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Increased Activation of Synapsin 1 and Mitogen-Activated Protein Kinases/Extracellular Signal-Regulated Kinase in the Amygdala of Maternal Separation Rats

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Keywords
Amygdala; Early life stress; Maternal separation; Mitogen-activated protein kinase; Synapsin 1.

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SUMMARY

Background: Early life stress (ELS) causes alterations in emotionality and anxiety levels as a significant risk factor for psychiatric problems, and these alterations have been associated with amygdala activity. **Aims:** To elucidate the molecular mechanism on the development of psychiatric problems following ELS, we identified the alteration of molecules in the amygdala using maternal separation (MS; pnd 14–21) rats through gene expression and DNA methylation microarray analysis, and studied the involvement of candidate genes using a Western blot and immunohistochemistry analysis. **Results:** Through a microarray analysis, in the amygdala of MS rats, we found a downregulation of mRNA expression of synapsin 1 (Syn1) gene with hypermethylation of its transcription start site (TSS), and the alterations of mRNA expressions of Syn1 activation-related kinase genes including mitogen-activated protein kinases (Mapks) with change of their TSS methylation. In addition, MS increased not only Syn1 phosphorylation at the phosphorylation sites by Mapk/extracellular signal-regulated kinase (Erk), but also Mapk/Erk phosphorylation in the amygdala. Furthermore, double immunofluorescence staining showed that MS could elevate phospho-Mapk/Erk immunoreactivity (IR) in Syn1-expression puncta. **Conclusion:** These findings indicated that the activation of Mapk/Erk and Syn1 may be a key mechanism modulating synaptic neurotransmition in the amygdala of MS rats.

Introduction

Early life stress (ELS) such as child abuse, social isolation, and parental loss is a significant risk factor for the development of neuropsychiatric diseases like cognitive deficits, anxiety disorders, and depression [1,2]. In addition, human and experimental animal studies have shown that ELS can affect the nervous system development, neuroendocrine, and neuroimmune responses [3–5].

Maternal separation (MS) in rats during the early postnatal period has been established as an experimental model for ELS and has been also served as a model of psychopathology for anxiety disorders and depression [6–9]. Indeed, previous studies showed increased anxiety- and depression-related behaviors, and alterations of stress-related neurobiological parameters such as elevated corticotropin-releasing hormone (CRH) expression and increased glucocorticoid receptor density in the brain in MS rats by various MS protocols such as a single or repeated separation and the duration of separation (e.g., 3-h or 15-min daily MS during postnatal day [pnd] 0-21, 3-10 or 2-14) [6–9]. Lee et al. [10] introduced MS animal model totally separating from pups' respective mothers and littermates from pnd 14. They described that the third postnatal week would represent a crucial period in the maturation of the rat nervous system, and also this MS paradigm could avoid the stress hyporesponsive period (pnd 2-14) [10,11]. MS rats by Lee et al. [10] have also shown increased anxiety- and depression-related behaviors and alterations of stress-related neurobiological parameters [10–16].

Amygdaloid complex plays a critical role in the formation and storage of memories associated with emotional events, including fear and anxiety [17,18]. The function of the amygdala is involved in the processing of incoming emotional information and to the execution of emotional responses such as an increased heart rate, increased startle responses, freezing, and increased skin conductance [19]. Clinical evidence also showed an involvement of the amygdala in the etiology of psychiatric diseases. For example, hyperactivation of the amygdala was observed in major depression patients through fMRI study [20,21]. A change of amygdala volume, lower serotonin transporter binding potential, and abnormal elevations of resting glucose metabolism in the amygdala was also shown in depression patients [22–25]. Furthermore, the amygdala is intimately connected to regions, such as the frontal lobe, which are involved in mood disorders, thereby influencing the storage and processing of emotional stimuli in cortical areas

[26]. Consequently, structural alterations in amygdaloid regions may lead to dysfunction of other key neuronal systems implicated in affective and behavioral regulation. Considering these previous reports, the amygdala may be a crucial brain region for the investigation of causation of psychiatric diseases. However, the molecular bases related to psychiatric diseases, and in particular, ELS-induced psychiatric diseases remain largely unknown in the amygdala.

Here, in the amygdala, we assessed the alteration of molecules in development of psychiatric diseases following ELS using MS rats by Lee et al. [10]. First of all, for comprehensive evaluation of the molecular changes following ELS, we studied alteration of mRNA expression and DNA methylation in the amygdala of MS rats, using microarray method. In microarray data, we particularly paid attention to the expression change of synapsin 1 (Syn1) gene. MS altered mRNA expression and DNA methylation of not only Syn1, but also v-src sarcoma viral oncogene homolog (Src), cyclin-dependent kinase 5 (Cdk5), protein kinase gamma 1 (Prkg1), and mitogen-activated protein kinases (Mapks), which are kinases for Syn1 phosphorylation [27]. Syn1 can affect synaptic function through activation by the phosphorylation [27]. Thus, we expected that Syn1 and its activation by Src, Cdk5, Prkg1, and Mapks might play a role in the pathophysiology of psychiatric diseases following ELS. Therefore, we further studied and discussed the possible involvement of Syn1 in the psychiatric diseases.

Materials and Methods

Animals and Maternal Separation

Timed-pregnant Sprague-Dawley rats were provided on gestation day 17 from Orient Bio (Seoul, South Korea). The rats were individually housed in standard rat cages and maintained under a 12-h light/dark cycle at a standard temperature (22 ± 3°C) with food and water freely available.

The day of delivery was designated as pnd 0. On pnd 14, pups were randomly assigned to one of the two groups, the control or MS group. Each group consisted of the same number of pups per litter with the same proportion of males and females in each litter as possible. Pups of the control group were housed with their mothers under standard conditions. Those of the MS group were maintained individually for 7 days (pnd 14-21) in a new cage with free access to food and water [10,12,14,15]. Then, rats of each group were submitted to the elevated plus-maze (EPM) test (n = 7 per group), forced swim test (FST) (n = 7 per group), or sacrificed for the biochemical studies (n = 22–23 per group) on pnd 21. All animal experiments were conducted in accordance with the animal care guidelines of the National Institute for Health (NIH) Guide and the Korean Academy of Medical Sciences, and approved by the Animal Care and Use Committee at Kyung Hee University.

Elevated Plus-maze Test

The EPM test was performed as described previously on pnd 21 [12,16]. The plus-maze was constructed from black wood and elevated to a height of 50 cm. It consisted of two open arms (30 × 10 cm) and two enclosed arms (30 × 10 × 30 cm, with an open roof). After habituated to the testing room for at least 20 min, each rat was placed in the center of a cross-maze facing an open arm. Time spent and the numbers of entries into the open or closed arms were recorded during a 5-min test session. As parameters of anxiety-related behavior, the percentage of time spent in the open arms and the percentage of open arm entries were measured.

Forced Swim Test

On pnd 21 [28], the FST was conducted using a modification of the method of Porsolt [29]. Briefly, two swimming sessions were conducted: an initial 15-min pretest followed 24 h later by a 5-min test. Rats were placed individually in Plexiglas cylinders (height 50 cm; diameter 20 cm) containing water at 25 ± 2°C for 15 min (pretest session). After 24 h, 5-min test session, was conducted. During the test session, the climbing, swimming, and immobility times were analyzed using a Smart version 2.5 video tracking system (Panlab, Barcelona, Spain).

Tissue Collection

For gene expression and DNA methylation microarray analysis (n = 5 per group for each analysis), and Western blot analysis (n = 6 per group), the amygdala region of each rat in both groups was dissected out. Tissue samples were weighted and kept frozen until analysis.

For the immunohistochemistry, rats (n = 6–7 per group) were transcardially perfused with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and then with chilled 4% paraformaldehyde. After post-fixation, the brains were cryoprotected in 30% sucrose, sectioned coronally (40 μm) on a freezing microtome, and collected in cryoprotectant.

Gene Expression Microarray Analysis

Gene expression was analyzed using MYcroarray's Rat 40K Microarray (MYcroarray.com). Total RNA was extracted from each individual amygdala (yield; 50–55 μg as total RNA, A260/A230 > 2.0; 28 s/18 s > 1.6). For extracted RNA, target cRNA probes (Cy3-labeled and Cy5-labeled cRNA for control and MS, respectively) were synthesized and were hybridized to microarray using Agilent's Low Input QuickAmp Labeling Kit (Agilent Technology, Palo Alto, CA, USA) according to the manufacturer's instructions. Then, the hybridized microarrays were scanned with Agilent DNA microarray scanner (Agilent Technology).

The data quantification was performed using Agilent Feature Extraction software 9.3.2.1 (Agilent Technology). The average fluorescence intensity for each spot was calculated, and local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3.1 (Agilent Technology). Genes were filtered with removing flag-out genes in each experiment. Intensity-dependent normalization (Lowess) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

Functional annotation of genes was performed according to Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3.1.

Immunoprecipitation and Microarray Analysis of Methylated DNA

The methylated DNA immunoprecipitation (MeDIP) assay was performed as described [30]. Briefly, genomic DNA was extracted from each individual amygdala (yield: 52–58 μg, A260/A280 > 1.8). Extracted DNA was sheared into 300–1200-bp fragments by sonication and was incubated with anti-5-methylcytidine mAb (Eurogentec, Liège, Belgium). Antibody-bound DNA was precipitated with Dynabeads (M-280 sheep antibodies to mouse IgG, Dynal Biotech). The immunoprecipitated DNA (Cy5) and sonicated input DNA (Cy3) on control and MS groups were differentially labeled with fluorescent dyes and hybridized to microarray. Additionally, to investigate the deference of DNA methylation between the control and MS groups, immunoprecipitated DNA of the control and MS groups were labeled with Cy3 (control) and Cy5 (MS), and hybridized to microarray.

We used NimbleGen Rat DNA methylation 3 × 720 K CpG Island Plus RefSep Promoter Arrays. The array contains 720,000 probes with an average length of 60 bp tiled in 100-bp steps along the upstream promoter regions. We applied standard normalization methods for two-channel microarrays: within (Lowess-based) and between (Quantile-based) normalization available in the DEVA software (Roche Nimblegen). The probe-level log ratio was determined as the log2 of the Cy5/Cy3 channels (immunoprecipitated DNA/input DNA or MS/control) and used as a measure of MeDIP enrichment. To compare the difference of DNA methylation between MS and control groups, we calculated log2 ratio of MS to control, and to remove false-positive data, we referred log2 ratio of immunoprecipitated DNA to input DNA on each group. The position of the center of each probe on the array was compared with the transcription start site (TSS) of known RefSeqs retrieved from UCSC Rat genome annotations (Rn34). Multiple annotations of a probe in association with different RefSeq IDs were allowed.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was performed to confirm the results obtained in the microarray analysis. The PCR reaction was conducted using first-strand cDNA, each gene-specific primers (Table S1), and SYBR Green I master (Roche, Mannheim, Germany). Each cDNA template was analyzed for quantitation in duplicate. Relative differences in the initial amount of each cDNA were determined by comparing cycle threshold values. The one-peak melting curve for detecting the SYBR Green-based objective amplicon was confirmed.

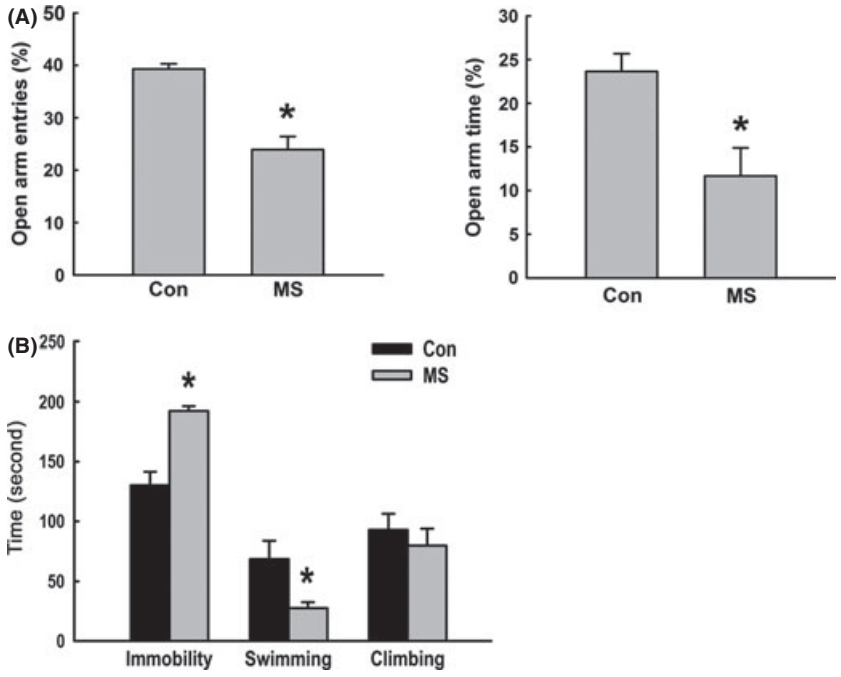

Figure 1 Behavioral characterization of maternal separation (MS) rats. (**A**) Effects of MS on the elevated plus-maze (EPM) test. An EPM test was performed to assess anxiety-related behavior. Anxiety-related behavior is indicated by the percentage of time spent in the open arms and percentage of open arm entries. (**B**) Effects of MS on the forced swim test (FST). The FST was performed to assess depression-related behavior. The following behaviors were measured as follows: (1) immobility: measured when no additional activity is observed other than that required to keep the rat's head above the water; (2) swimming: defined as horizontal movements throughout the cylinder including crossing across quadrants of the cylinder; and (3) climbing: defined as upward directed movements of the forepaws along the side of the cylinder. Results are expressed as immobility, swimming, and climbing time. Data are expressed as mean ± SEM. *Significant difference between groups, $P < 0.05$. (n = 7 per each group).

Western Blot Analysis

Equal amounts of proteins (50 μg) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). After blocking with 5% skim milk, membranes were probed with each antibody (Table S2) overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The results of a Western blot analysis were quantified using ImageJ image analysis software (NIH, Bethesda, MD, USA).

Immunohistochemistry

Floating sections, involving the amygdala region, were blocked with 10% normal goat serum for 1 h and were incubated overnight with rabbit Syn1 or phospho-Mapk/Erk antibodies (Table S2) at 4°C. The sections were incubated with biotinylate-conjugated goat anti-rabbit IgG for 2 h and then with Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 1 h. The sections were stained using a DAB substrate kit (Vector Laboratories), mounted onto gelatin-coated slides, and covered with coverslips.

Double Immunofluorescence Staining

For the double immunofluorescence staining, free-floating sections of the amygdala were incubated overnight with mouse phospho-Mapk/Erk and rabbit Syn1 antibodies (Table S2) at 4°C. The sections were subsequently incubated concurrently with Alexa 488-conjugated anti-mouse and Alexa 594-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 2 h. The sections were mounted onto gelatin-coated slides and covered with coverslips.

Quantification of Immunohistochemistry and Statistical Analysis

Immunohistochemical images were visualized using a light microscope (Olympus, Tokyo, Japan). Quantification was performed using an Image-Pro Plus computer-assisted image analysis system (Media Cyberbetics Inc., Bethesda, MD, USA). For immunostaining analysis of Syn1, the immunoreactivity (IR) level was measured quantitatively in terms of optical density on the central (CeA), lateral (LA), and basolateral amygdala (BLA). For immunostaining analysis of phospho-Mapk/Erk, the number of phospho-Mapk/Erk-positive cells in the CeA was counted from each section, and cell counts were presented as the mean number of cells per unit area (mm^2).

To visualize immunofluorescence images, an Olympus FluoView FV10i confocal microscope (Olympus, Tokyo, Japan) was used. The images were acquired and analyzed using Olympus Fluoview FV10-ASW 3.0 software (Olympus). Colocalization analysis for phospho-Mapk/Erk and Syn1 was performed using the JaCoP plug-in in ImageJ [31], and the percentage of Syn1 expression to phospho-Mapk/Erk expression was calculated. Mander's overlap coefficient (MOC) [32] was also employed. The MOC indicates an overlap of the signals and, thus, represents a true degree of colocalization.

All values are presented as the mean ± standard error of the mean (SEM). The data were analyzed by one-tailed Student's *t*-test using the SPSS software (release 18.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Effect of MS on Anxiety-related Behavior

The EPM test was performed to assess anxiety-related behavior. As shown in Figure 1A, the MS rats had a significantly lower

Table 1 Synapsin 1 (Syn1) and Syn1 activation-related genes altered by maternal separation (MS) in the rat amygdala

				MS/Con	
Gene	Location	Accession	Description	Gene expression array (ratio)	DNA methylation array (log2 ratio)
Upregulation					
Src	3q42	NM_031977	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	1.70	−2.19
Skap1	10q31	NM_173311	Src kinase-associated phosphoprotein 1	1.77	−2.14
Map4k2	1q43	NM_001106329	Mitogen-activated protein kinase kinase kinase kinase 2	2.07	−2.15
Prkag1	7q36	NM_013010	Protein kinase, AMP-activated, gamma 1 noncatalytic subunit	2.20	−2.14
Map4k1	1q21	NM_001106243	Mitogen-activated protein kinase kinase kinase kinase 1	3.33	−2.71
Downregulation					
Cdk5	4q11	NM_080885	Cyclin-dependent kinase 5	0.22	2.72
Syn1	Xq12	NM_019133	Synapsin I	0.45	2.28

Accession means GenBank accession number of the transcripts.

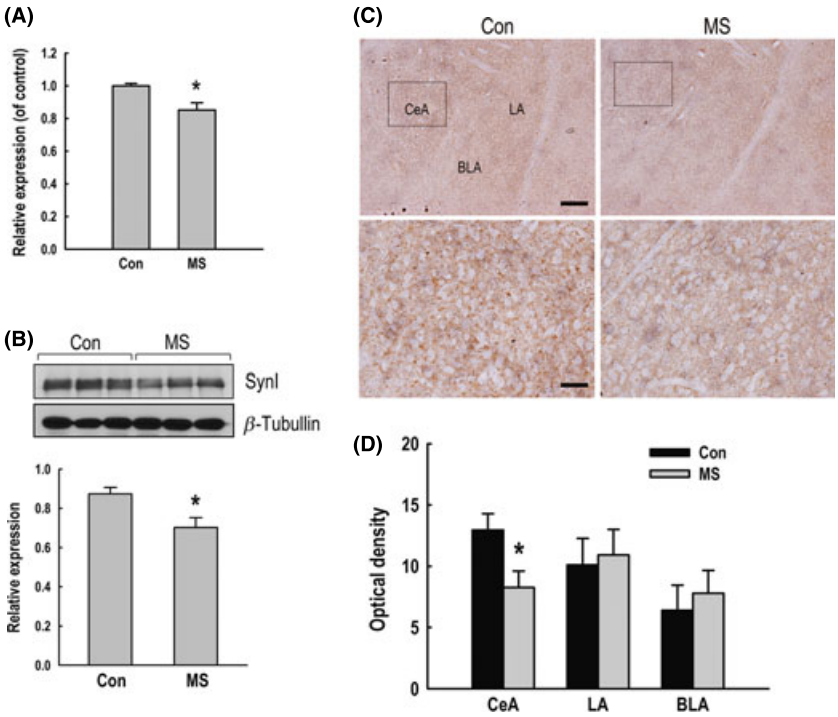

Figure 2 mRNA and protein expression of synapsin 1 (Syn1) in the amygdala of maternal separation (MS) rats. (**A**) Syn1 mRNA expression in the amygdala of control and MS rats. Expression of mRNA was normalized to the expression of Gapdh measured in the same RNA preparation. Results are presented as the mRNA level of Syn1 in the MS group divided by it in the control group, as the mean ± SEM. The experiments were performed using 5 mice per group. (**B**) Syn1 protein expression in the amygdala of control and MS rats. β-Tubulin was used as an internal control. Optical density of Syn1 protein expression is shown as the expression level of Syn1 divided by the expression level of β-tubulin, as the mean ± SEM. The experiments were performed using six mice per group. (**C**) Immunoreactivity (IR) of Syn1 in the amygdala of control and MS rats. Scale bars indicate 20 μm (upper panel) and 5 μm (lower panel). (**D**) Optical density of Syn1. The experiments were performed using six to seven mice per group. Results are presented as the mean ± SEM. *Significant difference between the groups, $P < 0.05$. CeA, central amygdala; LA, lateral amygdala; BLA, basolateral amygdala. Independent experiments were performed three times.

percentage of open arm entries and spent less time in the open arms than the control rats. This result indicated that anxiety-related behavior was increased in MS rats.

Effect of MS on Depression-related Behavior

According to the criteria of Porsolt et al. [29], the duration of immobility in the test session of the FST indicates a state of "despair". In this sense, depressive behavior is related to prolonged immobility times. As shown in Figure 1B, MS rats displayed significantly less swimming and more immobility times than control rats.

Altered Gene Expression by MS in the Rat Amygdala

To assess the gene expression profiles in the amygdala of control and MS rats, a microarray was used. We selected genes for which expression was upregulated or downregulated more than 1.5-fold by MS [1.5 ≤ gene expression ratio (MS group/control group) or 0.7 > gene expression ratio]. MS downregulated the level of 1138 gene transcripts and upregulated the level of 1311 gene transcripts in the amygdala compared with the control (data not shown).

Early life stress could change DNA methylation patterns, which could cause permanent alterations of the gene expression in the brain [33,34]. Thus, we explored the role of changes in DNA methylation through microarray analysis. To investigate DNA methylation pattern on altered gene expression by MS, we assembled the results of DNA methylation and gene expression microarray. In particular, we focused on methylation of TSS for each gene. Among the 2449 genes showed the altered expression, we selected genes for which DNA methylation was upregulated or downregulated more than twofold in the amygdala of MS rats compared with the control rats (2 ≤ log2 ratio or −2 > log2 ratio). MS downregulated expression of 50 genes together with hypermethylation of TSS (Supplemental Table S3) and upregulated expression of 79 genes together with hypomethylation of TSS (Supplemental Table S4). Interestingly, we found that MS changed the expression level of Syn1 gene and Syn1 activation-related genes, which are kinase genes that contribute to the modulation of Syn1 function [27]. As shown in Table 1, the level of Syn1 and Cdk5 genes was decreased in the amygdala of MS rats with hypermethylation, whereas the expression of Src, src kinase-associated phosphoprotein 1 (Skap1), Map4k2, Prkag1, and Map4k1 genes was increased with hypomethylation. Thus, we selected Syn1

from altered genes by MS and further studied the effect of MS on Syn1 and its activation.

mRNA and Protein Expression of Syn1 in the Amygdala of MS Rats

To confirm the decrease in Syn1 gene expression, we performed real-time quantitative RT-PCR in the amygdala of control and MS rats. As shown in Figure 2A, the mRNA expression of Syn1 was decreased in the amygdala of MS rats compared with the control.

Maternal separation also significantly attenuated Syn1 protein expression in the amygdala (Figure 2B). In particular, Syn1 protein expression was attenuated in the CeA of MS rats, but not in the LA and BLA (Figure 2C and D).

Syn1 Phosphorylation in the Amygdala of MS Rats

Syn1 is phosphorylated by several protein kinases (Figure 3A), and its phosphorylation/dephosphorylation state can influence Syn1 function, and thus synaptic plasticity [27]. Interestingly, our microarray analysis showed that the expression of several kinase genes related with Syn1 phosphorylation such as Src, Mapks, Prkag1 and Cdk5 was changed in the amygdala of MS rats. Thus, we examined Syn1 phosphorylation in the amygdala of control and MS rats. As shown in Figure 3B, MS significantly increased Syn1 phosphorylation at the phosphorylation site 4, 5 and 6, 7. Serines 62 and 67 (4, 5P) in domain B and serines 549 and 551 (6, 7P) in domain D were phosphorylated by Mapk/Erk (site 7 also by Cdk1/5) [27]. Thus, we expected that Mapk/Erk may play a role in Syn1 activation by MS in the rat amygdala.

Mapk/Erk Activation in the Amygdala of MS Rats

In our microarray data, we found that MS upregulated Map4k2 (also known as Mapk/Erk kinase kinase kinase 2) and Map4k1 (also known as Mapk/Erk kinase kinase kinase 1) gene expression in the rat amygdala. The expression change of these genes also was confirmed through real-time quantitative RT-PCR (Figure S1). We speculated that Map4k1 and Map4k2 increased by MS may affect the activation of Mapk/Erk, which plays an important role in modulation of synaptic function [35,36]. Thus, we examined the protein expression and phosphorylation of Mapk/Erk in the amygdala of control and MS rats. MS could increase the Mapk/Erk phosphorylation, but it did not affect the level of Mapk/Erk protein expression (Figure 4A). The immunostaining also showed that MS elevated phospho-Mapk/Erk IR in the amygdala, and in particular CeA, compared with control (Figure 4B and C).

Colocalization of Phospho-Mapk/Erk with Syn1 in the Amygdala of MS Rats

To determine the cellular localization of phospho-Mapk/Erk, double immunofluorescence labeling of phospho-Mapk/Erk with Syn1 (also known as a presynaptic marker) was performed in the CeA of control and MS rats. As shown in Figure 5, the phospho-Mapk/Erk IR was pronounced in the cell bodies and dendritic areas. Phospho-Mapk/Erk IR was colocalized with several Syn1-positive puncta at dendrites, and also Syn1-positive puncta surrounded phospho-Mapk/Erk-positive cells in the cell body. The colocalization of phospho-Mapk/Erk and Syn1 IR was increased in the CeA of MS rats, compared with the control. The MOC for phospho-Mapk/Erk and Syn1 IR was 0.424 and 0.674 in the CeA of control and MS rats, respectively. The colocalization percentage of Syn1 IR to phospho-Mapk/Erk IR was 1.95 ± 0.54% in control rats and 5.93 ± 0.76% in MS rats. This result indicates that the activated Mapk/Erk could contribute to Syn1 phosphorylation and that Syn1 activation by Mapk/Erk may more increased in MS rats than control rats.

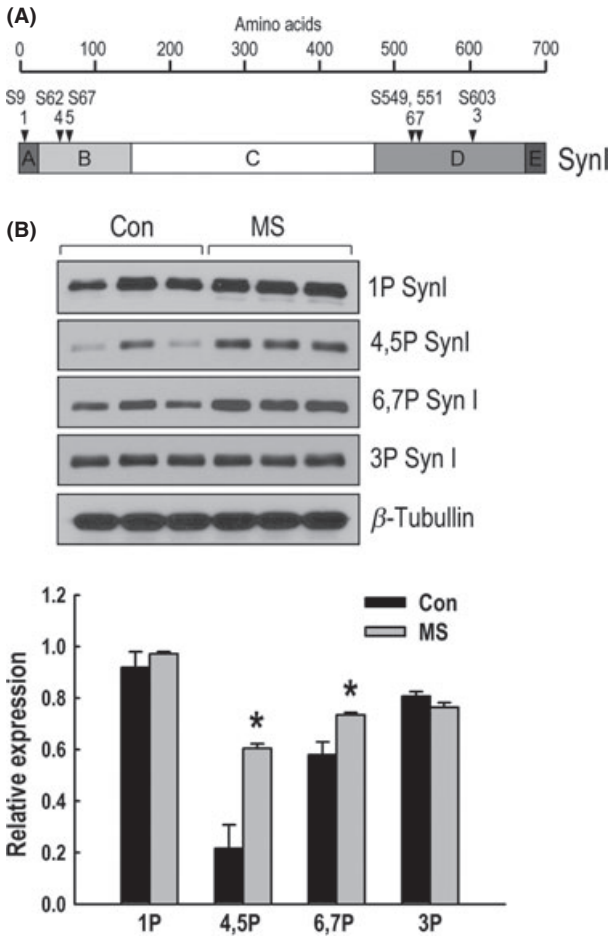

Figure 3 Effect of maternal separation (MS) on synapsin 1 (Syn1) and Mapk/Erk activation in the rat amygdala. (**A**) Syn1 phosphorylation sites. Amino acid numbers of rat Syn1 sequence are indicated at the top. Site 1 in domain A (Ser9 of rat Syn1), phosphorylation site by PKA/Camk-I,-IV; sites 4, 5 in domain B (Ser62,67), phosphorylation site by Mapk/Erk; sites 6, 7 in domain D (Ser549,551), phosphorylation site by Mapk/Erk and Cdk5; site 3 in domain D (Ser603), phosphorylation site by Camk-II [27]. (**B**) Syn1 phosphorylation in the amygdala of control and MS rats. β-Tubullin was used as an internal control. Optical density of the phosphorylated Syn1 expression is shown as the expression level of phospho-Syn1 divided by the expression level of β-tubullin, as the mean ± SEM. *Significant difference between groups, $P < 0.05$. The experiments were performed using 6 mice per group, and independent experiments were performed two times.

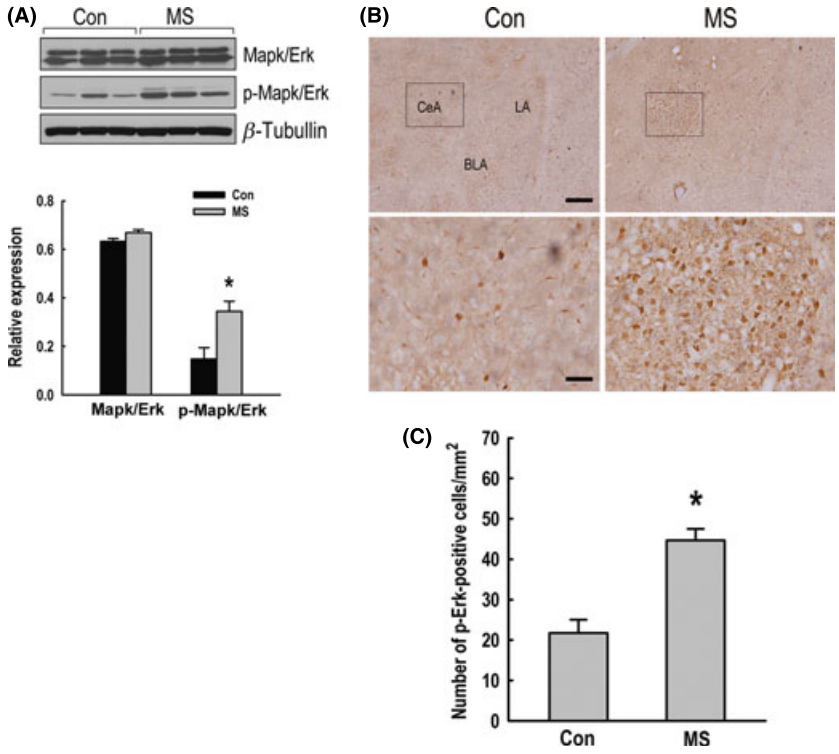

Figure 4 Mapk/Erk activation in the amygdala of maternal separation (MS) rats. (**A**) Total protein expression and phosphorylation of Mapk/Erk in the amygdala of control and MS rats. β-Tubulin was used as an internal control. Optical density of the expressions of Mapk/Erk and phospho-Mapk/Erk is presented as the expression levels of Mapk/Erk and phosphor-Mapk/Erk divided by the expression level of β-tubulin, as the mean ± SEM. The experiments were performed using 6 mice per group. (**B**) Immunoreactivity (IR) of phospho-Mapk/Erk in the amygdala of control and MS rats. Scale bars indicate 20 μm (upper panel) and 5 μm (lower panel). (**C**) Optical density of phospho-Mapk/Erk in the central amygdala (CeA). The experiments were performed using six to seven mice per group. Results are presented as the mean ± SEM. *Significant difference between groups, $P < 0.05$. Three independent experiments were performed.

Discussion

The present study was designed to identify the alteration of molecules following ELS in the amygdala using MS rats. In this study, MS changed not only mRNA expression of Syn1 and Syn1 activation-related kinase genes, but also DNA methylation of these genes. In addition, we found that Syn1 phosphorylation by Mapk/Erk, one of Syn1 activation kinases, was markedly increased. In the amygdala of MS rats, the increase in Mapk/Erk phosphorylation was also shown. Furthermore, MS could elevate the phospho-Mapk/Erk IR in Syn1-expressing puncta.

Maternal separation rats have shown the reduced frequencies of open arm entries and amount of time spent in the open arms in EPM test [7,13], and the increased immobility time in FST [6,13] by various MS protocols. Similar results were also observed in our MS rat. These results indicated that our MS protocol also induced anxiety- and depressive-related behaviors in rats.

Recent studies have shown that ELS in rodents and humans can change methylation patterns at specific loci of the genomic DNA, which in turn permanently alter gene expression in the brain and induce increases in anxiety and depressive behaviors in the adult [33,34,37]. In our study, we found that ELS by MS decreased the expression of 50 genes by TSS hypermethylation in the rat amygdala and increased expression of 79 genes by TSS hypomethylation through microarray analysis. In particular, among these genes, we paid attention to Syn1 gene. MS could not only reduce Syn1 gene expression with hypermethylation of its TSS, but also change expressions of Syn1 activation-related kinase genes such as Cdk5, Mapks, and Src with alteration of their TSS methylation. We also confirmed reduction in Syn1 gene expression through real-time quantitative RT-PCR and found the decrease in Syn1 protein expression in the amygdala, and in particular, in the CeA.

Syn1, which is localized to presynaptic terminals, tethers synaptic vesicles to the actin filaments, thereby forming a reserve pool of vesicles away from the docking site [27,38,39]. Syn1 is the substrate of several protein kinases, including Src, Prkaca (also known as PKA), Ca^{2+}/calmodulin-dependent protein kinases (Camks), Mapk/Erk, and Cdk5, which phosphorylate Syn1 on distinct serine residues and contribute to the modulation of Syn1 function. Phosphorylation of Syn1 reduces its binding to synaptic vesicles and cytoskeleton, which allows vesicles to enter the readily releasable pool and to be released during a presynaptic depolarization [27]. Regulation of the relative number of vesicles in the releasable and reserve pools represents an important mechanism by which neurons regulate neurotransmitter release, and therefore, the efficiency and strength of synaptic signaling [40].

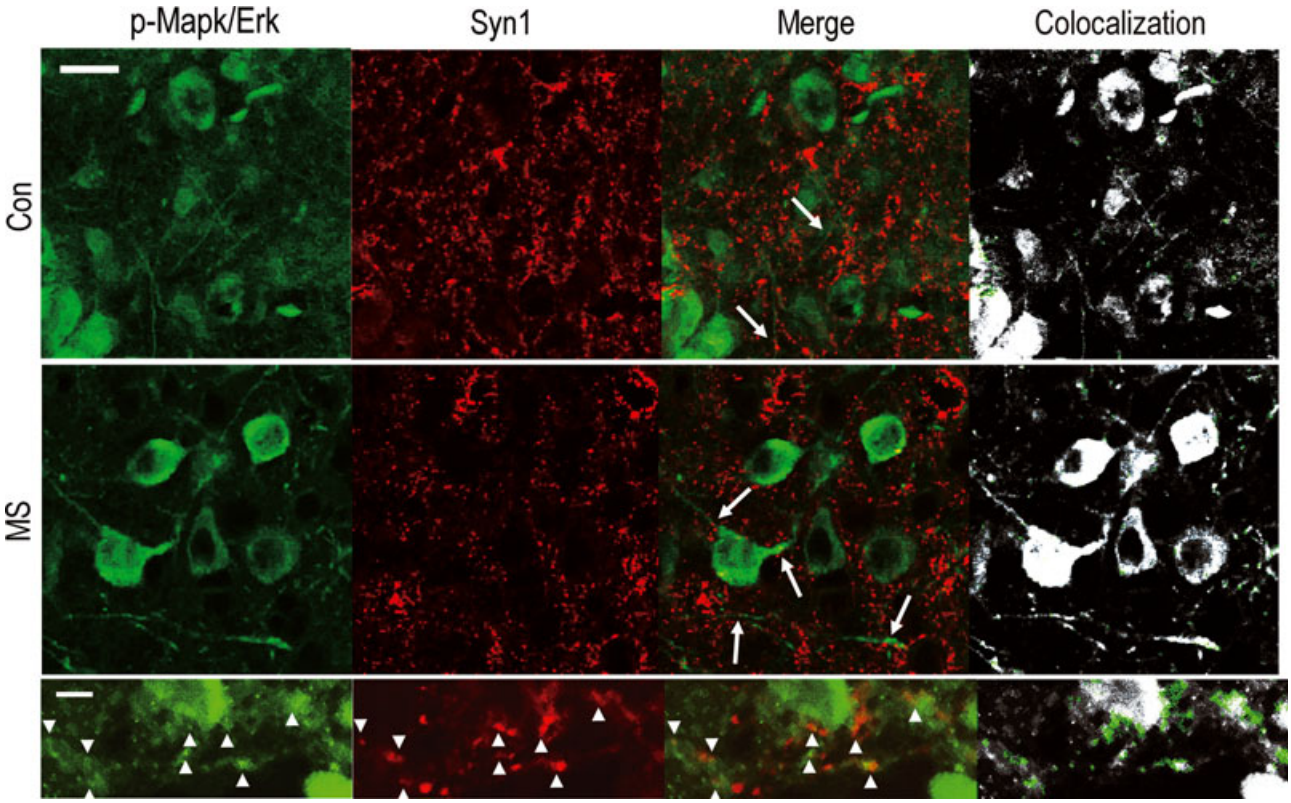

Figure 5 Colocalization of phospho-Mapk/Erk and synapsin 1 (Syn1) in the amygdala of maternal separation (MS) rats. Colocalization of Syn1 (red) on phospho-Mapk/Erk (green) was detected in the central amygdala (CeA) of control and MS rats. Arrows and arrowheads show the immunoreactivity of Syn1 merged with phospho-Mapk/Erk. Three separate experiments were performed, respectively, using six to seven mice per group. Lower panel presents higher magnification of colocalization of phospho-Mapk/Erk and Syn1 in the CeA of MS rats. Scale bars indicate 20 μm (upper panel) and 5 μm (lower panel). *Significant difference between groups, $P < 0.05$.

Previously, several studies showed the change of Syn1 protein expression in the brain of stress-induced anxiety and depression animals. Indeed, ELS-induced reduction in Syn1 expression was reported in the prefrontal cortex (PFC) of rats [41]. A decreased level of Syn1 was also shown in the cingulated cortex and hippocampus of chronic mild stress-induced depression mice [42]. Moreover, some studies reported the reduction in other synaptic markers like synaptogyrin 1 and synaptophysin in the hippocampus or amygdala of stress-induced depression rats and humans [43,44]. Our microarray result also showed decreased expressions of other synaptic markers such as Syn3, synaptotagmin 6 (Syt6), and Syt12 genes in MS rats (data not shown). However, DNA hypermethylation by MS was induced in the only Syn1 gene. Methylation of DNA at CpG dinucleotides may inhibit local gene transcription by interfering with transcription factor binding or by recruiting methylated-DNA-binding proteins that alter transcription efficiency [33,34,37]. Thus, DNA methylation may be a robust mechanism for inducing long-term changes in gene transcription and animal behavior. Interestingly, our microarray data also showed that MS could change transcription level of Syn1 phosphorylation-related kinase genes such as Cdk5, Map4k2, Map4k1, and Src with methylation of their TSS. Thus, we examined whether these Syn1 activation kinases could affect the Syn1 phosphorylation. In our study, MS markedly increased the Syn1 phosphorylation at the site 4, 5 and 6, 7 phosphorylated by Mapk/Erk, although site 6, 7 is also the site phosphorylated by Cdk5, and transcription level of Cdk5 was decrease in our microarray data. Considering that Map4k2 and Map4k1 are kinases of Mapk/Erk, and thus, could influence to Mapk/Erk activation, we expected that MS might affect Mapk/Erk activation in the amygdala. Interestingly, we found the elevation of Mapk/Erk phosphorylation in the amygdala (especially CeA) of MS rats.

The Mapk/Erk has an important role in synaptic plasticity. In synaptic terminals, activated Mapk/Erk could modulate synaptic function through phosphorylation of synaptic targets, including synapsins during short- and long-term plasticity [35,36]. In our study, phospho-Mapk/Erk IR was observed in both cell bodies and dendrites and could also be located in presynapses, colocalizing with Syn1 (it is also presynaptic marker). Interestingly, the presynaptic phospho-Mapk/Erk expression was increased in the CeA of MS rats. This result indicates that activated Mapk/Erk could contribute to the Syn1 phosphorylation, and the Syn1 activation by Mapk/Erk may be increased more in MS rats. In addition, MS decreased total Syn1 and increased phosphorylated Syn1. Because phosphorylated Syn1 allows vesicle release [27,40], it is tempting to speculate that the increase in the ratio of Syn1 phosphorylation

resulting from ELS may lead to a higher vesicle release. Thus, we speculated that the synaptic transmission may be increase in the amygdala of MS rats.

Conclusion

Our results showed that the transcription level of Syn1 and Syn1 activation-related kinase genes was altered in the amygdala of MS rats, and the alteration was induced by change of TSS methylation of the genes. Syn1 phosphorylation was increased, and the increase was potent at the phosphorylation sites by Mapk/Erk. Mapk/Erk phosphorylation was also elevated in the amygdala of MS rats. Moreover, MS could elevate the phospho-Mapk/Erk IR in presynapses. These findings suggested that ELS by MS could affect the synaptic transmission through activation of Mapk/Erk and Syn1 in the amygdala, and Mapk/Erk-Syn1 may be robust mechanism on synaptic modulation induced in ELS, considering that the expression change of Mapk/Erk-related kinase genes and Syn1 gene was induced by the alteration of DNA methylation. These results may contribute to identifying the molecular mechanism of psychiatric diseases following ELS.

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Conflict of Interest

The authors declare no conflict of interest.

Disclosure

The authors have not published or submitted the manuscript elsewhere.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. mRNA expression of mitogen-activated protein kinase (Mapk) genes in the amygdala of maternal separation (MS) rats.
Table S1. Sequences of the primers used for real-time RT-PCR analysis.
Table S2. Antibodies used for Western blot analysis, immunohistochemistry, and double immunofluorescence staining.
Table S3. List of genes downregulated by maternal separation in the rat amygdala.
Table S4. List of genes upregulated by maternal separation in the rat amygdala.