| Title          | Maternal Separation Enhances Conditioned Fear and Decreases the mRNA Levels of the Neurotensin Receptor 1 Gene with Hypermethylation of This Gene in the Rat Amygdala |
|---------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Author(s)     | Toda, Hiroyuki; Boku, Shuken; Nakagawa, Shin; Inoue, Takeshi; Kato, Akiko; Takamura, Naoki; Song, Ning; Nibuya, Masashi; Koyama, Tsukasa; Kusumi, Ichiro |
| Citation      | PLOS One, 9(5), e97421                                                                                                                |
| Doc URL       | http://hdl.handle.net/2115/57877                                                                                                     |
| Rights URL    | http://creativecommons.org/licenses/by/3.0/                                                                                           |
| Type          | article                                                                                                                              |
| File Information | PLoS One_9(5)_e97421.pdf                                                                                                             |

Hokkaido University Collection of Scholarly and Academic Papers: HUSCAP
Maternal Separation Enhances Conditioned Fear and Decreases the mRNA Levels of the Neurotensin Receptor 1 Gene with Hypermethylation of This Gene in the Rat Amygdala

Hiroyuki Toda1,2, Shuken Boku3, Shin Nakagawa2*, Takeshi Inoue2, Akiko Kato2, Naoki Takamura4, Ning Song2,5, Masashi Nibuya1, Tsukasa Koyama2, Ichiro Kusumi2

1 Department of Psychiatry, National Defense Medical College, Tokorozawa, Japan, 2 Department of Psychiatry, Hokkaido University Graduate School of Medicine, Sapporo, Japan, 3 Department of Psychiatry and Behavioral Sciences, Albert Einstein College of Medicine, Bronx, New York, United States of America, 4 Pharmaceutical Laboratories, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan, 5 Department of Psychiatry, The first hospital of China Medical University, Shenyang, China

Abstract

Stress during postnatal development is associated with an increased risk for depression, anxiety disorders, and substance abuse later in life, almost as if mental illness is able to be programmed by early life stressors. Recent studies suggest that such “programmed” effects can be caused by epigenetic regulation. With respect to conditioned fear, previous studies have indicated that early life stress influences its development in adulthood, whereas no potential role of epigenetic regulation has been reported. Neurotensin (NTS) is an endogenous neuropeptide that has receptors densely located in the amygdala and hippocampus. Recently, NTS systems have constituted an emerging target for the treatment of anxiety. The aim of the present work is to clarify whether the NTS system is involved in the disturbance of conditioned fear in rats stressed by maternal separation (MS). The results showed that MS enhanced freezing behaviors in fear-conditioned stress and reduced the gene expression of NTS receptor (NTSR) 1 but not of NTS or NTSR2 in the amygdalas of adult rats. The microinjection of a NTSR1 antagonist into the amygdala increased the percentage of freezing in conditioned fear, whereas the microinjection of NTSR1 agonist decreased freezing. These results suggest that NTSR1 in the amygdala may play a role in the effects of MS on conditioned fear stress in adult rats. Moreover, MS increased DNA methylation in the promoter region of NTSR1 in the amygdala. Taken together, MS may lead epigenetic marks in the NTSR1 gene in the amygdala, which may enhance conditioned fear in adulthood. The MS-induced alternations of DNA methylation in the promoter region of NTSR1 in the amygdala may be associated with vulnerability to the development of anxiety disorders and depression in adulthood.

Citation: Toda H, Boku S, Nakagawa S, Inoue T, Kato A, et al. (2014) Maternal Separation Enhances Conditioned Fear and Decreases the mRNA Levels of the Neurotensin Receptor 1 Gene with Hypermethylation of This Gene in the Rat Amygdala. PLoS ONE 9(5): e97421. doi:10.1371/journal.pone.0097421

Editor: Judith Homberg, Radboud University, Netherlands

Received February 10, 2014; Accepted April 17, 2014; Published May 15, 2014

Copyright: © 2014 Toda et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by JSPS KAKENHI Grant Numbers 18591269 (Shin Nakagawa) and 24791248 (Hiroyuki Toda). http://kaken.nii.ac.jp/d/60360905/ja.html http://research-ejr.jp/researchers/view/00610677/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Hiroyuki Toda has received honoraria from GlaxoSmithKline, Pfizer, Astellas, Eli Lilly, and Otsuka Pharmaceutical. Shin Nakagawa has received honoraria from GlaxoSmithKline, Eisai, Pfizer, Daiichi-Sankyo, Meiji Seika Pharma, and Otsuka Pharmaceutical. Naoki Takamura has received honoraria from GlaxoSmithKline, Pfizer, Astellas, Eli Lilly, Mitsubishi Tanabe Pharma, and Meiji Seika Pharma. Takeshi Inoue has received honoraria from GlaxoSmithKline, Pfizer, Astellas, Eli Lilly, Mitsubishi Tanabe Pharma, and Otsuka Pharmaceutical. Masashi Nibuya has received honoraria from Dainippon Sumitomo Pharma and Astellas. Takeshi Inoue has received research/grant support from Pfizer, Eli Lilly, Eisai, and Ono Pharmaceutical. Ning Song has received research/grant support from GlaxoSmithKline and Mitsubishi Tanabe Pharma. Tsukasa Koyama has received research grants from GlaxoSmithKline, Eli Lilly, and Pfizer. Ichiro Kusumi has received research grants from Eli Lilly and Mitsubishi Tanabe Pharma. Dr. Ichiro Kusumi has received honoraria from Eli Lilly and Mitsubishi Tanabe Pharma. Tsukasa Koyama has received research grants from GlaxoSmithKline and Mitsubishi Tanabe Pharma. Dr. Ichiro Kusumi has received research grants from Eli Lilly and Dainippon Sumitomo Pharma and Mitsubishi Tanabe Pharma. Shuken Boku and Akiko Kato and Ning Song and Masashi Nibuya reported no conflicts of interest in this work. Naoki Takamura is employed by Dainippon Sumitomo Pharma. This does not alter the authors’ adherence to PLOS ONE policies on sharing data and materials.

* E-mail: snakaga@med.hokudai.ac.jp

Introduction

Past clinical studies have shown that exposure to stress during the postnatal development periods is associated with an increased risk for depression, anxiety disorders, and substance abuse later in life [1,2]. Similar to humans, other mammals suffering from early life stress (ELS) in the postnatal period have a vulnerability towards anxiety states and depression-like syndromes [3–7]. These findings suggest that variations in one’s early environment may be associated with changes in gene expression and biological function that persist into adulthood. Such “programmed” effects may derive from epigenetic regulation, which causes structural alterations in genomic DNA [4,8].

The conditioned fear stress (CFS) paradigm is based on Pavlovian aversive conditioning. An emotionally neutral stimulus (e.g., a tone, figure, light, or context) is paired with an emotionally potent and innately aversive unconditioned stimulus (e.g., an electric shock) during a conditioning phase. The assessment of
conditioning then involves measuring a conditioned response elicited by the neutral stimulus. CFS is regarded as a psychological stress without physical stimuli and as a simple animal model of anxiety or fear [9–12]. With respect to conditioned fear, previous studies have indicated that ELS continues its influence into adulthood [13,14]; however, no potential role of epigenetic regulation in fear learning and memory caused by ELS has been reported.

Our previous study using a DNA microarray showed that neurotransmitter (NTS) is the only gene that had its expression changed by CFS. This change in NTS can be overcome by a selective serotonin reuptake inhibitor treatment, which is also effective for the treatment of various anxiety disorders [15]. NTS is an endogenous neuropeptide that closely interacts with monoamine neurotransmitter systems [16], and NTS receptor (NTSR) 1 and 2 are densely located in structures that are important for anxiety, including the amygdala (AMY) and hippocampus (HIP) [17]. The systemic administration of a NTSR1 agonist significantly decreased conditioned footshock-induced ultrasonic vocalization in rats [18], even while decreasing fear-potentiated startling in rats [19]. Moreover, NTSR1 knockout mice showed higher freezing rates than wild-type mice in contextual fear stress situations [11]. However, a role for NTS in conditioned fear induced by ELS has not been documented.

The aim of the present work is to clarify whether the NTS system is involved in the disturbance of conditioned fear in rats stressed by maternal separation (MS), which is one of the most commonly used procedures for inducing ELS in rodents. Our results showed that the MS and NTSR1 antagonist enhanced freezing behaviors in conditioned fear stress. In addition, MS reduced NTSR1 gene expression and increased DNA methylation in the promoter region of AMY.

Materials and Methods

Animals

Adult male Sprague–Dawley (SD) rats, each weighing 230–270 g, were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were housed in groups of four. Timed pregnant SD rats (Shizuoka Laboratory Animal Center) were delivered on gestation day 14 and were single-housed. All rats were housed in standard animal cages and received ad libitum access to food and water in a temperature-controlled environment (22±1°C) on a 12 h light/dark cycle (light phase: 0600–1800). All procedures were approved by the Hokkaido University Graduate School of Medicine Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals, Hokkaido University Graduate School of Medicine.

MS procedure

MS was conducted according to the method of Plotsky and Meaney [20]. When a litter was born between 0900 and 1700, its day of birth was designated as postnatal day (PND) 0. When a litter was born between 1700 and 0900, its day of birth was designated as PND 1. After the pups were cross-fostered to minimize litter differences, eight males and two females were selected on PND 2. Each litter was assigned for either MS or the control group. Handling and separation were performed from 0930–1230 daily from PND 2 to PND 14. In the MS group, a dam was removed from the cage and placed into an individual cage during the separation period. The pups were then removed from the cage and placed into a clean plastic cage lined with wood-chip bedding. Then, they were taken to another room and placed in an incubator set to maintain an ambient temperature at 27–30°C for 3 h. At the end of the separation period, the pups were returned to the cage, and then, the dam was returned. Pups in the MS group were permitted to huddle with their littermates during the separation period. AFR rats were briefly handled once a week by an animal care technician. Only male pups were used for the present study. Behavioral, molecular, and neurochemical experiments were performed during the postadolescent period (10–14 weeks old).

Dexamethasone/corticotropin releasing hormone test

The dexamethasone (DEX)/corticotropin releasing hormone (CRH) test was conducted according to the method described by Hatzinger et al. [21]. Surgery was performed under sodium pentobarbital (40 mg/kg, i.p.) anesthesia using aseptic procedures. Rats were chronically catheterized in the jugular vein for subsequent blood sampling six days before the experiment. The catheter was exteriorized at the neck of the animal and filled with sterile saline containing gentamicin (50,000 IU/rat); 0.2 ml was infused into the animal. Rats were weighed at 0700 on the day of the experiment. The jugular venous catheter was connected via free-moving devices (Eicom, Kyoto, Japan) to a plastic syringe filled with sterile heparinized saline (50 IU/ml) at 0800. DEX (Sigma, St. Louis, MO) (30 μg/kg, 0.5 ml/kg) was administered intravenously (i.v.) at 1200. Numerous 0.2-ml blood samples were collected at 1800, 1830, 1900 and 1930 to monitor the effects of the DEX treatment on the basal plasma concentrations of corticosterone during the diurnal acrophase. At 1931, human–CRH (kindly donated from Yoshitomi Pharma, Osaka, Japan) (50 ng/kg, 0.5 ml/kg i.v.) was injected. To assess the CRH-stimulated corticosterone secretion, further blood samples were taken at 1940, 2000, 2020, and 2040. Corticosterone was measured by an enzyme immunoassay using a corticosterone ELISA kit (Cayman, Ann Arbor, MI).

Open field test

The open field box (90-cm length × 90-cm width × 40-cm height) was divided into 81 squares of equal size by stripes of black paint, and the lighting at the center of the area was set to 200 lx. The arena was divided into two sections, and the outer edge of the 20-cm width was measured from the walls (outer zone) and the center square (inner zone). Rats were placed singly in the center of the field, and their behavior was recorded for 30 min. The total distance travelled and time spent in the inner zone were assessed using tracking software (LimeLight; Actimetrics, Wilmette, IL). Total movement in the field reflected their general activity, and relative movement in the inner zone was correlated with the anxious state of the rat. All tests were performed between 1400 and 1600 to minimize Circadian influences.

Elevated plus maze

The apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) surrounded by 40 cm high side walls that extended from the central platform (10 × 10 cm). The maze was elevated 50 cm above the floor, and the lighting at the center of the area was set to 200 lx. Rats were placed singly on the central platform facing an open arm, and their behavior was recorded for 5 min. The total distance moved in the maze and the number of entries for each arm were assessed using LimeLight (Actimetrics). The total distance moved in the maze was measured as the locomotor activity. The number of entries in the open arms was measured as memory-independent fear because rats innately avoid open spaces [22]. All tests were performed between 1400 and 1600 to minimize Circadian influences.
Footshock sensitivity test
The effects of MS on footshock-induced pain were examined. Four behaviors, including vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw and jumping, were used as indicators of nociception. Generally, each was used as an endpoint in the hot plate procedure [23]. The rats were individually placed in a shock chamber with a grid floor (19 × 22 × 20 cm). After a 5-min adaptation period, the rats were subjected to 15 series of scrambled electric footshocks. Each series had 10-s and 40-s intervals and ranged from 0.4 mA to 3.2 mA in 0.2 mA steps, which are presented in ascending order. The responses of the rats to each shock were recorded, and the minimal intensities of the electric footshocks at which each of the four behaviors appeared were determined.

Condition fear stress paradigm
Rats individually underwent inescapable electric footshocks for a total of 5 min in a shock chamber with a grid floor (30 × 24 × 30 cm, Med Associates Inc., USA) [24]. Five footshocks (2.0 mA scrambled shock, each of 30 s duration) were delivered at shock intervals of 30 s using an ENV-410 shock generator (Med Associates Inc., USA). Twenty-four hours later, the rats were exposed to the chamber without footshocks to assess their contextual fear memory for 5 min (first exposure), as measured by their freezing behaviors. On the next day, rats were exposed to the chamber for 5 min again (second exposure). During the observation period, the duration of the freezing behavior was recorded, as previously described [24]. Freezing was defined as the absence of all observable movements of the skeleton and the vibrissae, except for movements related to respiration. Rats were classified as either freezing or active according to their behavior during a 5-s period. The percentage score of freezing represents the number of 5-s periods during which the rats froze for 5 s.

Quantitative RT-PCR Analysis
Rats were deeply anesthetized with sodium pentobarbital and decapitated. The brains were removed and washed with ice-cold phosphate-buffered saline (PBS; pH 7.4). Coronal sections with a thickness of 1 mm were cut using a Brain Slicer (Muromachi, Tokyo, Japan) and immersed into dishes containing ice-cold PBS. The regions containing AMY and HIP were dissected carefully with a blade. Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) from these regions. Total RNA was converted to cDNA with the Quantitect Reverse transcription kit (Qiagen). Quantitative PCR was performed with the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in the ABI PRISM 7600 Sequence Detection System (Applied Biosystems, Foster, CA). All standards and unknown samples were assayed in triplicate. The conditions of PCR were the following: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of forward and reverse primers used were as follows: AGC TGG TCA TCA ATG GGA AA and ATT TGA TGT TAG CGG GAT CG for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GCT GAG GGT ATT CCA ACT CG and CAT TGC CAT GAT CGA GGA TA for NTS, AAG CAG GCA CCC TTC ATC T and GGA GGC TGG ATG GTT CTG T for NTSR, CCT GGT GAG ACA CAA GGA TG and ACG ATG GCT CTG AGA AAA CCT G for NTSR2. GAPDH was used as a control. PCR reaction assays for unknown samples were performed simultaneously with the same standard samples (cDNA derived from rat HIP) to construct a standard curve. The relative concentrations of GAPDH and of NTS, NTSR1 or NTSR2 in unknown samples were calculated from this standard curve, and we calculated the ratio of the relative concentrations of NTS, NTSR1 or NTSR2 (already normalized by GAPDH expression) to the relative concentration of GAPDH.

Analysis of DNA methylation in the promoter of NTSR1
Genome DNA of MS and AFR rats were isolated from AMY with a DNeasy Blood & Tissue Kit (Qiagen). A MethylCollector™ Ultra Kit (Active Motif, Carlsbad, CA) was used to enrich CpG methylated DNA fragments. The method was based on the Methylated CpG Island Recovery Assay (MIRA), which utilizes the high affinity of the MBDBh/MBD3L1 complex for methylated DNA. Genomic DNA was digested with Bfa I (New England Biolabs, Ipswich, MA) because the promoter region of NTSR1 contains the recognition site of Bfa I. The MBDBh/MBD3L1 protein-DNA complex was added to the DNA fragments, specifically binding to CpG-methylated DNA. These protein-DNA complexes were then captured with nickel-coated magnetic beads, and subsequent wash steps were performed to remove fragments with little or no methylation (unbound fragment; UF). The methylated DNA was then eluted from the beads in the presence of Proteinase K (eluted fragment; EF). Both UF and EF were purified using a MinElute PCR purification kit (Qiagen). PCR primers were designed to target the three DNA fragments (fragment A, B and C) within the CpG islands, which contain the promoter region of NTSR1 gene. The fragments were located −969 to −674 (fragment A), −673 to −283 (fragment B) and −273 to +263 (fragment C) from the first translational ATG codon. Quantitative PCR was performed with the SYBR GreenER qPCR SuperMix in the ABI PRISM 7600 Sequence Detection System. All samples were assayed in triplicate. The percentage enrichment, which represented the degree of methylation of each fragment, was calculated as follows: enrichment (%) = 2(CT (EF) − CT (UF))/1 + 2(CT (EF) − CT (EF)) × 100. The conditions for the PCR were the following: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used were as follows: CCG AGC CAG CTG TAC AAA G and GGC AGC ACA ATC TTC TGC TT for NTSR1-fragment A, CAA GGA GAA GAG GGA GAA CG and TGC TAC GGA CCT CCA GAT TC for NTSR1-fragment B, AAG AAG AGT GGA TCC CTG AGC and TAT GCT GCT TGT TCC TGC AC for NTSR1-fragment C.

Microinjections
Surgery was performed using sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally) under aseptic conditions. The head position was adjusted to place the bregma and lambda on the same horizontal plane in a stereotaxic frame. Naïve rats were implanted with a bilateral 26-gauge guide cannula (Plastics One, Roanoke, VA) directed toward AMY (immediately the central nucleus of AMY). The coordinates were −2.5 mm posterior to the bregma, 4.7 mm bilateral to the midline, and 7.2 mm ventral from the surface of the skull. Dummy stylets were inserted into guided cannulae, and the rats were allowed a 10–12-day post-recovery period. After surgery, the rats were housed individually. During the recovery period, the rats were handled 4 times before the microinjection procedure. PD149163, a specific agonist of NTSR1, was kindly donated by the NIMH Chemical Synthesis and Drug Supply Program (Washington D.C.) and SRI International (Menlo Park, California), and SR146992 was purchased from Tocris Biosciens (Bristol, UK). PD149163 was prepared in 0.9% physiologic saline as a 1 mM stock solution, and SR146992 was prepared in DMSO as 1 mM stock solution. They were stored at −20°C. Twenty-four hours, after fear conditioning, rats were injected bilaterally into AMY with 0.5 μl/side of saline or DMSO.
PD149163 (10, 100, or 1000 nM) or SR48692 (10, 100, or 1000 nM). After the removal of the dummy stylets, bilateral infusions were given simultaneously for 30 sec using 33-gauge stainless steel cannulae projecting 1.0 mm beyond the tips of the guide cannulae. Each injector was connected by polyethylene tubing to a 100-μl syringe that was driven at a rate of 1 μl/min by an infusion pump. Injectors remained in place for an additional 1 min to confine the drug to the target site. Ten minutes after the infusion, the rats were again placed in the shock chamber and observed for 5 min without shocks to assess their contextual fear.

Figure 1. Effect of maternal separation (MS) on body weight, hypothalamic-pituitary-adrenocortical system and anxiety behaviors. 

A, Body weight change of rats in MS and animal facility rearing (AFR) groups (MS, n = 22; AFR, n = 17). B, Plasma corticosterone concentrations of MS (n = 8) and AFR (n = 6) rats between 1800 and 2040 h on the dexamethasone/corticotropin-releasing hormone (DEX/CRH) test. All rats were pretreated with DEX (30 μg/kg i.v.) at 1200 h and administered with CRH (500 ng/kg i.v.) at 1931 h. MS rats showed significant increases in plasma corticosterone levels (#p < 0.05 vs AFR rats, *p < 0.05, **p < 0.01 vs baseline). C, Open field test. Total distance and % time in center during the 30 min test were analyzed. D, Elevated plus maze test. Total distance and % number of open arm entries during the 5 min test were analyzed. Error bars represent SEM. doi:10.1371/journal.pone.0097421.g001

MS Enhances Conditioned Fear and Decreases Methylation of NTSR1

Figure 2. Effect of MS on freezing behavior in conditioned fear stress (CFS). A, Sensitivity to pain induced by electrical stimulation. The minimal levels of the current required to elicit the stereotypical response of vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw and jumping were determined. Data are represented as pain thresholds (mA) (n = 8 per each group). B, Freezing rates of the MS and the AFR rats in the first exposure of 24 h after and the second exposure of 48 h after the fear conditioning by a 2.0 mA footshock. Two-way repeated ANOVA revealed significant main effects of the group (*p < 0.05) and session (**p < 0.01) but no significant interactions between the group and session (n = 10–12 per group). doi:10.1371/journal.pone.0097421.g002
decreased in AMY compared to AFR rats (n = 7 – 9 per group; *p < 0.05). Error bars represent SEM.

**MS Enhances Conditioned Fear and Decreases Methylation of NTSR1**

Figure 3. Effect of MS on the mRNA expression of neurotensin receptor 1 (NTSR1). A. Dissected regions of the amygdala (AMY) and hippocampus (HIP) (heavy black line) in coronal sections with a thickness of 1 mm from the rat brain atlas of Paxinos and Watson [69] (2.56 and 3.30 mm posterior to bregma). B. Comparative mRNA expression levels of neurotensin, NTSR1 and NTSR2 in the AMY and HIP of MS or AFR rats. NTSR1 mRNA levels in MS rats were significantly decreased in AMY compared to AFR rats (n = 7 – 9 per group; *p < 0.05, **p < 0.01). Error bars represent SEM. doi:10.1371/journal.pone.0097421.g003

MS affected the hypothalamic-pituitary-adrenocortical (HPA) system but not body weight or anxiety behaviors

No significant difference was found between the AFR and MS groups in body weight at 14 PND, at which point the maternal separation procedure had finished (Fig. 1A). A two-way repeated measure ANOVA revealed that MS had no significant main effect of the group (F(1, 62.5) = 0.010, p > 0.05, Fig. 1A) and no interaction (F(1, 41.7) = 1.758, p > 0.05, Fig. 1A). The effect of MS on plasma corticosterone levels was examined in the DEX/CRH test (Fig. 1B). A two-way repeated measure ANOVA revealed a significant main effect of group (F(1, 41) = 5.389, p < 0.05) and time (F(7, 286) = 15.75, p < 0.001) but no significant interaction between group and time. The release of corticosterone stimulated by CRH was significantly greater in MS rats than in AFR rats, as was reflected by the higher AUC values (MS: 14650 ± 2584; AFR: 8032 ± 1372; p < 0.05). Tukey’s post hoc test revealed that the maximum rise of corticosterone was significantly greater in MS over AFR rats (p < 0.05). Basal corticosterone levels between 1800 and 1930 h did not differ between the MS and AFR rats.

We examined the effects of MS on memory-independent anxiety behaviors in the open field test and elevated plus maze test. In the open field test (Fig. 1C), no significant difference was found between the two groups in the total distance moved in the field or in the % time spent in the center field. In the elevated plus maze (EPM) test (Fig. 1D), no significant difference was found between the two groups in the total distance moved in the maze or in the % of entries into the open arms.

MS enhanced freezing behavior in CFS

Figure 2A shows the minimal intensities of the electric footshocks at which pain-related behaviors first appeared. There were no significant differences between the two groups in vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw or jumping. These data indicate that the sensitivity to the footshock was not different between the AFR and MS groups.

Next, we examined memory-dependent behaviors in CFS (Fig. 2B). Twenty-four hours after the conditioning session for the 2.0 mA footshock, a two-way repeated ANOVA revealed a significant main effect for the group (F(2, 19) = 37.17, p < 0.05) and no significant interaction between the group and session.

MS decreased the mRNA expression of NTSR1 in AMY

AMY and HIP play a central role in fear learning and memory [25,29]. Previous findings have suggested that the NTS system is involved in fear memory processes [18,29,30]. Therefore, we examined the effects of MS on the mRNA expressions of NTS, NTSR1 and NTSR2 in AMY and HIP with quantitative RT-PCR. Figure 3A indicates the dissecting regions as HIP (upper coronal section) and AMY (lower coronal section). The bilateral AMY and HIP of adult rats were dissected, as indicated with heavy black lines. Figure 3B shows the mRNA expression levels of NTS, NTSR1 and NTSR2 in AMY and HIP of MS or AFR rats. In AMY, a significant decrease in mRNA expression of NTSR1 was observed in MS rats (p < 0.05), but no significant differences

memory (first exposure). On the next day, the rats were exposed to the chamber for 5 min again (second exposure). The 0.5-μl injection volume was based on a previous study [25]. The injection was aimed at the central nucleus because this area contains many NTS-immunoreactive neurons [26]. However, the entire region of AMY might be influenced by these drugs, as it has been reported that a volume of 0.5 μl is likely to infiltrate the other areas of AMY [27]. Injection placements were verified immediately after the second exposure session, as described previously [25]. Only the data from animals with the correct placement of the cannulae were analyzed.

Statistics

Data were analyzed using SPSS Statistics version 21 (SPSS, Chicago, IL). Numerical data were analyzed by an unpaired t-test, two-way repeated ANOVA or one-way ANOVA, followed by Tukey’s post hoc test. In all cases, the significance level was set at p < 0.05. All data are shown as the means ± S.E.M.
were found between the two groups in the mRNA expressions of NTS or NTSR2. In HIP, no significant differences were found between the two groups in mRNA expressions.

Microinjections of NTSR1 antagonist or agonist in AMY showed opposite effects on freezing behaviors in CFS

To determine whether NTSR1 in AMY is critically involved in CFS, we examined the effects of microinjections of an antagonist or agonist of NTSR1 into AMY of naïve rats on CFS. SR48692, which has a higher affinity for NTSR1 compared to NTSR2 [31], was used as an antagonist of NTSR1. PD149163, which selectively binds to the NTSR1 with no affinity for NTSR2 [32], was used as an agonist of NTSR1. CFS and microinjections of SR48692 or PD149163 were applied, as shown in Fig. 4A. The cannula placements for rats injected with SR48692 and PD149163 into AMY are shown in Figures 4B and 4D, respectively. Tissue damage was not apparent in either of the treatment groups or in the saline groups.

Figure 4C shows the freezing rate (% the DMSO group) of rats with the bilateral injections of saline or SR48692 (10 nM – 1000 nM) into AMY. One-way ANOVA indicated the significant effects of drug treatment at the first exposure ($F_{3, 27} = 3.26, p < 0.05$) and at the second exposure ($F_{3, 27} = 3.19, p < 0.05$). Tukey’s post hoc test showed that all drug treatment groups were not significantly higher than the DMSO group at the first exposure ($p > 0.05$), but the freezing rate at a dose of 1000 nM was significantly higher than that of the DMSO group at the second exposure ($p < 0.05$). Figure 4E shows the freezing rate (% the saline group) of the rats with bilateral microinjections of saline or PD149163 (10 μM – 1000 μM) into AMY. One-way ANOVA indicated no significant effect of drug treatment in the first exposure ($F_{3, 25} = 0.104, p > 0.05$) but a significant effect in the second exposure ($F_{3, 25} = 5.45, p < 0.05$). Tukey’s post hoc test showed that the freezing rate at a dose of 100 μM was significantly lower than that with saline at the second exposure ($p < 0.05$). These data indicate that the microinjection of a NTSR1 agonist (PD149163) into AMY disrupted the conditioned freezing and that the microinjection of NTSR1 antagonist (SR48692) into AMY potentiated it.

MS increased DNA methylation in the promoter region of the NTSR1 gene in AMY

MS decreased the expression of NTSR1 mRNA in adulthood (Fig. 3B). Such long-lasting effects are often mediated by epigenetic
Figure 5A illustrates a diagram of the NTSR1 gene and the sites of CpG islands. Figure 5B shows nucleotide sequences of the promoter region of NTSR1 and the potential binding sites for following transcription factors: GATA, activating protein 2 (AP2), specificity protein 1 (SP1), CAAT box, cAMP response elements (CRE), and nuclear factor-interleukin 6 (NF IL6) [34]. The coding sequence of the first exon is indicated by an asterisk. A large G+C-rich domain with characteristics in common with a CpG island (64.5% over 700 nt: −648 to 52) extended from the first translation ATG codon (+1) in the promoter region and into part of the coding region of the NTSR1 gene. The promoter region between −662 and −470 from the first translation codon contained strong positive regulatory elements that drove the expression of NTSR1 gene [34]. This region lacked a typical TATA or CAAT box but contained a CRE-like half site and putative Sp1 binding sites, suggesting the possible involvement of these factors in the positive regulation of NTSR1 expression. The locations of the fragments A, B and C are indicated in figures 5A and 5B. The underlined sequences in figure 5B show the position of the primers of fragment A, B and C. The % enrichment represents the degree of methylation of each fragment. The analysis of real time PCR revealed that the % enrichment of the fragments B and C was significantly increased in the MS group compared with the AFR group (unpaired t-test; p<0.01 and p<0.05, respectively) (Fig. 5C).

**Discussion**

We have shown that MS enhances conditioned fear in adulthood and increases the DNA methylation in the promoter region on the NTSR1 gene in AMY. In addition, the pharmacological blockade of NTSR1 in AMY activated conditioned fear, whereas the activation of NTSR1 disrupted it. These findings suggest that MS may enhance conditioned fear via DNA methylation of the promoter region of NTSR1 in AMY, underlying its decreased mRNA expression.

Our experiment showed that a remarkable increase in corticosterone levels following CRH stimulation could be observed with the DEX/CRH test in MS group, affirming that a long-term dysregulation of the HPA axis is induced by ELS in animal and human studies [33,35–37]. MS did not affect the body weight, anxiety behaviors in the elevated plus maze test or locomotor activity in the open field test. Otherwise, the freezing rate of MS rats was higher than that of AFR rats in the context of exposure without the different sensitivities to pain induced by footshock. Taken together, it has been suggested that MS may disturbance the processes of conditioned fear memory in adulthood. Memory retrieval may initiate two potentially dissociable but opposite processes: reconsolidation and extinction [38–40]. Previous studies have shown that the length of re-exposure determines, in part, the extinction of fear memory because our experiments were not sufficient to discriminate between these two processes.

Studies in developmental psychobiology and physiology are replete with examples of the environmental programming of gene expression. Such studies have commonly reported that a variation in the early environment is associated with changes in gene expression, including DNA methylation [33]. Therefore, we examined whether MS affected DNA methylation in the promoter region of the NTSR1 gene in AMY.
expression and function that persist into adulthood and thus well beyond the duration of the relevant environmental event [5,6,47]. Our results showed that MS decreased the expression of NTSR1 mRNA in AMY, but not in HIP, in adulthood. In addition, the pharmacological blockade of NTSR1 in AMY increased the freezing rate in the second exposure to CFS, whereas the activation of NTSR1 decreased the freezing rate. These results suggest the involvement of NTSR1 located at AMY in conditioned fear. The systemic administration of an NTSR1 agonist was reported to have an anxiolytic profile in a conditioned footshock-induced ultrasonic vocalization model [48]. NTSR1 knockout mice showed a higher freezing rate than wild-type mice in contextual fear memory [49]. Our study using microinjections of NTSR1 agonist or antagonist to the AMY agrees with these past studies.

It has been shown that NTS and its receptor play a role in pain transmission [50–54]. This may indicate that NTSR1 agonism or antagonism can change the freezing rate in CFS by modulating the pain transmission instead of affecting the fear memory. To exclude this possibility, NTSR1 agonists or antagonists were microinjected before the first exposure, but they did not influence the threshold of pain induced by footshock in the conditioning session. Therefore, it is unlikely that the observed effects on learning fear memory are due to changes in the pain threshold or other nonspecific performance variables associated with memory acquisition. The concentration of PD149163 (NTSR1 agonist) used in this study was in the range of pmol (10 pmol–1000 pmol), comparable to the dose range used by others for intracerebral injections [51,52]. On the other hand, the concentration of SR48692 (NTSR1 antagonist) was in the range of fmol (10 fmol–1000 fmol). Because SR 48692 has an agonistic effect at high doses [55,56], we used a low concentration of SR48692 compared to PD149163, which was also comparable to the dose range used by others for intracerebral injections [29,51,57,58].

We examined the effect of MS on DNA methylation in the promoter region of the NTSR1 gene in AMY. DNA methylations of fragments B and C were significantly increased in the MS group. Fragment B is located 673–283 bp upstream from the first translational codon, which includes the core promoter region of the NTSR1 gene. Increased methylation of CpG dinucleotides, particularly in the 5′-promoter regions, is generally associated with a decrease in gene expression [59]. Therefore, these results suggest that DNA hypermethylation at the promoter region of the NTSR1 gene decreased mRNA expression. Whereas the function of this hypermethylation of the coding regions is not clear, it was difficult to explain why the methylation of the fragment C in MS rats was increased. Taken together, MS enhanced conditioned fear in adulthood, which may be induced from the DNA methylation of the promoter region of the NTSR1 gene in AMY.

Recent studies suggest that the DNA methylation induced by ELS plays a role in the development of psychiatric illness [36,60]. Moreover, human studies have proven that individuals who are exposed to ELS are more likely to develop post-traumatic stress disorder (PTSD) and phobias [61–63]. Our results suggest the possibility that the DNA methylation of the promoter region of NTSR1 gene in AMY may induce a vulnerability to anxiety disorders, such as PTSD and phobia, as disturbances of the fear memory processes play an important role in the development of these diseases [9,66,67]. However, we were unable to verify whether the relationship between the MS-induced down-regulation of NTSR1 mRNA and impaired fear memory processes was causative or correlative. This is one important limitation of this study. To prove this, for example, we would have to confirm the overexpression of NTSR1 in AMY of MS rats recovering from the MS-enhanced conditioned fear memory. The other limitation of this study is that we did not examine the protein levels of NTSR1. Various genes have been reported to be associated with fear memory. For example, the decrease in the Ghn2b subtype of the NMDA subunit of the glutamate receptor in AMY would serve to inhibit the reconsolidation of the contextual memory [60]. Thus, it was possible to be associated with other genes than NTSR1 in the processes of fear memory in this study. Additionally, we examined the effect of drug microinjection before the first exposure test to clarify the role of NTSR1 in fear because the effect of drugs on the expression of conditioned fear anticipates the drug's effect on this response in many cases [11]. However, we did not administer the agonist or antagonist of NTSR1 before fear conditioning. Accordingly, future studies to examine the effect of NTSR1 agonism and antagonism administered before fear conditioning or before both fear conditioning and first exposure are needed.

In conclusion, we showed that MS leaves epigenetic marks in the promoter region of the NTSR1 gene in AMY that may enhance conditioned fear memory in adulthood. These results provide the first evidence that differential DNA methylation in the promoter region of NTSR1 gene in AMY may be associated with the conditioned fear memory, although epigenetic alteration is a correlate, and a no cause-effect relation can be established by this study. Because ELS plays an important role in the clinical vulnerability to the development of anxiety disorders and depression in adulthood, our results suggest the hypothesis that manipulating the NTSR1 gene in AMY may have a therapeutic action to improve from these diseases. To clarify this hypothesis, it would be useful to investigate whether the overexpression of NTSR1 in AMY of MS rats can recover MS-enhanced conditioned fear memory.

Acknowledgments
We thank Drs. Izumi T, Yamaguchi T, Masuda T and Nishikawa H for their helpful critiques. We received English editing assistance in the preparation of the present paper by the Elsevier English Language Editing Service.

Author Contributions
Conceived and designed the experiments: HT SB SN TK. Performed the experiments: HT SB AK NT NS. Analyzed the data: HT AK. Wrote the paper: HT SB SN TI MN TK IK.

References
1. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. (2008) Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359: 61–73.
2. Wise LA, Ziedler S, Krieger N, Harlow BL. (2001) Adult onset of major depressive disorder in mice: a case-controlled study. Lancet 358: 881–887.
3. Kalinichenk M, Easterling KW, Plotzky PM, Holtzman SG (2002) Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol Biochem Behav 73: 131–140.
4. Meaney MJ, Szyf M, Sekl J. (2007) Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. Trends Endocrinol Metab 18: 269–277.
5. Kavano K, Morinobu S, Sawada T, Tsuji S, Erabi K, et al. (2008) Prior neonatal isolation reduces induction of NGF mRNA and decreases GDNF mRNA in the hippocampus of juvenile and adult rodents subjected to immobilization stress. Synapse 62: 259–267.
6. Erabi K, Morinobu S, Kavano K, Tsuji S, Yamawaki S. (2007) Neonatal isolation changes the expression of IGF-IR and IGFBP-2 in the hippocampus in isolation changes the expression of IGF-IR and IGFBP-2 in the hippocampus in
response to adulthood restraint stress. Int J Neuroopharmacol 10: 369–381.

7. Imanaka A, Morimou S, Toki S, Yamawaki S (2006) Importance of early environment in the development of post-traumatic stress disorder-like behavior. Behav Brain Res 173: 129–137.

8. Jirle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. Nat Rev Genet 8: 253–262.

9. Cuthbert BN, Lang PJ, Strauss C, Dobres D, Patrick CJ, et al. (2007) The psychopharmacology of anxiety disorder: fear memory imagery. Psychopharmacology 40: 407–422.

10. Ressler KJ, Mayberg HS (2007) Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. Nat Neurosci 10: 1116–1124.

11. Inoue T, Kitaichi Y, Koyama T (2011) SSRIs and conditioned fear. Prog Neuropsychopharmacol Biol Psychiatry 35: 130–139.

12. Inoue T, Koyama T, Yamashita I (1993) Effect of conditioned fear stress on serotonin metabolism in the rat brain. Pharmacol Biochem Behav 44: 571–574.

13. Stevenson CW, Spicer CH, Mason R, Marsden CA (2009) Early life alterations in fear conditioning and extinction in adult male rats. Behav Brain Res.

14. Matsumoto M, Togashi H, Konno K, Koscki R, Hirata R, et al. (2008) Early postnatal stress alters the extinction of context-dependent conditioned fear in adult female mice. Behav Brain Res 199: 247–252.

15. Inoue T, Masui T, Kitachiy T, Tanaka T, Isumi T, et al. (2006) DNA methylation study on the mechanism of anxiolytic action of selective serotonin reuptake inhibitors (SSRIs). Annual Report of Pharmacopsharmacology Research 30: 23–27 (in Japanese).

16. Binder EB, Kinkaid B, Owens MJ, Nemeford CB (2001) Neurotransmitters and dopamine interactions. Pharmacol Rev 53: 453–486.

17. Alexander MJ, Leeman SE (1996) Widespread expression in adult rat forebrain of mRNA encoding high-affinity serotonin receptor. J Comp Neurol 402: 475–500.

18. Prus AJ, Hillhouse TM, Lacrosse AL (2014) Acute, but not repeated, administration of the neurotensin NTS1 receptor agonist PD149163 decreases conditioned footshock-induced ultrasonic vocalizations in rats. Prog Neuropharmacol Biol Psychiatry 49: 78–84.

19. Shilling PD, Feil D (2008) The neurotransmitter-1 receptor agonist PD194163 blocks fear-potentiated startle. Pharmacol Biochem Behav 90: 748–752.

20. Plostry PM, Meyase MJ (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res Mol Brain Res 18: 195–200.

21. Hatzinger M, Reim JM, Landgraf R, Hofbauer F, Neumann I (1996) Combined dexamethasone/CRH test in rats: hypothalamic-pituitary-adrenocortical system alterations in aging. Neuroendocrinology 64: 349–356.

22. Treit D, Menard J, Rocy C (1993) Anxiogenic stimuli in the elevated plus-maze. Pharmacol Biochem Behav 44: 463–469.

23. Carter RB (1991) Distinguishing genetic and non-genetic drug activities on rat hot plate: effect of behavioral endpoint. Pain 47: 211–220.

24. Hashimoto T, Inoue T, Koyama T (1996) Serotonin reuptake inhibitors reduce conditioned fear through its effect in the amygdala. Eur J Pharmacol 497: 311–316.

25. Inoue T, Kitaichi Y, Koyama T (2011) SSRIs and conditioned fear. Prog Neuropsychopharmacol Biol Psychiatry 35: 130–139.

26. McGowan PO, Sasaki A, D’Alessio AG, Dymov S, Labonte B, et al. (2009) Epigenetic regulation of the glucocorticoid receptor in human brain with childhood abuse. Nat Neurosci 12: 342–348.

27. Rao U, Hammen C, Ortiz LR, Chen LA, Poland RE (2008) Effects of early and recent adverse experience on adult response to psychosocial stress in depressed adolescents. Biol Psychiatry 64: 521–526.

28. Nader K, Schafe GE, Le Doux JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature 406: 722–728.

29. Myers KM, Davis M (2002) Behavioral mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33: 56–72.

30. Maren S (2011) Seeking a spool wise mind: extinction, deconsolidation, and erasure of fear memory. Neuron 70: 540–545.

31. Parson RG, Ressler KJ (2013) Implications of memory modulation for post-traumatic stress and fear disorders. Nat Neurosci 16: 146–153.

32. Sala SJ (2006) Retrieval and reconsolidation: toward a neurobiology of remembering. Learn Mem 7: 73–84.

33. Eisenberg M, Kobolo T, Berman DE, Dudai Y (2003) Stability of retrieved memory: inverse correlation with trace dominance. Science 301: 1102–1104.

34. Suri D, Veinot V, Sarkar A, Thagaraman D, Kumar A, et al. (2013) Early stress evokes age-dependent biphasic changes in hippocampal neurogenesis, BDNF expression, and cognition. Biol Psychiatry 73: 651–666.

35. Schmelzer DS, Mozer DA, McCaw JE, Myers MM (2013) Autonomic functioning in mothers with interpersonal violence-related posttraumatic stress disorder in response to separation-reunion. Dev Psychobiol.

36. Yamada D, Wada E, Amano T, Wada K, Sekiguchi M (2010) Lack of neurotransmitter type 1 receptor facilitates contextual fear memory depending on the memory strength. Pharmacol Biochem Behav.

37. Clineschmidt BV, McGuffin JC, Bunting PB (1979) Neurotransmitter: anticonipsive action in rodents. Eur J Pharmacol 59: 129–139.

38. Buhler AV, Chou J, Proudfoot HK, Gehart GF (2005) Neurotransmitter activation of the NTR1 on spinal-proprioceptive neurons in the rostral ventromedial medulla is anticonipsive. Pain 118: 285–294.

39. Buhler AV, Proudfoot HK, Gehart GF (2008) Neurotransmitter-produced anticonipsion in the rostral ventromedial medulla is partially mediated by spinal cord norepinephrine. Pain 135: 280–290.

40. Roussy G, Danuser MA, Bauduson S, Euzzouba B, Felveille K, et al. (2009) Evidence for a role of NTS2 receptors in the modulation of tonic pain sensitivity. Mol Pain 5: 30.

41. Roussy G, Danuser MA, Dorre-Savard L, Belleville K, Beauden N, et al. (2008) Spinal NTS1 receptors regulate neurotensin receptor-negative signaling in a rat formalin tonic pain model. J Neurosci 105: 1100–1114.

42. Yamada M, Lombert A, Forgez P, Rostene W (1998) Distinct functional characteristics of levoebutamine sensitive rat neurotransmitter NT2 receptor expressed in Chinese hamster ovary cell. Life Sci 62: 375–380.

43. Sarret P, Gendron L, Kilian P, Nguyen HM, Gallo-Payet N, et al. (2002) Pharmacological and functional properties of NTS2 neurotransmitter receptors in cerebellar granule cells. J Biol Chem 277: 36323–36324.

44. Laszlo K, Toth K, Kettes E, Peczely I, Lenard L (2010) The role of neurotransmitter in positive reinforcement in the rat central nucleus of amygdala. Behav Brain Res 204: 430–435.

45. Laszlo K, Toth K, Kettes E, Peczely I, Ollmann T, et al. (2010) Effects of neurotransmitters in amygdaloid spatial learning memories. Behav Brain Res 210: 280–285.

46. Rakanay VN, Preis J, Morgan HD, Whitehead E (2001) The marks, mechanisms and memory of epigenetic states in mammals. Biochem J 356: 1–10.

47. Muragroyd C, Patchev AV, Wu Y, Miracle V, Beckmull Y, et al. (2009) Dynamic DNA methylome patterns persistent adverse effects of early-life stress. Nat Neurosci 12: 1559–1566.

48. Lang AJ, Aarons GA, Gearyr J, Lafaye C, Satz L, et al. (2008) Direct and indirect links between childhood maltreatment, posttraumatic stress disorder, and women’s health. Behav Med 33: 125–135.

49. Coughler JK, Timpano KR, Sado-Ericson N, Koeough ME, Riccardi CJ (2010) Examining the unique relationships between anxiety disorders and childhood physical and sexual abuse in the National Comorbidity Survey-Replication. Psychiatr Res 177: 130–135.

50. Brewin CR, Andrews B, Valentine JD (2000) Meta-analysis of risk factors for posttraumatic stress disorder in trauma-exposed adults. J Consult Clin Psychol 68: 748–766.

51. Nishith P, Mechanic MB, Resick PA (2000) Prior interpersonal trauma: the contribution to current PTSD symptoms in female rape victims. J Abnorm Psychol 109: 20–25.

52. Claessen CC, Paleos OG, Aggarwal R (2005) Sexual victimization: a review of the empirical literature. Trauma Violence Abuse 6: 103–129.
66. Bentz D, Michael T, de Quervain DJ, Wilhelm FH (2010) Enhancing exposure therapy for anxiety disorders with glucocorticoids: from basic mechanisms of emotional learning to clinical applications. J Anxiety Disord 24: 223–230.

67. Yamamoto S, Morimobu S, Takei S, Fuchikami M, Matsuki A, et al. (2009) Single prolonged stress: toward an animal model of posttraumatic stress disorder. Depress Anxiety 26: 1110–1117.

68. Wang SH, de Oliveira Alvares L, Nader K (2009) Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. Nat Neurosci 12: 905–912.

69. Paxinos G, Watson C (1997) The Rat Brain in Stereotaxic Coordinates. 3rd ed. San Diego: Academic Press.