Aging-Dependent Downregulation of SUV39H1 Histone Methyltransferase Increases Susceptibility to Stress-Induced Depressive Behavior

Jung-Eun Lee1 · So-Young Park1 · Pyung-Lim Han1

Received: 19 May 2021 / Accepted: 7 August 2021 / Published online: 18 September 2021
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Abstract
Aging induces cellular and molecular changes including gene expression alteration in the brain, which might be associated with aging-induced decrease in stress coping ability. In the present study, we investigate how aging changes the ability to cope with stress and increases sensitivity to stress. Aged mice show decreased expression of SUV39H1 histone methyltransferase and increased expression of Mkp-1 in the hippocampus. The siRNA-mediated knockdown of SUV39H1 increases Mkp-1 expression and suppresses p-CREB and Bdnf expression in HT22 cells and in the hippocampus of mice. Chromatin immunoprecipitation assays indicate that the levels of SUV39H1 and methylated histone-H3 bound to the promoter of the Mkp-1 in the hippocampus are reduced in aged mice. Aged mice exhibit depression-like behavior following weak stress that does not induce depressive behavior in young mice. Rosmarinic acid, a phenolic compound that increases SUV39H1 expression, reverses stress-induced changes of SUV39H1, Mkp-1, and Bdnf expression in the hippocampus via an overlapping but distinct mechanism from those of fluoxetine and imipramine and produces anti-depressive effects. These results suggest that aging increases susceptibility to stress via downregulation of SUV39H1 and resulting changes in SUV39H1-regulated signaling pathways in the hippocampus.

Keywords Aging · SUV39H1 · Mkp-1 · p-CREB · BDNF

Introduction
Physiological responses of stress proceed with a neuroendocrine reaction, resulting in the release of glucocorticoids (GC) into the blood. GC release in a homeostatic range is vital to activate global metabolic activities of the body and helps individuals cope with intrinsic or extrinsic adverse stimuli [1–3]. However, when the stress response is activated excessively, it may cause maladaptive changes including structural and functional changes in various brain regions such as the hippocampus, prefrontal cortex, and amygdala [4–7].

Elderly people have increased basal GC levels in the blood [8, 9] and show reduced GC release in response to stressors [10, 11]. Brain aging advances with cellular and molecular changes including accumulation of oxidative stress, increased mitochondrial dysfunction, reduced expression of neurotrophic factors, and impaired cellular stress responses [9, 12]. These aging-related changes in the brain are likely to be associated with GC-induced events, and aged brains might have reduced stress coping ability. However, the detailed mechanisms by which aging
and stress interactively produce maladaptive changes in the brain and promote depressive behaviors are not clearly understood.

Mitogen-activated protein kinase (MAPK) phosphatase-1 (Mkp-1) plays an important role in stress-induced adaptive changes in the brain. Chronic unpredictable mild stress ([13], chronic social defeat stress [14], and chronic restraint stress [15] all increased Mkp-1 expression in the brain. Mkp-1 is an important factor regulating the activity of ERKs, p38 kinases, and JNKs [16]. Acute restraint stress increased p-p38 levels in the hippocampus [17], whereas chronic restraint stress opposingly decreased p-p38 and p-ERK levels in the hippocampus ([15, 17]). Chronic social defeat stress also reduced the levels of p-ERK, p-CREB, and BDNF in the medial prefrontal cortex and hippocampus [18], which is consistent with increased Mkp-1 expression [14]. Stress-induced decreased ERK activity can reduce the expression of brain-derived neurotrophic factor (BDNF) via CREB [19, 20]. Aged animals show increased expression of Mkp-1 in the hippocampus [21] and reduced levels of p-ERK and p38 MAPK in the frontal cortex [22]. Aged animals also had reduced levels of p-CREB and BDNF expression in the hippocampus [23–25]. These results suggest that aging-induced increased expression of Mkp-1 likely produces decreased p-CREB and BDNF expression in the hippocampus and affects stress-induced behavioral changes. However, MAPK activity and the expression of neurotrophic factors can be affected by multiple pathways [26, 27]. Most of the previous stress-related studies have been conducted on young animals. Therefore, it needs to study whether age-induced change of Mkp-1 expression reduces p-CREB and BDNF expression and if aged animals with increased Mkp-1 facilitate stress-induced depressive behavior.

Epigenetic factors are critical players in the sustained maintenance of depressive behaviors induced by chronic stress [28, 29]. The histone methyltransferase SUV39H1, which adds tri- or dimethyl groups to histone-3 lysine 9 (H3K9), was downregulated in the hippocampus following chronic stress, and the reduced expression of SUV39H1 in the hippocampus caused sustained increase of Mkp-1 expression and facilitated depressive-like behaviors [15]. Aged mice had increased p47phox and gp91phox expression in the hippocampus, which was associated with aging-induced reduced SUV39H1 expression and reduced SUV39H1 binding to the promoters of p47phox and gp91phox genes [21]. On the contrary, SUV39H1 inhibition using chaetocin A (an inhibitor of histone lysine methyltransferase family) in aged mice increased GluR1 and BDNF expression in hippocampal synaptosomes and reversed age-dependent deficits in hippocampal memory [30]. These results raise the question of whether aging-induced decreased expression of SUV39H1 increases Mkp-1-related factors and facilitates stress-induced depressive behavior.

In the present study, we investigated whether GC- and aging-dependent downregulation of SUV39H1 increases Mkp-1 expression and decreases Bdnf expression in the hippocampus and demonstrated that a strategy promoting SUV39H1 expression rescues stress-dependent adaptive changes in aged brains and stress-induced behavioral deficits.

**Materials and Methods**

**Materials**

Corticosterone, RU486, imipramine, and fluoxetine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rosmarinic acid was purchased from Tocris (Bristol, UK). They were dissolved in 0.9% saline.

**Methods**

**Animals**

Seven-week-old male C57BL/6 mice were purchased from Daehan BioLink Inc. (Eumseong, Chungbuk, Korea) and used for young mice. Aged mice were prepared by crossing C57BL/6 J mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and were raised to the indicated ages in the laboratory. Mice were housed in pairs in a standard clear plastic cage filled with chopped wood particles (TAPVEI, Paekna, Estonia). They had free access to food and water at a temperature (23 °C)- and humidity (50–60%)-controlled environment under 12-h light/dark cycle conditions (from 7:00 a.m. to 7:00 p.m.). All animals were handled in accordance with the animal care guidelines of Ewha Womans University (IACUC 16–018).

**Restraint Stress**

Mice were subjected to restraint stress as described previously [31, 32]. Mice were randomly assigned into experimental groups and housed in pairs in a plastic cage 5 days before placing in the restraint regimen. Mice were individually placed head first into a well-ventilated 50-ml polypropylene conical tube with holes for ventilation and restrained against back-and-forth movement. This treatment was applied for 2-h per day and was repeated for 14 days or
the number of days indicated. After each daily session of restraint, the mice were returned to their home cages.

**Drug Administration**

Rosmarinic acid (30 mg/kg/injection), fluoxetine (20 mg/kg/injection), or imipramine (20 mg/kg/injection) was administered by intraperitoneal (ip) injection in a volume of 100 μl 30 min before the start of each 2-h restraint stress in the co-treatment paradigm. RA, FLX, and IMI were treated for 14 days of the stress phase.

**Cell Culture and Drug Treatment**

HT22 mouse hippocampal cells were cultured as described previously [15, 21]. HT22 cells were cultured in DMEM (LM-001–05; Welgene, Gyeongsan, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FB02-500; Serum Source, NC, USA) and antibiotics (penicillin and streptomycin) (LS-202–02; Welgene) at 37 °C in a humidified incubator gassed with 95% air and 5% CO2.

Cells grown to 70–80% confluence were treated with corticosterone, RU486, or drugs in DMEM containing 1% fetal bovine serum. After 24 h, cells were washed with phosphate-buffered saline (PBS) and harvested for experiments. Corticosterone, RU486, imipramine, fluoxetine, and rosmarinic acid were treated at the dose indicated.

HT22 cells were transfected with siRNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) as described previously [15]. siRNA transfection was carried out in DMEM containing 1% FBS. The final concentration of siRNA was 100 nM, and the concentration of Lipofectamine-2000 was 7.5 μl/well.

The siRNAs used were siRNA-control (SN-1012), siRNA-Mkp-1 (#1,351,203, NM_011514.1), which were obtained from Bioneer Co. (Daejeon, Korea).

**Measurement of Corticosterone Levels**

Corticosterone levels were measured using an ELISA kit (ADI-901–097; Enzo Life Science, Farmingdale, NY, USA) as described previously [21]. Briefly, immediately after the 2-h restraint, mice were anesthetized with 2.5% avertin (intraperitoneal injection) at a dose of 20 μg/g body weight. Blood was collected from the heart between 9:00 a.m. to 12:00 p.m. Blood samples were centrifuged at 3000 rpm at 4 °C for 15 min in a microcentrifuge, and the supernatant was collected. The supernatant (100 μL) of each sample was diluted with the steroid displacement reagent solution of the ELISA kit. They were then dispensed into new wells of the ELISA kit plate and incubated with antibody reagent on a plate shaker. After a 2-h reaction, each well was washed 3 times with wash solution from the ELISA kit. Then, p-nitrophenyl phosphate solution was added and incubated for 1 h. The reaction was stopped with a stop solution, and the plate was read immediately at 405 nm.

**Immunohistochemistry**

Immunohistochemical analyses were carried out as described previously [15]. Briefly, mice were perfused with 4% paraformaldehyde by a trans-cardiac method, and the brains were surgically removed and immersed in the same fixative overnight at 4 °C. Brains were coronally cut into 40-μm thick sections using a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Brain sections were incubated at RT for 1 h with 5% BSA using a free-floating method and were reacted with primary antibody in 5% BSA overnight at 4 °C. After washing 3 times with 1×PBST, the sections were incubated with a secondary antibody for 1 h. After washing with 1×PBST, stained sections were mounted on a gelatin-coated slide with a DAPI staining mounting solution (H-1200; Vector Lab.). The stained sections were analyzed using an Olympus BX 51 microscope equipped with a DP71 camera and MetaMorph Microscopy Automation and Image Analysis software (Molecular Devices, San Jose, CA, USA).

The primary antibodies used were p-CREB (06–519, Millipore, Billerica, MA, USA; 1:500; RRID:AB_310153), p-ERK (4370 s, Cell Signaling, Danvers, MA, USA; 1:500; RRID:AB_2315112), MKP-1 (sc-271684, Santa Cruz, TX, USA; 1:200; RRID:AB_10708413), MAP2 (05–346; Millipore, 1:1000; RRID: AB_309685), Doublecortin (sc-8066, Santa Cruz; 1:1000; RRID:AB_2088494), and Ki67 (VP-K451, Vector Lab.; 1:1000; RRID:AB_2314701). The secondary antibodies used were anti-mouse IgG tagged with DyLight 488 (DI-2488, Vector Lab., Burlingame, CA, USA) or rabbit IgG–conjugated DyLight 594 (DI-1094, Vector Lab.).

**Western Blot Analyses**

Western blot analysis was performed as described previously [33]. The dorsal hippocampal tissue or cultured HT22 cells were homogenized in homogenization buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.1% sodium deoxycholate) containing a cocktail of protease inhibitors (Roche, Mannheim, Germany) on ice using an EpiShear probe sonicator (Active Motif, Carlsbad, CA, USA) at a 40% power output with two rounds of 10-s pulses. The homogenate was centrifuged at 13,000 g at 4 °C for 10 min, and the supernatant was collected. Protein concentration was measured by the Bradford method (Bio-Rad Laboratories, Hercules,
CA, USA). Tissue or cell proteins (20 μg) were mixed with 6 × sample loading buffer and boiled for 5 min. The samples were resolved on SDS-PAGE, and then they were transferred onto a PVDF membrane (Bio-Rad Laboratories). Blots were incubated with blocking solution containing 1% BSA in TBST (150 mM NaCl, 50 mM TRIS–Cl buffer, pH 7.4, 0.1% Tween 20) for 1 h, followed by incubation with a primary antibody in blocking solution for 3 h at RT. The blots were washed 3 times with TBST and then incubated with a secondary antibody of anti-mouse IgG-HP (GTX213111-01; 1:1,000, GeneTex, Irvine, CA, USA) dissolved in TBST at RT for 1 h. Immunoblots were visualized using a PicoEED Western Reagent Kit (EBP-1073; ELpis Biotech, Daejeon, Korea). Western blot images were quantified using Image-ProPremier 6.0 (MediaCybernetics, MD, USA).

The primary antibodies used were anti-p-CREB (06–519, Millipore; 1:1000; RRID:AB_3015153), anti-CREB (sc-186, Santa Cruz; 1:1000; RRID:AB_2086021), and anti-β-actin (sc-47778; 1:1,000, Santa Cruz). The secondary antibodies used were goat anti-mouse IgG (GTX213111, GeneTex, Irvine, CA, USA) and goat anti-rabbit IgG (GTX213110, GeneTex).

**Quantitative Real-Time PCR**

Quantitative real-time PCR was carried out as described previously [15, 21]. Briefly, total RNA from the dorsal hippocampus and cultured HT22 cells was isolated with TRIzol reagent (15,596–018; Invitrogen). The concentration of isolated RNA was measured with NanoDrop (Thermo Fisher Scientific). Successful chromatin shearing samples was quantified using NanoDrop (Thermo Fisher Scientific). Successful chromatin shearing was confirmed by agarose gel electrophoresis. Sheared chromatin samples were stored at −80°C until use.

For the immunoprecipitation (IP) reaction, 10 μg of sheared chromatin of each sample was mixed with 2 μg of primary antibody, 20 μl of Protein G magnetic beads, 10 μl of 10× ChiP buffer 1 from the ChiP Assay Kit (Active Motif), and 1 μl of proteinase inhibitor cocktail in a volume of 100 μl, and was incubated at 4 °C overnight. The primary antibodies used were SUV39H1 (05–615, Millipore; RRID:AB_2196724), dimeH3K9 (ab1220, Abcam, Cambridge, UK; RRID:AB_449854), or trimeH3K9 (ab8898, Abcam; RRID:AB_306848).

The reacted IP products were washed with ChiP buffer I, followed by ChiP buffer II twice using a magnetic rack (Active Motif). After the final wash, the beads were eluted with 50 μl of elution buffer and incubated at RT for 15 min with intermittent agitation. Then, each sample was added to 50 μl of reverse cross-linking buffer, and the supernatant containing eluted chromatin was carefully taken on a magnetic rack. The input DNA (10 μl) was also prepared as the mixture with 88 μl of ChiP buffer II and 2 μl of 5 M NaCl.

Chromatin Immunoprecipitation (ChIP)-qPCR analyses

Chromatin immunoprecipitation (ChIP)-qPCR assays were carried out as described previously [15, 21] using a ChIP-IT® Express Kit (102,026; Active Motif). Hippocampal tissue (40–50 mg) obtained from 6 mice was pooled and minced with a razor blade. Samples were then incubated in 7 ml of 1% formaldehyde (F1635; Sigma-Aldrich) in 1× PBS for 8 min to cross-link the proteins and DNA. Cross-linking reaction between proteins and DNA was stopped by adding 5 ml of 0.125 M glycine and incubating for 5 min. They were then homogenized using a Dounce homogenizer (357,542; Wheaton, Millville, NJ, USA), and the homogenate was centrifuged at 1250 g for 5 min. The pellet was collected and resuspended in 500 μl of lysis buffer containing proteinase cocktail (0.5x, final) and PMSF (0.5 mM, final) and incubated on ice for 30 min. The lysates then were homogenized again and washed with 1× PBS twice. The pellet containing fixed chromatin was resuspended in 350 μl of shearing buffer containing proteinase inhibitors and PMSF. They were then sheared into 200–800 bp fragments by sonication on ice using an Episheer probe sonicator (Active Motif) with 20-s pulses at 50-s intervals at 35% power, repeated 20 times. The sheared chromatin was centrifuged at 22,000 g for 10 min, and the supernatant was saved for immunoprecipitation. The amount of DNA in the sheared chromatin samples was quantified using NanoDrop (Thermo Fisher Scientific).
After incubation at 95 °C for 15 min, 1 μg of Proteinase K was added to the reaction mixture and incubated at 37 °C for 1 h. Followed by the addition of 2 μl of proteinase K stop solution, the resulting reaction was regarded as immunoprecipitated DNA and used for real-time PCR.

The real-time PCR reaction was carried out with 4 μl of the immunoprecipitated DNA, 10 μl of 2×iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), and 1 μl each of 5 pmol/μl forward and reverse primers in a volume of 20 μl using the CFX 96 Real-Time PCR System Detector (Bio-Rad Laboratories). One-tenth of the input DNA was used for quantitative PCR to control the relative amounts of DNA fragments in immunoprecipitation and normalized for quantification. Real-time PCR was carried out using the following primers: 5′-CCAGAGGACAGGAGGTG-3′ and 5′-TTGAATGCGAGACGTGAC-3′ for Mkp-1 P1, for Mkp-1 P1, and 5′-AGTGGAGATGAAAGCAGA-3′ and 5′-GACTTTGGTAGAGCTCCACA-3′ for Mkp-1 P2.

**Stereotoxic Injections of siRNA**

Stereotoxic injection of siRNA was carried out as described previously [15]. Briefly, mice were anesthetized with a 3.5:1 mixture of ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (23.3 mg/ml) at a dose of 2.5 μl/g body weight. Each siRNA (50 ng/μl) was mixed with 2.5 volume of Neurofect transfection reagent (T800075; Genlantis, San Diego, CA, USA) and 0.5 volume of 50% sucrose, and the mixture was incubated for 20 min. A 1.8 μl volume of the siRNA mix (7.5 ng/μl) was injected into each CA3 region (stereotoxic coordinate: AP, −1.9; ML, ±2.1; DV, −2.1 mm) at 0.2 μl/min using a stereotoxic injection system (Vernier Stereotoxic Instrument, Leica Biosystems, Wetzlar, Germany) and a Hamilton syringe with a 30-G needle. The animals were sacrificed to analyze the gene expression at 72 h after siRNA injection.

siRNA-control (SN-1012), siRNA-Mkp-1 (#1,351,659, NM_011514.1) were purchased from the Bioneer Co (Daejeon, Republic of Korea). siRNA-SUV39H1 (#1,433,203, NM_013642.1), and siRNA-SUV39H1 (#1,433,203, NM_011514.1) purchased from the Bioneer Co (Daejeon, Republic of Korea).

**Behavioral Analyses**

Behavioral tests were performed during the light cycle (9 a.m. ~ 4 p.m.) as described previously [15, 31]. Mice were brought to the behavior testing room 30 min before the start of each behavioral test. Background sound in the testing room was masked with 65 dB of white noise by a white noise generator (HDT Korea, Seoul, Korea). The light was lit with indirect illumination by 20 lx for the U-shaped two-choice field and 250 lx for the tail suspension test (TST) and forced swimming test (FST). Animal behaviors in the tests were recorded using a computerized video tracking system (SMART, PanLab, Spain) or a webcam recording system (HD Webcam C210, Logitech, USA).

Social interaction time and distance in the U-shaped two-choice field were measured using an equipped program (SMART, PanLab). Immobility time in the tail suspension test and forced swimming test was recorded in a blind manner. All behavioral tests were conducted in a randomized fashion and/or in an alternative manner depending on test order and position in the U-shaped two-choice field (e.g., left vs. right).

**Sociability Test**

The sociability test using a U-shaped two-choice field was performed as described previously [15, 31]. The U-shaped two-choice field consisted of symmetrical two-spaces with a partitioning wall (40 cm in the open field (45×45 cm). Each symmetrical space had both closed and open square zones, forming a “U-shaped two-choice field” [26]. Each side of the closed square contained a circular grid cage (12 cm in diameter×33 cm in height) with either a social target or an empty cage. The space containing a social target was defined as the target field, and the other side with an empty cage was defined as the non-target field. Subject mice were individually introduced in the middle of the U-shape field and allowed to freely explore 5 min before the test. After habituation, the mice were returned to their home cage for 1 min and then exposed again for 10 min to the U-shaped field with a social target in a grid cage on one side and an empty cage on the other. Age-matched normal B6 male mice were used as a social target, and each target mouse served 3–4 times. All animals, including social targets, were housed in pairs in standard plastic home cages.

**Tail Suspension Test**

The tail suspension test (TST) was performed as described previously [15]. Mice were individually suspended by fixing their tails to the ceiling of a white wooden box using adhesive tape, thereby being held 50 cm above the surface of a table. During a 6 min period, the cumulative immobility time of each mouse was counted.

**Forced Swimming Test**

The forced swimming test (FST) was carried out as described previously [15]. Mice were placed individually in a Plexiglas cylinder (15 cm in diameter×27 cm in height) filled with water at 24 °C at a depth of 14 cm. During a 6 min period, mice were put in the Plexiglas cylinder and their escape-related mobility behavior was measured during...
the last 5 min. Immobility time was defined as the sum of
time during the floating behavior in which all limbs were
motionless.

**K-Means Clustering**

K-means cluster analyses were carried out using SPSS statis-
tics 25 software (IBM SPP Statistics, NY, USA) as described
previously [21]. K-means clustering is an unsupervised
machine-learning algorithm that classifies data points into
similar groups. Depending on the $k$ values, all individual
points are assigned to the nearest cluster.

**Statistical Analyses**

GraphPad PRISM 6.0 software (GraphPad Software, Inc.,
CA, USA) was used for statistical analyses. Two-sample
comparisons were carried out using the Student’s t-test, and
multiple comparisons were made using a one-way ANOVA
followed by the Newman-Keuls post hoc test or two-way
ANOVA followed by the Bonferroni post hoc test. All data
are presented as mean ± SEM, and statistical significance
was accepted at the 5% level.

**Results**

**Aging Decreased SUV39H1 Expression while Increasing Mkp‑1 Expression in the Hippocampus**

Recently, we reported that young mice exposed to chronic
stress have decreased expression of SUV39H1 in the hip-
pocampus, which causes sustained upregulation of Mkp-1
[15]. In the present study, we continued to investigate
whether SUV39H1 and Mkp-1 have a role in the aging brain.
The transcript levels of Mkp-1 increased in the hippocampus
in an aging-dependent manner, which was in contrast to the
aging-dependent decrease of SUV39H1 expression in the

![Fig. 1](image)

Fig. 1 Aging upregulated Mkp-1 expression via SUV39H1 downreg-
ulation in the hippocampus. a, b Transcript levels of Mkp-1 (a) and
SUV39H1 (b) in the hippocampus of mice at 2, 7, 14, and 18 months
of age ($n=6$, each; one-way ANOVA, $F(3,20)=12.72$, $p<0.0001$ for
Mkp-1; $n=6$, each; One-way ANOVA, $F(3,20)=9.562$, $p=0.0004$
for SUV39H1). c, d Experimental design (c, left panel) for stere-
otaxic injection of siRNA-SUV39H1 and siRNA-CON in CA3 region
(red arrows). Blue arrow, tissue preparation point. Transcript levels of
and SUV39H1 (c, right panel) and Mkp-1 (d) after siRNA-mediated
knockdown of SUV39H1 in the CA3 region ($n=6$, each). e–h Dia-
grams (e) showing the promoter region of the $Mkp-1$ and the process
of immunoprecipitation, DNA purification, and following qPCR anal-
ysis. GRE, glucocorticoid-responsive element (red box). P1 and P2,
the promoter regions used for ChIP-qPCR analysis. ChIP-qPCR data
showing the levels of SUV39H1 (f), trim3H3K9 (g), and dime3K9
(h) binding to the promoter of the $Mkp-1$ in the hippocampus of
mice at 2 and 18 months of age ($n=8$, each). Data are presented as
mean ± SEM. * and **, differences between indicated groups at
$p<0.05$ and $p<0.01$, respectively (Student’s t-test, and one-way
ANOVA followed by Newman-Keuls post hoc test).
hippocampus (Fig. 1a, b). The siRNA-mediated knockdown of SUV39H1 in the hippocampus increased the expression of Mkp-1 (Fig. 1c, d). Chromatin immunoprecipitation (ChIP) analysis indicated that the level of SUV39H1 bound to the promoter region of the Mkp-1 was significantly reduced in the hippocampus of aged mice (18 months or 18 M) compared to that of young mice (2 M) (Fig. 1e, f). Consistently, the levels of tri- and di-methylation of histone H3 lysine 9 bound to the promoter region of the Mkp-1 were also reduced in aged mice compared to those in young mice (Fig. 1g, h). These results suggest that the aging-dependent decrease in the expression of SUV39H1 could result in increased Mkp-1 expression in the hippocampus.

**Glucocorticoid Caused Reduced SUV39H1 Expression, which Resulted in Increased Mkp-1 Expression and Decreased p-CREB and Bdnf Expression**

Aged mice (18 M) had increased basal GC levels in the blood compared to those of young mice (2 M) (Fig. 2a), and this result is consistent with the previous report [21]. Aged mice (18 M) had reduced levels of p-CREB and Bdnf expression in the hippocampus (Fig. 2b, c). Therefore, we asked whether the reduced expression of CREB and Bdnf results from GC-induced changes in the expression of SUV39H1 and Mkp-1. GC treatment in HT22 hippocampal cells decreased the levels of p-CREB and Bdnf expression (Fig. 2d, e). Moreover, GC treatment in HT22 cells decreased the expression of SUV39H1 and increased the expression of Mkp-1, in which both GC-induced changes were blocked by co-treatment with RU486, a GR antagonist (Fig. 2f, g). Consistently, siRNA-mediated knockdown of SUV39H1 in HT22 cells increased the expression of Mkp-1 and decreased the levels of p-CREB and Bdnf expression (Fig. 2h–j). In contrast, siRNA-mediated knockdown of Mkp-1 in HT22 cells increased the levels of p-CREB and Bdnf expression (Fig. 2k–m).

ERK1/2 can regulate BDNF expression via CREB [34, 35]. Indeed, inhibition of ERK1/2 using U0126 (an ERK inhibitor) in HT22 cells reduced the Bdnf expression (Fig. 2n). GC treatment in HT22 cells increases Mkp-1 expression and GR binding to its proximal promoter [15]. The siRNA-mediated knockdown of CREB in HT22 cells decreased Bdnf expression (Fig. 2o, p). Together, these results suggest that GC-indcated downregulation of SUV39H1 in HT22 cells induces Mkp-1 expression, which in turn negatively regulates p-CREB and Bdnf expression (Fig. 2q).

Next, we examined if Mkp-1 could regulate p-CREB and Bdnf expression in the hippocampus of mice. Immunohistochemical analysis indicated that Mkp-1 and p-CREB were co-localized at the single-cell level in CA3 pyramidal neurons of the hippocampus, and their expression levels were negatively correlated (Fig. 3a, b). The siRNA-mediated knockdown of Mkp-1 in the hippocampus increased Bdnf expression (Fig. 3c, d) and increased the level of p-CREB (Fig. 3e, f). These results suggest that Mkp-1 negatively regulates Bdnf expression via p-ERK and p-CREB (Fig. 2q).

**Rosmarinic Acid, Imipramine, and Fluoxetine Blocked Stress-Induced Changes of SUV39H1, Mkp-1, and Bdnf Expression in an Overlapped, but Slightly Different, way**

Rosmarinic acid (RA) is a phenolic compound that functions as a transcription inducer of SUV39H1 [15]. In HT22 cells, RA treatment (50 and 100 μM) blocked GC-induced reduced expression of SUV39H1 (Fig. 4a), GC-induced increased expression of Mkp-1 (Fig. 4b), and GC-induced reduced expression of Bdnf (Fig. 4c). Imipramine (IMI; 50 and 100 μM) or fluoxetine (FLX; 5 and 10 μM) treatment also reversed the GC-induced downregulation of SUV39H1 and Bdnf expression (Fig. 4a, c), but not the GC-induced upregulation of Mkp-1 (Fig. 4b). We found that high doses of FLX (15 μM and higher) produced cytotoxic effects in HT22 cells, and therefore, FLX was used at the concentrations ranging 5 μM to 10 μM in the present study. Overall, these results suggest that IMI and FLX can reverse the GC-induced downregulation of SUV39H1 and Bdnf, but not the GC-induced upregulation of Mkp-1, whereas RA can reverse the GC-induced downregulation of SUV39H1 and Bdnf, and the GC-induced upregulation of Mkp-1 (Fig. 4d).

Next, we examined whether and how the expression of SUV39H1, Mkp-1, and Bdnf is changed by the amount of days of restraint exposure. A single 2-h restraint event to mice was sufficient to decrease the expression of SUV39H1 and Bdnf and to increase the expression of Mkp-1 in the hippocampus. The stress-induced changes of those genes were augmented more than those by a single 2-h restraint after treatment with 5 days of 2-h restraint and even further after treatment with 14 days of 2-h restraint (Fig. 4e–h). These results mirrored the transcriptional changes of the same genes after GC-treatment over time in HT22 cells (Fig. 2e–g). These results suggest that the stress-induced decrease in the expression of SUV39H1 and Bdnf and the increased expression of Mkp-1 in the hippocampus are likely regulated by the GC-dependent mechanism.

Next, we examined whether RA, IMI, and FLX treatment modifies stress-induced changes of SUV39H1, Mkp-1, and Bdnf expression in the brain of CRST mice. Mice exposed to daily 2-h restraint for 14 days (RST14d) had reduced expression of SUV39H1 and Bdnf and increased expression of Mkp-1 in the hippocampus. In contrast, mice treated with RA during the RST14d phase (RST14d + RA) had control
Fig. 2 Glucocorticoid or aging-induced changes in the SUV39H1-Mkp-1-p-CREB-Bdnf pathway. a Serum corticosterone levels of mice at 2 months and 18 months of age (n=20, each). b Western blot data showing p-CREB and CREB levels in the hippocampus of mice at 2 months and 17 months (n=6, each). c Transcript levels of Bdnf in the hippocampus of mice at 2, 7, 14, and 18 months of age (n=6, each; one-way ANOVA, F(3,20)=25.62, p<0.0001). d Western blot data showing p-CREB and CREB levels in HT22 cells treated with GC (400 ng/ml) for 24 h (n=4, each). e–g Transcript levels of Bdnf (e), SUV39H1 (f), and Mkp-1 (g) in HT22 cells treated with GC (400 ng/ml) for 6 h or 24 h, or GC (24 h) plus RU486, and RU486 alone. Bdnf (n=6, each; CON vs. GC6h vs. GC24h, one-way ANOVA, F(2,15)=10.28, p=0.0044), SUV39H1 (n=6, each; CON vs. GC6h vs. GC24h, one-way ANOVA, F(2,15)=10.26, p=0.0001), and Mkp-1 (n=6, each; CON vs. GC6h vs. GC24h, one-way ANOVA, F(2,15)=40.21, p=0.0001; CON vs. GC24h vs. CON+RU486 vs. GC+RU486, two-way ANOVA, GC main effects, F(1,20)=43.29, p<0.0001; RU486 main effects, F(1,20)=52.94, p<0.0001; GC×RU486, F(1,20)=56.14, p<0.0001). h Transcript levels of SUV39H1 in HT22 cells treated with siRNA-SUV39H1 or siRNA-CON (H) (n=6, each). i Transcript levels of Mkp-1 and Bdnf after SUV39H1 knockdown in HT22 cells. (n=6, each). j Western blot data showing p-CREB and CREB levels after SUV39H1 knockdown in HT22 cells (n=4, each). k Transcript levels of Mkp-1 in the HT22 cells treated with siRNA-Mkp-1 or siRNA-CON. (n=6, each). l Western blot data showing p-CREB and CREB levels after Mkp-1 knockdown in HT22 cells (n=4, each). m Transcript levels of Bdnf after Mkp-1 knockdown in HT22 cells (n=6, each). n Transcript levels of Bdnf after Mkp-1 knockdown in HT22 cells (n=6, each). o Transcript levels of Creb in the HT22 cells treated with siRNA-Creb or siRNA-CON (n=6, each). p Transcript levels of Bdnf changed by Creb knockdown in HT22 cells (n=6, each). q A summary of the SUV39H1, Mkp-1, p-ERK, p-CREB, and BDNF signaling pathway. Data are presented as mean±SEM. * and **, differences between indicated groups at p<0.05 and p<0.01, respectively (Student’s t-test, and one-way ANOVA or two-way ANOVA followed by Newman-Keuls post hoc test)
levels of SUV39H1 and Bdnf expression, and a control level of Mkp-1 expression (Fig. 4i–l). Mice treated with FLX during the RST14d phase (RST14d + FLX) had control levels of SUV39H1, Bdnf, and Mkp-1 expression in the hippocampus in the same way as in those treated with RA (Fig. 4i–l). Mice treated with IMI during the RST14d phase (RST14d + IMI) had control levels of SUV39H1 and Bdnf expression in the CA3 regions infused with siRNA-Mkp-1 and siRNA-CON (e, f). Scale bars, 100 μm (left); 20 μm (right). Quantification levels of p-CREB expression in CA3 pyramidial neurons (f). (n = 8, each). Data are presented as mean ± SEM. * and **, differences between indicated groups at p < 0.05 and p < 0.01, respectively (Student’s t-test, and one-way ANOVA followed by Newman-Keuls post hoc test).

In the behavioral tests, mice treated with RST14d + RA had increased sociability in the social interaction test (SIT), and reduced immobility time in the TST and FST (Fig. 4m–o). Mice treated with RST14d + IMI had increased sociability in the SIT and reduced immobility time in the TST and FST. Mice treated with RST14d + FLX also had increased sociability in the SIT, and reduced immobility time in the TST, but they had increased immobility in the FST (Fig. 4m–o).

Chronic stress produces hippocampal dendritic retraction and reduced branches [36–38] and stress-dependent atrophy in dendritic processes of hippocampal pyramidal neurons are regulated by BDNF [39, 40]. We investigated whether RA, IMI, and FLX can reverse stress-induced changes of dendritic processes of hippocampal pyramidal neurons. Immunohistochemical analysis revealed that mice subjected to RST14d had reduced levels of MAP-2 staining, a marker of dendritic processes, in pyramidal neurons in the stratum radiatum and stratum lucidum of the CA1 and CA3 regions, respectively, of the hippocampus, whereas mice that received RA injection during the RST14d phase (RST14d + RA) had increased levels of SUV39H1 and Bdnf expression, and a control level of Mkp-1 expression (Fig. 4i–l). Mice treated with FLX during the RST14d phase (RST14d + FLX) had control levels of SUV39H1, Bdnf, and Mkp-1 expression in the hippocampus in the same way as in those treated with RA (Fig. 4i–l). Mice treated with IMI during the RST14d phase (RST14d + IMI) had control levels of SUV39H1 and Bdnf expression in the hippocampus in the same way as in those treated with RA and FLX, but they had increased Mkp-1 expression (Fig. 4i–l).
enhanced MAP2-staining levels in dendritic processes of CA1 and CA3 pyramidal neurons (Fig. 5a–d).

Mice subjected to RST14d had a reduced number of new neurons stained by anti-Ki67, a cell proliferation marker (Fig. 5e, f) and a reduced number and reduced levels of dendritic processes of developing neurons stained by anti-doublecortin (DCX), a marker for differentiating neurons (Fig. 5g–i), in the dentate gyrus of the hippocampus compared to control mice. In contrast, mice treated with RA during the RST14d phase (RST14d + RA) had an increased number of Ki-stained cells and an increased number of DCX-stained neurons in the dentate gyrus relative to RST14d-treated mice. These results suggest that RA protects the stress-induced decrease in MAP2-staining levels in dendritic processes of CA1 and CA3 pyramidal neurons and blocks stress-induced reduction in neurogenesis in the dentate gyrus.
Aged Mice Were Highly Sensitive to Stress-Induced Depression, which Was Moderated by RA

Next, we examined whether RA can be used for the treatment of stress-induced depressive behaviors in aged mice. In the pilot study, we found that 14 M mice had significantly reduced expression SUV39H1 and increased expression of Mkp-1 in the hippocampus. Therefore, we investigated the stress coping ability of aged mice at 14 M of age. Naïve aged mice (14 M) showed control levels of social interaction in the SIT and control levels of immobility time in the FST, which were comparable to those of naïve young mice (2 M) (Fig. 6a–d). Aged mice treated with daily 2-h restraint for 5 days (RST5d), a condition which did not produce depressive-like behavior in young mice, exhibited a slightly reduced sociability, although the difference was not statistically significant, however, immobility time in the FST was significantly increased. In contrast, aged mice treated with RA before the 2-h restraint for 5 days (RST5d + RA) exhibited increased sociability in the SIT and reduced immobility time in the FST (Fig. 6a–d).

Analysis of the behavioral performance of individual animals distributed in the sociability × FST matrix followed by K-means clustering, an unsupervised machine-learning algorithm, indicated that the members of the young mouse control, young mice with RST5d, aged mouse control, and the aged mice with RST5d were grouped into two clusters; cluster 1 contained the individuals distributed mostly in the quadrants 1 and 4 in the sociability × FST matrix, and cluster 2 contained the individuals distributed mostly in the quadrants 2 and 3 (Fig. 6e). Individuals of the aged mouse group subjected to RST5d were located in cluster 1, which exhibited low sociability and high immobility, while most individuals of the aged mice treated with RA were in cluster 2, which contained the young mouse control, the young mice with RST5d, and the aged mouse control (Fig. 6e). The % composition of young control mice and aged control mice in cluster 1 was 57% for both groups, although the detailed distribution of individuals within cluster 1 was not overlapped. After RST5d treatment, the % composition of young mice and aged mice in cluster 1 shifted from 57 to 43% and from 57 to 100%, respectively (that is, 57% and 100%, respectively, of the group members were positioned in cluster 2 after RST5d treatment) (Fig. 6e, f). More interestingly, after RST5d treatment, the % composition of young mice with RST5d and aged mice with RST5d in cluster 1 shifted from 43 to 100% and from 100 to 50%, respectively (that is, 100% and 50%, respectively, of the group members were positioned in cluster 2 after RA treatment) (Fig. 6e, f).

The transcript levels of SUV39H1 and Mkp-1 in the hippocampus were decreased and increased, respectively, in aged mice (14 M) compared to those in young mice (2 M) (Fig. 6g, h). Bdnf expression in aged mice was lower than that in young mice (Fig. 6i). After RST5d treatment, the transcript levels of SUV39H1 were reduced and the transcript levels of Mkp-1 were enhanced in both young and aged mice (Fig. 6g, h), and the transcript level of Bdnf was reduced in aged mice compared to that in young mice (Fig. 6i). In contrast, RA treatment before the 2-h restraint for 5 days blocked the RST5d-induced reduction in the expression of SUV39H1 and Bdnf and the increase in the expression of Mkp-1 in both young mice and aged mice (Fig. 6g–i).
Discussion

The present study demonstrates that aged mice had reduced expression of SUV39H1, which resulted in the increased expression of Mkp-1 and reduced expression of p-CREB and Bdnf in the hippocampus (Figs. 1 and 6). Aging-dependent changes in the expression of SUV39H1, Mkp-1, p-CREB, and Bdnf mirrored the changes in the expression of those genes in HT22 cells treated with GC (Fig. 2) and the changes of the same factors in the hippocampus in mice exposed to chronic stress (Figs. 3 and 4). These results suggest that aging-induced changes of SUV39H1, Mkp-1, p-CREB, and
Bdnf expression is associated with GC-induced changes. Indeed, aged mice (18 M) had increased basal GC levels (Fig. 2a), and SUV39H1, Mkp-1, p-CREB, and Bdnf levels in the hippocampus of aged mice were further changed after exposure to moderate stress (Fig. 6). Mkp-1 expression is also increased by GC [15]. In fact, the Mkp-1 gene proximal promoter regions contain potential glucocorticoid receptor (GR) binding sites between −1421 and −1118, and between −717 and −702 [15]. GC-induced SUV39H1 downregulation increases Mkp-1 expression and, conversely, GC-induced Mkp-1 upregulation decreases SUV39H1. Therefore, GC-induced changes of these factors lead to amplify a vicious cycle of the SUV39H1- and Mkp-1-regulated pathway [15]. Furthermore, SUV39H1 downregulation could change the expression of PP2A, p-AMPK, PPARY, p-CREB, and NADPH oxidase [15, 21]. These results suggest that stress-induced downregulation of SUV39H1 in aged mice might change divergent signaling pathways, which could affect reduced stress coping ability.

Non-stressed aged mice had decreased SUV39H1 expression and increased Mkp-1 expression in the hippocampus, and those expression levels were, respectively, lower than and equivalent to those changed by chronic stress in young mice (Fig. 6). However, naïve aged mice did not exhibit depressive-like behavior, although they exhibit depressive-like behavior following moderate stress that did not induce depressive-like behavior in young mice (Fig. 6). These results suggest that although SUV39H1 and Mkp-1 are important players for stress-induced depressive behavior in aged brains, the detailed action mechanisms of these factors in regulating depressive behavior in aged brains are slightly different from those in young mice. Aging-dependent changes advance slowly and progressively with other alterations including increased accumulation of oxidative stress, increased mitochondrial dysfunction, and impaired cellular responses to stress [9, 12]. It could be possible that SUV39H1 and Mkp-1-dependent changes are affected by other aging-dependent accumulated factors in the brain. Chronic stress decreased p-ERK and p-CREB levels in the hippocampus in a GC- and Mkp-1 dependent manner [15]. These p-ERK and p-CREB levels can be also affected by other factors, such as oxidative stress and increased levels of cytokines, and those changes could in turn reduce Bdnf expression [41, 42]. Considering these results, it will be worth to test whether a strategy targeting Mkp-1 inhibition in aged mice could suppress stress-induced depressive-like behavior.

Our results suggest RA offers anti-depressant effects in a stress-induced model of depression as did IMI and FLX, although their underlying mechanisms are slightly different. IMI and RA reversed the stress-induced changes of SUV39H1 and BDNF in the hippocampus. Unlike RA, however, IMI did not block GC-induced Mkp-1 upregulation in HT22 cells and stress-induced Mkp-1 upregulation in the hippocampus (Figs. 4 and 6). FLX reversed the stress-induced changes in SUV39H1, Mkp-1, and BDNF expression in the hippocampus. However, FLX did not reverse the GC-induced Mkp-1 upregulation in HT22 cells (Fig. 4b). FLX and IMI are a selective serotonin reuptake inhibitor and tricyclic anti-depressant, respectively, and their primary mode of action is known to increase monoamine action in synaptic sites. As demonstrated in the present study, FLX and IMI reversed the GC-induced changes of SUV39H1 and BDNF in HT22 cells (Fig. 4a, c). Further studies are needed to establish how FLX and IMI reverse GC-induced changes of SUV39H1, CREB, and BDNF but produced no effect on Mkp-1 expression, and why FLX produces cytotoxicity in neuronal cells. In a CUS-induced model of depression, FLX reversed the increased level of Mkp-1 in the dentate gyrus, partially blocked increased Mkp-1 in CA1, but not in CA3 cell layers [13]. IMI or FLX treatment for 14 days in normal mice (2 M) decreased immobility time in the TST and FST, and their antidepressant effects can be extended beyond the monoamine hypothesis, and if targeting of SUV39H1 and Mkp-1 systems can be used to treat clinical depression.

As demonstrated in the present study, RA blocked the GC-induced changes of SUV39H1, Mkp-1, and Bdnf
expression and overall RA was more effective than IMI and FLX in remediating stress-induced changes of SUV39H1 and Mkp-1 expression (Figs. 4 and 6). Furthermore, RA treatment rescued stress-induced dendritic morphology changes and restored decreased neurogenesis in the hippocampus (Fig. 5). In a chronic stress model of depression, RA increased ERK activity and BDNF expression in the hippocampus, which protects against stress-induced depression-like behaviors [44]. RA is known as a phenolic carboxylic acid that produces antioxidant and anti-inflammatory effects in various cell types [45, 46]. The results of the present study and those of our previous one [15] suggest that RA increases SUV39H1 independently from its antioxidant property or anti-inflammatory effects. RA upregulated SUV39H1 expression and increased SUV39H1 binding to the proximal promoter of the Mkp-1, and thereby reduced Mkp-1 expression [15]. In aged mice, the RA-dependent decrease of Mkp-1 expression is likely to be induced via a SUV39H1-dependent mechanism (Figs. 1 and 2). These results suggest that RA produces antidepressant-like effects by modulating SUV39H1 and Mkp-1-regulated signaling pathways in aging and stress-induced depressive behaviors.

**Author Contribution** JEL and SYP carried out the experiments; JEL and PLH designed the experiments, performed the statistical analysis, and wrote the manuscript.

**Funding** This research was supported by a grant (2021R1A2B5B02002245) from the Ministry of Science, ICT and Future Planning, Republic of Korea.
Consent for Publication All authors consent to the publication of the manuscript in Mol Neurobiology, should the article be accepted by the Editor-in-chief.

Conflict of Interest The authors declare no competing interests.

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