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Archival Report

Biological Psychiatry

Neonatal Maternal Separation Alters the Capacity of Adult Neural Precursor Cells to Differentiate into Neurons Via Methylation of Retinoic Acid Receptor Gene Promoter

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ABSTRACT

BACKGROUND: Early life stress is thought to contribute to psychiatric disorders, but the precise mechanisms underlying this link are poorly understood. As neonatal stress decreases adult hippocampal neurogenesis, which, in turn, functionally contributes to many behavioral phenotypes relevant to psychiatric disorders, we examined how in vivo neonatal maternal separation (NMS) impacts the capacity of adult hippocampal neural precursor cells via epigenetic alterations in vitro.

METHODS: Rat pups were separated from their dams for 3 hours daily from postnatal day (PND) 2 to PND 14 or were never separated from the dam (as control animals). We isolated adult neural precursor cells from the hippocampal dentate gyrus at PND 56 and assessed rates of proliferation, apoptosis, and differentiation in cell culture. We also evaluated the effect of DNA methylation at the retinoic acid receptor (RAR) promoter stemming from NMS on adult neural precursor cells.

RESULTS: NMS attenuated neural differentiation of adult neural precursor cells but had no detectible effect on proliferation, apoptosis, or astroglial differentiation. The DNA methyltransferase (DNMT) inhibitor, 5-aza-dC, reversed a reduction by NMS of neural differentiation of adult neural precursor cells. NMS increased DNMT1 expression and decreased expression of RARα. An RARα agonist increased neural differentiation and an antagonist reduced retinoic acid-induced neural differentiation. NMS increased the methylated portion of RARα promoter, and the DNMT inhibitor reversed a reduction by NMS of RARα messenger RNA expression.

CONCLUSIONS: NMS attenuates the capacity of adult hippocampal neural precursor cells to differentiate into neurons by decreasing expression of RARα through DNMT1-mediated methylation of its promoter.

Keywords: Adult neurogenesis, Dentate gyrus, DNA methylation, DNA methyltransferase, Maternal separation, Retinoic acid receptor

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One of the fundamental issues in neurobiology is how environmental factors alter molecular states in the brain, ultimately leading to behavioral phenotypes. Neonatal and postnatal stress are thought to have long-lasting effects on individuals, resulting in heightened risk for many psychiatric disorders, including schizophrenia, substance abuse disorders, personality disorders, and mood and anxiety disorders (1). The precise mechanisms of this process are still poorly understood in humans.

In rodents, neonatal maternal separation (NMS) alters behavioral phenotypes related to neuropsychiatric disorders later in life. Defective prepulse inhibition (PPI) is nonselectively associated with many neuropsychiatric disorders, including schizophrenia, bipolar disorder, schizotypal personality disorder, obsessive-compulsive disorder, and panic disorder in humans (2). NMS reduces PPI from adolescence to adulthood but not before puberty in rats (3–7). Moreover, NMS exacerbates stress responses and anxiety-like behaviors (8–10), heightens preference for ethanol (8,11), and induces cognitive impairments (5,12) in rats by the time they reach adulthood.

NMS induces a host of neuronal phenotypes in many rodent brain regions (13), but neuronal alterations in the hippocampus are likely to mediate some of the long-lasting effects of NMS on behaviors (14). Indirect evidence suggests that adult neurogenesis in the hippocampus contributes to the behavioral effects of NMS. First, NMS reduces adult neurogenesis in the rat hippocampal dentate gyrus in vivo (15). Second, direct alterations in adult neurogenesis in the hippocampus affect PPI (16), mood-related behaviors (17), and fear-related memory (18–20).

Epigenetic alterations in hippocampal neural precursor cells are increasingly appreciated as contributors to many aspects of adult neurogenesis (21,22). Methyl-CpG binding domain

SEE COMMENTARY ON PAGE 307

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protein 1, a member of the methylated DNA-binding protein family, binds methylated gene promoters and facilitates transcriptional repression. Loss of this gene reduces neural differentiation in vivo and in vitro (23) through a basic fibroblast growth factor 2 promoter in vitro (24) and induces PPI deficits and defective fear conditioning and heightens anxiety- and depression-related behaviors in vivo (25).

We hypothesized that NMS alters the rate of adult neurogenesis in the hippocampal dentate gyrus via methylation of a neurogenesis-related gene. Because adult neural precursor cells represent a small fraction of the total hippocampal cell population, in vivo analysis cannot identify an epigenetic modification for this specific cell population. To circumvent this technical obstacle, we evaluated the impact of in vivo environmental stress on adult neural precursor cells in the hippocampal dentate gyrus, using our in vitro cell culture system. Our cell culture system uses adult dentate gyrus-derived neural precursor cells (ADP) and does not include ependymal cells (26). Pups were separated from their dams on postnatal days (PNDs) 2 to 14, and we evaluated how this environmental stress altered the capacity of in vitro adult neural precursor cells and DNA methylation at PND 56. Rats become sexually mature by 6 weeks of age (i.e., enter adolescence). They are considered to be young adult from PND 63, reaching socially mature adulthood around 6 months of age (27). We focused on young adulthood, because onset of many neuropsychiatric disorders occurs during the period from late adolescence through young adulthood.

METHODS AND MATERIALS

Animals

Pregnant Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were delivered on gestation day 14 and singly housed. All rats were housed in standard animal cages with ad libitum access to food and water in a temperature-controlled environment (22°C ± 1°C) on a 12-hour light/dark cycle (light phase: 6:00 AM–6:00 PM). All procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

Neonatal Maternal Separation

We used a brief maternal separation procedure previously reported by Plotsky and Meaney (28). Pups were cross-fostered on PND2 to minimize litter differences; eight male and two female pups were placed in each litter. Ten pups per litter were assigned to neonatal maternal separation or typical animal facility rearing (AFR) groups. Because cross-fostering could have long-lasting effects on emotional behaviors (29), this factor was held constant for both groups. Under the cross-fostering condition, NMS, but not AFR, results in reduced adult neurogenesis (15). Maternal separation took place for 3 hours per day (9:30 AM to 12:30 PM each day) from PND 2 to PND 14. Dams were removed from the cage and placed in a separate cage; pups were also removed from the cage, placed in a clean plastic cage with wood-chip bedding in an incubator to maintain an ambient temperature at 27°C to 30°C in another room, and returned 3 hours later to the original cage with the dams. Pups in the NMS group were permitted to position themselves, which included huddling with littermates, during the separation period. Pups in the AFR group were not disturbed and were maintained with dams. Bedding for both AFR and NMS groups was changed once a week by an animal care technician.

The same pool of animals that simultaneously underwent NMS was randomly divided into two subgroups. One subgroup was tested for fear conditioning and the other for the present cell culture analysis. The efficacy of our NMS procedure was validated as NMS-treated rats showed fear-related phenotypes (30). We removed all pups from the dam for 3 hours each day. Other published procedures keep two to three pups with the dam to minimize her stress and subsequent maternal abuse. The precise environmental factor in the NMS procedure that causes behavioral phenotypes cannot be easily or definitively isolated. Nonetheless, the version we employed has been demonstrated to cause robust behavioral phenotypes (30) and alteration in adult neurogenesis (15). In the literature, control for NMS is our AFR, brief handling, or both. A brief-handing group is handled for 30 seconds to 15 minutes; however, this also inevitably results in maternal separation during handling. Thus, this control does not isolate the impact of handling alone. As pointed out by Matthews and Robbins (31), it is not realistic to apply a pure experimental condition that would permit definitive descriptions of the effects of handling or maternal separation. In reality, the AFR and brief isolation with handing do not result in consistently different behavioral phenotypes (8,31–35). We conducted a pilot study to compare the impact of the AFR and 15-minute handling but did not find phenotypic differences in anxiety-related behaviors between these two groups and thus did not include the handling control.

Isolation and Culture of ADP Cells

At weaning, male and female rats were separated and group-housed. At PND 56, all eight male rats from each of the NMS groups and AFR groups were used to dissect the dentate gyrus. We used four NMS groups and four AFR groups as one set. Tissues from 32 rats of each treatment group (NMS or AFR) were pooled and digested using proteases and DNase (Worthington Biochemical Corp., Lakewood, New Jersey). ADP cells were isolated using Percoll-gradient centrifugation and then prepared in monolayer culture in nonserum medium with basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, California), using our standard procedure (26). Each cell culture was derived from 32 rats (8 rats per foster mother and 4 foster mother lines per treatment group). As each assay was repeated in three to six cell cultures, the sample size ranged from three to six.

Drugs

We used retinoic acid (Invitrogen), Ro 41-5213 (Enzo Life Sciences, Farmingdale, New York), and 5-aza-dC (Sigma, St. Louis, Missouri). Staurosporine was kindly donated by Asahi-Kasei Corporation (Tokyo, Japan) and CD1556 was kindly donated by Garderma (Sophia-Antipolis, France).

Proliferation Assay

ADP cells (1×10^4 per well) were placed on laminin-ornithine coated Lab-Tek II eight-chamber slides (Nalge Nunc International, Naperville, Illinois) with .5% fetal bovine serum medium (Invitrogen). After 24 hours, cells were treated with 5 ng/mL bFGF, a major stimulator of proliferation in neural precursor cells (36). Bromodeoxyuridine (BrdU) (Sigma) was added 24 hours later (10 nmol/L). Immunocytochemistry assays were conducted with anti-BrdU antibody 24 hours later as described in our previous study (37). Fluorescent signals were detected using an IX-71 fluorescence microscope (Olympus, Tokyo, Japan). We evaluated BrdU and 4′,6-diamidino-2-phenylindole (DAPI) signal in four randomly selected fields per well and then calculated the ratio of BrdU-derived signals to DAPI signals.

Apoptosis Assay

ADP cells (2×10^4 per well) were placed on laminin-ornithine coated Lab-Tek II eight-chamber slides with medium including 20 ng/mL bFGF, and 24 hours later, apoptosis was induced using staurosporine (300 nmol/L) (38). While apoptosis could be mediated by tumor necrosis factor alpha (TNF-α)- and staurosporine-dependent pathways, we previously demonstrated that in adult neural progenitor cells, TNF-α does not induce apoptosis but staurosporine does at this concentration (38). Two days later, we performed a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay using the DeadEnd Fluorometric TUNEL System (Promega, Madison, Wisconsin), as described in our previous study (38). We detected fluorescent signals using an IX-71 fluorescence microscope (Olympus); we then counted the number of TUNEL and DAPI-positive cells in four randomly selected fields per well and calculated the signal ratio of TUNEL to DAPI.

Differentiation Assay

ADP cells (2×10^4 per well) were placed on laminin-ornithine coated Lab-Tek II eight-chamber slides with medium including .5% fetal bovine serum. Following overnight incubation, differentiation was induced using 1 μmol/L retinoic acid. Little is known about potential multiple pathways for differentiation of adult neural progenitor cells. However, we previously demonstrated that retinoic acid is one of the most likely endogenous factors that induces differentiation of adult neural progenitor cells (38). Seven days later, we performed immunocytochemistry assays using anti-Tuj1 antibody (Covance Inc., Princeton, New Jersey), a marker of immature neurons, and anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Glostrup, Denmark), a marker of glial cells, as described in our previous study (38). It is difficult to induce complete differentiation of adult neural progenitor cells to the extent that they assume features of genuinely mature neurons. Microtubule-associated protein 2 is a marker of mature neurons and use of this marker would not identify all differentiating cells, including immature neurons. Because Tuj1 is a marker of immature neurons, we used it as a marker to evaluate the rate of differentiation. We counted the numbers of cells positive for markers in four randomly selected fields per well and calculated the ratio of each cell marker to DAPI.

To evaluate the role of retinoic acid receptor (RAR)α and DNA methyltransferase (DNMT) in differentiation of ADP cells, we used CD1556, a selective RARα agonist; Ro 41-5213, a selective RARα antagonist; and 5-aza-dC, a DNMT inhibitor. In a pilot study, we used a wide range of concentrations based on published studies and chose 2 μmol/L for CD1556, 1 μmol/L for Ro 41-5213, and 10 μmol/L for 5-aza-dC, because they induced the expected effect without toxicity. To evaluate the effect of 5-aza-dC on RARα messenger RNA (mRNA) expression, we used the same concentration (10 μmol/L).

Total RNA Isolation and Quantitative Real Time Polymerase Chain Reaction

ADP cells (2×10^5) were placed on laminin-ornithine coated 35-mm dishes or six-well plates in medium 20 ng/mL bFGF. For 35-mm dishes, after 24 hours, we isolated total RNA using All Prep DNA/RNAMini (Qiagen, Hilden, Germany). For six-well plates, drugs were added 24 hours later, and we isolated total RNA 3 days later. We performed RNA isolation and quantitative real time polymerase chain reaction (RT-PCR) 24 hours after cell seeding and quantitative RT-PCR using our standard procedure (26), using a glyceraldehyde 3-phosphate dehydrogenase as a control. The results were analyzed by using SDS 2.0 software (Applied Biosystems, Foster, California).

Western Blotting

ADP cells (2×10^5) were placed on laminin-ornithine coated 35-mm dishes or six-well plates in medium with 20 ng/mL bFGF. We prepared cells 24 hours after cell seeding. Total protein was prepared using the Mammalian Cell Lysis Kit (Sigma). We performed western blotting using anti-DNMT1 antibody (1:1000) (Active Motif, Carlsbad, California) and anti-RARα antibody (1:1000) (Cell Signaling, Danvers, Massachusetts) as described in our previous study (26). Protein expression was detected using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Milwaukee, Wisconsin) and Amersham Hyperfilm ECL (GE Healthcare). Images were converted to digital files and the intensity of bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Methylation Analysis of RARα Promoter

ADP cells (2×10^5) were placed on laminin-ornithine coated 35-mm dishes in medium with 20 ng/mL bFGF. Twenty-four hours later, we isolated genome DNA of ADP cells using Allprep DNA/RNA Mini (Qiagen) and digested with Mse 1 (New England Biolabs, Ipswich, Massachusetts). We enriched CpG-methylated DNA using Mse 1-digested DNA fragments with MethylCollector Ultra (Active Motif) and performed PCR with AmpliTaq Gold 360 Master Mix (Applied Biosystems). PCR conditions were 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. Primers for semiquantitative PCR were designed to cover 340 base pair (bp) of the CpG island of RARα promoter (Figure S1 in Supplement 1). We used the following sequences of forward and reverse primers: TAG GGG CTG GAA TCC CAG AG and AAG TTG TGC AGG TTG GAG GAA G. PCR products were electrophoresed with

2% agarose gel. Digital images of this gel were acquired and we analyzed the intensity of each band using ImageJ (National Institutes of Health).

Statistical Analysis

We conducted statistical analyses using unpaired *t* test or analysis of variance followed by Bonferroni post hoc comparisons. Significance was set as $p < .05$. Data are expressed as the means ± SEM.

RESULTS

Neonatal Maternal Separation Attenuates Neural Differentiation of ADP Cells

To identify if neonatal maternal separation, from neonatal days 2 to 14, has a long-lasting effect on ADP cells at PND 56, we evaluated the rates of proliferation, apoptosis, and differentiation into neurons and astrocytes. We found no difference in the numbers of BrdU-positive cells in AFR group compared with the NMS group (Figure 1B). Using staurosporine-induced apoptosis, cells were examined with TUNEL staining. We found no difference in the numbers of TUNEL-positive cells between the AFR and NMS groups (Figure 1C). We next examined the rates of differentiation of ADP cells. Because ADP cells lose their capacity to spontaneously differentiate as rats age, we used retinoic acid to induce differentiation (38). We evaluated neural and astroglial differentiation using immunocytochemistry assays with anti-Tuj1 antibody and anti-GFAP antibody, respectively (38). While the number of Tuj1-positive cells was significantly reduced in the NMS group compared with the AFR group, there was no difference between groups for the number of GFAP-positive cells (Figure 1D). Here, we showed that the impact of neonatal stress applied in vivo can be evaluated using in vitro experimental methods. Taken together, these data indicate that neonatal maternal separation has a long-lasting (>42 days) effect on the capacity of ADP cells to differentiate into neurons but has no detectable effect on proliferation, apoptosis, or differentiation into astrocytes.

Neonatal Maternal Separation Attenuates Neural Differentiation of ADP Cells via DNA Methylation

We hypothesized that the long-lasting effect might be mediated by epigenetic alterations. Histone acetylation acts as an epigenetic modification to mediate neural differentiation of

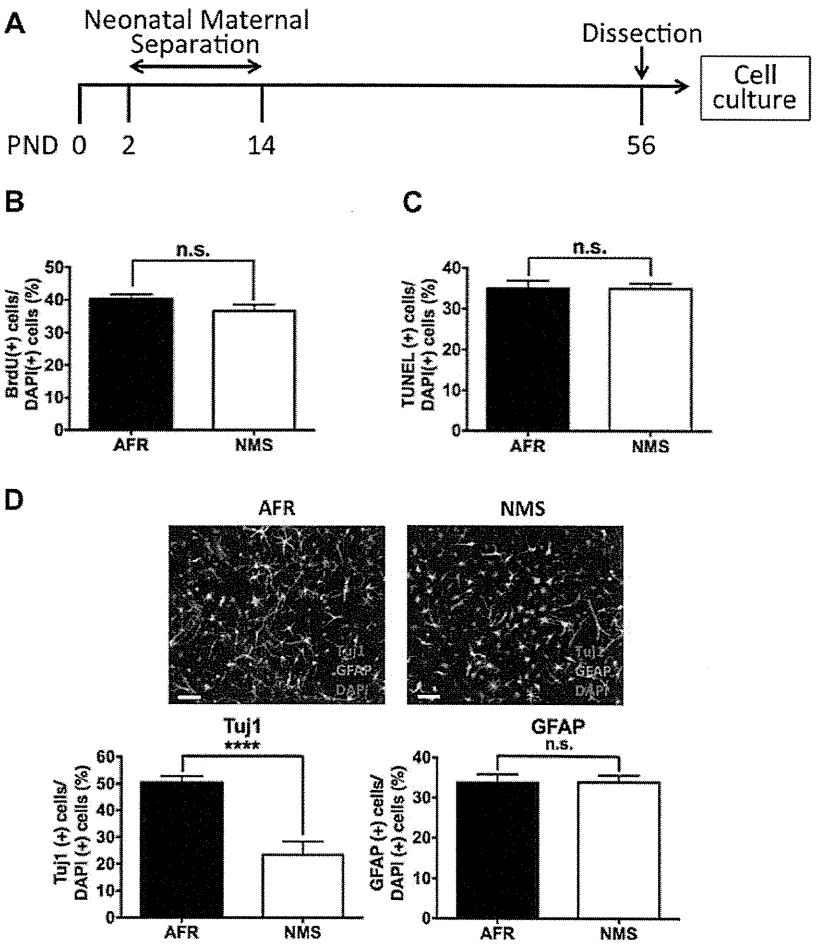

Figure 1. Effects of neonatal maternal separation (NMS) on proliferation, apoptosis, and differentiation of adult dentate gyrus-derived precursor cells in vitro. **(A)** Rats underwent NMS between postnatal days (PND) 2 and 14. Adult dentate gyrus-derived precursor cells were collected from the hippocampal dentate gyrus at PND 56. **(B)** NMS had no effect on proliferation, as assessed by bromodeoxyuridine (BrdU)-positive cells ($t_{10} = .149$, n.s.) or **(C)** staurosporine-induced apoptosis, as assessed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay ($t_{10} = .9954$, n.s.). **(D)** NMS reduced retinoic acid-induced neural differentiation, as assessed by Tuj1-positive cells ($t_{10} = 11.96$, $p < .0001$) but had no effect on astroglial differentiation, as assessed by glial fibrillary acidic protein (GFAP)-positive cells ($t_{10} = .062$, n.s.). Data are shown as the means ± SEM. ****Statistically significant difference at $p < .0001$. AFR, animal facility reared; DAPI, 4′,6-diamidino-2-phenylindole; n.s., no statistically significant difference.

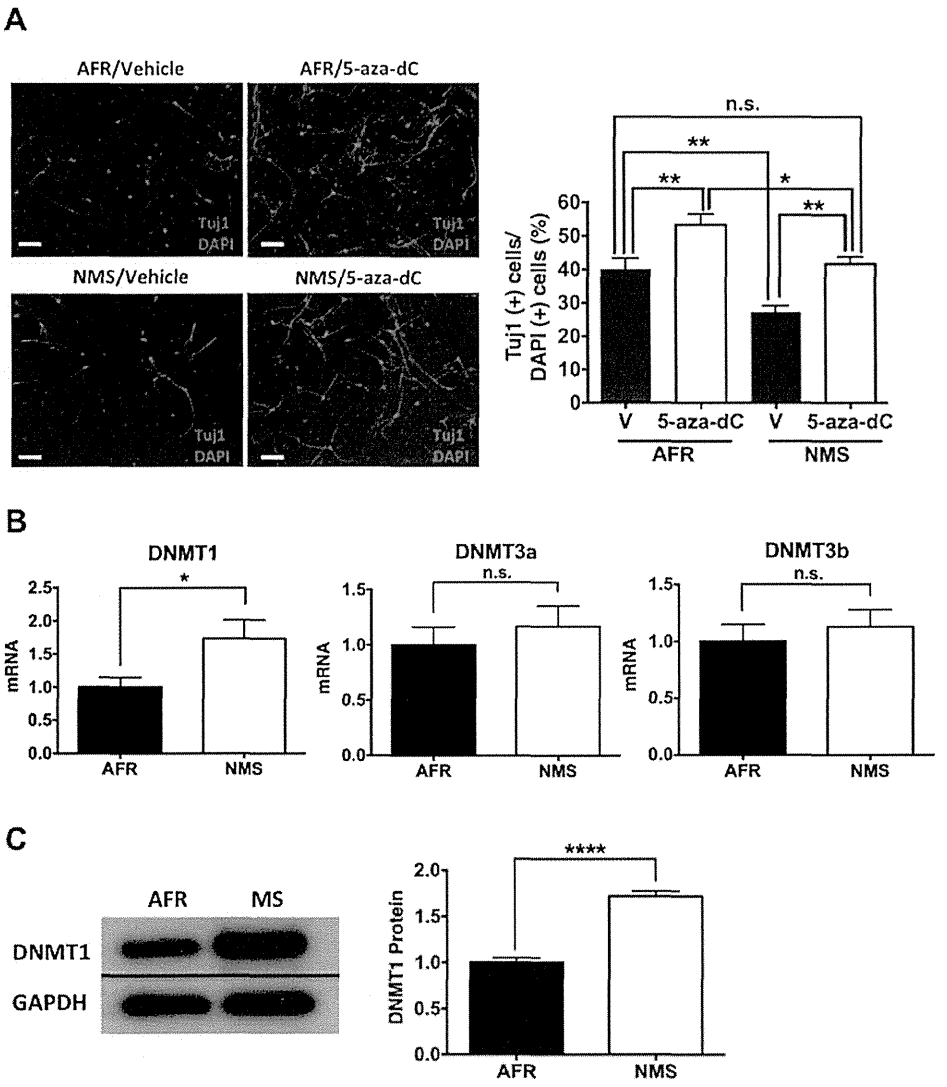

Figure 2. Effects of neonatal maternal separation (NMS) on neural differentiation of adult dentate gyrus-derived neural precursor cells via DNA methyltransferase (DNMT) and on regulation of DNMT subtypes in vitro. **(A)** A reduction in neural differentiation following NMS was reversed by 5-aza-dC (10 μmol/L), a DNMT inhibitor. Immunocytochemistry assays were performed 7 days after drug treatment. Two-way analysis of variance showed that the main group (animal facility reared [AFR] vs. NMS) effect ($F_{1,60}$ = 17.95, p < .0001) and the drug (vehicle [V] vs. 5-aza-dC) effect ($F_{1,60}$ = 22.76, p < .0001) were significant without an interaction effect ($F_{1,60}$ = .101, n.s.). Post hoc comparison of the vehicle-treated AFR and 5-aza-dC-treated NMS groups showed that the groups did differ. **(B)** NMS increased messenger RNA (mRNA) levels of the DNMT1 subtype (t_{10} = 2.306, p < .05) but had no effect on DNMT3a (t_{10} = .678, n.s.) or DNMT3b (t_{10} = .609, n.s.). **(C)** NMS increased protein levels of DNMT1 (t_6 = 9.801, p < .0001). Data are shown as the means ± SEM. A statistically significant difference is indicated at *p < .05, **p < .01, and ****p < .0001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; n.s., no statistically significant difference.

neural progenitor cells (39). However, histone acetylation might not account for a reduction in neural differentiation of ADP cells. Valproate, a histone deacetylase inhibitor, promotes neural differentiation of cells derived from embryonic rat hippocampus (39) and facilitates astroglial differentiation and attenuates neural differentiation of ADP cells (38). In the current study, we thus examined the role of DNA methylation as an epigenetic alteration, because previous reports show that DNA methylation at a CpG island decreases expression of a globin gene (40,41).

We examined the effect of 5-aza-dC, a common inhibitor of DNA methyltransferases (DNMTs), on the diminished rate of neural differentiation of ADP cells. If DNA methylation was involved, then its inhibition by 5-aza-dC would restore neural differentiation that was decreased due to neonatal maternal separation. The number of Tuj1-positive cells was equally increased by 5-aza-dC in the AFR and NMS groups; the number of Tuj1-positive cells in the NMS group increased to normal levels of the AFR group (Figure 2A). We observed a small variance (~10%) in differentiation rates, as judged byTuj1, among different cell lines (Figures 1D and 2A). However, NMS consistently decreased the rate of neural differentiation. These results suggest that increased DNA methylation by DNMTs was involved in the diminished neural differentiation of ADP cells. Given that there are three DNMT subtypes, namely DNMT1, 3a, and 3b (42), using quantitative RT-PCR, we next examined whether neonatal maternal separation induces long-lasting effects on expression of the three DNMT subtypes in ADP cells. DNMT1 mRNA, but not DNMT 3a or 3b, was selectively increased in ADP cells from the NMS group (Figure 2B). We additionally confirmed that DNMT1 protein was also increased in ADP cells from the NMS animals (Figure 2C), suggesting that neonatal maternal separation has a long-lasting effect on DNMT1 expression in ADP cells.

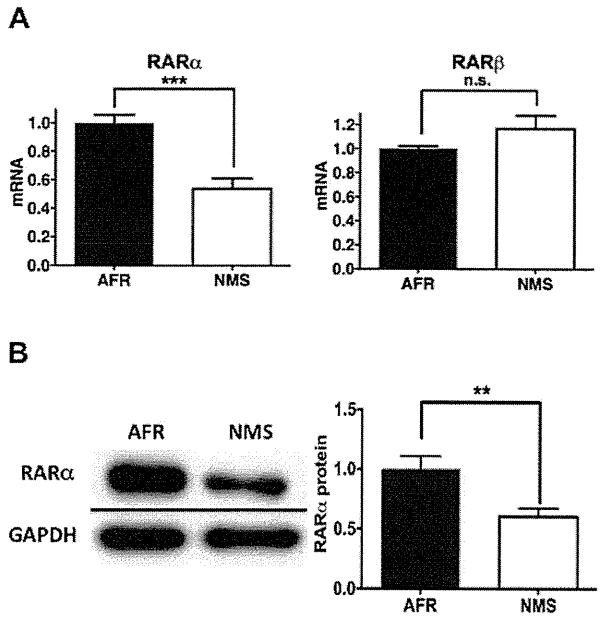

Figure 3. Effects of neonatal maternal separation (NMS) on expression of retinoic acid receptor (RAR)α and RARβ subtypes in adult dentate gyrus-derived neural precursor cells in vitro. **(A)** NMS decreased messenger RNA (mRNA) expression of RARα (t_{10} = 4.991, $p < .001$) but not RARβ (t_{10} = 1.513, n.s.). **(B)** NMS decreased expression of RARα protein (t_{14} = 3.088, $p < .01$). Data are shown as the means ± SEM. A statistically significant difference is indicated at $^{**}p < .01$ or $^{***}p < .001$. AFR, animal facility reared; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; n.s., no statistically significant difference.

Neonatal Maternal Separation Selectively Reduces Retinoic Acid Receptor α Subtype Expression in ADP Cells

Because endogenous retinoic acid is involved in neural differentiation in adult hippocampus in vivo (43,44) and we used retinoic acid to induce differentiation, we hypothesized that the receptor for this ligand might mediate the impact of neonatal maternal separation. Among the three known subtypes of retinoic acid receptors, RARα, RARβ, and RARγ (45), RARα and RARβ, but not RARγ, mediates neural differentiation of embryonic neural progenitor cells (46). RARα mRNA, but not that of RARβ, was decreased in ADP cells of the NMS group (Figure 3A); RARγ mRNA was not detectable in this cell population (data not shown). Moreover, immunoblotting analysis confirmed that RARα protein was similarly reduced in ADP cells of the NMS group (Figure 3B), indicating that neonatal maternal separation selectively reduces RARα mRNA and protein in ADP cells.

RARα Expression Is a Determinant of the Balance of Differentiation of ADP Cells into Neurons and Astrocytes

To more directly evaluate if RARα is functionally involved in neural differentiation of ADP cells, we examined the effect of the RARα-selective agonist CD1556 and the RARα-selective antagonist Ro 41-5253 on neural differentiation using ADP cells from the AFR group. CD1556 more robustly increased Tuj1-positive cells than retinoic acid (Figure 4A). Conversely, Ro 41-5253 reduced retinoic acid-induced differentiation into Tuj1-positive neurons (Figure 4B). These data suggest that RARα activation is a determinant for differentiation of ADP cells into neurons.

Neonatal Maternal Separation Increases Methylated RARα Promoter Levels and Reduces RARα mRNA Expression in ADP Cells

We next examined the level of DNA methylation in a *RARα* gene promoter in the AFR and NMS groups. *RARα* gene promoter was more highly methylated in the NMS group than the AFR group (Figure 5A). If DNA methylation is causally involved in diminished expression of RARα in ADP cells of the NMS group, inhibition of DNA methylation would be expected to increase RARα expression. To test this hypothesis, we added 5-aza-dC, an inhibitor of DNA methylation, to ADP cells. This treatment increased expression of RARα mRNA in ADP cells of both the AFR and NMS groups, thereby normalizing the diminished level of RARα mRNA in the NMS group (Figure 5B). These data suggest that maternal separation reduces RARα expression by increasing methylation of its promoter.

DISCUSSION

Our in vitro analyses showed that neonatal maternal separation diminishes the capacity of adult neural precursor cells to differentiate into neurons; increases expression of DNMT1, but not 3a or 3b; reduces expression of RARα, but not β subtype; and increases methylation of RARα promoter. Functional analysis showed that direct activation of RARα increased neural differentiation and blockade of RARα reduced neural differentiation induced by retinoic acid. Finally, inhibition of DNMT methylation reversed the reduction of neural differentiation and RARα expression seen following neonatal maternal separation (Figure 6). Taken together, our data suggest that neonatal maternal separation reduces the capacity of adult hippocampal neural precursor cells to differentiate into neurons and this effect is dependent on a reduction in RARα expression through methylation of its promoter.

Among the three DNMT subtypes, neonatal maternal separation selectively decreased DNMT1 expression, but not DNMT3a or DNMT3b. DNMT1 is highly expressed in the central nervous system of adult rodents (47), specifically in the hippocampus (48), and especially in the hippocampal dentate gyrus (49). However, DNMT3a is also highly expressed in adult hippocampal dentate gyrus (49) and mediates neural differentiation in embryonic neural stem cells (50) and in vivo adult dentate gyrus (51). DNMT3b also is present in the rat hippocampal dentate gyrus (52) and thought to contribute to embryonic neurogenesis (53). As DNMT inhibitor 5-aza-dC does not differentiate among the three DNMT subtypes, we cannot rule out the possibility that other DNMT subtypes functionally contribute to the impact of neonatal maternal separation on adult hippocampal neurogenesis.

It remains unclear exactly how NMS increases DNMT1 expression. While it is possible that NMS increased DNMT1 gene expression by decreasing methylation of the DNMT1

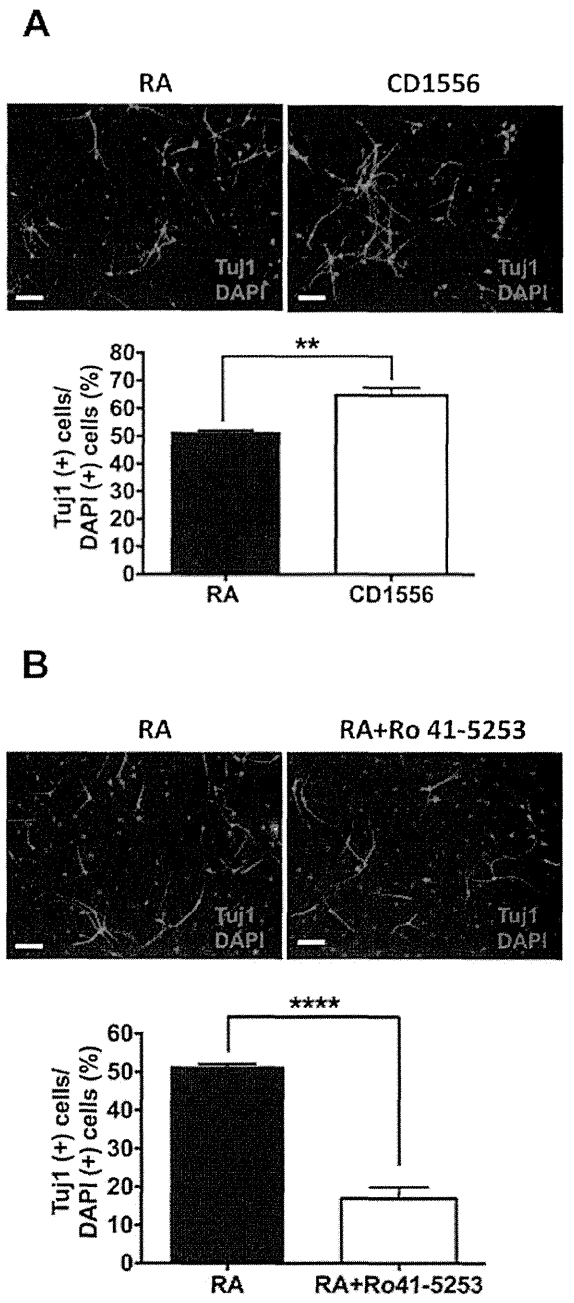

Figure 4. Effects of a retinoic acid receptor (RAR)α agonist (CD1556) and antagonist (Ro 41-5253) on neural differentiation of adult dentate gyrus-derived neural precursor (ADP) cells in vitro. **(A)** Retinoic acid (RA) and CD1556 (2 μmol/L), a selective RARα agonist, induced neural differentiation but the latter had a more robust effect (t_6 = 4.651, $p < .01$) in ADP cells from the animal facility reared group. **(B)** Ro 41-5253 (1 μmol/L), a selective RARα antagonist, attenuated neural differentiation induced by RA of ADP cells in the animal facility reared group (t_6 = 11.370, $p < .0001$). Data are shown as the means ± SEM. Statistical significance is shown at $^{**}p < .01$ or $^{****}p < .0001$. DAPI, 4′,6-diamidino-2-phenylindole.

promoter, this is highly unlikely because there is no CpG island up to 1 kilobase of the transcription start site of DNMT1. Indirect evidence suggests that glucocorticoids might mediate this link. We recently reported that NMS resulted in increased basal and inducible corticosterone (30). More work is needed to further elucidate the upstream mechanisms of DNMT1 regulation.

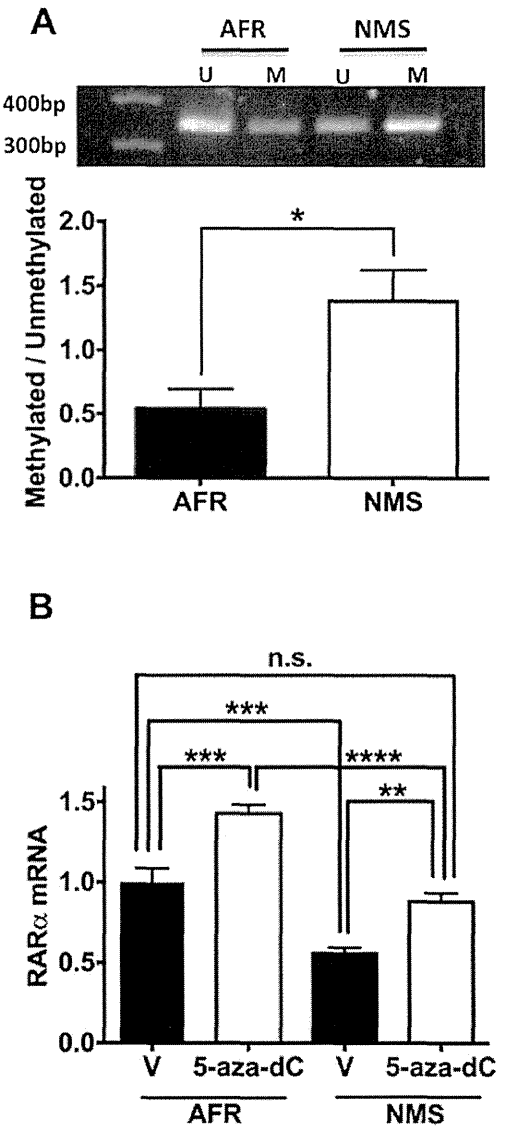

Figure 5. Effects of neonatal maternal separation (NMS) on methylation and methylation-dependent messenger RNA (mRNA) expression of retinoic acid receptor (RAR)α in adult dentate gyrus-derived neural precursor cells in vitro. **(A)** NMS increased the ratio of methylated (M) fraction to unmethylated fraction (U) of RARα promoter (t_4 = 2.956, $p < .05$). **(B)** The DNA methyltransferase inhibitor 5-aza-dC (10 μmol/L) increased expression of RARα mRNA in adult dentate gyrus-derived neural precursor cells from the animal facility reared (AFR) and NMS groups (AFR vs. NMS, $F_{1,20}$ = 67.160, $p < .0001$; vehicle [V] vs. 5-aza-dC, $F_{1,20}$ = 39.090, $p < .0001$; interaction, $F_{1,20}$ = .780, n.s.). Post hoc comparison of vehicle-treated AFR and 5-aza-dC-treated NMS groups showed that the groups did differ. Data are shown as the means ± SEM. Statistical significance is shown at $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, or $^{****}p < .0001$. n.s., no statistically significant difference.

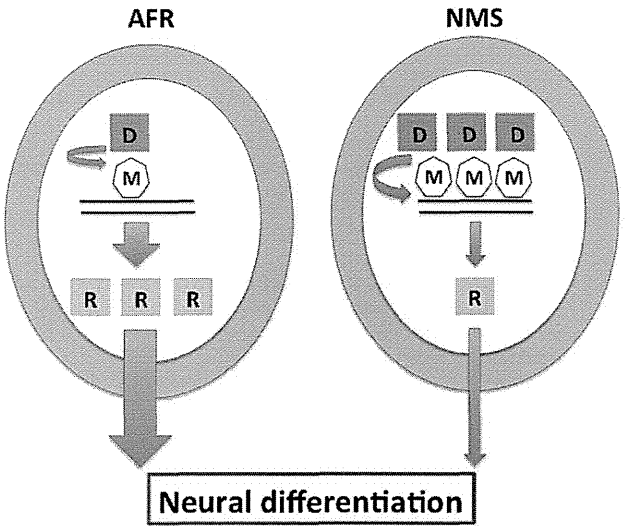

Figure 6. Hypothetical epigenetic mechanisms underlying the effects of neonatal maternal separation (NMS) on neural differentiation of adult dentate gyrus-derived neural precursor cells. NMS reduces neural differentiation by decreasing retinoic acid receptor α (R) expression via increased DNA methyltransferase 1 (D) expression and DNA methylation (M) at a retinoic acid receptor α promoter. AFR, animal facility reared.

We showed that neonatal maternal separation selectively decreased RARα, but not RARβ, in ADP cells. Further, an RARα agonist and antagonist facilitated and decreased, respectively, neural differentiation of this cell population. Neonatal maternal separation increased methylation at a promoter region of this gene and 5-aza-dC, a DNA methylation inhibitor, diminished methylation of this gene. Together with our observation that the methylation inhibitor also increased neural differentiation of adult hippocampal neural precursor cells, we submit that neonatal maternal separation increases methylation of RARα promoter, resulting in reduced levels of RARα expression and neural differentiation.

Given that neonatal maternal separation increased DNMT1 mRNA and protein levels and a common inhibitor of DNMTs reversed the reduction by NMS of RARα expression, the activity of DNMT1 is likely to be increased. However, there is no currently available reliable method to differentially detect activities of the three DNMT subtypes. Analysis of total DNMT activities is not suitable for validation of DNMT1-specific mRNA and protein regulation at the activity level. We also caution that because many genes are likely to contribute to neural differentiation (54), neonatal maternal separation might additionally affect differentiation of adult neural precursor cells of the hippocampal dentate gyrus through DNA methylation of other genes.

While the in vitro molecular and cellular events observed in this study could in theory be examined in vivo by knocking down genes by a viral vector, promoters designed to affect a specific cell population often do not confer intended cell specificity (55,56). Such preparation would include effects of gene knockdown in the target cells and other cell types. Moreover, in vivo isolation of a small fraction of cells (i.e., adult neural progenitor cells) and detection of epigenetic alterations in that cell population alone from tissue pose another technical challenge. A future challenge involves development of a reliable technique to validate in vitro mechanisms under in vivo conditions. Notwithstanding this challenge, our work provides an alternative to circumvent these technical difficulties in vivo and has an innovative translational value in psychiatry. Our in vitro cellular model makes it possible to delve into precise molecular mechanisms underlying neonatal stress in a select population of cells, thereby providing an assay system for development of novel therapeutic options. Drugs and other therapeutic options developed using this assay could then be directly tested, as a means of validation, for their effects on behavioral abnormalities caused by neonatal stress in rodents and ultimately in humans. Moreover, the molecular and cellular outcomes of such therapeutic options applied to rodents in vivo could be validated using our in vitro assay.

The recent discovery of many copy number variants (e.g., 22q11.2), which are robustly associated with schizophrenia and autism, as well as mood and anxiety disorders, has made it possible to establish reliable genetic mouse models of these variants (57,58). Given that these genetic variants do not show complete penetrance, genetic and environmental modifiers are likely to contribute to variability. Adult neurogenesis in the hippocampus is a potential intermediate substrate, which is altered by many environmental factors (e.g., environmental enrichment) (59,60), in addition to stress. Our in vitro assay protocol provides novel technical methods to elucidate epigenetic and molecular mechanisms underlying the impact of environmental factors on adult neurogenesis and behavioral phenotypes relevant to neuropsychiatric disorders.

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ARTICLE INFORMATION

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Research report

Does temperature or sunshine mediate the effect of latitude on affective temperaments? A study of 5 regions in Japan

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ABSTRACT

Background: Previously, we compared the hyperthymic scores of residents in Sapporo, Koshigaya, and Oita (which are located at latitudes of 43°N, 36°N, and 33°N in Japan, respectively) using the Temperament Evaluation of Memphis, Pisa, Paris and San Diego-auto questionnaire version (TEMPS-A). We found that residents who lived at lower latitudes had higher hyperthymic temperament scores; however, the mechanism of the effect of latitude on hyperthymic temperament remained unclear. The current study examined the mediators of the latitude effect in additional regions with different annual temperatures and amounts of ambient sunshine.

Methods: The Japanese archipelago stretches over 4000 km from north to south and has four large islands: Hokkaido, Honshu, Shikoku, and Kyushu. In addition to the TEMPS-A previously reported data collected at Sapporo (latitude 43°N), Koshigaya (36°N), and Oita (33°N), we collected the TEMPS-A data of 189 and 106 residents from Takaoka (36°N) and Obihiro (42°N), respectively. Taken together, these five regions have different patterns (i.e., highs and lows) of annual ambient total sunshine (hours) and mean temperature (°C). The effect of latitude, sunshine, and temperature on affective temperaments was analyzed for five Japanese regions.

Results: Multiple regression analyses revealed that latitude predicted significant variance in hyperthymic temperament. Ambient temperature, but not sunshine, significantly affected hyperthymic temperament.

Limitations: The light exposure that residents actually received was not measured. The number of regions studied was limited. The findings might not generalize to residents across Japan or other countries.

Conclusions: The present findings suggest that latitude affects hyperthymic temperament, and ambient temperature might mediate this effect.

1. Introduction

People with hyperthymic temperaments display extroversion, a high energy level, emotional intensity and little need for sleep; furthermore, this temperament is an adaptive and desirable condition outside of the boundaries of mood disorders (Rovai et al., 2013). Conversely, four additional affective temperaments, cyclothymic, depressive, irritable and anxious (Akiskal et al., 2005), are more closely related to mood, anxiety and substance use disorders. Moreover, they imply difficulties in adapting emotionally and behaviorally to somatic diseases and life stressors (Rovai et al., 2013). A gap between the hyperthymic temperament and other four is associated with stress vulnerability and experiences of childhood abuse (Nakai et al., 2014; Rovai et al., 2013; Sakai et al., 2005). However, other factors that explain this gap have not been well clarified.

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Within the series of our studies, Kohno et al. (2012) reported that latitude has a significant effect on the hyperthymic temperament (but not other four) among residents living in two regions. Lower and higher latitudes are associated with higher and lower hyperthymic temperament scores, respectively. This interesting "latitude effect" on affective temperaments was recently confirmed by our subsequent extension study of three regions with different latitudes that indicated a dose–response relationship between hyperthymic temperament and sunshine and latitude (Kohno et al., 2014) (our unpublished data). Hoaki et al. (2011) reported that more hyperthymic participants receive more light. In addition, more hyperthymic participants prefer brightness and dislike darkness compared with less hyperthymic participants, suggesting the presence of heliotropism (Harada et al., 2013). These findings suggest that the sunshine mediates the positive effect of latitude on the hyperthymic temperament. However, another possibility should be considered: because latitude is generally correlated with ambient temperature in Japan (despite certain exceptions), temperature might mediate the effect of latitude on the hyperthymic temperament.

To determine which factor, sunshine or ambient temperature, mediates the effect of latitude on the hyperthymic temperament, it is useful to examine the hyperthymic temperament scores of residents living in regions with various combinations of higher or lower temperatures and more or less sunshine. The present study used multiple regression analyses to investigate affective temperaments (including the hyperthymic temperament) across five regions with various combinations of ambient temperament and sunshine (see Fig. 1).

2. Subjects and methods

2.1. Subjects

Totals of 189 and 106 medical personnel from Kouseiren Takaoka Hospital in Takaoka and National Hospital Organization Obihiro Hospital in Obihiro, respectively, volunteered for the present study. Their data were combined with those of 94 medical students and personnel from Hokkaido University in Sapporo, 95 medical students and personnel from Oita University in Oita, and 125 medical students and personnel from Juntendo Koshigaya Hospital in Koshigaya (Kohno et al., 2014). The data of these 609 participants were collected, and their mean age was 33.4 years ± 9.1 (SD). There were 270 males and 339 females. All participants were screened for present and past psychiatric disorders, and no participants suffered from psychiatric disorders. Their demographic data are shown in Table 1. Written informed consent was obtained from all participants, and the 3 universities and 2 hospital ethical committees approved the study.

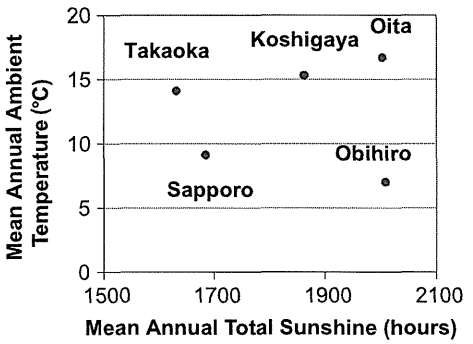

Fig. 1. The relationship between the annual means of total sunshine and ambient temperature for the 5 regions.

Japan has 4 large islands that extend north to south: Hokkaido, Honshu, Shikoku, and Kyushu. The northernmost island, Hokkaido, and its cities Sapporo and Obihiro are located at latitude of 43°N and 42°N respectively, whereas Oita of the southern island Kyushu is at 33°N. Honshu, whose cities Koshigaya and Takaoka are located at 36°N, is located between Hokkaido and Kyusyu. When three cities, Sapporo, Koshigaya, and Oita, were compared in our previous study (Kohno et al., 2014) (our unpublished data), lower latitudes were associated with higher temperatures and more sunshine; however, it was impossible to analyze the effect of sunshine and temperature as the predictors of hyperthymic temperament scores because sunshine and temperature are strongly correlated with each other (r=0.95), thereby causing multicollinearity. To avoid this problem in the current study, 2 regions with different sunshine and temperature data (Obihiro and Takaoka) were added so that sunshine and temperature would be weakly correlated across the 5 regions of this study.

2.2. Temperament assessment

The participants completed the Japanese version of the Temperament Evaluation of Memphis, Pisa, Paris and San Diego-auto questionnaire (TEMPS-A). This 110-item true–false questionnaire measures the following temperament dimensions: depressive, cyclothymic, hyperthymic, irritable and anxious (Akiskal et al., 2005; Matsumoto et al., 2005). TEMPS-A was translated into Japanese, and the reliability and validity of the Japanese version have been established (Akiyama et al., 2005; Kawamura et al., 2010; Matsumoto et al., 2005).

2.3. Illuminance and temperature

The mean annual total sunshine (in hours) was used as the illuminance parameter for Sapporo, Obihiro, Koshigaya, Takaoka, and Oita. The mean annual ambient temperatures of the 5 regions from 1993 to 2012 were denoted "temperature".

2.4. Data analysis

First, participant age, sunshine, and TEMPS-A scores were compared across Sapporo, Obihiro, Koshigaya, Takaoka, and Oita using a one-way analysis of variance (ANOVA). Post-hoc analyses were performed using Fisher's protected least significant difference (PLSD) test. The gender ratio was compared among the 5 cities using the Kruskal–Wallis test followed by the Steel–Dwaas test. Second, Pearson's correlation coefficients were used to examine the relationships among the 5 temperament scores, age, gender, temperature, sunshine, and latitude. Finally, a multiple regression analysis was used to identify the variables associated with hyperthymic scores based on TEMPS-A. Finally, multicollinearity was investigated.

3. Results

3.1. Age, gender, latitude, sunshine, and temperature

As Table 1 shows, significant differences were observed with regard to age and the gender ratio among the participants of the 5 regions. Obihiro and Oita had significantly more mean annual total sunshine than Sapporo, Koshigaya, and Takaoka, and Koshigaya had significantly more sunshine than Sapporo and Takaoka. Based on mean annual ambient temperature, the warmest to the coldest cities were Oita, Koshigaya, Takaoka, Sapporo, and Obihiro. The relationship between mean annual total sunshine and mean ambient temperature is illustrated in Fig. 1. Obihiro had more

Table 1
Participant demographics and the annual means of climatic variables from 1993 to 2012 for Sapporo, Obihiro, Koshigaya, Takaoka, and Oita.

Variables	Sapporo (a)	Obihiro (b)	Koshigaya (c)	Takaoka (d)	Oita (e)	*F* (ANOVA) or *H* (Kruskal–Wallis) value	#Post-hoc comparison
N	94	106	125	189	95		
Age	29.4 ± 4.9	32.6 ± 7.4	36.6 ± 9.4	37.1 ± 9.9	26.8 ± 5.8	35.9 (*F*)***	*a vs e **a vs b, c, d; b vs c, d, e; c vs e; d vs e
Gender (M/F)	66/28	48/58	42/83	47/142	67/28	86.7 (*H*)***	**a vs b, c, d; b vs d, e; c vs e; d vs e
TEMPS-A scores							
Depressive	5.8 ± 3.4	7.3 ± 3.2	7.3 ± 3.1	7.9 ± 3.8	6.8 ± 3.2	6.3 (*F*)***	**a vs b, c, d; d vs e
Cyclothymic	3.3 ± 3.8	3.9 ± 3.6	3.8 ± 3.1	5.0 ± 3.9	4.4 ± 3.5	4.0 (*F*)**	*a vs e; b vs d **a vs d; c vs d
Hyperthymic	3.8 ± 3.2	3.5 ± 2.8	3.8 ± 2.9	4.3 ± 3.5	5.0 ± 3.9	3.2 (*F*)*	*a vs e; **b vs e; c vs e
Irritable	2.7 ± 3.7	2.7 ± 2.9	2.3 ± 2.4	3.1 ± 3.3	3.0 ± 3.0	1.6 (*F*)	
Anxious	3.9 ± 4.2	4.7 ± 4.4	4.8 ± 3.8	5.2 ± 4.6	4.5 ± 3.7	1.5 (*F*)	
Latitude	43°N	42°N	36°N	36°N	33°N		
Sunshine (h)	1684.6 ± 98.4	2008.4 ± 104.5	1862.9 ± 189.9	1631.3 ± 121.3	2002.9 ± 127.9	35.1 (*F*)***	**a vs b, c, e; b vs c, d; c vs d, e; d vs e
Temperature (°C)	9.1 ± 0.4	7.0 ± 0.5	15.3 ± 0.5	14.1 ± 0.5	16.7 ± 0.5	1527 (*F*)***	**a vs b, c, d, e; b vs c, d, e; c vs d, e; d vs e

Mean ± SD.
Fisher's PLSD test after ANOVA or Steel–Dwaas test after the Kruskal–Wallis test among Sapporo (a), Obihiro (b), Koshigaya (c), Takaoka (d), and Oita (e).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Pearson's correlations among age, gender, the 5 temperaments, sunshine, temperature, and latitude for 609 participants.

	Age	Gender	Dep	Cyc	Hyp	Irr	Anx	Sunshine	Temperature	Latitude
Age	1	−0.09	0.09*	−0.09*	0.06	−0.04	0.03	−0.22***	0.07	−0.07
Gender		1	−0.10*	−0.08*	0.08*	0.01	−0.04	0.16***	− 0.08	0.10*
Dep			1	0.42***	−0.07	0.39***	0.54***	−0.04	0.07	−0.10*
Cyc				1	0.24***	0.63***	0.57***	−0.06	0.08*	−0.10**
Hyp					1	0.23***	0.04	0.00	0.10*	−0.11**
Irr						1	0.57***	−0.03	0.02	−0.03
Anx							1	−0.02	0.04	−0.05
Sunshine								1	− 0.10*	−0.03
Temperature									1	−0.97***
Latitude										1

Dep: depressive temperament, Cyc: cyclothymic temperament, Hyp: hyperthymic temperament, Irr: irritable temperament, Anx: anxious temperament, Gender: female=1, male=2.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

sunshine than Sapporo even though Obihiro and Sapporo are located at almost the same latitude and had similar mean annual ambient temperatures. Takaoka had less sunshine than Koshigaya even though Takaoka and Koshigaya are located at the same latitude and had similar mean annual ambient temperatures.

3.2. Correlations among age, gender, the 5 temperaments, sunshine, temperature, and latitude

As Table 2 shows, several significant associations were observed among the affective temperaments. Moreover, depressive, cyclothymic and hyperthymic temperament scores were significantly and negatively associated with latitude. Latitude was strongly and negatively associated with temperature but not with sunshine when comparing the 5 regions investigated in this study; however, temperature and sunshine were significantly and negatively associated with each other. Temperature, but not sunshine, was positively associated with cyclothymic and hyperthymic temperament scores.

3.3. Multiple regression analyses

Table 3 shows the results of the multiple regression analyses where individual temperament score was the dependent variable and the other 4 temperament scores, age, gender (female=1, male=2), and latitude were independent variables. Only the hyperthymic temperament scores among the 5 affective temperaments were significantly and negatively independently associated with latitude.

When both sunshine and temperature were included as the independent variables in place of latitude in a multiple regression analysis, hyperthymic temperament scores were significantly and positively associated with cyclothymic and irritable temperament scores, age, gender, and ambient temperature as well as significantly and negatively independently associated with depressive and anxious temperament scores (Table 4). According to these datasets, hyperthymic temperament scores were not significantly associated with sunshine.

Multicollinearity was not found in these multiple regression analyses.

Table 3
Multiple regression analysis using the 5 affective temperaments for 609 participants.

Independent variable	Dependent variable				
	Dep	Cyc	Hyp	Irr	Anx
Dep	n.t.	0.14***	−0.21***	0.08*	0.29***
Cyc	0.19***	n.t.	0.29***	0.13***	−0.09**
Hyp	−0.16***	0.17***	n.t.	0.40***	0.29***
Irr	0.10*	0.38***	0.21***	n.t.	0.29***
Anx	0.37***	0.27***	−0.14**	0.30***	n.t.
Age	0.10**	−0.12***	0.12**	−0.01	0.05
Gender	−0.04	−0.08**	0.10**	0.05	0.03
Latitude	−0.06	−0.05	−0.11**	0.04	0.005
ANOVA	$F=45.9$***	$F=92.2$***	$F=16.1$***	$F=80.0$***	$F=81.3$***
R^2 (adjusted R^2)	0.35 (0.34)	0.52 (0.51)	0.16 (0.15)	0.48 (0.48)	0.49 (0.48)

Beta values (standardized partial regression coefficients) are presented except in cases of F values and R^2 (adjusted R^2).
Dep: depressive temperament, Cyc: cyclothymic temperament, Hyp: hyperthymic temperament, Irr: irritable temperament, Anx: anxious temperament, Gender: female=1, male=2, n.t.: not tested.

* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

Table 4
Multiple regression analysis of hyperthymic temperament scores for 609 participants.

Independent variable	Beta	p
Depressive temperament	−0.21	<0.0001
Cyclothymic temperament	0.29	<0.0001
Irritable temperament	0.21	<0.0001
Anxious temperament	−0.15	0.005
Age	0.13	0.001
Gender (female=1, male=2)	0.09	0.018
Sunshine (h)	0.04	0.37
Temperature (°C)	0.10	0.01
ANOVA	$F=13.9$	<0.0001
R^2 (adjusted R^2)	0.16 (0.15)	

Beta values (standardized partial regression coefficients) are presented except in cases of F values and R^2 (adjusted R^2).

4. Discussion

The major findings of the present study are that latitude predicted significant variance in hyperthymic temperament via multiple regression analyses. Participants who live in regions at lower latitudes showed higher hyperthymic temperament scores. This finding confirmed the results of our previous study that compared the hyperthymic temperament scores of residents from 2 regions in Japan suggesting that lower latitude induces a hyperthymic temperament (Kohno et al., 2012). Of the two possible mediators of the effect of latitude on hyperthymic temperament scores (sunshine and temperature), temperature predicted significant variance in hyperthymic temperaments of the participants of this study. Thus, this study extends our previous findings and suggests a possible mechanism of the latitude effect on hyperthymic temperament.

To the best of our knowledge, no prior study has found an association between ambient temperature and hyperthymic temperament. Interestingly, Gonda et al. (2011) found significant differences among 6 cultural and national samples with regard to the hyperthymic temperament. The frequencies of participants with dominant hyperthymic temperament from high to low lived in Portugal (3.3%), Hungary (3.0%), Korea (2.8%), Germany (2.1%), Argentina (0.2%), and Lebanon (0%). These international differences suggest that uncertainty avoidance is involved in the hyperthymic temperament. However, this study did not examine the associations of other geographical factors (Gonda et al., 2011). Although lower hyperthymic temperaments tended to be associated with lower latitudes in their study (unlike the current study), factors such as culture, gender and age might also affect this tendency. Comparisons of hyperthymic temperaments within the same ethnic group among regions with a wider range of latitudes will be necessary for future studies.

Although the current study found a negative relationship between sunshine and hyperthymic temperament, our previous study suggested the presence of a dose-dependent effect of light (ambient sunshine) and latitude on hyperthymic temperament among residents of 3 regions at different latitudes (Kohno et al., 2014) (our unpublished data). Different datasets might account for this discrepancy regarding the role of sunshine between our present and previous studies. On the other hand, the low ambient temperatures in Sapporo and Obihiro might preclude residents from going outside and receiving sunshine, especially during cold winters. Thus, the light exposure that residents actually receive (rather than ambient sunshine) should be measured in a study of the effect of light on hyperthymic temperament in future studies, representing a limitation of the current study. This study cannot deny the possibility that illuminance affects the hyperthymic temperament. Ambient light exposure is most likely associated with mood, mood disorder, or both (reference citation in Kohno et al. (2014)). More importantly, Hoaki et al. (2011) reported that the greater illuminance of daytime independently predicted higher hyperthymic temperament scores via a multiple regression analysis. That study measured the illuminance that each participant received using actigraphy.

Another limitation is the limited number of regions studied: our findings might not generalize to participants across Japan or other countries.

In conclusion, the present findings suggest that latitude affects the hyperthymic temperament, and ambient temperature might mediate this effect.

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Conflict of interest

All authors declare that they have no conflicts of interest that are relevant to the subject of this article.

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Behavioural pharmacology

Subchronic lithium treatment increases the anxiolytic-like effect of mirtazapine on the expression of contextual conditioned fear

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ABSTRACT

Lithium not only has a mood-stabilizing effect but also the augmentation effect of an antidepressant, the mechanism of which remains unclear. Although lithium may augment the effect of mirtazapine, this augmentation has not been confirmed. Using a contextual fear conditioning test in rats, an animal model of anxiety or fear, we examined the effect of subchronic lithium carbonate (in diet) in combination with systemic mirtazapine on the expression of contextual conditioned fear. Mirtazapine (10 mg/kg) reduced freezing one day after fear conditioning dose-dependently, whereas the anxiolytic-like effect of mirtazapine (10 mg/kg) diminished seven days after fear conditioning. When the interval between fear conditioning and testing was seven days, only the combination of subchronic 0.2% Li_2CO_3 but not 0.05% Li_2CO_3 with acute mirtazapine (10 mg/kg) reduced freezing significantly. These results indicate that subchronic 0.2% Li_2CO_3 treatment enhanced the anxiolytic-like effect of systemic mirtazapine. This augmentation therapy might be useful for the treatment of anxiety disorders.

1. Introduction

Most patients have a favorable response to antidepressant drugs for anxiety and depressive disorders, but approximately 30% to 40% do not respond adequately to first-line antidepressant medication (Kornstein and Schneider, 2001; Zamorski and Albucher, 2002). Recently, many augmentation strategies have been developed to increase the effectiveness of antidepressant drugs. One such approach used in the treatment of psychiatric disorders is the addition of lithium to antidepressant drugs (Bauer et al., 2010; Chenu and Bourin, 2006).

Preclinical and clinical studies have demonstrated that lithium modifies serotonergic neurotransmission and increases extracellular serotonin (5-hydroxytryptamine; 5-HT) levels in the brain through several mechanisms, such as increased 5-HT synthesis, increased 5-HT turnover, and increased 5-HT release from nerve endings (Eroglu and Hizal, 1987; Kitaichi et al., 2004, 2006; Muraki et al., 2001; Price et al., 1990; Wegener et al., 2003). Preclinically, the combinations of lithium with selective serotonin reuptake inhibitors (SSRIs) or monoamine oxidase inhibitors have been reported to increase the effects of these antidepressants on extracellular 5-HT concentrations and on anxiety-like behaviors in the contextual fear conditioning test (Kitaichi et al., 2006; Muraki et al., 1999, 2001). Therefore, these findings indicate that lithium augmentation of the antidepressant effect may occur via a direct and/or indirect effect on 5-HT.

Recent clinical evidence has shown that mirtazapine is effective in the treatment of anxiety disorders as well as in depressive disorders (Davidson et al., 2003; Gambi et al., 2005). In addition, animal behavioral studies have shown that systemic administration and local administration to the median raphe nucleus of mirtazapine decreased contextual conditioned freezing behavior, an index of fear or anxiety (An et al., 2013; Kakui et al., 2009). An in vivo microdialysis study reported that mirtazapine increased extracellular 5-HT concentrations in the hippocampus of rats (Yamauchi et al., 2012). These results suggest that the anxiolytic and antidepressant effects of mirtazapine might be mediated by the facilitation of central 5-HT neurotransmission.

Although a few clinical studies have shown that the combination of mirtazapine and lithium is safe and well-tolerated and might be more efficacious (Bruijn et al., 1998; Sitsen et al., 2000), to the best of our knowledge, the behavioral effects of adding lithium to mirtazapine have not yet been examined. Based on the mechanism of the anxiolytic actions of lithium and mirtazapine described above, we hypothesized that the combination of lithium and mirtazapine would have a superior effect on anxiety-like behavior as measured in the contextual fear conditioning test. As described above, a number of

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studies have demonstrated the reliability of the use of the contextual fear conditioning test as a behavioral paradigm to show the anxiolytic-like effect of mirtazapine, other serotonergic anxiolytics/antidepressants and lithium augmentation and to clarify the mechanisms of drug interactions that are mediated by serotonin (Inoue et al., 2011). To verify the above hypothesis, the present study assessed the anxiolytic-like effect of the combination of the subchronic lithium and systemic mirtazapine treatment in rats using the contextual fear conditioning test as an animal model of fear and anxiety.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 260–320 g at the beginning of the experiments were used. The rats were housed in polypropylene cages (four animals per cage) with wood shavings on the floor. The room temperature was kept at 22 ± 2 °C. The subjects were maintained on a 12-h light/dark cycle (light phase: 06:30–18:30). The experiments began after a two-week period of acclimatization. The animals were maintained on a diet of standard laboratory rat chow or rat chow containing 0.05% or 0.2% of Li_2CO_3 for seven days. In the lithium experiments, the lithium-treated rats and the control rats were given 10 mM NaCl instead of tap water to prevent lithium-induced hyponatremia (Thomsen and Olesen, 1974). The rest of the time, all animals had free access to food and water. All experiments were performed between 08:00 and 13:00. All procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

2.2. Drug

Mirtazapine (obtained from Merck & Co. Inc., Whitehouse Station, NJ, U.S.A.) was suspended in 0.15% tartaric acid. The vehicle alone was administered as a control. Mirtazapine was injected intraperitoneally (i.p.) in a volume of 2 ml/kg.

The doses of mirtazapine used in this study were based on previous reports (Kakui et al., 2009; the company's drug information). The dose of mirtazapine (10 mg/kg) used in this study produces plasma mirtazapine concentrations that are comparable to or greater than the clinically therapeutic plasma concentrations observed after administration of the clinically maximal dose (45 mg/day) of mirtazapine (company's drug information). Accordingly, 10 mg/kg of mirtazapine in rats approximately corresponds to a clinically maximal dose.

The concentrations of lithium carbonate in the rat chow were chosen based on our previous study in which we found that plasma lithium levels are 0.71 ± 0.05 mEq/l after 1 week of 0.2% Li_2CO_3 treatment and 0.26 ± 0.01 mEq/l after 1 week of 0.05% Li_2CO_3 treatment (Muraki et al., 1999). The plasma levels of lithium after 1 week of 0.2% Li_2CO_3 treatments are within the recommended therapeutic range (0.5–1.2 mEq/l) (Suppes et al., 2008: Bauer et al., 2010).

2.3. Contextual conditioned fear stress model

In this study, rats were individually subjected to a total of 2.5 min of inescapable electric footshocks [five footshocks (2.5 mA scrambled footshocks, pulse wave, 30-s duration) that were delivered at intershock intervals from 35 to 85 s (mean 60 s)] in a shock chamber with a grid floor (19 × 22 × 20 cm^3, Medical Agent, Kyoto, Japan). Electric shocks were produced by a Model SGS-02D Shock Generator (Medical Agent). This generator provides a circuit with resistance controlled by dial settings calibrated by the manufacturer in a short circuit current. At the setting of 2.5 mA, this generator delivered a 0.2-mA shock intensity to the rats. One or seven days after the electric footshock, the rats were again placed in the shock chamber and observed for 5 min, but no current was applied to the floor of the chamber. The behavior was video taped and scored later by human observation. During the observation period, the duration of the freezing behavior was recorded using a modified time-sampling procedure as previously described (An et al., 2013). Every 10 s, the behavior in which the animal was currently engaged was classified as either "freezing" or "activity". Freezing was defined as the absence of any observable movement of the skeleton and the vibrissae with the exceptions of those related to respiration. All other behaviors were scored as activity. The animal was classified as either freezing or active according to its behavior throughout the entire 10-s period. We observed the rats for successive 10-s periods over 5 min (i.e., 30 successive sampling periods). If a rat exhibited any activity during the 10-s sampling period, we considered that period as active. The percentage freezing score [freezing (%)] was computed as the proportion of the 10-s periods during which the animal remained frozen for the entire period.

2.4. Experimental design

2.4.1. Effect of acute systemic mirtazapine treatment on the expression of contextual conditioned freezing: a dose–response study and a study with different intervals between fear conditioning and exposure to conditioned fear

The rats were subjected to inescapable electric footshocks in a chamber with a grid floor. In the dose–response study, 24 h after the footshock, the rats received a single intraperitoneal injection of mirtazapine at doses of 0, 1, 3 and 10 mg/kg 30 min before testing. In the study with different intervals, one and seven days after the footshock, the rats received a single intraperitoneal injection of mirtazapine at 10 mg/kg 30 min before testing.

2.4.2. Effect of subchronic lithium with acute systemic mirtazapine treatment on the expression of contextual conditioned freezing

Immediately after the footshock, the rats received standard laboratory rat chow (0% Li_2CO_3) or rat chow containing 0.05% or 0.2% of Li_2CO_3 for 7 days. On the eighth day, the rats received a single intraperitoneal injection of mirtazapine at 10 mg/kg 30 min before testing.

2.5. Motor activity

Motor activity was measured for mirtazapine (10 mg/kg) with or without subchronic 0.2% Li_2CO_3 treatment in unshocked rats. The rats were housed individually for three days before testing. During the testing, rats were individually placed in a testing cage (38 × 33 × 17 cm^3), and motor activity was automatically recorded as described previously (Ohmori et al., 1994) using infrared sensors between 08:00 and 13:00. Mirtazapine was administered i.p. at 30 min before testing for 5 min. Horizontal movement was digitized and uploaded to a computer. Locomotion was responsible for most of the count, though other body movements also contributed when they included a substantial horizontal component.

2.6. Data analysis

All the data are presented as the means ± S.E.M. of the individual values of the rats from each group. The statistical analyses of the data were performed using one- and two-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons as a post-hoc test when the interaction was significant. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of acute mirtazapine treatment on the expression of contextual conditioned freezing: a dose–response study

Acute mirtazapine caused a dose-dependent reduction in freezing (Fig. 1). The higher doses of mirtazapine, 3 mg/kg ($P<0.05$) and 10 mg/kg ($P<0.05$), reduced the expression of contextual conditioned freezing significantly, while a lower dose, 1 mg/kg, showed no significant effect compared with the vehicle ($P=0.27$).

3.2. Effect of different intervals between fear conditioning and exposure to contextual conditioned fear on acute inhibition of conditioned freezing by mirtazapine

Two-way ANOVA revealed significant main effects of the interval and acute challenge of mirtazapine and a significant interaction [Effect of interval: $F(1,28)=4.62$, $P<0.05$; Effect of acute challenge of mirtazapine: $F(1,28)=8.39$, $P<0.05$; Effect of interaction: $F(1, 28)=6.81$, $P<0.05$] (Fig. 2). One day after the footshock, an acute challenge of mirtazapine (10 mg/kg) significantly reduced the expression of contextual conditioned freezing compared with the respective vehicle group ($P<0.01$). However, mirtazapine (10 mg/kg) did not affect contextual conditioned freezing compared with the respective vehicle group when the rats were exposed to conditioned fear seven days after footshock.

3.3. Effect of subchronic lithium with acute mirtazapine (10 mg/kg) treatment on the expression of contextual conditioned freezing

Subchronic treatment with 0.2% Li_2CO_3 significantly enhanced the effect of mirtazapine (10 mg/kg) on the expression of contextual conditioned freezing (Fig. 3A). Two-way ANOVA revealed significant main effects of mirtazapine and 0.2% Li_2CO_3 on freezing behavior. In addition, a significant interaction between mirtazapine and 0.2% Li_2CO_3 was identified [Effect of mirtazapine: $F(1,28)=5.62$, $P<0.05$; Effect of 0.2% Li_2CO_3: $F(1,28)=6.24$, $P<0.05$; Effect of interaction: $F(1,28)=5.82$, $P<0.05$]. Post-hoc analysis showed that the mirtazapine–0% Li_2CO_3 or vehicle–0.2% Li_2CO_3 treatments had no significant effect on freezing behavior compared with the untreated group, while the mirtazapine–0.2% Li_2CO_3 treatment significantly reduced freezing compared with the vehicle–0% Li_2CO_3 ($P<0.01$), mirtazapine–0% Li_2CO_3 ($P<0.01$) and vehicle–0.2% Li_2CO_3 ($P<0.01$) groups.

In contrast to the effect of subchronic treatment with 0.2% Li_2CO_3, subchronic treatment with 0.05% Li_2CO_3 did not change the inhibitory effect of mirtazapine on the expression of contextual conditioned freezing (Fig. 3B). Two-way ANOVA revealed no significant effects of mirtazapine or 0.05% Li_2CO_3 and no significant interaction [Effect of mirtazapine: $F(1,28)=0.002$, $P=0.97$; Effect of 0.05% Li_2CO_3: $F(1, 28)=0.15$, $P=0.71$; Effect of interaction: $F(1, 28)=3.11$, $P=0.09$].

3.4. Motor activity

Both acute systemic mirtazapine (10 mg/kg) treatment alone and subchronic 0.2% Li_2CO_3 treatment alone failed to affect motor activity in the home cages. Furthermore, the combination of acute systemic mirtazapine (10 mg/kg) and subchronic 0.2% Li_2CO_3 also failed to affect motor activity in the home cages compared with the vehicle–0% Li_2CO_3 group (Table 1). Two-way ANOVA revealed no significant effect of mirtazapine or 0.2% Li_2CO_3, and there was no significant interaction [Effect of mirtazapine: $F(1, 28)=2.43$, $P=0.13$; Effect of 0.2% Li_2CO_3: $F(1, 28)=2.90$, $P=0.10$; Effect of interaction: $F(1, 28)=0.03$, $P=0.85$].

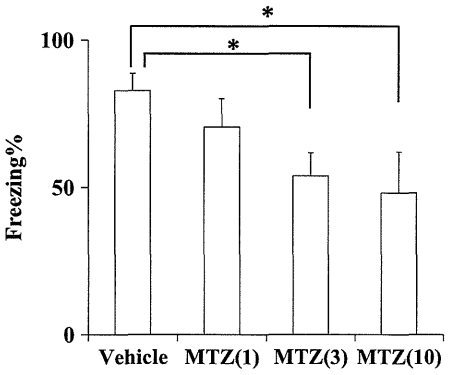

Fig. 1. Effect of acute mirtazapine (MTZ) treatment on the expression of contextual conditioned freezing. Mirtazapine was administered intraperitoneally 1 day after footshock and 30 min before testing. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled in 10-s intervals. *$P<0.05$. $N=8$–12 per group.

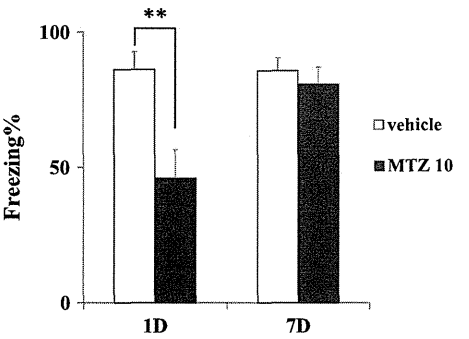

Fig. 2. Effect of acute mirtazapine treatment on the expression of contextual conditioned freezing with various intervals (1 and 7 days) between fear conditioning (footshock) and testing. Mirtazapine (MTZ, 10 mg/kg) or the vehicle was administered intraperitoneally 30 min before testing. Data are represented as the mean ± S.E.M of freezing scored for a 5-min observation period. Behavior was sampled in 10-s intervals. **$P<0.01$. $N=8$ per group. 1D, 1 day; 7D, 7 days.

4. Discussion

In this study, subchronic 0.2% Li_2CO_3 treatment in the diet for one week significantly enhanced the inhibitory effect of mirtazapine on contextual conditioned freezing. Moreover, subchronic 0.2% Li_2CO_3 treatment with mirtazapine did not affect motor activity compared with the vehicle controls, thereby excluding the possibility of nonspecific motor interference as the main factor accounting for its effect in the conditioned fear test. Because freezing behavior induced by contextual conditioned fear has been used as an animal model of anxiety or fear (Inoue et al., 2011), these results indicate that subchronic 0.2% Li_2CO_3 treatment enhanced the anxiolytic-like effect of acute mirtazapine.

Generally, it is believed that the serotonergic system is involved in the pathophysiology and treatment of anxiety disorders (Graeff et al., 1996). Moreover, increased 5-HT neurotransmission decreases contextual conditioned fear in animal experiments (Inoue et al., 2011). In vivo microdialysis studies reported that the systemic administration of mirtazapine increased extracellular 5-HT concentrations in the hippocampus of rats (Yamauchi et al., 2012). In addition, subchronic lithium treatment increased extracellular 5-HT levels in the medial prefrontal cortex and hippocampus (Kitaichi et al., 2004, 2006; Muraki et al., 2001; Wegener et al., 2003) and additively increased the elevating effect of SSRI and MAOI on extracellular 5-HT concentrations (Kitaichi et al., 2006; Muraki et al., 2001). Furthermore, subchronic lithium increased the anxiolytic-like effect of SSRI and MAOI in contextual conditioned fear (Kitaichi et al., 2006; Muraki