 18, 19, 22, and 30) was likely responsible for the decreased mGluR5 protein and mRNA expression after exposure to repetitive stress. Particularly, the CpG 24 site was the only site in the HE group showing decreased methylation post-stress, because this site may be important of increasing the expression of mGluR5 protein and mRNA. These results indicate that alterations in the methylation of the mGluR5 gene are associated

with changes in mGluR5 expression.

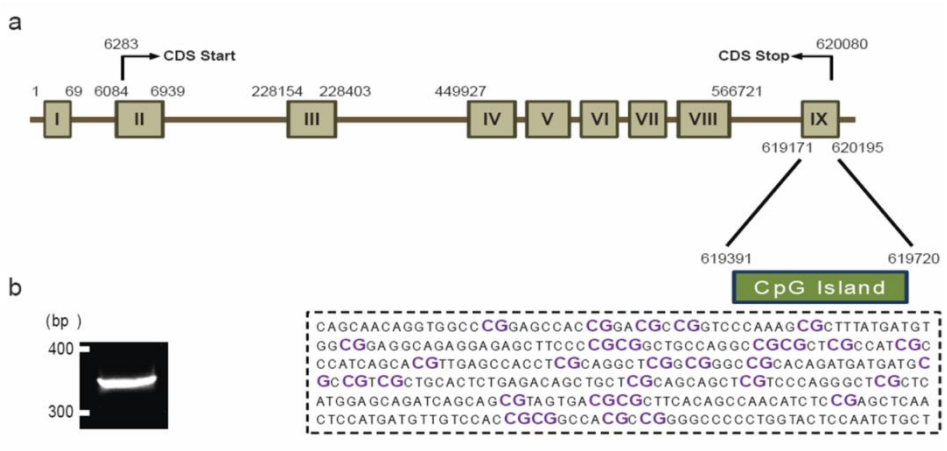
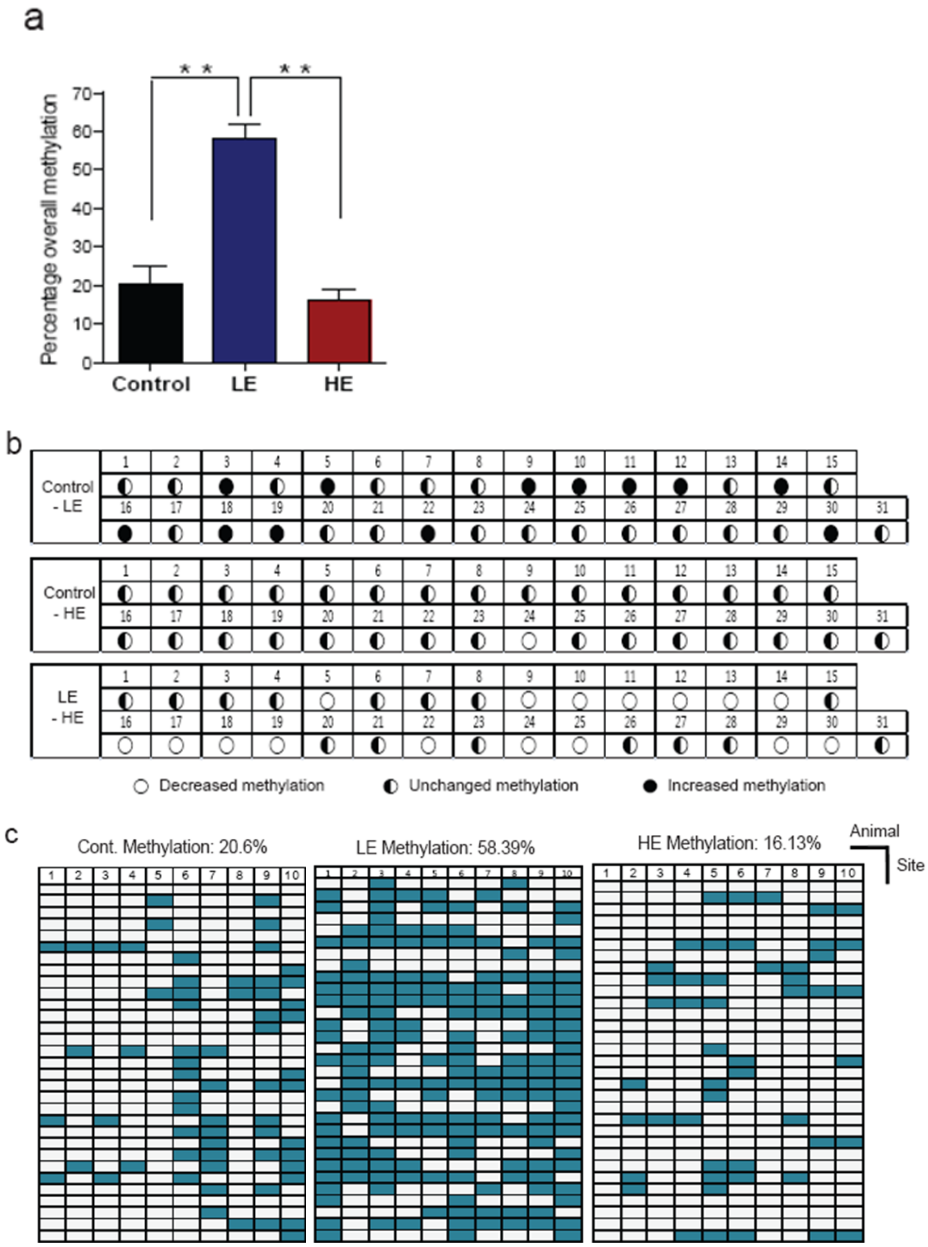


Figure 5. Prediction of CpG islands in the mGluR5 gene. (a) Location of predicted CpG island in the mGluR5 gene. Computational analysis predicted one CpG island in the mGluR5 gene, which contains 31 CpG sites within a stretch of 330 bp in exon IX. (b) PCR product of CpG island. For the investigation of DNA methylation patterns, bisulfite conversion and PCR amplification of the CpG island were conducted. PCR product size is 330 bp.


a
Percentage overall methylation
70
60
50
40
30
20
10
0
* *
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Control
LE
HE
b
Control - LE
Control - HE
LE - HE
Decreased methylation
Unchanged methylation
Increased methylation
c
Cont. Methylation: 20.6%
LE Methylation: 58.39%
HE Methylation: 16.13%
Animal
Site

Figure 6. DNA methylation pattern of the mGluR5 gene after 6 days of repetitive restraint stress. (a) Percentage of overall mGluR5 gene methylation. The control group showed methylation of about 20% of the CpG island. The overall methylation of the CpG island increased in the low expression (LE) group compared to the control and high expression (HE) groups. $^{**}p<0.01$, one way ANOVA followed by Tukey-HSD test. (b) Site specific analysis of GluR5 gene methylation patterns. Compared to the control group, the LE group exhibited 12 hypermethylated CpG sites (#3, 5, 9, 10, 11, 12, 14, 16, 18, 19, 22, and 30), and the HE group showed one hypomethylated site (#24). The LE group showed 16 hypermethylated CpG sites (# 5, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 22, 24, 25, 29, and 30) compared to the HE group (Pearson's Chi-square test, $p<0.05$ is the significance level). (c) CpG island methylation pattern for each rat from the three groups. The X axis indicates animal numbers (n=10 per group), and the Y axis indicates the CpG sites (31 sites). The white square denotes unmethylated CpG site, and dark square shows methylated CpG site.

4. Theta power in electroencephalograms after repetitive exposure to restraint stress

To determine whether different levels of mGluR5 expression after repetitive stress are associated with varied brain activities, EEG using a wireless telemetry system was performed. The real-time EEG responses of animals were recorded. Positive and negative poles for the EEG recordings were placed on the surface of the dura mater inside a drilled hole, which was located 2 mm lateral to the sagittal suture and 2 mm anterior to the lambda suture (**Figure 7a**). Circadian rhythms were monitored 2 weeks after the implantation of the telemetry device for EEG recordings. The EEG total power rose during the dark phase and declined toward the end of the dark phase. The total power during the light phase was lower, with occasional higher values (**Figure 7b**). This pattern allowed us to confirm that the recording device was implanted correctly and the general condition of the rats after surgery was appropriate. The EEGs were monitored during the pre-exposure period (5 min immediately before stress exposure) and the exposure period (60 min exposure to the stress) to restraint stress for all 6 days (**Figure 8a**). The EEG recording from the pre-exposure period on Day 1 were considered to represent basal brain activity, and the EEGs from the pre-exposure period on Day 6 were considered basal brain activity after 5 days of repetitive exposure to the stress.

When the rats were restrained, the EEG waves exhibited unique peak deviants from those detected during the pre-exposure period on Day 1 (**Figure 9a,b**). To investigate which frequencies of brain waves (e.g., delta, theta, alpha, beta, or gamma) were responsible for this unique peak, the raw EEG data was analyzed by using the MATLAB software. The analysis revealed that the EEG theta frequency showed increased power at the peak during the stress exposure condition (**Figure 9a,b**). Then EEG analysis was focused on the theta power in the pre-exposure and exposure periods on Days 1 and 6. The theta power during the pre-exposure period on Day 1 did not differ between the LE and the HE groups (control: 0.62 ± 0.01 mV^2, LE: 0.61 ± 0.01 mV^2, HE: 0.62 ± 0.013 mV^2). Interestingly, after exposure to repetitive stress, two different EEG patterns were observed during the pre-exposure period. On Day 6, the theta power in the HE group was reduced by 21% compared to Day 1 ($p<0.01$), whereas the theta power in the LE group was not changed (control: 0.63 ± 0.013 mV^2, LE: 0.61 ± 0.015 mV^2, HE: 0.48 ± 0.016 mV^2) (**Figure 9c**). When the effect of stress exposure was plotted as individual changes in EEG theta power, 9 out of 10 rats showed decreased power after repetitive exposure to stress, whereas the LE group showed variable directions of change in theta power (**Figure 9d**).

The theta power was analyzed during the exposure periods on Days 1 and 6. These data were divided into 5 min intervals for the entire 60 min exposure period. On Day 1, in the LE group, an exposure to stress initially decreased the

theta power to 35% of pre-exposure period levels followed by a subsequent gradual increase; however, the theta power of the HE group decreased late in the 6^{th} block to 11% of pre-exposure period levels and gradually increased. On Day 6, the theta power of the HE group, compared to the control group, decreased in almost all blocks during the exposure period and that of the LE group decreased only in the 5^{th}-8^{th} blocks during the exposure period. Moreover, the theta power of the HE group was less than that of the LE group in the 1^{st} and 5^{th} blocks (**Figure 8b,c**). These EEG data indicate that brain activity can be altered by repetitive exposure to stress, and the theta power is negatively correlated with the mGluR5 expression after repetitive exposure to stress.

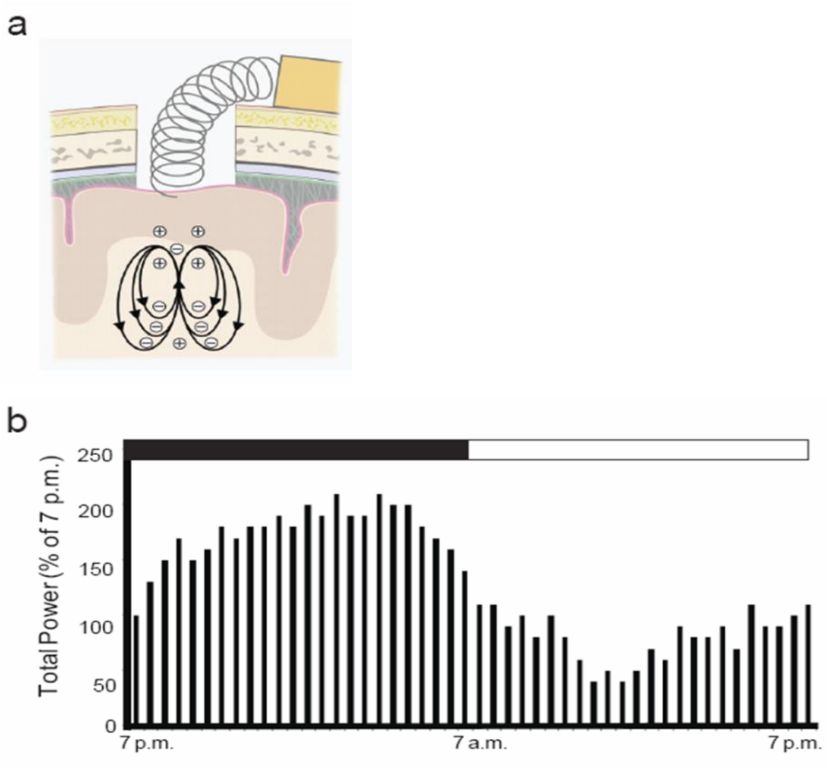

Figure 7. Schematic diagram of brain EEG telemetry implantation and EEG recording to monitor circadian rhythms. (a) Diagram of lead implantation. Positive and negative poles were placed on the surface of the dura mater inside the drilled hole located 2 mm lateral to the sagittal suture and 2 mm anterior to the lambda suture. (b) Rat circadian rhythm. Two weeks after device implantation, circadian rhythm was monitored by EEG. During the dark phase, the total EEG power initially rose and eventually declined toward the end of the dark phase. During the light phase, the total power was low with occasional higher values.

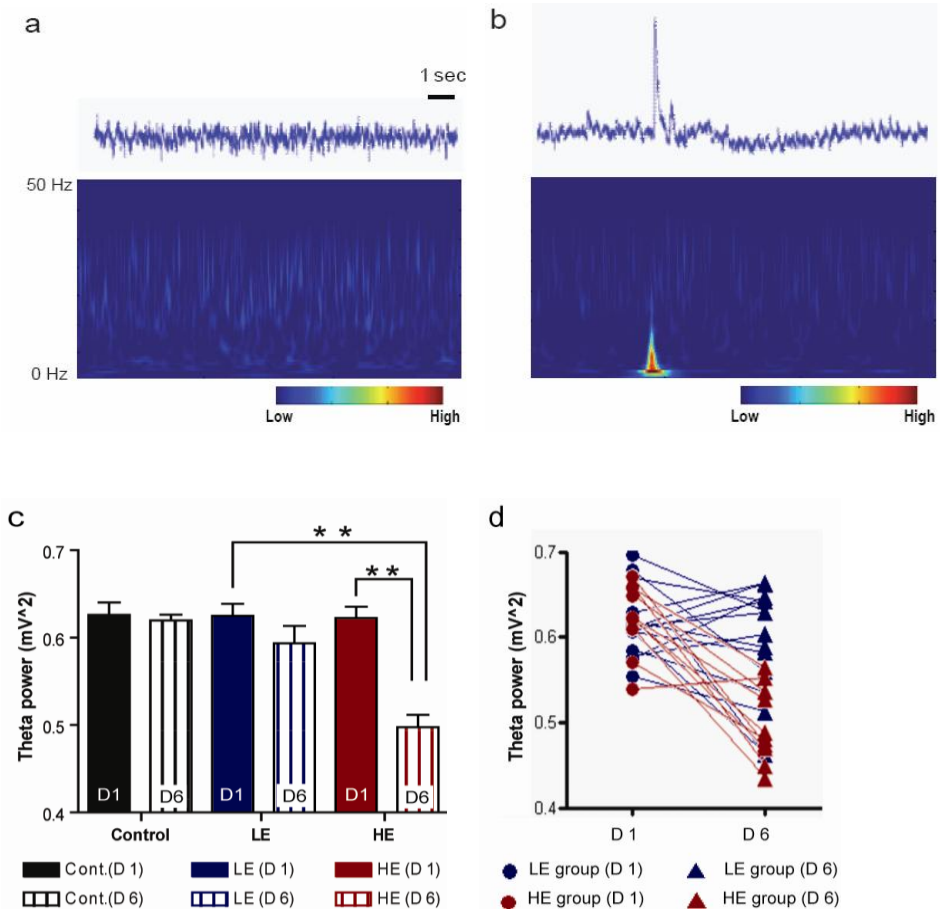

Figure 8. Theta power in electroencephalograms after exposure to repetitive restraint stress. (a) EEG trace and spectral analysis during the pre-exposure period on Day 1. The EEG waves indicated basal condition when animals were not stressed. The upper panel shows the raw EEG trace, and the lower panel represents spectral analysis using MATLAB. (b) EEG trace and spectral analysis during the exposure period on Day 1. When rats were restrained, the raw EEG

trace shown in the upper panel depicted a unique deviant peak from those detected during the exposure period at Day 1. This peak was closely associated with increased theta power. (c) Theta power in the pre-exposure period on Days 1 and 6. Theta powers between the low expression (LE) and the high expression (HE) groups were not different during the pre-exposure period on Day 1. On Day 6, the HE group theta power was reduced, while theta power in the LE group was not changed. (d) Spaghetti plot for individual tracing on Days 1 and 6. In the HE group, the theta power decreased in 9 out of 10 rats after 5 days of repetitive stress. In contrast, the theta power changed variably in the LE group. $^{**}p<0.01$, one way ANOVA followed by Tukey-HSD test.

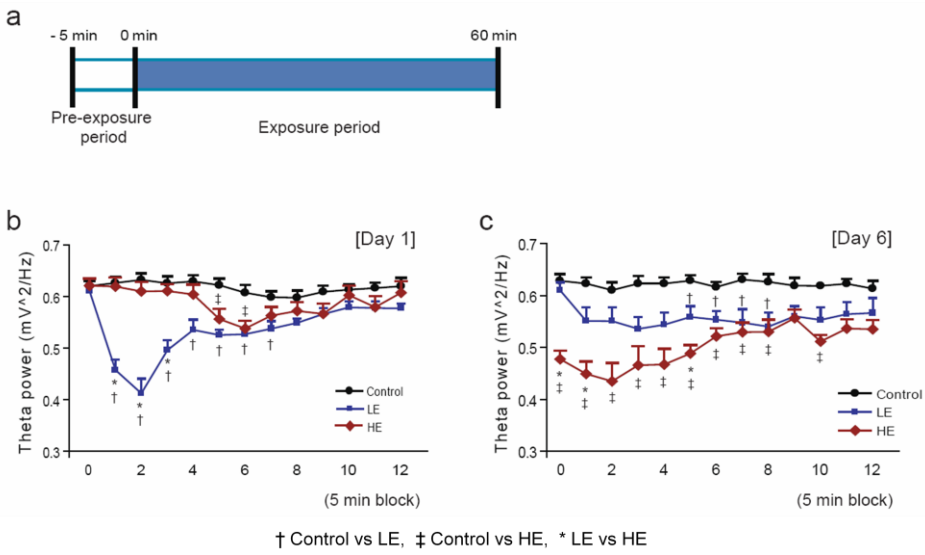

Figure 9. Real-time EEG monitoring on Days 1 and 6. (a) Schedule of monitoring EEG. EEGs were monitored during the pre-exposure period (5 min immediately preceding stress exposure) and during the exposure period (60 min) to restraint stress for 6 days. Data were divided into 5-min intervals, as represented by 5-min blocks numbered 0 to 12. The zero block indicates the 5-min pre-exposure period (from -5 min to 0 min), 1st block represents 0 min to 5 min, and the 12th block indicates the last 5 min of the exposure period (from 55 min to 60 min). (b) Theta power during the pre-exposure and the exposure periods on Day 1. During the pre-exposure period, the theta power did not differ between the low expression (LE) and the high expression (HE) groups. The LE group showed low EEG band power in the 2nd block after the restraint, then

gradually increased throughout the remaining exposure period. The theta power of the HE group decreased a little late in the 5th and the 6th blocks and gradually increased. The control group showed no changes. (c) Theta power during the pre-exposure and the exposure periods on Day 6. The HE group showed decreased theta power during the pre-exposure period. During the exposure period, the decreased theta power maintained in the HE group. However, in the LE group, a decreased theta power was observed only in the 5th-8th blocks. One way ANOVA followed by Tukey-HSD test. Control vs. LE; † $p<0.05$, Control vs. HE; ‡ $p<0.05$, LE vs. HE; *$p<0.05$, **$p<0.01$.

5. Blood concentrations of corticosterone after repetitive exposure to restraint stress

Blood corticosterone concentrations were measured 1 hr after to the completion of stress exposure on Day 6. I hypothesized that different coping strategies result in different hormonal responses after repetitive exposure to stress, if differences in blood corticosterone concentrations were observed. Interestingly, the corticosterone levels were higher in the HE group than the control group (Control: 167.6 ± 7.36 μg/㎖, HE: 263.1 ± 7.24 μg/㎖, $p<0.01$); however, no changes were found in the LE group (190.4 ± 8.74 μg/㎖) (**Figure 10**). By considering the hormone and EEG changes together, the HE group showed decreased EEG theta power and increased blood corticosterone concentrations, suggesting negative adaptation. In contrast, the LE group did not show changed EEG and corticosterone level despite being exposed to the same repetitive stress, suggesting positive adaptation.

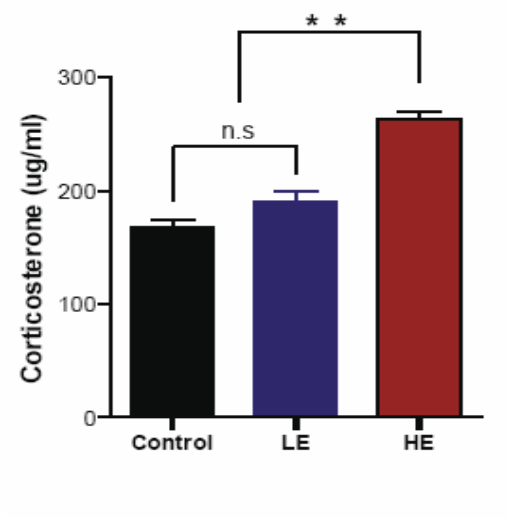

Figure 10. Corticosterone blood concentrations after exposure to repetitive restraint stress. The corticosterone levels increased in the HE group, while no change was observed in the LE group. ** $p<0.01$, one way ANOVA followed by Tukey-HSD test.

6. The mGluR5 antagonist, MPEP, blocks variations in coping responses after repetitive exposure to restraint stress.

To confirm the role of mGluR5 in establishing coping strategies, the stress was administered in combination with blockade of mGluR5 activity. The selective mGluR5 antagonist, 2-Methyl-6-(phenylethynyl) pyridine (MPEP), was injected ICV (3.6 μg in 10μℓ saline) 30 min prior to the stress exposure from Day 2 to Day 6 (**Figure 11a**). After five daily MPEP injections, the observed group differences after repetitive exposure to stress were abolished in terms of mGluR5 protein expression and EEG patterns. After MPEP, the mGluR5 protein expression levels in the stress exposed group ranged from 80-121.7% (99.7 ± 3.04%) of the control group (**Figure 11b,c**). Similarly, I could not group the theta power patterns based on EEGs from the pre-exposure period on Day 6 after MPEP (**Figure 11d**). The effects of MPEP on mGluR5 expression and theta EEG power indicated that mGluR5 is directly associated with the individual differences after repetitive exposure to stress.

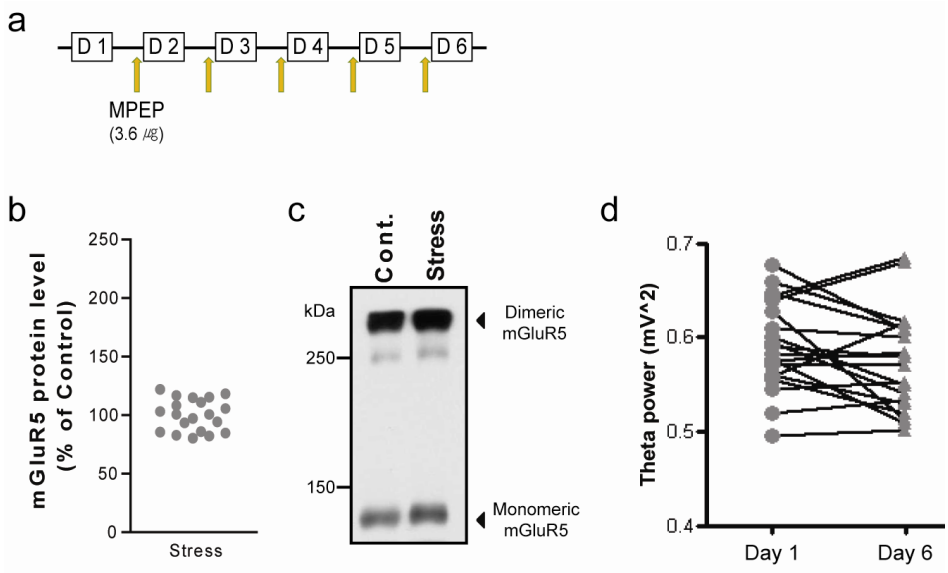

Figure 11. The mGluR5 antagonist, MPEP, blocks responses after repetitive restraint stress. (a) Schedule of the antagonist injection. MPEP was injected i.c.v. 30 min prior to each stress exposure from Days 2 to 6. (b) Individual plot of mGluR5 protein expression levels in the hippocampus. After MPEP, the mGluR5 protein levels were not different between stressed and unstressed rats. (c) Representative blot for hippocampal mGluR5 protein expression. Two mGluR5 bands were detected by western blot: the upper corresponds to dimeric mGluR5 and the lower one to monomeric mGluR5. (d) Spaghetti plot for individual tracing on Days 1 and 6. After MPEP, the theta power did not change in individual tracings on Days 1 and 6.

IV. **DISCUSSION**

This study suggests that the mGluR5 play a pivotal role in establishing individual differences in coping strategies in response to the same environmental stressful stimulus. In the Experiment 1, after exposure to repetitive electric footshock stress, mGluR5 protein expression level increased in the HL (+) group and decreased in the HL (-) group. Here, HL (+) represented uncontrolled stress due to negative coping and HL (-) due to positive coping. And, in the Experiment 2, after exposure to repetitive restraint stress, broad individual differences were observed in the hippocampal expression of mGluR5. The HE group showed a high mRNA expression of mGluR5, a decreased methylation site in the CpG island of the mGluR5 gene, and a decreased theta power on basal EEG analysis. However, the LE group exhibited low mGluR5 mRNA expression, an increased methylation sites in the CpG island of the mGluR5 gene, and no changes in basal EEG theta power. Furthermore, individual differences in mGluR5 expression and theta power after repetitive exposure to the stress were all abolished by the specific mGluR5 antagonist, MPEP.

Recently, mGluRs emerged as critical molecules for the mechanism of learning and memory[44-47], as well as ionotropic glutamate receptors that used to be targeted frequently in learning and memory research[48-50]. Learning and

memory acquired from the past are important to respond to later stressful situation. To date, no previous research has examined neurobiological components responsible for stress controllability, although a few studies have reported that catecholamines and corticotropin-releasing factors may affect individual variations in behavioral response[51,52]. More recently, mGluR5 has been reported to be crucial for the acquisition of new learning, which is altered by prior exposure to stress depending on its nature. mGluR5 knock-out mice showed impaired learning when the location of the platform was changed after the acquisition phase in the Morris water maze task, suggesting that mGluR5 may be important for the adaptation process that is required to alter preexisted behavior through new learning[5,53]. This study revealed the importance of mGluR5 for establishing either positive or negative coping strategies in response to a stressor that has been repeatedly experienced by the organism.

One intriguing finding in this study was that mGluR5 protein and mRNA levels in the hippocampus were widely altered by repetitive exposure to the stress. Furthermore, the increased level or the decreased level of mGluR5 was accompanied by altered brain functions. Dysfunction of mGluR5 has been implicated in numerous central nervous system disorders, including anxiety, depression, epilepsy, neuropathic pain, drug addiction, fragile X syndrome, and Parkinson's disease[54-60]. Some study indicating a possible link between mGluR5 and stress, showed a decreased mGluR5 protein expression in rats after chronic

injection with corticosterone, a representative stress hormone in rodents, or electric footshock stress[38,61,62].

To investigate the effect of expression level changes of the mGluR5 protein on behavioral response, the occurrence of the HL behavior, corticosterone concentration, and EEG changes were observed. The results showed that an increased mGluR5 protein expression level is related to HL (+) behavior, a decreased EEG theta power and an increased blood corticosterone concentration. In contrast, decreased mGluR5 protein expression level is accompanied by HL (-) behavior, no changed EEG and no changed corticosterone level. From these findings, it can be deduced that the coping mechanism in different groups is closely related with mGluR5 expression levels. The HL paradigm was designed in order to examine stress controllability by administering electric foot shocks. HL (+) behavior was interpreted as a negative adaptive response, which could result in depressive-like behavior according to the helplessness paradigm[63]. And, theta activity is well known as a measure of gating function in information processing, specifically to permit selective attention for memory formation in limbic regions[64]. Therefore, it is possible that a subject with low theta power has impaired information processing and memory formation. Furthermore, decreased theta power is an EEG pattern that in observed clinically in patients with depression. Indeed, the EEGs of patients with seasonal affective disorder, a form of depression, showed

decreased theta power in the parietal and the temporal regions[65]. Moreover, the functional connection of theta power between brain regions like the anterior cingulate cortex - prefrontal cortex, or the anterior cingulate cortex - orbitofrontal cortex was found to be reduced in depression[66]. The elevated blood corticosterone levels observed in patients with depression[67], because blood corticosterone levels are elevated by prolonged exposure to intensive stressors. Therefore, the group, which showed increased mGluR5 expression level, utilized negative adaptation and has similar characteristics to patients with depression. Thus, it appears that pattern changes of mGluR5 protein expression may be necessary to cope and positively respond to the stressor.

To investigate the underlying mechanism of expression level changes of the mGluR5 protein, I have demonstrated a novel evidence that altered mGluR5 expression is accompanied by the epigenetic modulation of mGluR5 gene after repetitive exposure to stress. This epigenetic modulation correlated positively with DNMT3a mRNA levels, which increased in the LE group that showed increased DNA methylation sites of the mGluR5 gene. I was unable to select specific methylation sites for a decreasing mGluR5 expression in this study because 12 out of 31 CpG sites exhibited increased methylation. However, one notable CpG site (CpG 24) was found in the HE group, which represented the only site with decreased methylation compared to the control group. I speculate that this CpG site (CpG 24) is closely linked with the observed increases in

mGluR5 expression. The PROMO program indicated that paired box protein 5, albumin D-box binding protein, zinc-finger factor 5 (ZF5), and Adh transcription factor 1 have potency for binding to this particular CpG site. Among these transcription factors, ZF5 was especially interested, because ZF5 negatively regulates fragile X mental retardation gene activity[68,69]. Interestingly, mental retardation is a major symptom of fragile X syndrome, and mentally retarded patients have shown poor adaptation to environments, which is a sign of negative adaptation to stress. Further study will be needed to demonstrate a conclusive underlying mechanism for these processes.

V. CONCLUSION

Present study demonstrated that the role of hippocampal mGluR5 in controllability-based coping strategies to the same environmental stressful experiences.

Behavioral changes according to the patterns of the mGluR5 expression level

(1) When exposed to electric footshock twice at PNDs 14 and 90, 81.8 % of them showed the same behavior during the second footshock as they did during the first. And among these, 54.5 % manifested helplessness positive (HL (+)) behavior during both electric footshock.

(2) mGluR5 expression increases when HL is present and decreases when absent.

(3) Exploratory activity decreased gradually as the trial was repeated in the control and the HL (-) group. However, the HL (+) group did not show any decrease in ambulatory activity during the repeated trials.

(4) The group of the increased mGluR5 expression level showed decreased theta power and increased blood concentration of corticosterone.

(5) The group of the decreased mGluR5 expression level showed no changed theta power and blood concentration of corticosterone.

(6) Individual differences in mGluR5 expression and theta power after repetitive exposure to the stress were all abolished by the specific mGluR5 antagonist, MPEP.

(7) The increased mGluR5 expression level suggests the negative adaptation with HL (+) behavior to stressor, while the decreased mGluR5 expression level with HL (-) behavior suggests the positive adaptation to stressor.

Underlying mechanisms of the patterns of the mGluR5 expression level

(1) The group of the increased mGluR5 expression level showed high mRNA expression of the mGluR5, and a decreased methylation site in the CpG island of the mGluR5 gene.

(2) The group of the decreased mGluR5 expression level exhibited low mGluR5 mRNA expression, and an increased methylation sites in the CpG island of the mGluR5 gene.

The results indicated that mGluR5 plays a critical role in the development of coping strategies for stress and suggest that targeting mGluR5 may be an effective way to treat neuropsychiatric conditions involving perseverance of inadequate ideas. This study was the first to ever link differential regulation of mGluR5 to stress coping strategies.

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Abstract (IN KOREAN)

스트레스 행동반응에서 metabotropic glutamate receptor 5 (mGluR5)의 역할: 생리학적 및 행동학적 분석

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임영신

동일한 스트레스 인자에 노출되었지만 서로 다른 반응을 보이는 개인차 현상은 유전적 요소로 선천적으로 타고나는 것과, 경험을 통하여 후천적으로 학습하는 것의 총화에 의해 결정된다. 유전과 경험의 총화에 의해 결정되는 대체전략은 스트레스 인자에 대한 행동 통제가능성을 결정할 수 있다. 본 연구에서는 개인차 현상을 동일한 스트레스 인자에 노출된 개체의 통제가능성에 따라서 분석하고자 하였다. 이를 위하여 무력 모델 (helplessness paradigm)과 반복 구금스트레스 모델 (repetitive restraint stress model)을 사용하였다.

mGluR5 단백질의 양적 변화의 행동학적 의미를 분석하기 위하여, 무력 행동 표출, 혈중 콜티코스테론 농도, 그리고 뇌파 변동을

측정하였다. mGluR5 단백질의 양이 증가된 경우, 무력 행동이 나타나며, EEG 의 theta 파가 감소하고, 혈중 콜티코스테론의 농도가 증가하였다. 이는 스트레스에 부정적 적응을 한 것을 의미한다. 반면에, mGluR5 단백질의 양이 감소한 경우, 무력 행동이 나타나지 않았으며, EEG 의 theta 파와 혈중 콜티코스테론의 농도에도 변화가 없었다. 이는 스트레스에 긍정적 적응을 한 것을 의미한다.

mGluR5 단백질의 양적 변화의 분자적 기전을 분석하기 위하여, mGluR5 단백질의 후생유전학적 변형 정도를 관찰하였다. 그 결과, mGluR5 단백의 양이 줄어든 경우, mGluR5 mRNA 양이 감소되어 있었으며, DNMT 3a 의 활성이 증가되어 있고, mGluR5 유전자의 메틸화가 증가되어 있었다. 반면에, mGluR5 단백질의 양이 증가된 경우, mGluR5 mRNA 양이 증가되어 있었으며, 특정 CpG site 에서 메틸화가 감소되어 있었다.

따라서, 본 연구는 반복적으로 노출되는 스트레스 인자에 대한 대체 전략 개발에 mGluR5가 관여함을 밝혔다. 그리고, 대체 전략에 따른 mGluR5의 발현 양 차이는 mGluR5 유전자의 후생유전학적 조절이 동반됨을 밝혔다.

핵심되는 말: mGluR5, 스트레스, DNA 메틸화, 대체 전략, 해마