Molecular analysis for brain epigenetic mechanisms

in aging and exogenous stimuli

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This dissertation is dedicated to my parents, my sister,
my grandmother and my friends.

Table of Contents

Abbreviations

• ***Chemical substances and drugs***

METH: methamphetamine

• ***Endogenous substances***

BDNF: brain-derived neurotrophic factor

PDGFA: platelet-derived growth factor, alpha

PDGFB: platelet-derived growth factor, B polypeptide

VEGF: vascular endothelial growth factor

NGF: nerve growth factor

EGF: epidermal growth factor

• ***Enzymes***

Mll1: myeloid/lymphoid or mixed-lineage leukemia 1

LSD1: lysine (K)-specific demethylase 1A

Jarid1a: Jumonji, AT rich interactive domain 1A

Jarid1b: Jumonji, AT rich interactive domain 1B

Jmjd2A: jumonji domain containing 2A

Jmjd2B: jumonji domain containing 2B

Jmjd2C: jumonji domain containing 2C

Jmjd2D: jumonji domain containing 2D

EZH2: enhancer of zeste homolog 2

UTX: ubiquitously transcribed tetratricopeptide repeat gene, X chromosome

Jmjd3: jumonji domain containing 3

• ***Brain region***

DG: Dentate gyrus

NAc: Nucleus accumbens

• ***Others***

ANOVA: Analysis of variance

CNS: Central nervous system

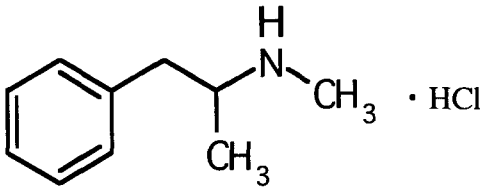
IR: Immunoreactivity

RT-PCR: Reverse transcription-polymerase chain reaction

S.E.M.: Standard error mean

Structures of drugs used in the present study

Methamphetamine hydrochloride



General introduction

Epigenetics

Epigenetic mechanisms typically involve heritable alterations in chromatin structure, which, in turn, regulate gene expression. Fundamental insights about epigenetic heritability have come from studies of cell division and development. However, there is increasing evidence that the regulation of chromatin structure through histone acetylation and DNA methylation might mediate long-lasting behavioral changes in the context of learning and memory. This idea is fascinating because similar mechanisms are used for triggering and storing longterm memories at the cellular level during, for example, cell differentiation. Another intriguing aspect of this hypothesis is that the storage of lifelong behavioral memory might involve lasting changes in the physical, three-dimensional structure of DNA itself.

Acetylation of histone lysine residues reduces the electrostatic interaction between histone proteins and DNA, which is thought to relax chromatin structure and make DNA more accessible to transcriptional regulators [46]. Histone methylation is particularly complex and can exist in mono-, di- (me2) or tri-methylated (me3) states, enabling each state to recruit unique coregulators and exert distinct effects on transcriptional activity [46]. Histone methylation is also unique because each lysine residue has distinct, and often opposite, effects on transcription. For example, H3K4 is highly associated with gene activation, whereas H3K9 and H3K27 are usually associated with repression [51].

Aging

Since the average human life span has increased dramatically over the last century, human society is becoming more concerned about malfunctions associated with aging. The dysfunction of neurotransmission in normal aging and neuropsychiatric diseases late in life may contribute to the behavioral changes commonly observed in the elderly population [60].

Aging is associated with a progressive accumulation of damaged molecules and impaired energy metabolism in brain cells [57]. Neurons and glial cells may adapt to the adversities of aging by compensating for lost or damaged cells by producing new neurons and glia, and remodeling neuronal circuits [33,68]. The aging hippocampal formation is affected by a number of structural and functional changes, including the loss of neurons in CA regions and the hilus [86], reduced synaptic densities [73], reduced postlesion sprouting [58], decreased glucose utilization [29] and reduced expression of growth factor and steroid receptors [9]. However, other changes are indicative of a compensatory increase in hippocampus function and include elevated postsynaptic efficacy of granule cell synapses [5,6], increased average dendritic extent in the dentate gyrus [28] and increased N-methyl D-aspartate receptor function [75]. These findings suggest that age-related functional decline in the dentate gyrus may not be mediated by a loss of granule cells, but rather by a decline in the birth of new granule cells.

Enriched environment

In an experimental setting, an enriched environment is 'enriched' in relation to standard laboratory housing conditions. Some have argued that experimental enrichment is merely a step up from standard laboratory impoverishment[83]. In general, the 'enriched' animals are kept in larger cages and in larger groups with the opportunity for more complex social interaction. The environment is complex and is varied over the period of the experiments: tunnels, nesting material, toys and (often) food locations are changed frequently. In addition, animals are often given the opportunity for voluntary physical activity on running wheels [89].

The standard definition of an enriched environment is "a combination of complex inanimate and social stimulation". This definition implies that the relevance of single contributing factors cannot be easily isolated but there are good reasons to assume that it is the interaction of factors that is an essential element of an enriched environment, not any single element that is hidden in the complexity. Controls for the importance of single variables on the effects of enriched environment have been tested, particularly for the effects of socialization and general activity. In general, the results have revealed that no single variable can account for the consequences of enrichment. For example, it has been shown that neither observing an enriched environment without being allowed active participation ('TV rat') nor social interaction alone can elicit the effects of enriched environments. Environmental enrichment and voluntary exercise produce notably similar effects on the brain. In a recent study, mice were assigned to groups with a learning task, wheel running, enrichment or standard housing. Voluntary exercise

in a running wheel enhanced the survival of newborn neurons in the dentate gyrus, which is similar to the effects of environmental enrichment, whereas none of the other conditions had any effects on cell genesis. This finding led to a comparison of the effects of enrichment and exercise on behavioural, morphological and molecular changes in the brain.

Environment enrichment appears as a broad noninvasive strategy of potential interest. Indeed, it has been shown to induce regional increases in neurotrophin levels in the rodent brain [36,69], and can also stimulate neurogenesis in healthy animals [40,41,83]. In pathological conditions, enrichment may promote neuronal survival and resistance to brain insult [89]. Furthermore, accumulating experimental evidence shows that experience and training are also important in the plasticity and functional recovery provided by neural grafts [22,23]. A growing body of evidence suggest that environmental enrichment and voluntary exercise enhances the proliferation of neural stem cells and neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice. Molecular regulation of stem cell fate involves a coordinated interaction between epigenetic[8,13,59,61], transcriptional [20,27,42,54,88] and translational [16,74] mechanisms. Despite the intense characterization and some molecular targets have been known to influence adult hippocampal neurogenesis, relatively little is known about the epigenetic mechanism governing hippocampal neurogenesis by enriched environment.

Drug addiction

Repeated exposure to many drugs of abuse results in a progressive and enduring enhancement in the motor stimulant effect elicited by a subsequent drug challenge. This phenomenon, termed behavioral sensitization, is thought to underlie certain aspects of drug addiction. Behavioral sensitization is the consequence of drug-induced neuroadaptive changes in a circuit involving dopaminergic and glutamatergic interconnections between the ventral tegmental area (VTA) and nucleus accumbens (NAc). Behavioral sensitization is known as one animal model for experience- and drug-dependent behavioral plasticity. This model corresponds to the intensification of drug craving in human addicts and is readily elicited by many drugs. Recently, genome-wide mRNA analyses have identified many more potential gene targets for drugs of abuse in distinct brain reward regions, and these targets might also contribute to long-lasting behavioral effects. Therefore, it has become of great interest to identify the underlying mechanisms by which chronic drug exposure promotes stable changes in gene expression and, ultimately, behavior. Recent evidence has suggested that epigenetic mechanisms – key cellular processes that integrate diverse environmental stimuli to exert potent and often long-lasting changes in gene expression through the regulation of chromatin structure – contribute to these drug-induced transcriptional and behavioral changes.

Aim and Scope

The aim of the present study was to examine these next levels of transcriptional mechanisms underlying aging, enriched environment and drug action. Epigenetic mechanisms, which are key cellular and molecular processes that integrate diverse environmental stimuli to exert potent and often long-lasting changes in gene expression through the regulation of chromatin structure, contribute to transcriptional and behavioral changes. Therefore, for the first time I investigated the involvement of chromatin remodeling in the neuromechanisms.

The specific aims of the proposed research are:

In Chapter 1:

The present study was undertaken to evaluate the age-related changes in the expression of doublecortin and NeuroD, which are markers for neuronal precursors, along with epigenetic modification in the hippocampus of aged mice. In addition, I investigated whether aging decreases neurogenesis accompanied by the activation of microglia and astrocytes, which increases the expression of IL-1β in the hippocampus, and whether *in vitro* treatment with IL-1β in neural stem cells directly impairs neurogenesis.

In Chapter 2:

Environmental enrichment is an experimental paradigm that increases brain-derived neurotrophic factor (BDNF) gene expression accompanied by neurogenesis in the hippocampus of rodents. In the present study, I investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice.

In Chapter 3:

In the present study, to further understand the molecular mechanisms that underlie methamphetamine-induced behavioral sensitization, I investigated changes in epigenetic modification at 14 chemokine and cytokine genes in the Nucleus Accumbens of mice that were intermittently treated with methamphetamine.

Ethics

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Hoshi University), as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Hippocampal epigenetic modification involved in the impairment of neurogenesis with aging

Introduction

Since the average human life span has increased dramatically over the last century, there are growing concerns about malfunctions associated with aging. The dysfunction of neurotransmission in normal aging and neuropsychiatric diseases late in life may contribute to the behavioral changes commonly observed in the elderly.

Neurogenesis occurs in specific areas in the adult brain throughout life, e.g. in the subventricular zone at the telencephalic level and in the dentate gyrus of the hippocampus [25,55]. The dentate gyrus, the hippocampus proper, and the subiculum constitute the hippocampal formation, which is critical for certain forms of learning and memory [10]. A positive correlation has been established between neurogenesis in the dentate gyrus and an animal's performance in behavioral tasks [41,83]. Aging is associated with a progressive accumulation of damaged molecules and impaired energy metabolism in brain cells. Neurons and glial cells may adapt to the adversities of aging by compensating for lost or damaged cells by producing new neurons and glia, and remodeling neuronal circuits. The influence of age on rates of neurogenesis has been studied by several groups [48,52]. Changes in the relative proportion of young dentate gyrus neurons may have important consequences for hippocampal function and could possibly contribute to age-dependent structural and functional hippocampal deficits [4,30].

Microglia are CNS-resident, macrophage-like cells of hematopoietic origin that play a role in the homeostasis of the healthy CNS and as immune surveillance cells in response to infection and injury. When neurons are injured as a result of aging or

neurodegeneration, microglia become activated via the release of ATP, neurotransmitters, growth factors or cytokines, chemokines, ion changes in the local environment, or a loss of inhibitor molecules displayed by healthy neurons [56].

In there role as cells that provide multiple forms of support to the CNS, astrocytes are beginning to be appreciated as suppliers of critical survival and differentiation factors to neurons and other glial cells. Recent studies have suggested that astrocytes may express cytokines, chemokines, and growth factors as well as neurotransmitters throughout life, and not only in the developing fetus. Further studies on brain damage have suggested that, like microglia, astrocytes may be over-activated with aging [50].

Epigenetic changes involve transmissible alterations in gene expression caused by mechanisms other than changes in the DNA sequence [32]. Epigenetic information is destined to change during development and in the course of essential somatic functions. This makes it a more likely candidate for errors than its more stable DNA-sequence counterpart, changes in which have been well-documented and increase during aging [32]. Indeed, epegenomic alterations are now becoming increasingly recognized as part of aging and its associated pathologic phenotypes [32].

To better understand such age-dependent epigenetic modification, in this study I focused on the decreased expression of doublecortin, which is frequently used as a marker of a migrated neuronal progenitor, and NeuroD, a neural progenitor cell marker, in the hippocampus of aged mice. Furthermore, I investigated whether the neuroimmune network driven by central glial cells could affect age-dependent structural and functional hippocampal deficits accompanied by epigenetic modification in aged mice.

Materials and Methods

Animals

Two- and from 24- to 28-month-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in the present study. Animals were kept in a room with an ambient temperature of 23 ± 1 °C and a 12-hr light/dark cycle (lights on 8:00 AM to 8:00 PM). Food and water were available *ad libitum*. This study was approved by the Animal Research Committee of Hoshi University.

Immunohistochemistry using hippocampus cold-slice sections

Mice were deeply anesthetized with isoflurane (3%) and perfusion-fixed with 4 % paraformaldehyde (pH 7.4). The brain was then quickly removed and the hippocampus was rapidly dissected and postfixed in 4% paraformaldehyde for 2 hr. The hippocampus was permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, and then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan). All samples were stored at –30 ˚C until use. The sections were cut transversely at a thickness of 8 μm on a cryostat (Leica CM1510, Leica Microsystems, Heidelberg, Germany). The hippocampus sections were blocked in 10% normal goat serum in 0.01 M phosphate-buffered saline (PBS) for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% normal goat serum [doublecortin (1:3500 Abcam Ltd., Cambridge, UK), glial fibrillary acidic protein, (GFAP) (1:20 Nichirei, Tokyo, Japan) or ionized calcium-binding adaptor molecule 1, (Iba1) (1:130 Wako Pure

Chemicals, Osaka, Japan)], and incubated for 2 days overnight at 4 °C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, U.S.A.) for 2 hr at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). Fluorescence of immunolabeling was detected using a light microscope (Olympus AX-70; Olympus, Co., Tokyo, Japan) and a Radiance 2000 laser-scanning microscope (BioRad, Richmond, CA, U.S.A.), and photographed with a digital camera (Polaroid PDMCII/OL; Olympus, Co., Tokyo, Japan).

Western blotting assay

Each hippocampus was individually homogenized in ice-cold buffer A containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg of leupeptin per ml, 0.1 mg of aprotinin per mL and 0.32 Msucrose. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was ultracentrifuged at 100,000 x g for 30 min at 4 °C. The pellets were washed with buffer B (buffer A without sucrose) and then ultracentrifuged at 100,000 x g for 30 min. The final pellets were retained as the membranous fraction for Western blotting at –80 °C until the assay. Protein concentration in the samples was assayed by the method of Bradford et al. [14]. An aliquot of tissue sample was diluted with an equal volume of 2 x electrophoresis sample buffer (Protein Gel Loading Dye-2 x; Amresco, Solon, OH) containing 2 % sodium dodecyl sulfate (SDS) and 10 % glycerol with 0.2 M dithiothreitol. Proteins (10-20 mg/lane) were separated by size on 4-20%

SDS-polyacrylamide gradient gel using the buffer system, and then transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection, the membranes were blocked in Tris-buffered saline (TBS) containing 1 % non-fat dried milk with 0.1 % Tween 20 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for 1 hr at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS containing 1 % non-fat milk with 0.1 % Tween 20 [1:1000 GFAP (Nichirei, Tokyo, Japan), 1:1000 Iba1 (Wako Pure Chemicals, Osaka, Japan)] overnight at 4 °C. The membrane was washed in TBS containing 0.05 % Tween 20 (TTBS), and then incubated for 2 hr at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, U.S.A.) diluted 1:10000 in TBS containing 1 % non-fat dried milk with 0.1 % Tween 20. To control for validation in loading, we assayed the expression of a housekeeping gene by Western blot analysis. After incubation with primary and secondary antibodies, the membrane was then re-probed with mouse anti-GAPDH polyclonal antibody [GFAP: 1:200000 in TBS containing 1 % non-fat milk with 0.1% Tween 20, Iba1: 1:50000 in 5 % non-fat milk (Chemicon International, Inc., Temecula, CA, U.S.A.)] for 1 hr, and then incubated with anti-mouse secondary antibody conjugated with horseradish peroxidase (1:10000) for 2 hr at room temperature. The antigen-antibody peroxidase complex was finally detected by enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, U.S.A.).

RNA preparation and semi-quantitative analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA in the hippocampus of aged mice was extracted using the SV Total RNA Isolation system (Promega, Madison, WI, U.S.A.) following the manufacturer's instructions. Purified total RNA was quantified spectrophotometrically at A_{260}. To prepare first-strand cDNA, 1 μg of RNA was incubated in 100 μL of buffer containing 10 mM dithiothreitol, 2.5 mM $MgCl_2$, dNTP mixture, 50 U of reverse transcriptase II (Invitrogen, Carlsbad, CA, U.S.A.), and 0.1 mM oligo-dT_{12-18} (Invitrogen, Carlsbad, CA, U.S.A.). Each gene was amplified in 50 μl of PCR solution containing 0.8 mM $MgCl_2$, dNTP mixture, and DNA polymerase with synthesized primers (Table 1). Samples were heated to 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. The final incubation was at 72°C for 7 min. The mixture was run on 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. The agarose gel was stained with ethidium bromide and photographed with UV transillumination. The intensity of the bands was analyzed and semiquantified by computer-assisted densitometry using ImageJ software.

Quantitative analysis by Real-time PCR

Fast SYBR Green Master Mix (Applied Biosystems, Inc., Foster City, CA, U.S.A.) was used as the basis for the reaction mixture in the real-time PCR assay. Each gene prepared by the above procedure was amplified in 20 mL of a PCR solution containing

10 ml of the Fast SYBR Green Master Mix with with synthesized primers (Table 1). In addition to each sample, each test run included a no-target control that contained reaction mixture and PCR-grade water. PCR with a StepOnePlus™ (Applied Biosystems, Inc., CA, U.S.A.) was performed with the following cycling conditions: 95°C for 20 sec, followed by 45 cycles of 95℃ for 3 sec and 60℃ for 30 sec. Fluorescence detection was conducted after each extension step.

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed as described previously [79,81] with minor modifications. Briefly, mouse hippocampus tissue was dissected as described above and cross-linked, and the tissue was then lysed. Fifteen mg of soluble chromatin was incubated with 2 μg of specific antibodies; against acetylation of histone H3 (AcH3) (Millipore, Billerica, MA); trimethylation of lysine 4 on histone H3 (H3K4) (Wako Pure Chemicals, Osaka, Japan); trimethylation of lysine 9 on histone H3 (H3K9) (Millipore, Billerica, MA, U.S.A.); or trimethylation of lysine 27 on histone H3 (H3K27) (Millipore, Billerica, MA, U.S.A.), overnight at 4°C. The immuno-complex was collected by Dynabeads Protein A (Invitrogen, Carlsbad, CA, U.S.A.), and DNA was recovered with RNaseA treatment, Proteinase K treatment and isopropanol precipitation. Immunoprecipitated DNA was dissolved in 50 μL of 1 x TE and 1 μL was used for quantitative PCR. Quantitative PCR was performed as described previously [64]. The primers used are listed in Table 1.

Preparation and differentiation of mice neural stem cells (NSCs)

Pregnant C57BL/6J mice were used to prepare NSCs. NSCs were obtained from whole brain of E14.5 mice and cultured. Briefly, the brain were triturated in serum-free medium: Dulbecco's modified Eagle's medium with 4500 mg/L glucose, 5 μg/mL insulin, 10 ng/mL EGF, 50 μg/mL transferrin, 10 ng/mL biotin and 30 nM Na_2SeO_3. EGF (10 ng/mL) was used to keep the cultures proliferating. For differentiation experiments, approximately 10 neurospheres of the same size were isolated with a pipette and deposited on 10 μg/mL laminin-coated glass slides with 400 μL of serum-free medium with 10 ng/mL EGF. The NSCs were incubated overnight in medium containing EGF, which was then replaced by medium without EGF but containing IL-1β (10, 100 ng/mL) for 7 days.

Immunohistochemistry using differentiated neurospheres

Differentiated neurospheres were fixed with 4% paraformaldehyde for 20 min at room temperature and processed for immunocytochemistry (ICC). The samples were rinsed with PBS twice and pre-treated with PBS containing 0.3% Triton-X100 for 5 min at room temperature for ICC. After blocking in blocking buffer (PBS containing 5% FBS and 0.3% TritonX) for 1 hr at room temperature, the samples were incubated at 4°C overnight with the following antibodies: anti-bIII-tubulin (mouse IgG, 1:1000, Sigma-Ardrich, St. Louis, MO, T8660), After three washes with PBS, the samples were incubated for 1 hr at room temperature with secondary antibodies conjugated with Alexa488 (Invitrogen, Carlsbad, CA, U.S.A.) After being washed with PBS, the

samples were mounted on slides and examined with microscope with a 10 x objective lens (IX 71, Olympus, Co., Tokyo, Japan) and photographed with a digital camera (VB-6000, Keyence, Co., Osaka, Japan).

Cell Viability Assay

NSCs (5 x 10^5 cells per well) were treated with IL-1β (10 ng/mL, 100 ng/mL) for 8 hr in 96-well plates. Afterwards, the numbers of viable cells in culture were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, U.S.A.), which evaluates the presence of ATP, an indicator of metabolically active cells, according to the manufacturer's instructions. All experiments were performed in triplicate wells.

Statistical analysis

The data are expressed as the mean ± S.E.M. The statistical significance of differences between groups was assessed with Student's t-test (comparison of two groups) or an analysis of variance [37] followed by the Bonferroni test (comparison among multiple groups).

Results

Changes in the expression of doublecortin in the hippocampus of aged mice

Doublecortin is a cytoskeletal protein that is transiently expressed only in newborn neurons, and is used as a marker of neural progenitors. In agreement with a previous report [35], we confirmed that doublecortin-positive cells were almost completely absent from the dentate gyrus of the hippocampus of 28-month-old mice (Figure 1-1-A). The level of doublecortin, the doublecortin mRNA was significantly decreased in the hippocampus of aged mice compared to that in young mice (Figure 1-1-B and 1-1-C, $P<0.001$ vs. young mice). In contrast, mRNA levels of glial fibrillary acidic protein (GFAP), a glial marker, and brain-derived neurotrophic factor (BDNF) in the hippocampus were not altered by normal aging.

Age-dependent epigenetic modification at doublecortin and NeuroD promoter in the hippocampus

I next evaluated whether changes in doublecortin mRNA expression accompanied by aging could be regulated through chromatin-specific events. In this study, I analyzed two active histone modifications (acetylation of histone H3, AcH3, and trimethylation of lysine 4 on histone H3, H3K4) and two repressive histone modifications (trimethylation of lysine 9 on histone H3, H3K9, and trimethylation of lysine 27 on histone H3, H3K27) at doublecortin promoter regions in the hippocampus. As a result, I detected a significant decrease in H3K4 trimethylation at the doublecortin

promoters with aging (Figure 1-2-A, $P<0.05$ vs. young mice). Furthermore, a significant increase in H3K27 trimethylation at the doublecortin gene was seen with aging (Figure 1-2-A, $P<0.05$ vs. young mice). Under these conditions, aging did not produce H3K9 trimethylation or hyperacetylation of H3 at the doublecortin gene (Figure 1-2-A). I also found that mRNA expression of NeuroD, a neural progenitor cell marker, was significantly decreased in the hippocampus of aged mice compared to that in young mice ($p<0.01$; Figure 1-2-B). In agreement with the PCR assay, I detected a significant increase in H3K9 trimethylation at the NeuroD promoter in the hippocampus of aged mice compared to that in young mice ($p<0.05$; Figure 1-2-C). In contrast, I observed no changes in other histone modifications at the NeuroD gene promoter (Figure 1-2-C). The methylation of H3K4, H3K9 and H3K27 can be directly modulated by histone methylases and demethylases that target specific lysine residues and methylation states. Thus, I investigated whether aging could alter the mRNA level of several histone methylases and demethylases in the hippocampus. I found that aging did not change the mRNA expression of MLL1 (a H3K4 methyltransferase), LSD1, Jarid1a or Jarid1b (H3K4 demethylases), jmjd2A, jmjd2B, jmjd2C or jmjd2D (H3K9 demethylases), EZH2 (a H3K27 methyltransferase), or UTX or jmjd3 (H3K27 demethylases) (Figure 1-3).

Changes in ionized calcium-binding adaptor molecule 1 (Iba1)- and glial fibrillary acidic protein (GFAP)-immunoreactivity (IR) in the hippocampus of aged mice

To investigate a possible change in glial cell activity in the hippocampus of aged

mice, immunohistochemical studies were performed. As shown in Figure 1-4, the IR of the specific microglial marker Iba1 was prominently observed in the DG of young mice (Figure 1-4-A-i). In the DG of aged mice, Iba1-IR was dramatically increased compared to that in young mice (Figure 1-4-A-ii). Western blots showed that the levels of Iba1 were significantly increased in the hippocampus of aged mice compared to those in young mice ($p<0.001$; Figure 1-4-A-iii). Astrocytes in the DG of the hippocampus were stained with GFAP antibody. These astrocytes were sparsely distributed in young mice (Figure 1-4-B-i). In the DG of aged mice, IR for GFAP was increased compared to that in young mice (Figure 1-4-B-iii). Each individual astrocyte labeled by GFAP was hypertrophied with an enlarged cell body (Figure 1-4-B-ii and -iv). Western blots showed that the level of GFAP was significantly increased in the hippocampus of aged mice compared to that in young mice ($p<0.001$; Figure 1-4-B-v).

Age-dependent increase of IL-1β mRNA without related histone modifications

I next investigated the changes in the mRNA expression of IL-1β in the mouse hippocampus of young and aged mice. The mRNA expression of IL-1β was significantly increased in the hippocampus of aged mice compared to that in young mice ($p<0.01$; Figure 1-4-C). To gain further insight into these phenomena, I next studied histone modifications at the promoter regions of the IL-1β gene (Figure 1-4-D). As a result, I detected no changes in two active histone modifications AcH3 and H3K4 or in two repressive histone modifications H3K9 and H3K27.

Effects of IL-1β on neural stem cell proliferation and neural differentiation

To further determine whether IL-1β could suppress neural stem cell proliferation and neural differentiation, I used neural stem cells prepared from whole brain of E14.5 mice. For the proliferation experiments, EGF (10 ng/mL) was used to keep the cultures proliferating. Under these conditions, treatment with IL-1β (10 to 100 ng/mL) failed to change the proliferation of neural stem cells as detected by a microtiter plate-based cell-survival assay (Figure 1-5-A). For the differentiation experiments, approximately 10 neurospheres of the same size were plated onto 10 μg/mL laminin-coated glass slides, and 400 μL of serum-free medium with 10 ng/mL EGF was then added to each well. The neural stem cells were incubated overnight in medium with 10 ng/mL EGF and then replaced with medium alone or medium containing IL-1β (10 to 100 ng/mL). Treatment with IL-1β for 7 days produced a significant decrease in IR for the differentiated neural cell marker β-tubulin (Figure 1-5-B).

Under this condition, I next investigated the effects of treatment with IL-1β in neural stem cells on NeuroD mRNA expression in neural progenitor cells. Treatment with IL-1β significantly decreased the mRNA expression of NeuroD ($p<0.05$; Figure 1-5-C). I next studied histone modifications at the NeuroD promoter. In agreement with the results in the aged brain, I found a significant increase in H3K9 trimethylation at the NeuroD promoter in neural stem cells that had been incubated with IL-1β compared to that in control groups ($p<0.05$, $p<0.01$; Figure 1-5-D).

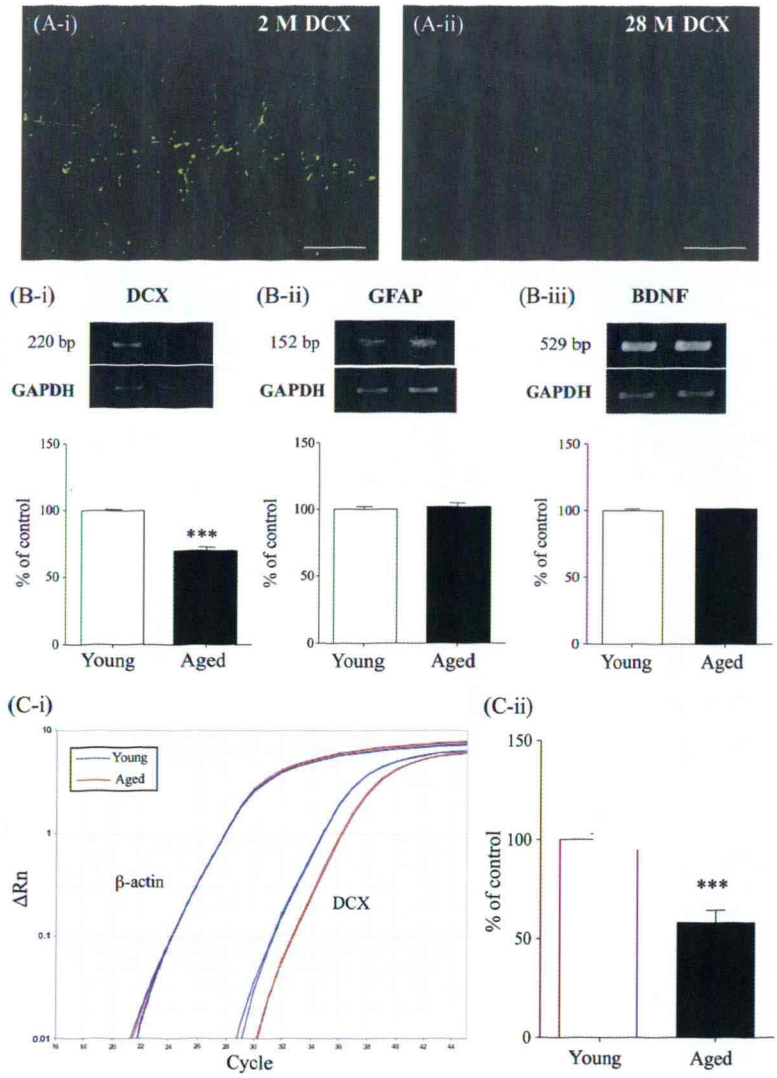


Figure 1-1. (A) Immunofluorescent staining for doublecortin in the dentate gyrus in young and aged mice. Doublecortin-like immunoreactivity in the dentate gyrus of 28-month-old mice (A-ii) was decreased compared to that in 2-month-old mice (A-i). Scale bar: 50 μm. (B) Upper: Representative RT-PCR for doublecortin (DCX; B-i), GFAP (B-ii) and BDNF (B-iii) mRNAs in the hippocampus obtained from young and aged mice. Lower: The intensity of the bands was semi-quantified using NIH Image software. The values for DCX, GFAP and BDNF mRNA were normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for aged mice is expressed as a percentage of the increase in young mice. Each column represents the mean ± S.E.M. 6 samples. ***P<0.001 vs. the young group. (C) Quantitative analysis of DCX mRNA in the hippocampus obtained from young and aged mice. (C-i) Amplification plots of fluorescence intensities versus PCR cycle numbers in each sample. (C-ii) Each column represents the mean ± S.E.M. of 3 samples. ***P< 0.001 vs. the young group.

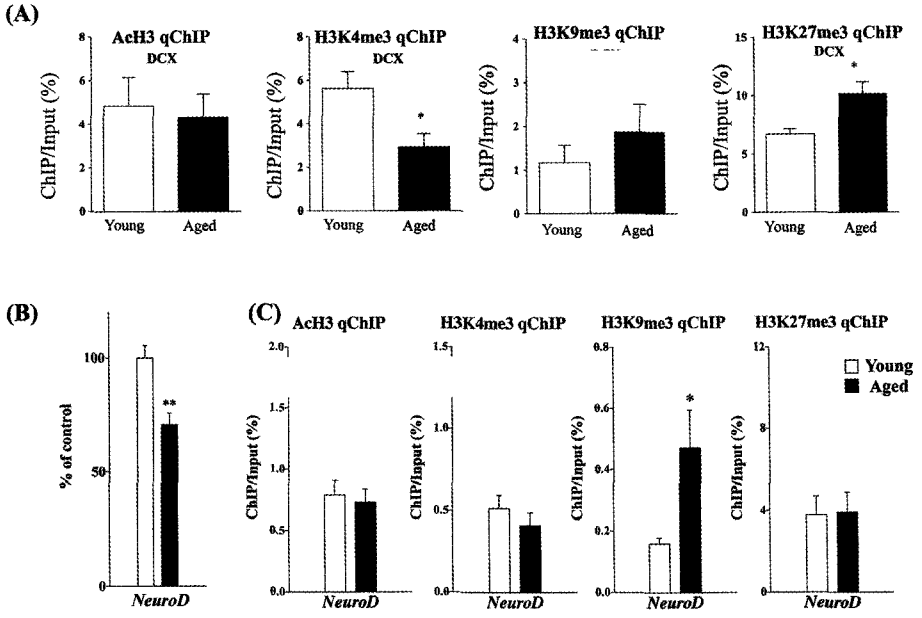


Figure 1-2. (A) Stable changes in histone modifications in the hippocampus in aged mice. ChIP assays were performed to measure the levels of several histone modifications at the doublecortin (DCX) promoter in the hippocampus using specific antibodies for each modification state. Levels of promoter enrichment were quantified by quantitative PCR. Histone H3 was not altered at DCX. Significant changes in acetylation (AcH3) or H3K9 trimethylation (H3K9me3) were not detected at the DCX promoter region. Histone H3K4 trimethylation (H3K4me3) was decreased at the DCX promoter region in aged mice. *P<0.05 vs. the young group. Histone H3K27 trimethylation (H3K27me3) was increased at the DCX promoter region in aged mice. *P<0.05 vs. the young group. (B) Quantitative analysis of NeuroD mRNA in the hippocampus obtained from young and aged mice. Each column represents the mean ± S.E.M. of 3 samples. (**P< 0.01 vs. the young group). (C) qChIP analysis of AcH3, H3K4me3, H3K9me3 and H3K27me3 at NeuroD loci in the hippocampus obtained from young and aged mice. Each column represents the mean ± S.E.M. of 6 samples (*P<0.05 vs. young group).

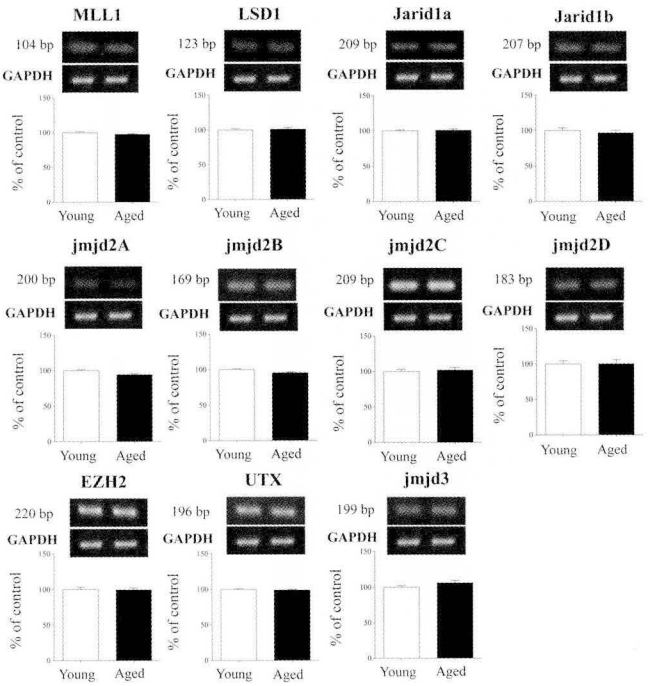


Figure 1-3. Upper: Representative RT-PCR for MLL1 (a H3K4 methyltransferase), LSD1, Jarid1a and Jarid1b (H3K4 demethylases), jmjd2A, jmjd2B, jmjd2C and jmjd2D (H3K9 demethylases), and EZH2 (a H3K27 methyltransferase), and UTX and jmjd3 (H3K27 demethylases). mRNAs in the hippocampus obtained from young and aged mice. Lower: The intensity of the bands was semi-quantified using NIH Image software. The value for mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for aged mice is expressed as a percentage of the increase in young mice. Each column represents the mean ± S.E.M. of 6 samples.

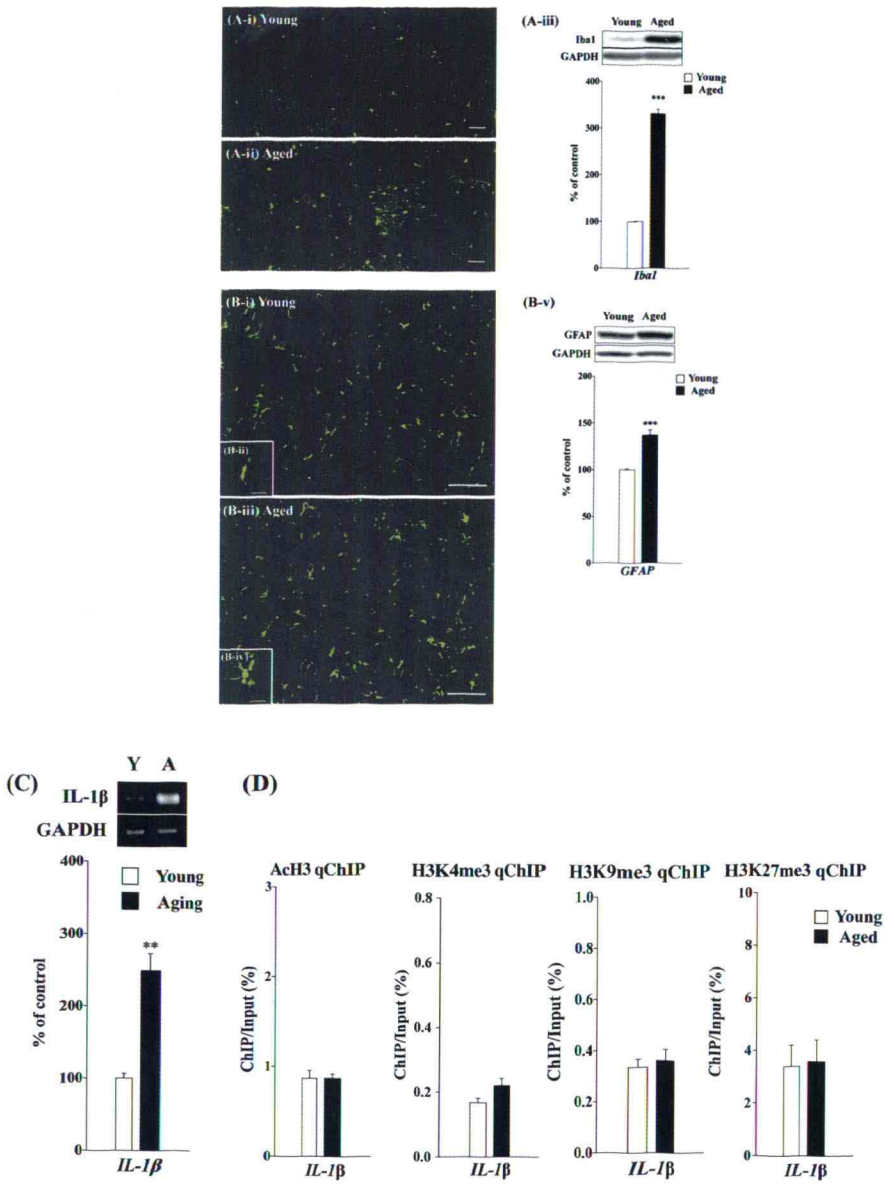


Figure 1-4. (A) Immunofluorescent staining for ionized calcium-binding adaptor molecule 1 (Iba1) in the dentate gyrus (DG) in young and aged mice. Iba1-like IR in the DG of aged mice (A-ii) was increased compared to that in young mice (A-i). Scale bar: 50 μm. (A-iii) *Upper*: Representative Western blot of Iba1. *Lower*: Changes in IR for Iba1 in the cytosolic fraction of hippocampus obtained from aged mice. Each column represents the mean ± S.E.M. of 6 samples (***p<0.001 vs. young group). (B) Immunofluorescent staining for glial fibrillary acidic protein (GFAP) in the DG in young and aged mice. GFAP-like IR in the DG of aged mice (B-iii, B-iv: high magnification) was increased compared to that in young mice (B-i, B-ii: high magnification). Scale bar: 50 μm (B-i,-iii). Scale bar: 10 μm (B-ii,-iv). (B-v) *Upper*: Representative Western blot of GFAP. *Lower*: Changes in IR for GFAP in the membranous fraction of hippocampus obtained from aged mice. Each column represents the mean ± S.E.M. of 6 samples (***p<0.001 vs. young group). (A) *Upper*: Representative RT-PCR for IL-1β mRNA in the hippocampus obtained from young and aged mice. *Lower*: The intensity of the bands was semi-quantified using NIH Image software. The value for mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for aged mice is expressed as a percentage of the increase in young mice. Each column represents the mean ± S.E.M. of 3 samples. (**P<0.01 vs. young group). (B) qChIP analysis of acetylated histone 3 (AcH3), histone 3 trimethylated at lysine 4 (H3K4me3), lysine 9 (H3K9me3), and lysine 27 (H3K27me3) at IL-1β loci in the hippocampus obtained from young and aged mice. Each column represents the mean ± S.E.M. of 3 samples.

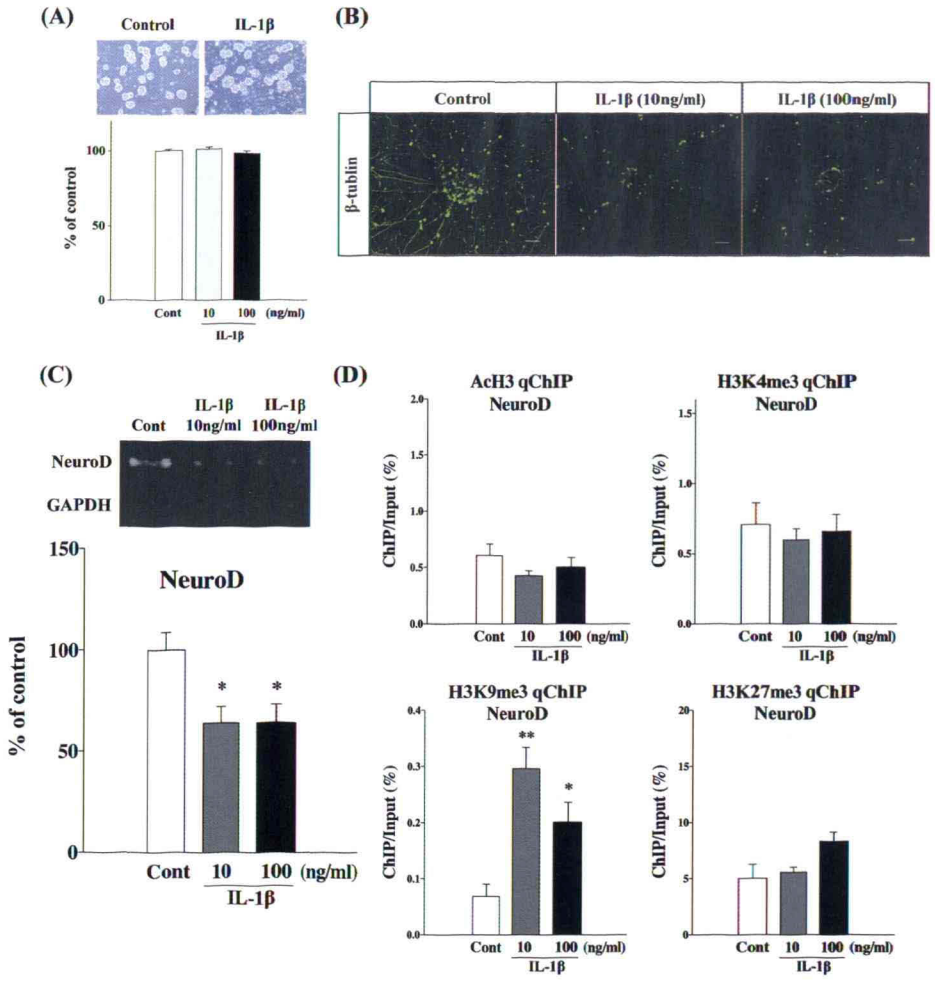


Figure 1-5. (A) Cell viability of neural stem cells after 1 day in the presence of 10 or 100 ng/ml IL-1β. Note that the IL-1β concentration has no effect on the survival of neural stem cells as indicated by Cell viability assay. (B) Effects of IL-1β on the differentiation of neural stem cells into β-tubulin-positive neurons. The cells were incubated overnight in medium containing epidermal growth factor (EGF) that was replaced with medium alone or medium containing IL-1β (10, 100 ng/ml). Treatment with 10 or 100 ng/ml IL-1β decreased β-tubulin-positive neurons when compared with control cultures. (C) *Upper*: Representative RT-PCR for NeuroD mRNA in neural stem cells. *Lower*: The intensity of the bands was semi-quantified using NIH Image software. The value for mRNA was normalized by that for the internal standard GAPDH mRNA. Each column represents the mean ± S.E.M. of 3 samples. (*P<0.05 vs. control group). (D) qChIP analysis of AcH3, H3K4me3, H3K9me3 and H3K27me3 at NeuroD loci in neural stem cells incubated with IL-1β compared to that in control groups. The value for ChIP/Input was normalized by that for the internal standard in each control. Each column represents the mean ± S.E.M. of 3 samples (*P<0.05, **P<0.01 vs. control group).

Table 1

Experiment	Name	Sequence
RT-PCR	DCX	F: CTTTTGGTTCAGCAGAAGGG R: CAAATGTTCTGGGAGGCACT
	GFAP	F: ACAACTTTGCACAGGACCTC R: CGATTCAACCTTTCTCTCCA
	BDNF	*F: TCACTGGCTGACACTTTTGAG* R: CTATCCTTATGAATCGCCAGC
	MLL1	F: AGCGGAGAGGATGAGCAGT R: CGAGGTTTTCGAGGACTAGC
	LSD1	F: TCAACGTCCTCAATAATAAACCTGT R: CCTGAGTTTTCACTATCTTCTTCCA
	Jarid1a	F: CCTCCATTTGCCTGTGAAGT R: CCTTTGCTGGCAACAATCTT
	Jarid1b	F: AGAGGCTGAATGAGCTGGAG R: TGGCAATTTTGGTCCATTTT
	jmjd2A	F: GACCACACTCTGCCCACAC R: TCCTGGGGTATTTCCAGACA
	jmjd2B	F: GGCTTTAACTGCGCTGAGTC *R: GTGTGGTCCAGCACTGTGAG*
	jmjd2C	F: CACGGAGGACATGGATCTCT R: CGAAGGGAATGCCATACTTC
	jmjd2D	F: GTCTTGGTCGTCGTCCTTGT R: AATCCCCCTTCAGAAGCTGT
	EZH2	F: GCCAGACTGGGAAGAAATCTG R: TGTGCTGGAAAATCCAAGTCA
	UTX	F: ATCCCAGCTCAGCAGAAGTT R: GGAGGAAAGAAAGCATCACG
	jmjd3	F: CCCCCATTTCAGCTGACTAA R: CTGGACCAAGGGGTGTGTT
	IL-1β	F: CACTAGGTTTGCCGAGTAGATCTC R: GTGCTGCCTAATGTCCCCTTG AATC
	NeuroD	*F: GCATGCACGGGCTGAACGC* R: GGG ATGCACCGGGAAGGAAG
	GAPDH	F: CCCCACGGCAAGTTCAACGG R: CTTTCCAGAGGGCCATCCA
Real-time PCR	DCX	F: CTTTTGGTTCAGCAGAAGGG R: CAAATGTTCTGGGAGGCACT
	NeuroD	F: GCATGCACGGGCTGAACGC R: GGGATGCACCGGGAAGGAAG
	β-actin	F: CAGCTTCTTTGCAGCTCCTT R: TCACCCACATAGGAGTCCTT
ChIP	DCX	F: AGCTTGCCTGTGCAATCTTT R: GAACACCCCCAACCTCCTAT
	IL-1β	F: TCCACCACGATGACACACTT *R: GGGAGAAGCTTGATGGGAAT*
	NeuroD	F: GCATGCACGGGCTGAACGC R: GGGATGCACCGGGAAGGAAG

Discussion

Doublecortin is a microtubule-associated protein that is expressed specifically in virtually all migrating neural precursors of the CNS and has been used as a candidate marker for neuronal migration and differentiation. In this study, aging caused a dramatic decrease in levels of doublecortin protein and mRNA in the hippocampus. In the dentate gyrus of the hippocampus, few or no doublecortin-positive cells were observed by aging. Furthermore, I also found that mRNA expression of NeuroD, another neural progenitor cell marker, was significantly decreased in the hippocampus of aged mice compared to that in young mice. These findings strongly suggest that an aging can stimulate the impairment of neuronal differentiation from precursors in the hippocampal dentate gyrus. These notions are supported by previous reports that aging promoted the impairment of neurogenesis [48,52].

Epigenetic alterations of DNA play key roles in determining gene structure and expression [38,82]. A major epigenetic modification, chromatin remodeling, modulates gene expression with high temporal and spatial resolution by permitting small groups of nucleosomes to become more or less open, which consequently enhances or inhibits access of the transcriptional machinery to specific promoter regions. The acetylation and methylation of histone proteins at specific residues play a major role in chromatin remodeling. Lysine acetylation almost always correlates with chromatin accessibility and transcriptional activity, whereas lysine methylation can have different effects depending on which residue is modified. Trimethylation of H3K4 is associated with

transcribed chromatin. In contrast, trimethylation of H3K9 and H3K27 generally correlates with repression [7]. In agreement with the PCR assay, aging caused a significant decrease in H3K4 trimethylation and a significant increase in H3K27 trimethylation at the doublecortin gene. Furthermore, a significant increase in H3K9 trimethylation at the promoter of NeuroD was also observed in the hippocampus. These findings suggest that aging produces a dramatic decrease in the expression of the neuronal progenitors, doublecortin and NeuroD, along with epigenetic modifications in the hippocampus.

Histone methylation is dynamically regulated by a plethora of methylases and demethylases [77]. In the present study, aging failed to change the mRNA expression of several methylases and demethylases including MLL1 (a H3K4 methyltransferase), LSD1, Jarid1a and Jarid1b (H3K4 demethylases), jmjd2A, jmjd2B, jmjd2C and jmjd2D (H3K9 demethylases), EZH2 (a H3K27 methyltransferase), and UTX and jmjd3 (H3K27 demethylases). These findings suggest that aging causes a dramatic decrease in neurogenesis accompanied by epigenetic modification related to the decreased expression of doublecortin or NeuroD without changing the expression of their associated histone methylases and demethylases in the hippocampus.

Microglia constantly survey the microenvironment for noxious agents or injurious processes [67]. With aging, microglia are thought to increase in number and become activated, and may enter a phagocytic or reactive stage [56]. In this study, Iba1-positive microglia were significantly increased in the DG of the hippocampus of aged mice.

Based on the activation stimuli, microglia are informed of the encountered problem

and instructed to act in an appropriate manner and perform a defined task. If the disturbance is relatively minor, microglia may secrete anti-inflammatory cytokines and supportive growth factors [56]. If the disturbance poses a serious threat, such as a pathogen invasion, microglia may release toxic factors to kill the pathogen and recruit help by releasing proinflammatory cytokines. If we consider the continuum of potential microglial responses, how a given insult is interpreted can mean the difference between a beneficial outcome or a detrimental outcome if the response is either too aggressive or too passive.

As well as microglia, aging caused a dramatic increase in GFAP-positive IR, which is located in dendritic astrocytes, with an expanding distribution in the hippocampus of aged mice. Each individual astrocyte labeled by GFAP was branched, which indicates the activation of astrocytes in the hippocampus of aged mice. This notion is supported by a previous finding that in humans the GFAP level increases dramatically after the age of 65 years, and more specifically in the hippocampal formation [21]. Furthermore, aging had a differential effects on astrocytic and microglial hyperactivity in gray vs. white matter areas. (see, Figure 1) This mosaic of glial aging suggests that multiple mechanisms are at work during aging.

The activation of microglia or astrocytes that secrete and express numerous regulatory ligands can affect cells of immune or nonimmune tissues such as neurons through the release of cytokines and chemokines. Cytokines and chemokines have been classified as molecules that coordinate inflammatory and immune responses, and also mediate normal, ongoing signaling between cells of nonimmune tissues [11]. In the

present study, the mRNA level of IL-1β in the hippocampus was significantly increased by aging. This result is partly supported by previous findings [63]. However, aging did not cause any changes in the acetylation or methylation of histone proteins at IL-1β. These findings suggest that aging produces a dramatic increase in the expression of IL-1β without related histone modifications.

It has been reported that IL-1β suppresses the proliferation of hippocampal progenitor cells [45]. Thus, I finally investigated whether IL-1β could affect neural progenitor cells through epigenetic regulation. *In vitro* treatment with IL-1β in neural stem cells prepared from whole brain of E14.5 mice significantly increased H3K9 trimethylation at the NeuroD promoter. These findings suggest that IL-1β, which may be produced by aging, decreases neural progenitor cells through epigenetic modulation.

In conclusion, although further investigation is still required, the present findings suggest that decreased expression of the neural progenitors, doublecortin and NeuroD, may be, at least in part, associated with an aging-dependent repressive epigenetic modulation at each gene in the hippocampus. Furthermore, aging significantly increased mRNA levels of IL-1β without related histone modifications and increased H3K9 trimethylation at the promoter of NeuroD in the hippocampus of aged mice. These findings suggest that aging may decrease hippocampal neurogenesis via epigenetic modifications accompanied by the activation of microglia and astrocytes with an increased expression of IL-1β in the hippocampus. In addition, the present finding of the epigenetically repressive modulation of neural progenitors could allow us to better understand the mechanism of aging-dependent hippocampal dysfunction.

Chapter 2

Hippocampal epigenetic modification at the brain-derived neurotrophic factor gene induced by an enriched environment

Introduction

Over the past few decades, exposure to an enriched environment, which consists of housing groups of animals together in a complex environment with various toys to provide more opportunity for learning and social interaction than standard laboratory living conditions, has been shown to enhance behavioral performance in various learning tasks. Consistent with these behavioral tests, exposure to an enriched environment has been shown to induce biochemical and structural changes in the hippocampal DG and CA1 region, such as an increased number of dendritic branches and spines, enlargement of synapses and an increased number of glial cells. Moreover, exposure of adult rodents to increased environmental complexity induces hippocampal progenitor proliferation and neurogenesis [66,83]. However, the detailed mechanisms that control neurogenesis in the hippocampus of animals housed in an enriched environment are still unclear.

It has been reported that BDNF promotes neuronal differentiation from endogenous progenitor cells in the ventricular wall of the adult forebrain [1,44], and the increased expression of BDNF is required for the environmental induction of hippocampal neurogenesis in rodents [72]. The BDNF gene and the regulation of its expression are highly complex, and have been examined in both human and rodent brains [2,53,70,80]. The mouse BDNF gene, which a shows high degree of sequence homology to its human congener, contains multiple 5' noncoding exons and a single 3' coding exon for the mature BDNF protein [2]. These noncoding exons undergo alternative splicing with the

common coding exon to produce multiple exon-specific BDNF transcripts. Nine BDNF promoters have been previously identified in the mouse [2], and each drives the transcription of BDNF mRNAs containing one of the four 5' noncoding exons (I, II, III, IV, V, VI, VII or VIII) spliced to the common 3' coding exon.

Chromatin remodeling at gene promoter regions is becoming increasingly recognized as a key control point of gene expression. Histone modification represents one prominent form of chromatin remodeling. According to the "histone code theory", different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state that encodes gene activation versus gene silencing [39]. Intriguing correlations have been found between cellular plasticity, including transformation and such epigenetic modification at a specific gene [12,46], indicating that possible epigenetic modification at BDNF gene promoters may partly contribute to adult neurogenesis. Therefore, in the present study, I evaluated whether an enriched environment could induce histone modification at several BDNF gene promoters in mice.

Materials and Methods

Animals

Male C57BL/6J mice (Jackson Laboratory), weighing 18-23 g were used in the present study. Animals were kept in a room with an ambient temperature of 23 ± 1℃ and a 12-hr light-dark cycle (lights on 8:00 AM to 8:00 PM). Food and water were available ad libitum. This study was approved by the Animal Research Committee of Hoshi University.

Housing environment

Control mice were housed 4 per standard (16.5 x 26.5 x 13.5cm) plexiglass cage. Mice of the enriched environment group were kept 8 mice per large (25.5 x 42.5 x 39cm) wire mesh two-storied cage which contained tunnels and running wheels for 4 weeks (Figure 1).

Drugs and injection procedure

BrdU (Sigma-Aldrich, St Louis, MO, U.S.A.) was dissolved in saline. For analysis of BrdU-positive cells, mice were administered BrdU (6 x 50 mg/kg every 2 hr) before housed in enriched environment. To evaluate cell differentiation activity, 4 weeks after the BrdU injection, animals were killed, and the brains were processed for immunohistochemistry.

RNA preparation and quantitative analysis by reverse transcription-PCR

Total RNA in the hippocampus of mice housing in the enriched environment was extracted using the SV Total RNA Isolation system (Promega, Madison, WI, U.S.A.) following the manufacturer's instructions. Purified total RNA was quantified spectrophotometrically at A_{260}. To prepare first-strand cDNA, 1 μg of RNA was incubated in 100 μL of buffer containing 10 mM dithiothreitol, 2.5 mM $MgCl_2$, dNTP mixture, 50 U of reverse transcriptase II (Invitrogen, Carlsbad, CA, U.S.A.), and 0.1 mM oligo-dT_{12-18} (Invitrogen). Each gene was amplified in a 50 μL PCR solution containing 0.8 mM $MgCl_2$, dNTP mixture, and DNA polymerase, with synthesized primers. Primers were designed complementary to exon IX to measure levels of total BDNF mRNA: total BDNF IX (529 bp), GAPDH (427 bp) (Table 2). Samples were heated to 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final incubation was 72°C for 7 min. The mixture was run on 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. The agarose gel was stained with ethidium bromide and photographed with UV transillumination. The intensity of the bands was analyzed and semiquantified by computer-assisted densitometry using ImageJ software. Values represent the mean ± SEM of three independent experiments.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described previously [79,81] with minor modifications. Briefly, mouse hippocampus tissue was dissected as described above and cross-linked,

and then tissue was lysed. 15 μg of soluble chromatin was incubated with 2 μg of specific antibodies; against acetylated histone H3 (Millipore); H3K4 trimethylation (Wako Pure Chemicals, Osaka, Japan); H3K9 trimethylation (Millipore); H3K27 trimethylation (Millipore), overnight at 4°C. The immuno-complex was collected by Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), and DNA was recovered with RNaseA treatment, Proteinase K treatment followed by isopropanol precipitation. Immunoprecipitated DNA was dissolved in 50 μL of 1 x TE and 1 μL was used for quantitative PCR. Quantitative PCR was performed as described previously [64]. Using primers were listed in Table S1.

MicroRNA

In brief, TaqMan MicroRNA assays include two steps: stem-loop RT followed by real-time PCR. Each 7.5 μL RT reaction that includes 3.75 ng purified total RNA, RT primer, RT buffer, 250 μM each of dNTPs, 0.25 U/μL RNase inhibitor, and 3.33 U/L MultiScribe Reverse Transcriptase from the TaqMan MicroRNA Reverse Transcription Kit (Part No. 4366597, Applied Biosystems) were incubated for 30 min each at 16℃ and at 42℃. Realtime PCR was performed using standard protocol on an Applied Biosystems 7900HT Sequence Detection System. All TaqMan miRNA assays were performed in triplicate. Total RNA input was normalized based on the CT values of the TaqMan snoRNA (GeneBank ID: HUMUR6) assay as an endogenous control (Part No. 4373381). The fold change was calculated based on Δ Ct between endogenous snoRNA control and individual miRNA.

Immunohistochemistry

Mice were deeply anesthetized with isoflurane (3 %) and perfusion-fixed with 4 % paraformaldehyde (pH 7.4). The brain was then removed quickly and the hippocampus was rapidly dissected and postfixed in 4% paraformaldehyde for 2 hr. The hippocampus was permeated with 20 % sucrose for 1 day and 30 % sucrose for 2 days, then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan). All samples were stored at –30 ˚C until use. The sections were cut transversely at a thickness of 8 μm on a cryostat (Leica CM1510, Leica Microsystems, Heidelberg, Germany). The hippocampus sections were blocked in 10% normal horse serum, 5% normal goat serum and 10 % normal goat serum in 0.01 M phosphate-buffered saline (PBS) for 1 hr at room temperature. For the BrdU staining assay, to detect BrdU-labeled cells, we incubated coronal sections for 90 min with citrate buffer (10 mM, 90 ℃) before blocking. Each primary antibody was diluted in 0.01 M PBS containing either 5 % normal goat serum (guinea pig polyclonal antibody to doublecortin [1:3500 Abcam Ltd., Cambridge, UK]), 10% normal goat serum (rat monoclonal antibody to BrdU [1:150 Abcam Ltd., Cambridge, UK]), goat polyclonal antibody to NeuroD [1:500 Santa Cruz Biotechnology, CA, U.S.A.]) and incubated for 2 days overnight at 4 °C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with either Alexa 488 or Alexa 546 for 2 hr at room temperature. For BrdU/NeuN double-labeling, Alexa 488-conjugated goat anti-rat IgG was used for BrdU, and after washing out and challenging the mouse monoclonal antibody to NeuN

[1:250 Chemicon International Inc., CA, U.S.A.], Alexa 546-conjugated goat anti-mouse IgG was used. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA, USA). Fluorescence of immunolabeling was detected using a light microscope (Olympus AX-70; Olympus, Tokyo, Japan, and a Radiance 2000 laser-scanning microscope; BioRad, Richmond, CA, U.S.A.), and photographed with a digital camera (Polaroid PDMCII/OL; Olympus). The number of NeuroD-labeled cells in the DG was counted and the mean (± SEM) number of cells was calculated. BrdU-positive cells located in the DG were counted in six sections per animal that were spaced 100 μm apart and corresponded to coronal coordinates of bregma from 1.46-2.30 mm. Sections were taken by sampling at equal intervals from the hippocampus region nearer to the septal end for more consistent BrdU labelling. The number of BrdU-positive cells in both side of the DG in a section was counted using a light microscope. This reference sample volume was 450 μm thick. The number of BrdU cells in the DG was divided by the thickness of the section to obtain the average number of labelled cells per traced area. The estimated number of BrdU cells in the hippocampus sample volume per brain is obtained by multiplying the average number of labelled cells per area by the reference sample volume.

Neural stem cells (NSCs) culture

Pregnant C57BL/6J mice were used to prepare NSCs. NSCs were prepared from whole brain of E14.5 mice and culture. Briefly, the brain were triturated in serum-free medium: Dulbecco's modified Eagle's medium with 4500 mg/L glucose, 5 μg/mL

insulin, 10 ng/mL EGF, 50 μg/mL transferrin, 10 ng/mL biotin and 30 nM Na_2SeO_3. EGF (10 ng/mL) was used to keep the cultures proliferating. For differentiation experiments, approximately 10 neurospheres of the same size were isolated with a pipette and deposited on 10 μg/mL laminin-coated glass slides with 400 microl of serum-free medium with 10 ng/mL EGF. The NSCs were incubated overnight in medium containing EGF, and was then replaced on medium without EGF containing BDNF (100 ng/mL) for 7 days. Seven days after drug treatment, *in vitro* cells were washed in PBS, and fixed in 4 % paraformaldehyde in phosphate buffer at pH 7.4 and room temperature for 30 min. After the cells were washed in PBS, added PBS containing 0.3% Triton X-100 to each well and incubated for 5 min at room temperture for permeabilization. The NSCs were blocked in 20 % blockace in PBS for 5 min at room temperature. Thereafter, the neural stem cells were immunostained for microtubule-associated protein 2ab (MAP2ab), a marker for neurons. MAP2ab antibody was diluted in PBS containing 20 % blockace [1:500 MAP2ab (CHEMICON International, Inc., Temecula, CA, U.S.A.)] and incubated for 2 hr at 37 ˚C. The cells were then rinsed and incubated with the followed by incubation with Alexa 546-conjugated goat anti-mouse IgG (1:2000) for 30 min at 37 ˚C. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA, U.S.A.). Fluorescence of immunolabelling was detected using a microscope with a 10 x objective lens (IX 71, Olympus, Co., Tokyo, Japan) and photographed with a digital camera (VB-6000, Keyence, Co., Osaka, Japan).

Statistical analysis

The data are expressed as the mean ± S.E.M. The statistical significance of differences between groups was assessed with Student's t-test (comparison of two groups) or an analysis of variance [37] followed by the Bonferroni test (comparison among multiple groups).

Results

Increase in the immunoreactivity for doublecortin and NeuroD in the hippocampal dentate gyrus of mice housed in the enriched environment

Control mice were housed 4 per standard plexiglass cage. Mice in the enriched environment group were kept 8 per large wire-mesh, two-storied cage, which contained tunnels and running wheels, for 4 weeks (Figure 2-1-A).

In the DG of mice housed in the enriched environment for 4 weeks, immunoreactivity (IR) for doublecortin, which is a microtubule-associated protein that is expressed specifically in virtually all migrating neuronal precursors of the CNS and which has been used as a candidate marker for neural migration and differentiation, was increased compared to that in mice housed in the standard cage (Figure 2-1-B). Furthermore, IR for NeuroD, which is another marker for the differentiation of granule cells in the hippocampus [62], was clearly increased in the DG of mice housed in an enriched environment (Figure 2-1-C). Additionally, the number of BrdU-positive cells in the DG that were classified as newly dividing cells was markedly increased in mice housed in an enriched environment (Figure 2-1-D-ii,-iii), and theses were clearly colocalized with the neuronal marker NeuN (Figure 2-1-D-vii).

Expression of BDNF in the hippocampal dentate gyrus of mice housed in the enriched environment

In paralleled with adult neurogenesis, the expression of BDNF mRNA in the

hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks (Figure 2-2-A). In contrast, mRNA levels of GFAP, platelet derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF) and epidermal growth factor (EGF) in the hippocampus were not altered by exposure to an enriched environment for 4 weeks (Figure 2-2-B).

Under these conditions, a significant increase in H3K4 trimethylation at the BDNF P3 and P6 promoters was observed upon exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in H3K9 trimethylation at the BDNF P4 promoter, and H3K27 trimethylation at the BDNF P3 and P4 promoters were seen in the hippocampus of mice under an enriched environment. In contrast, an enriched environment did not produce the hyperacetylation of H3 in the hippocampus of enriched mice (Figure 2-2-C).

No change in the levels of histone modification enzymes and microRNA

In terms of changes in mRNA levels of several histone methylases and demethylases in the hippocampus, an enriched environment failed to change the mRNA expression of MLL1, LSD1, Jarid1a, Jarid1b, Jmjd2B, Jmjd2C, Jmjd2D, EZH2, UTX or Jmjd3 (Figure 2-3-A).

As with histone methylases and demethylases, no significant changes in microRNA9 (miR9), miR124a, miR132, miR133b or miR145 were observed in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in

a standard cage (Figure 2-3-B).

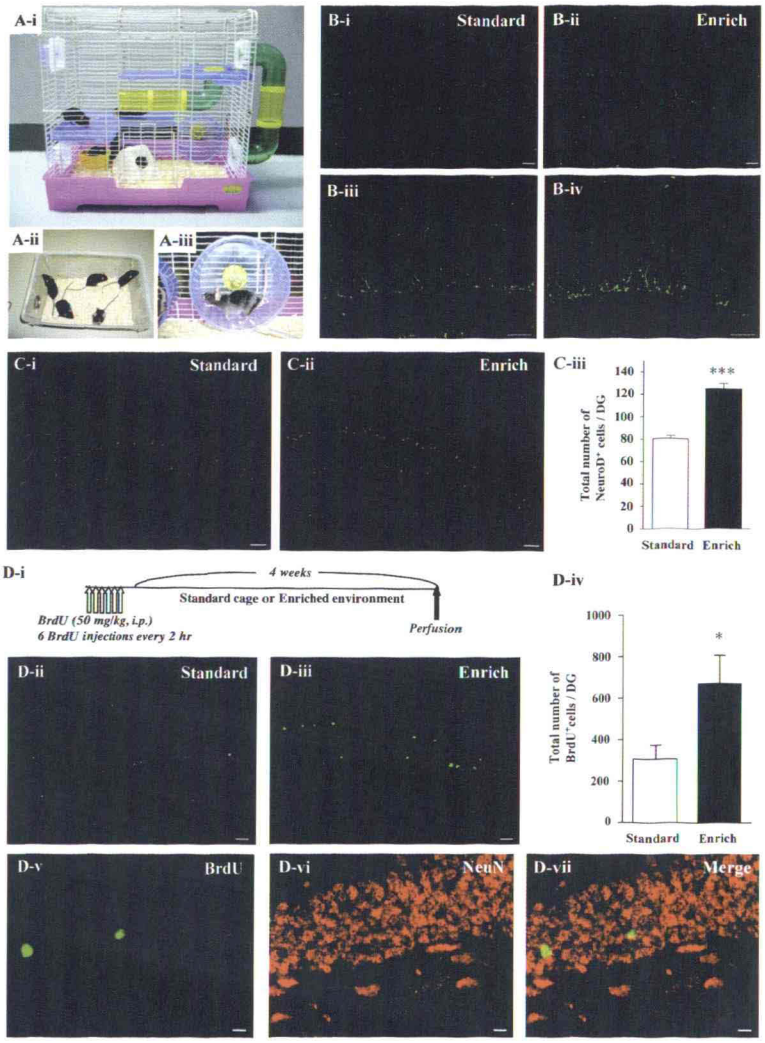


Figure 2-1. (A-i) Cage equipped with an enriched environment. Enrichment consisted of greater social interaction (8 mice in the large cage vs. 4 mice in the standard cage), greater potential for exploratory behavior with objects such as toys and a rearrangeable set of tunnels, and physical activity in a running wheel (A-iii). (A-ii) Standard cage. (B) Immunofluorescent staining for doublecortin in the dentate gyrus in mice housed under their respective experimental conditions for 4 weeks. Doublecortin-like immunoreactivity in the dentate gyrus of enriched mice (B-ii, B-iv: high magnification) was increased compared to that in standard mice (B-i, B-iii: high magnification). Scale bar: 50 μm. (C) Immunofluorescent staining for NeuroD in the dentate gyrus in mice housed under standard or enriched conditions for 4 weeks. NeuroD-like immunoreactivity in the dentate gyrus of enriched mice (C-ii) was increased compared to that of standard mice (C-i). (C-iii) Results of the stereological counting of NeuroD-positive cells in the dentate gyrus of standard and enriched mice. The total number of NeuroD-positive cells was significantly increased in the enriched group compared to the standard group. Each column represents the mean ± S.E.M. $^{***}p<0.001$ vs. the standard group. Scale bar: 50 μm. (D) Time line for the experimental design (D-i). Representative photomicrographs of BrdU labeling in the dentate gyrus of standard (D-ii) and enriched mice (D-iii). (D-iv) Results of the stereological counting of BrdU-positive cells in the dentate gyrus of standard and enriched mice. The survival of newly generated cells was significantly increased by environmental enrichment. Each column represents the mean ± S.E.M. $^{*}p<0.05$ vs. the standard group. Phenotype of BrdU-positive cells in the dentate gyrus. Immunofluorescence double-labeling for BrdU (D-v) and NeuN, a neuron-specific nuclear marker (D-vi). (D-vii) Colocalization of BrdU with NeuN in the dentate gyrus of mice housed in an enriched environment for 4 weeks after the last injection of BrdU. Scale bar: 100 μm.

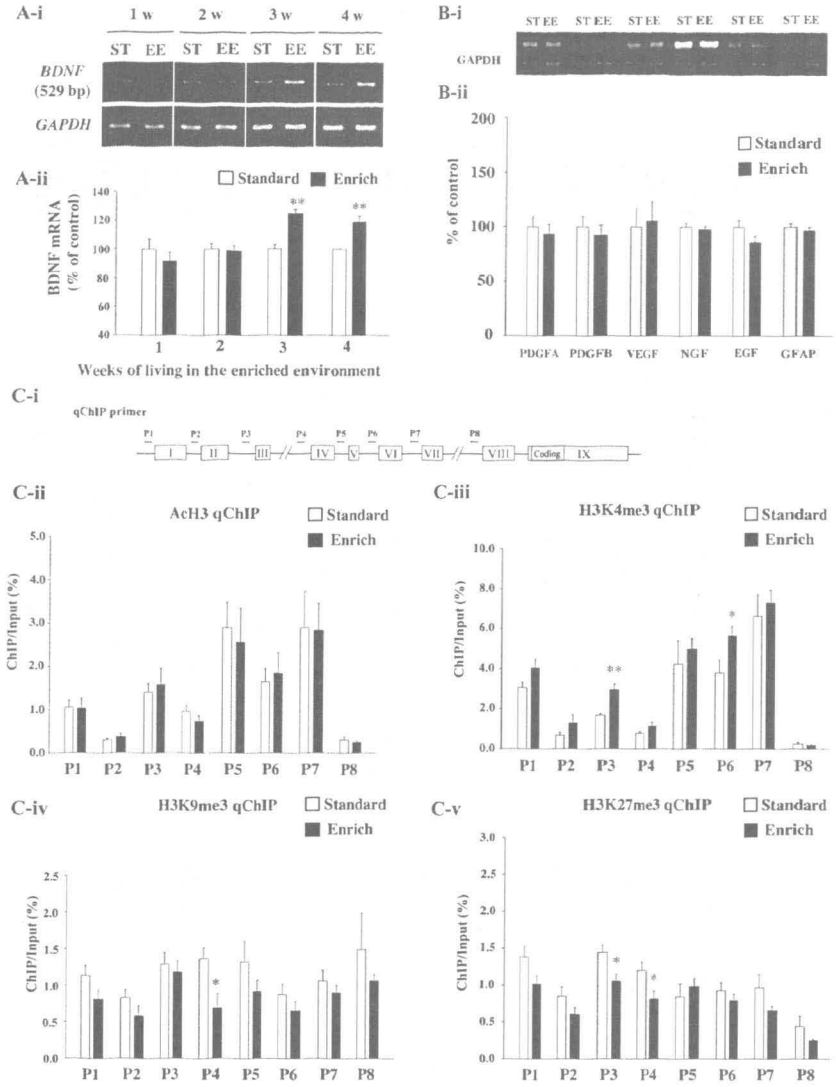


Figure 2-2. (A) Time course of changes in the expression of BDNF mRNA in the hippocampus. (A-i) Representative RT-PCR for BDNF mRNA in the hippocampus obtained from standard or enriched mice. (A-ii) The intensity of the bands was semi-quantified using NIH Image software. The value for BDNF mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for enriched mice is expressed as a percentage of the increase in standard mice. Each column represents the mean ± S.E.M.of 6 samples. **p<0.01 vs. the standard group. (B) Upper: Representative RT-PCR for PDGFA, PDGFB, VEGF, NGF, EGF and GFAP mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The values for mRNAs were normalized by that for GAPDH mRNA. Each column represents the mean ± S.E.M. of 6 samples. (C-i) Schematic of the BDNF gene: The BDNF gene contains eight noncoding exons I-VIII upstream of the coding exon IX in mouse. Exons I-VIII can each be alternatively spliced next to exon IX, from the 5' UTR region of different mRNA splice variants, BDNF I-VIII, which can promote the expression of their corresponding transcript variants. For an mRNA analysis of total BDNF, primers were used to amplify exon IX. For ChIP analysis, primers were designed around the putative promoters, P1-P8, which are located upstream of exons I-VIII. (C-ii, iii, iv, v) Stable changes in histone modifications in the hippocampus in mice housed under standard or enriched conditions for 4 weeks. ChIP assays were performed to measure the levels of several histone modifications at the eight BDNF promoters Ex1-Ex8 (P1-8) in the hippocampus using specific antibodies for each modification state. Levels of promoter enrichment were quantified by quantitative PCR. (C-ii) Histone H3 was not altered at BDNF P1-P8. Significant changes in acetylation or methylation were not detected at other BDNF promoter regions. (C-iii) Histone H3K4 trimethylation was increased at BDNF P3 and P6 in mice housed under standard or enriched conditions for 4 weeks. *P<0.05 vs. the standard group, **p<0.01 vs. the standard group. (C-iv) H3K9 trimethylation was decreased at BDNF P4 in mice housed under standard or enriched conditions for 4 weeks. *P<0.05 vs. the standard group. (C-v) Histone H3K27 trimethylation was decreased at BDNF P 3 and P4 in mice housed under standard or enriched conditions for 4 weeks. *P<0.05 vs. the standard group.

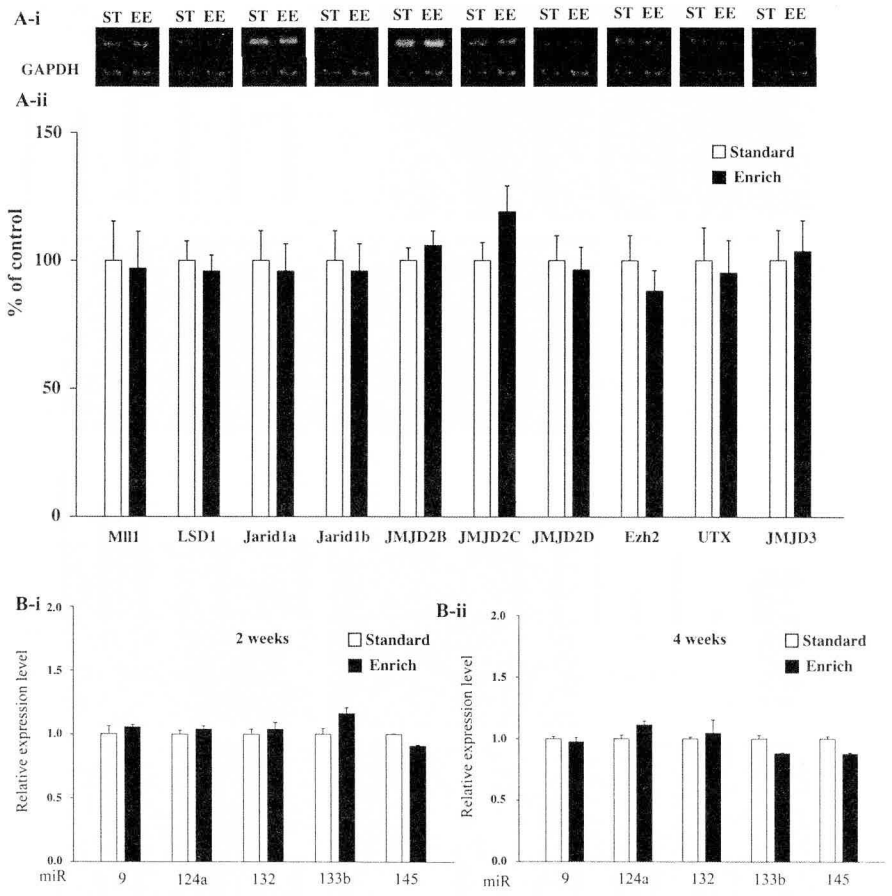


Figure 2-3. (A) Upper: Representative RT-PCR for MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a and Jarid1b (H3K4 demethylases), JMJD2B, JMJD2C and JMJD2D (H3K9 demethylases), Ezh2 (H3K27 methyltransferase), and UTX and JMJD3 (H3K27 demethylases). mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The value for mRNA was normalized by that for GAPDH mRNA. Each column represents the mean ± S.E.M. of 6 samples. (B) Expression levels of miRNAs were measured in the hippocampus of mice housed in ~~the~~ an enriched environment for 2 weeks (B-i) and 4 weeks (B-ii). The value for miRNA was normalized by that for the internal standard snoRNA202.

Table

Experiment	Name	Sequence
mRNA	Total BDNF	F: TCA CTG GCT GAC ACT TTT GAG R: CTA TCC TTA TGA ATC GCC AGC
	PDGFA	F: CTG TGC CCA TTC GCA GG R: ACC GCA CGC ACA TTG
	PDGFB	F: GCA ATA ACC GCA ATG TGC AAT GCC R: CGC CTT GTC ATG GGT GTG CTT AAA
	VEGF	F: CCT GGT GGA CAT CTT CCA GGA GTA CC R: GAA GCT CAT CTC TCC TAT GTG CTG GC
	NGF	F: GCC CAC TGG ACT AAA CTT CAG C R: CCG TGG CTG TGG TCT TAT CTC
	EGF	F: ACC AGA CGA TGA TGG GAC AG R: GCC AGC ACA CAC TCA TCT AT
	GFAP	F: ACA ACT TTG CAC AGG ACC TC R: CGA TTC AAC CTT TCT CTC CA
	Mll1	F: AGC GGA GAG GAT GAG CAG T R: CGA GGT TTT CGA GGA CTA GC
	LSD1	F: TCAACGTCCTCAATAATAAACCTGT R: CCTGAGTTTTCACTATCTTCTTCCA
	Jarid1a	F: CCT CCA TTT GCC TGT GAA GT R: CCT TTG CTG GCA ACA ATC TT
	Jarid1b	F: AGA GGC TGA ATG AGC TGG AG R: TGG CAA TTT TGG TCC ATT TT
	JMJD2B	F: GGC TTT AAC TGC GCT GAG TC R: GTG TGG TCC AGC ACT GTG AG
	JMJD2C	F: CAC GGA GGA CAT GGA TCT CT R: CGA AGG GAA TGC CAT ACT TC
	JMJD2D	F: GTC TTG GTC GTC GTC CTT GT R: AAT CCC CCT TCA GAA GCT GT
	Ezh2	F: GCC AGA CTG GGA AGA AAT CTG R: TGT GCT GGA AAA TCC AAG TCA
	UTX	F: ATC CCA GCT CAG CAG AAG TT R: GGA GGA AAG AAA GCA TCA CG
	JMJD3	F: CCC CCA TTT CAG CTG ACT AA R: CTG GAC CAA GGG GTG TGT T
	GAPDH	F: CCC CAC GGC AAG TTC AAC GG R: CTT TCC AGA GGG CCA TCC A
ChIP	BDNF P1	F: TGA TCA TCA CTC ACG ACC ACG R: CAG CCT CTC TGA GCC AGT TAC G
	BDNF P2	F: CCG TCT TGT ATT CCA TCC TTT G R: CCC AAC TCC ACC ACT ATC CTC
	BDNF P3	F: GTG AGA ACC TGG GGC AAA TC R: ACG GAA AAG AGG GAG GGA AA
	BDNF P4	F: CTT CTG TGT GCG TGA ATT TGC T R: AGT CCA CGA GAG GGC TCC A
	BDNF P5	F: TTC CCG GCT AGA AGC AGT AA R: TCG GCT TAA CCA GAG TTT GC
	BDNF P6	F: ACT CAC ACT CGC TTC CTC CT R: GCA CTG GCT TCT CTC CAT TT
	BDNF P7	F: CAA GGG AAG AGG ACG ACT TG R: CCC AAC AGC TGT CGC TCT AT
	BDNF P8	F: GCT TAT GTG GGA GTG GCA TT R: GTC ACA ATC CAT GTG GGT CA

Discussion

In the present study, I demonstrated hippocampal neurogenesis in mice that were exposed to an enriched environment. This notion is supported by previous reports that exposure of adult rodents to an enriched environment increased neurogenesis in the hippocampus [41,66].

During development, growth factors provide important extracellular signals that regulate the proliferation and differentiation of neuronal stem cells in the CNS [15]. Several investigations have examined the role of these factors in the adult brain [15,49]. Furthermore, it has been shown that exposure to an enriched environment increased the expression of BDNF genes [26]. In support of these findings, the present study showed that the expression of BDNF mRNA in the hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks. In contrast, mRNA levels of GFAP, PDGF-A, PDGF-B, VEGF, NGF and EGF in the hippocampus were not altered under the present conditions. In our *in vitro* study using neural stem cells cultured from the mouse embryonic forebrain, neuronal differentiation was clearly observed following exposure to recombinant BDNF. These findings raise the possibility that an enriched environment may stimulate expression of the BDNF gene in the hippocampus and, in turn, the enhanced BDNF protein may lead to neuronal differentiation from its precursors in the hippocampal DG.

I next evaluated whether an enriched environment increases BDNF gene expression through chromatin-specific events that promote the expression of distinct transcript

variants. In this study I analyzed two active histone modifications AcH3 and H3K4 and two repressive histone modifications H3K9 and H3K27 at different BDNF promoter regions in the hippocampus. As a result, I detected a significant increase in H3K4 trimethylation, an activated histone modification marker, at the BDNF P3 and P6 promoters after exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in H3K9 trimethylation, a repressive histone modification marker, at the BDNF P4 promoter, and H3K27 trimethylation, another repressive histone modification marker, at the BDNF P3 and P4 promoters were seen after exposure to an enriched environment. Under these conditions, I observed that an enriched environment did not produce the hyperacetylation of H3 in enriched mice.

The methylation of H3K9 and H3K27 can be directly modulated by histone methylases and demethylases that target specific lysine residues and methylation states [39,46]. Thus, I investigated whether an enriched envirnment could alter the mRNA level of several histone methylases and demethylases in the hippocampus. I found that an enriched environment did not change the mRNA expression of MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a or Jarid1b (H3K4 demethylases), jmjd2B, jmjd2C or jmjd2D (H3K9 demethylases), EZH2 (an H3K27 methyltransferase), or UTX or jmjd3 (H3K27 demethylases).

Recently, miRNAs, a class of small, noncoding RNAs, have been identified as important regulators of many biological processes, including organogenesis and disease development [17,34,43]. Indeed, it has been shown that epigenetic factors such as DNA methylation, histone modification and regulatory noncoding RNAs affect the fate

of neural stem cells [19]. miRNAs have the potential to specifically regulate a large set of target molecules, which may affect the cell fate in a programmatic way, and the role of miRNAs in stem cell gene networks is being actively explored. Their ability to potentially regulate large numbers of target genes simultaneously suggests that they may be important sculptors of transcriptional networks. In this study, I found that miR9, miR124a, miR132, miR133b and miR145 are expressed in the hippocampus of adult mice. It has been reported that miR145 regulates Oct4, Sox2 and Klf4 and suppresses the potential of human embryonic stem cells to generate any differentiated cell type (pluripotency) [87]. miR124, one of these signature miRNAs that is enriched in the brain, regulates adult neurogenesis in the subventricular zone stem cell niche [18]. miR132 is localized and synthesized, in part, at synaptic sites in dendrites to regulate synaptic formation and plasticity [85]. miR9 is expressed specifically in the hippocampus and may be involved in neural stem cell self-renewal and differentiation [3,47]. In the present study, there were no significant changes in miR9, miR124a, miR132, miR133b or miR145 in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in a standard cage. Although further studies are required to investigate the molecular mechanism of hippocampal neurogenesis induced by an enriched environment, we propose that an enriched environment may increase BDNF expression accompanied by histone modification without directly changing the expression of histone H3 methylases and demethylases, and miRs in the hippocampus.

In conclusion, the present study demonstrated that an enriched environment stimulates neuronal differentiation from precursors in the hippocampal DG.

Furthermore, the increased expression of BDNF was observed in the hippocampus of mice that had been exposed to an enriched environment. This enrichment induced a significant increase in H3K4 trimethylation at the BDNF P3 and P6 promoters and a significant decrease in H3K9 trimethylation at the BDNF P4 promoter and H3K27 trimethylation at the BDNF P3 and P4 promoters in the hippocampus of mice. These results suggest that an enriched environment may increase BDNF expression with notable sustained chromatin regulation in the mouse hippocampus. This phenomenon could partly explain the hippocampal neurogenesis induced by an enriched environment in mice.

Chapter 3

Epigenetic modulation at the CCR2 gene correlates with the maintenance of behavioral sensitization to methamphetamine

Introduction

Methamphetamine is a strongly addictive psychostimulant that dramatically affects the CNS and is highly abused worldwide. The abuse of psychostimulants leads to the development of psychotic symptoms that resemble those of paranoid schizophrenia [78]. In rodents, it has been shown consistently that repeated exposure to psychostimulants results in a progressive and enduring enhancement of the motor-stimulant effect elicited by a subsequent drug challenge, which is called behavioral sensitization [84]. Many studies have suggested that the mesolimbic dopaminergic system, which projects from the ventral tegmental area (VTA) to the nucleus accumbens, is critical for the initiation of methamphetamine-induced hyperlocomotion. A growing body of evidence suggests that the behavioral sensitization induced by psychostimulants may be accompanied by long-lasting neural plasticity [71] that may involve structural modifications in the dopaminergic system [76].

This dopaminergic neuronal plasticity response has been believed to require diverse alterations in gene expression. Although some of the candidate genes that are involved in behavioral sensitization to methamphetamine have been identified, an important step toward unraveling the complex machinery of methamphetamine -induced behavioral sensitization is multiplex analysis for both gene-expression profiling and epigenetic modifications, which exert lasting control over gene expression without altering the genetic code.

Recent evidence has suggested that epigenetic mechanisms, which are key cellular

and molecular processes that integrate diverse environmental stimuli to exert potent and often long-lasting changes in gene expression through the regulation of chromatin structure, contribute to drug-induced transcriptional and behavioral changes. However, to date, all of the investigations of methamphetamine -induced changes in gene expression have focused, by necessity, on measures of steady-state mRNA levels, which may not always reflect the transcriptional regulation of the encoding genes. Histones are modified at many sites. There are over 60 different residues on histones where modifications have been detected either by specific antibodies or by mass spectrometry. The vast majority of reports have indicated that increased acetylation of histone H3 or methylation of H3 at K4 (Lys4) is highly predictive of gene activation, while increased methylation of H3 at K9 or K27 (Lys9 or 27) is predictive of gene repression. The triggering of signaling cascades in target neurons leads to more long-lasting effects, including changes in gene expression via the control of transcription and thereby chromatin remodeling.

In the present study, to further understand the molecular mechanisms that underlie methamphetamine-induced behavioral sensitization, I investigated changes in epigenetic modification at 14 chemokine and cytokine genes in the limbic forebrain including the nucleus accumbens of mice that were intermittently treated with methamphetamine.

Materials and Methods

Animals

In the present study, we used male C57BL/6J mice and CCR2 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a room maintained at 23 ± 1°C with a 12 hr light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available *ad libitum.*

Drugs

Methamphetamine hydrochloride (Dainippon-sumitomo Pharmaceutical Co. Ltd, Osaka, Japan) was diluted with sterile physiological saline (Fuso Pharmaceutical Co. Ltd, Osaka, Japan) and injected subcutaneously (s.c.) in a fixed volume of 0.1 mL/10 g body weight.

Locomotor assay

The locomotor activity of mice was measured by an ambulometer (ANB-M20, O'Hara, Tokyo, Japan) as described previously [65]. Briefly, male mice were individually placed in a tilting-type round activity cage of 20 cm in diameter and 19 cm in height. Any slight tilt of the activity cage caused by horizontal movement of the animal was detected by microswitches. Total activity counts in each 20-min segment were automatically recorded for 120 min following the administration of methamphetamine. To induce behavioral sensitization to methamphetamine, mice were given 5 treatments

with methamphetamine (2 mg/kg, s.c.) once every 96 hr. Their activities were counted after every injection to confirm the development of methamphetamine-induced sensitization. To investigate the maintenance of this sensitization, mice were again administered methamphetamine (2 mg/kg, s.c.) after 7 weeks of withdrawal.

RNA preparation and quantitative analysis by reverse transcription-PCR

Total RNA in the limbic forebrain of methamphetamine-treated mice was extracted using the SV Total RNA Isolation system (Promega, Madison, WI) following the manufacturer's instructions. Purified total RNA was quantified spectrophotometrically at A_{260}. To prepare first-strand cDNA, 1 μg of RNA was incubated in 100 μL of buffer containing 10 mM dithiothreitol, 2.5 mM $MgCl_2$, dNTP mixture, 50 U of reverse transcriptase II (Invitrogen, Carlsbad, CA), and 0.1 mM oligo-$dT_{12\text{-}18}$ (Invitrogen). The genes were amplified in 50 μL of PCR solution containing 0.8 mM $MgCl_2$, dNTP mixture, and DNA polymerase with synthesized primers (Table 3). Samples were heated to 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The final incubation was at 72°C for 7 min. The mixture was run on 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. The agarose gel was stained with ethidium bromide and photographed with UV transillumination. The intensity of the bands was analyzed and semiquantified by computer-assisted densitometry using ImageJ software. Values represent the mean ± SEM of three independent experiments.

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed as described previously [79] with minor modifications. Briefly, mouse limbic forebrain tissue was dissected as described above and cross-linked, and then tissue was lysed. Fifteen μg of soluble chromatin was incubated with 2 μg of specific antibodies against acetylated histone H3 (Millipore), H3K4 trimethylation (Wako Pure Chemicals, Osaka, Japan), H3K9 trimethylation (Millipore), and H3K27 trimethylation (Millipore), overnight at 4°C. The immuno-complex was collected by Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), and DNA was recovered by treatment with RNaseA, and Proteinase K followed by isopropanol precipitation. Immunoprecipitated DNA was dissolved in 50 μL of 1 x TE and 1 μL was used for quantitative PCR. Quantitative PCR was performed as described previously [79]. The primers used are listed in Table 3.

Statistical analysis

The data are expressed as the mean ± S.E.M. The statistical significance of differences between groups was assessed with Student's *t*-test (for RT-PCR or ChIP), or a one-way ANOVA test (for each session of the locomotor assay) or a two-way repeated-measures ANOVA test (for the time-course of the locomotor assay) followed by the Bonferroni test. A level of probability of 0.05 or less was considered significant.

Results

Development of behavioral sensitization to methamphetamine-induced hyperlocomotion

Intermittent injection of methamphetamine produced a progressive increase in methamphetamine-induced locomotion, indicating the development of sensitization to methamphetamine (Figure 3-1-A, $F_{(4, 95)}=4.940$, $p<0.01$, 1st session vs. 5 th session).

Changes in the mRNA expression of inflammatory cytokines and chemokine

A significant increase in mRNA of CCR2, but not of IL-1β, IL-4, IL-6, IL-10, TNFα, TGFβ1, TGFβ2, TGFβ3, MCP-1, RANTES, MCP-3, CXCL12 or CXCR4, was observed in the limbic forebrain, mainly including the nucleus accumbens, of mice that had shown behavioral sensitization to methamphetamine (Figure 3-1-B, $p<0.01$ vs. saline-treated mice).

Histone modifications at the promoter regions of the CCR2 gene

Treatment with methamphetamine caused a significant increase in the level of H3K4 trimethylation at the CCR2 promoter in the mouse limbic forebrain (Figure 3-2-A, $P<0.01$ vs. saline-treated mice).

Development of behavioral sensitization to METH-induced hyperlocomotion in CCR2 KO mice

The 5th injection of methamphetamine produced a dramatic and significant increase in methamphetamine-induced hyperlocomotion compared to the 1st injection in both C57BL/6J (wild-type) and CCR2 gene knockout mice to the same degree (wild-type: 1 st vs. 5 th, $F_{(1, 160)} = 12.39$, $p<0.01$, CCR2 knockout: 1 st vs. 5 th, $F_{(1, 30)} = 20.00$, $p<0.01$), indicating that lack of the CCR2 gene had little or no effect on the development of sensitization to methamphetamine-induced hyperlocomotion. Intriguingly, the sensitization to methamphetamine was maintained even after 7 weeks of withdrawal following intermittent administration of methamphetamine in wild-type mice (5 th vs. withdrawal, $F_{(1, 110)}=0.05$, no significant). However, the methamphetamine-induced sensitization was almost reversed after 7 weeks of withdrawal in CCR2 knockout mice (5 th vs. withdrawal, $F_{(1, 30)}=8.50$, $p<0.05$, Fig. 2B).

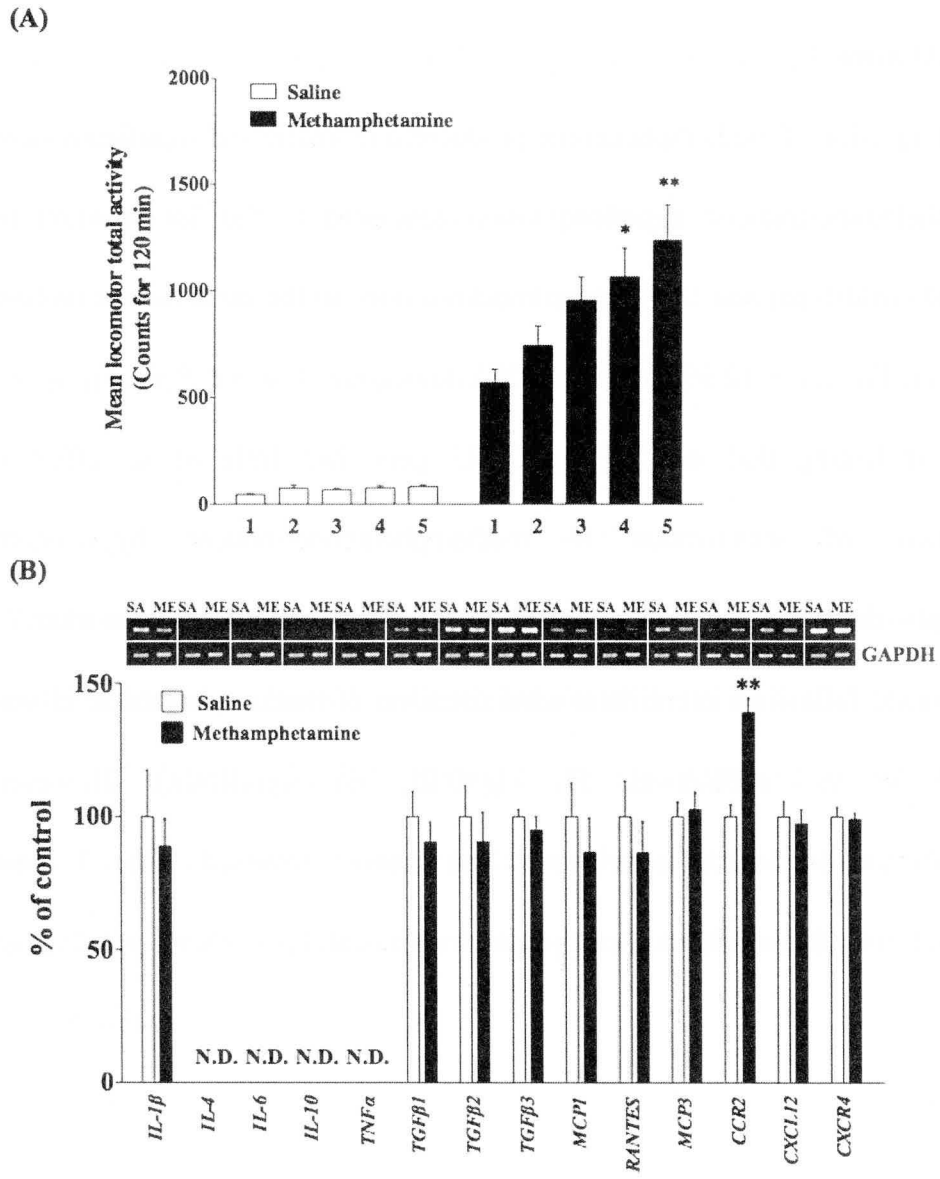


Figure 3-1. (A) Development of sensitization to methamphetamine in mice. Methamphetamine (2 mg/kg, s.c.) or saline was repeatedly given 5 times to mice every 96 hr. Total activity was counted for 120 min after each injection. Each column represents the mean total counts for 120 min with S.E.M. of 20 mice. (**P<0.01, ***P<0.001 vs. METH 1st trial). (B) Upper: Representative RT-PCR for IL1β, IL-4, IL-6, IL-10, TNFα, TGFβ1, TGFβ2, TGFβ3, MCP1, RANTES, MCP3, CCR2, CXCL12 and CXCR4 mRNAs in in the limbic forebrain of mice that have shown behavioral sensitization to methamphetamine (ME). The limbic forebrain sample was prepared 24 hr after the last injection of saline (SA) or ME. Lower: The intensity of the above bands was semi-quantified using Image J software. The value for mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for mice treated with methamphetamine is expressed as a percentage of the increase in mice treated with saline. Each column represents the mean ± S.E.M. (n=3 animals per group; three independent experiments). N.D.: not detectable. **p<0.01 vs. Saline-treated mice.

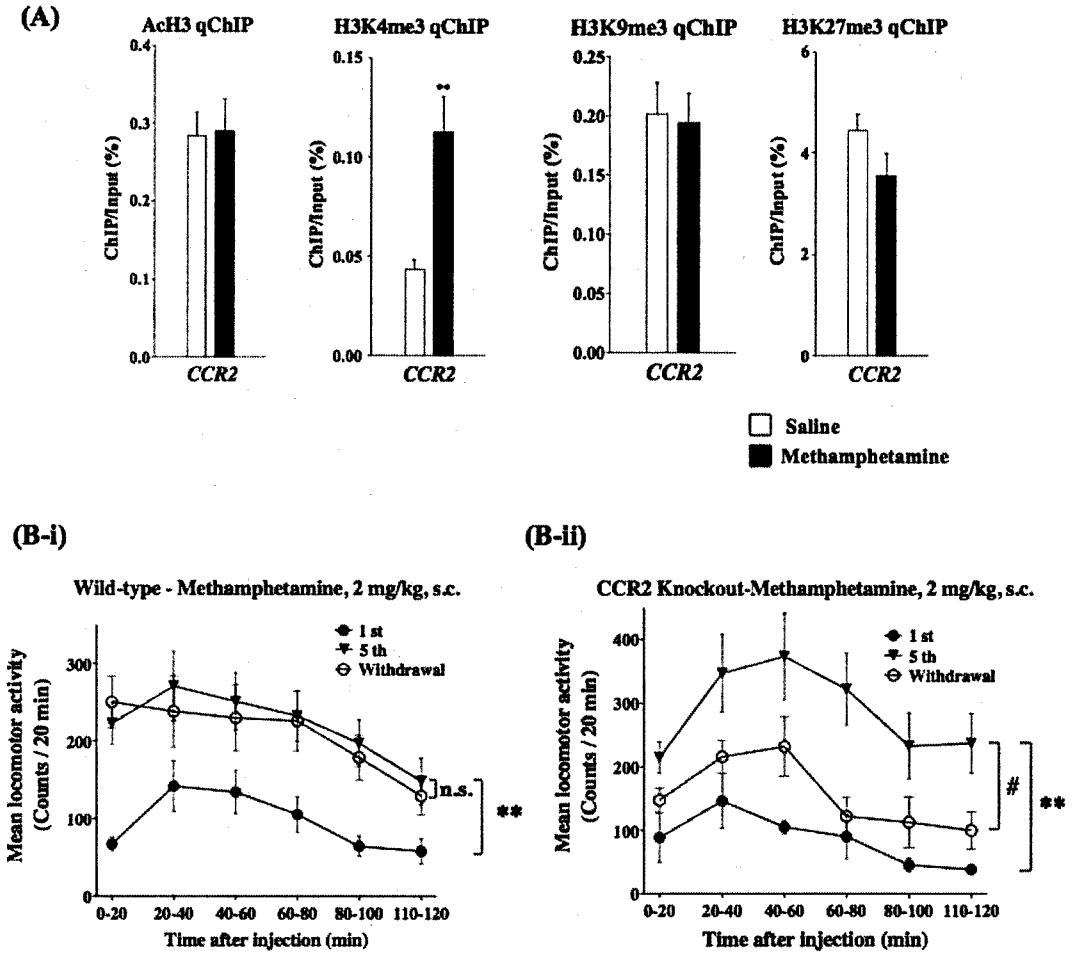


Figure 3-2. (A) qChIP analysis of acetylated histone H3 (AcH3), histone H3 trimethylated at lysine 4 (H3K4me3), lysine 9 (H3K9me3), and lysine 27 (H3K27me3) at CCR2 loci in the limbic forebrain of mice that had been intermittently treated with methamphetamine. Each column represents the mean ± S.E.M. (n=4 animals per group; three independent experiments). **p<0.01 vs. Saline-treated mice. (B) Change in locomotor activity (per 20 min time intervals) following intermittent administration of methamphetamine (2 mg/kg, s.c.) in wild-type mice (B-i) or CCR2 knockout mice (B-ii). Mice were treated intermittently with methamphetamine every 96 hr for 5 sessions. "1 st" represents the 1 st injection group, whereas 5 th shows the 5 th injection group. Mice described as "withdrawal" were again administered methamphetamine after 7 weeks of withdrawal. **p<0.01, 1 st vs. 5 th, #p<0.05, 5 th vs. withdrawal (two-way ANOVA). n.s. : not significant. Each point represents the mean ± S.E.M. (n= 4-17 mice).

Experiment	Name	Sequence	Product size (DNA Podition)
RT-PCR	IL-1β	F: CACTAGGTTTGCCGAGTAGATCTC R: GTGCTGCCTAATGTCCCCTTGAATC	388bp
	IL-4	F: CATCGGCATTTTGAACGAGGTCA R: CTTATCGATGAATCCAGGCATCG	239bp
	IL-6	F: ATGAAGTTCCTCTCTGCAAGAGACT R: CACTAGGTTTGCCGAGTAGATCTC	638bp
	IL-10	F: TACCTGGTAGAAGTGATGCC R: CATCATGTATGCTTCTATGC	251bp
	TNFα	F: ATGAGCACAGAAAGCATGATC R: ATGAGCACAGAAAGCATGATC	276bp
	TGFβ1	F: TACTATGCTAAAGAGGTCACCC R: TCCTTGGTTCAGCCACTGCC	327bp
	TGFβ2	F: TTTGCAGGTATTGATGGCACC R: ATCCATTTCCATCCAAGATCCC	257bp
	TGFβ3	F: AACTAGCTATCTCAGGTCCC R: ACTTCAGTCTGTGCATCTGG	318bp
	MCP1	F: AGCCAGATGCAGTTAACGC R: CTGATCTCATTTGGTTCCGA	221bp
	RANTES	F: GGTACCATGAAGATCTCTGCA R: AAACCCTCTATCCTAGCTCAT	293bp
	MCP-3	F: TCTGTGCCTGCTGCTCATAG R: CTTTGGAGTTGGGGTTTTCA	267bp
	CCR2	F: ATGTTACCTCAGTTCATCCACGGC R: GTAATGGTGATCATCTTGTTTGGA	587bp
	CXCL12	F: CACCTCGGTGTCCTCTTG R: GGTCAATGCACACTTGTCTG	280bp
	CXCR4	F: GTTCTGGAGACTATGACTCC R: CACAGATGTACCTGTCATCC	525bp
	GAPDH	F: CCCACGGCAAGTTCAACGG R: CTTTCCAGAGGGGCCATCCA	427bp
qChIP	CCR2	F: CAGACTGCTTGGTAGTAGGA R: GCATCCCTTAAGTGATAGCC	247bp (618 ~ 864)

Discussion

People take drugs of abuse to elevate their mood, but the repeated use of these drugs produces serious unwanted effects, including tolerance and sensitization, which are referred to as drug dependence. To date, several animal models have been created to investigate the mechanisms that underlie the behavioral sensitization to psychostimulants [31]. The repeated administration of psychostimulant drugs results in a progressive and enduring elevation in the motor response, which is accompanied by a long-lasting neural plasticity.

One important development in our understanding of the cellular and molecular processes that cause long-lasting changes in neuroplasticity concerns the role of the immune system, mainly driven by glial cells, in the CNS. Considerable evidence has recently demonstrated that glial cells such as astrocytes and microglia are key players in the modulation of brain functions. In the mammalian brain, astrocytes and microglia affect neuronal function through the release of neurotransmitters, neurotrophic factors, cytokines, chemokines, and extracellular matrix [24]. These glial cell-related soluble factors could amplify the development of the rewarding effect induced by methamphetamine in the limbic forebrain including the nucleus accumbens [65].

Recent studies have demonstrated that the chemokine monocyte chemoattractant protein-1 (MCP-1)/CCL2 and its receptor, CCR2, are responsible for various brain diseases. Taken together, these findings strongly suggest that CCR2-modulated signaling networks associated with the innate immune response in the limbic forebrain

including the nucleus accumbens may be responsible for the development of sensitization to methamphetamine-induced hyperlocomotion.

The key finding of the present study was that treatment with methamphetamine caused a significant increase in the level of H3K4 trimethylation at the CCR2 promoter in the limbic forebrain including the nucleus accumbens methamphetamine did not produce other histone modifications at the CCR2 gene promoter. To the best of our knowledge, the present data are the first to indicate that chronic treatment with METH induces a dramatic increase in the expression of the CCR2 gene along with epigenetic modifications in in the limbic forebrain including the nucleus accumbens.

In conclusion, the present study suggests that the intermittent administration of methamphetamine increases the mRNA level of CCR2 in association with epigenetic modification at its promoter in the limbic forebrain including the nucleus accumbens, and this may be responsible for the maintenance of sensitization to methamphetamine-induced hyperlocomotion.

General Conclusion

In Chapter 1:

In the present study, we investigated whether aging decreases neurogenesis accompanied by the activation of microglia and astrocytes, which increases the expression of IL-1β in the hippocampus, and whether *in vitro* treatment with IL-1β in neural stem cells directly impairs neurogenesis. The Iba1-positive microglia and GFAP-positive astrocytes were increased in the dentate gyrus of the hippocampus of 28-month-old mice. In contrast, the immunoreactivity for a marker for neuronal precursors doublecortin was almost completely absent from the dentate gyrus of the hippocampus of aged mice. Furthermore, the mRNA levels of doublecortin or another neural progenitor cell marker NeuroD were significantly decreased in the hippocampus of aged mice, whereas the mRNA levels of IL-1β were significantly increased without related histone modifications. Under these conditions, a significant decrease in H3K4 trimethylation and a significant increase in H3K27 trimethylation at doublecortin promoters were observed with aging without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. I also detected a significant increase in H3K9 trimethylation at the promoter of NeuroD in the hippocampus of aged mice. *In vitro* treatment with IL-1β in neural stem cells prepared from whole brain of E14.5 mice significantly increased H3K9 trimethylation at the NeuroD promoter. These findings suggest that aging may decrease hippocampal neurogenesis via epigenetic modifications accompanied by the activation of microglia

and astrocytes with the increased expression of IL-1β in the hippocampus.

In Chapter 2:

In the present study, I investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice. Exposure to an enriched environment for 3 to 4 weeks caused a dramatic increase in the mRNA expression of BDNF, but not PDGF-A, PDGF-B, VEGF, NGF, EGF or GFAP, in the hippocampus of mice. Under these conditions, exposure to an enriched environment induced a significant increase in H3K4 trimethylation at the BDNF P3 and P6 promoters, in contrast to significant decreases in H3K9 trimethylation of at the BDNF P4 promoter and H3K27 trimethylation at the BDNF P3 and P4 promoters without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. The expression levels of several microRNAs in the hippocampus were not changed by an enriched environment. These results suggest that an enriched environment increases BDNF mRNA expression via sustained epigenetic modification in the mouse hippocampus.

In Chapter 3:

In the present study, I found that repeated injection of methamphetamine produced a progressive increase in the locomotor-enhancing effect of methamphetamine, which confirms the development of sensitization to methamphetamine-induced hyperlocomotion. Under these conditions, RT-PCR analysis indicated that among the 14 genes annotated in the mouse genome, mRNA levels of CCR2 were significantly increased the limbic forebrain including the nucleus accumbens of mice that had been treated with methamphetamine. Rrepeated treatment with methamphetamine caused a significantly increase in the level of H3K4 trimethylation at the CCR2 promoter in the mouse the limbic forebrain including the nucleus accumbens. These findings suggest that increased CCR2 associated with epigenetic modification at the CCR2 promoter the limbic forebrain including the nucleus accumbens after intermittent administration of methamphetamine may be, at least in part, responsible for the development of sensitization to methamphetamine-induced hyperlocomotion.

Conclusive remarks:

There is now growing evidence that epigenetic mechanisms, such as histone acetylation and methylation, are involved in the regulation of the saliency of environmental stimuli or in severe diseases. Epigenetic mechanisms are attractive candidates for molecular substrates that mediate long-lived changes in brain, such as normal aging, enriched environment and drug addiction. However, it has yet to be determined whether similar long-lasting changes in chromatin re-structure are involved

in switching off the cell damage accompanied by normal aging and drug addiction. If this is the case, the ability to reverse the epigenetic signature of aging or addicted state would offer a fundamentally new approach for more effective treatments of progressive aging or drug relapse.

List of Publications

This dissertation is based on the following original publications:

1. Naoko Kuzumaki, Daigo Ikegami, Rie Tamura, Takuya Sasaki, Keiichi Niikura, Michiko Narita, Kazuhiko Miyashita, Satoshi Imai, Hideyuki Takeshima, Takayuki Ando, Katsuhide Igarashi, Jun Kanno, Toshikazu Ushijima, Tsutomu Suzuki and Minoru Narita: Hippocampal epigenetic modification at the doublecortin gene is involved in the impairment of neurogenesis with aging. *Synapse* (*in press*): **Chapter 1**

2. Naoko Kuzumaki, Daigo Ikegami, Satoshi Imai, Michiko Narita, Rie Tamura, Marie Yajima, Atsuo Suzuki, Kazuhiko Miyashita, Hideyuki Takeshima, Takayuki Ando, Toshikazu Ushijima, Tsutomu Suzuki and Minoru Narita: Enhanced IL-1β production in response to the activation of hippocampal glial cells impairs neurogenesis in aged mice. *Synapse* (*in press*): **Chapter 1**

3. Naoko Kuzumaki, Daigo Ikegami, Rie Tamura, Nana Hareyama, Satoshi Imai, Michiko Narita, Kazuhiro Torigoe, Keiichi Niikura, Hideyuki Takeshima, Takayuki Ando, Katsuhide Igarashi, Jun Kanno, Toshikazu Ushijima, Tsutomu Suzuki and Minoru Narita: Hippocampal epigenetic modification at the brain-derived neurotrophic factor gene induced by an enriched environment. *Hippocampus* (*in press*): **Chapter 2**

4. Daigo Ikegami, Minoru Narita, Satoshi Imai, Kazuhiko Miyashita, Rie Tamura, Michiko Narita, Shigemi Takagi, Akiko Yokomizo, Hideyuki Takeshima, Takayuki Ando, Katsuhide Igarashi, Jun Kanno, Naoko Kuzumaki, Toshikazu Ushijima and Tsutomu Suzuki: Epigenetic modulation at the CCR2 gene correlates with the maintenance of behavioral sensitization to methamphetamine. *Addiction Biology* (*in press*): **Chapter 3**

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