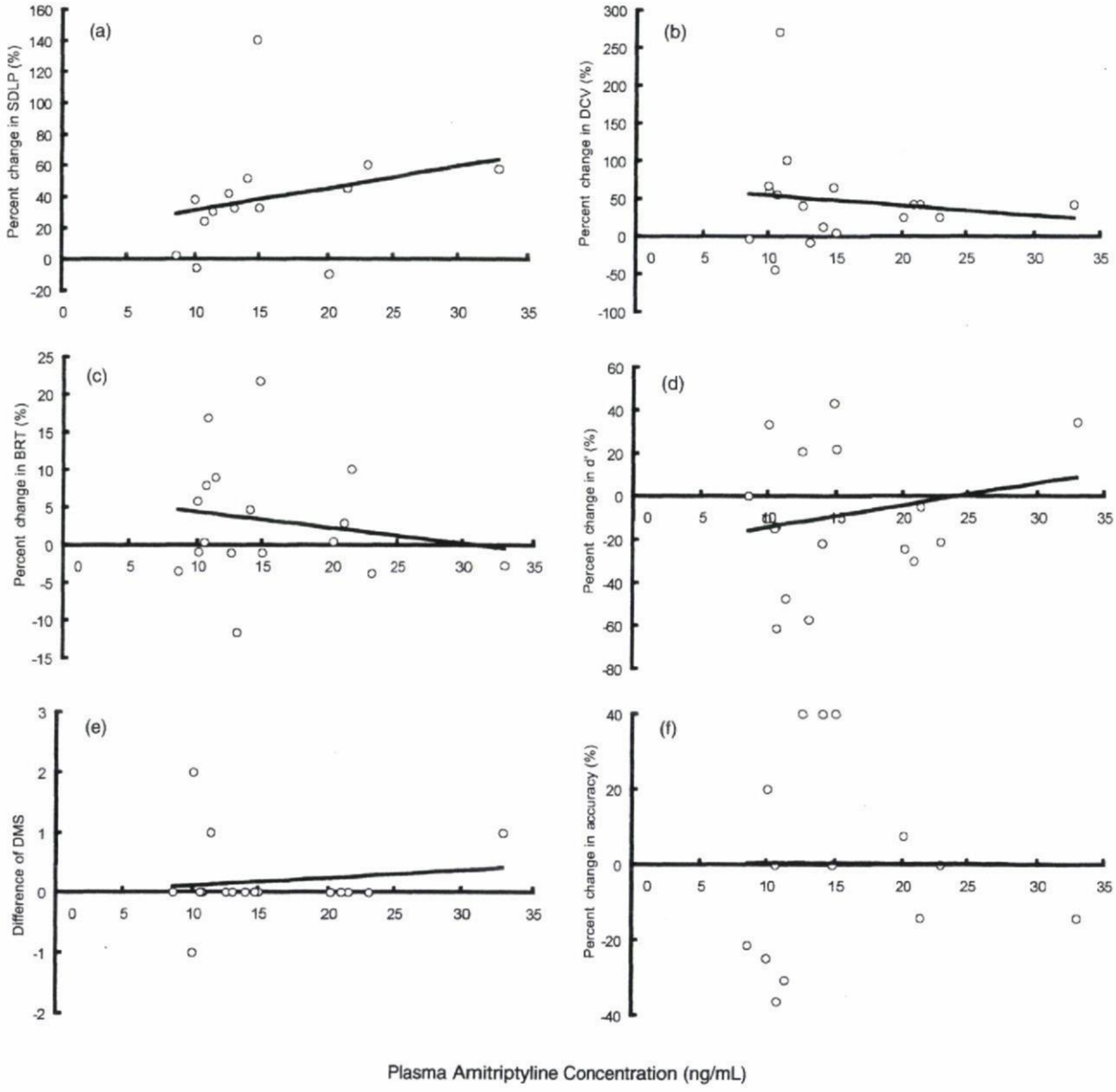

Figure 1. Relationship between plasma amitriptyline concentration and percent changes in the variables of driving performance, cognitive functions, and subjective somnolence. (Difference rather than percent change was used for (e) difficulty of maintaining set [DMS], because the baseline values of DMS can be 0 and hence, percent changes cannot be calculated.) (a) Percent change in standard deviation of the lateral position (SDLP; $r = 0.543$, $P = 0.045$); (b) percent change in distance coefficient of variation (DCV; $r = -0.110$, $P = 0.673$); (c) percent change in brake reaction time (BRT; $r = -0.163$, $P = 0.532$); (d) percent change in signal detection index d-prime (d') in the Continuous Performance Test ($r = 0.209$, $P = 0.420$); (e) difference of DMS in the Wisconsin Card-Sorting Test ($r = 0.132$, $P = 0.614$); (f) percent change in accuracy in the N-back test ($r = 0.260$, $P = 0.370$). Due to non-completion of the assigned task and technical malfunctions, three subjects were excluded from statistical analyses for SDLP and N-back test.

Effects of amitriptyline on driving performance, cognitive function, and subjective assessments

At 4 h after receiving the single dose of 25 mg amitriptyline, SDLP ($P = 0.003$), DCV ($P = 0.006$), CA ($P = 0.035$), and SSS score ($P = 0.0002$) were significantly impaired. The effect of amitriptyline on the remaining variables was not statistically significant. These data have been reported in our previous study.[20]

DISCUSSION

The present results demonstrated a significant linear correlation between plasma amitriptyline concentration and percent change in SDLP. Baseline SDLP was 38.9 ± 10.8 cm, and at 4 h it increased to 51.3 ± 12.7 cm. This increase of lateral swerving might lead to traffic accidents. The plasma amitriptyline concentration, however, did not show a significant relationship with (i) other driving performance parameters of DCV and BRT; (ii) cognitive functions measured using the WCST, CPT, and N-back test; or (iii) subjective somnolence, determined using the SSS.

In a previous study imipramine had a detrimental effect on driving performance measured as SDLP and caused slight cognitive impairment as assessed on a memory scanning test.[33] This memory test indicated that the plasma drug concentration significantly correlated with reaction time change but not with SDLP change. The present study found a significant correlation between plasma concentration of amitriptyline after a single dose and driving performance measured as SDLP. Amitriptyline may have a concentration-dependent detrimental effect on road-tracking ability. Therapeutic monitoring of amitriptyline would be useful for predicting the difficulties encountered while driving. The present results and those of the van Laar *et al.* study[33] do not agree, although both these studies used TCA. The methodological differences between the two studies might contribute to the discrepancy.

A previous review demonstrated that somnolence or sedation is the most important cause of driving impairment in patients treated with antidepressants.[10] In our previous simulator study we also confirmed a weak but significant association between the detrimental effects of antidepressants on driving performance and increased subjective somnolence.[20] In the present study an acute dose of 25 mg amitriptyline strongly increased the SSS scores, but no significant correlation was observed between plasma amitriptyline concentration and percent change in SSS scores. These values might be influenced by individual pharmacodynamic differences rather than individual pharmacokinetic differences. The same logic may be applied to the absence of correlations between plasma amitriptyline concentration and DCV and CA (WCST); therefore, further investigations should be conducted in this regard.

Several studies indicate cognitive impairments in major depression patients.[34–36] Richardson *et al.* reported that amitriptyline and fluoxetine showed equal clinical improvement but patients receiving amitriptyline did not perform as well on the verbal learning task.[37] The present results indicate that TCA including amitriptyline might affect recovered cognitive function, even though clinical depressive symptoms are successfully treated.

The present study has several limitations. First, it used a single, low dose of amitriptyline. Therefore, we could not investigate the steady state condition, in which amitriptyline and its active metabolites exert their influence. Second, the participants were limited to healthy adult male volunteers; therefore, women who are prone to develop depression and the elderly should be included in future studies. Third, the validity and sensitivity of the driving simulator used in the present study should be considered. Finally, we found a significant linear correlation between plasma amitriptyline concentration and percent change in SDLP, but it is necessary to investigate this relationship under clinical therapeutic dose and steady-state conditions.

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Social isolation rearing-induced impairment of the hippocampal neurogenesis is associated with deficits in spatial memory and emotion-related behaviors in juvenile mice

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Abstract

Experiences during brain development may influence the pathogenesis of developmental disorders. Thus, social isolation (SI) rearing after weaning is a useful animal model for studying the pathological mechanisms of such psychiatric diseases. In this study, we examined the effect of SI on neurogenesis in the hippocampal dentate gyrus (DG) relating to memory and emotion-related behaviors. When newly divided cells were labeled with 5-bromo-2′-deoxyuridine (BrdU) before SI, the number of BrdU-positive cells and the rate of differentiation into neurons were significantly decreased after 4-week SI compared with those in group-housed mice. Repeated treatment of fluoxetine prevented the SI-induced impairment of survival of newly divided cells and ameliorated spatial memory impairment and part of aggression in SI mice. Furthermore, we investigated the changes in gene expression in the DG of SI mice by using DNA microarray and real-time PCR. We finally found that SI reduced the expression of development-related genes *Nurr1* and *Npas4*. These findings suggest that communication in juvenile is important in the survival and differentiation of newly divided cells, which may be associated with memory and aggression, and raise the possibility that the reduced expression of *Nurr1* and/or *Npas4* may contribute to the impairment of neurogenesis and memory and aggression induced by SI.

Keywords: aggressive behavior, fluoxetine, learning and memory, neurogenesis, social isolation.
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Experiences during a critical period of brain development may affect structural and functional development and maturation of the brain (Mataga *et al.* 2004; Mirescu *et al.* 2004), and influence behavior including cognitive and emotional functions which could be attributable to the expression or exacerbation of developmental disorders (Castellanos and Tannock 2002). Rearing animals in isolation is a relevant paradigm to investigate the effect of early life stress and for understanding the pathogenesis of certain neurological and psychiatric diseases (Myhrer 1998; Whitaker-Azmitia *et al.* 2000). Behavioral changes induced by isolation rearing have been characterized, including

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Abbreviations used: 5-HT, 5-hydroxytryptamine; BrdU, 5-bromo-2′-deoxyuridine; DG, dentate gyrus; Flx, fluoxetine; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; GH, group-housed; NeuN, neuronal nuclei; Npas4, neuronal PAS domain 4;Nurr1, nuclear receptor subfamily 4, group A, member 2; PBS, phosphate-buffered saline; RT, reverse transcription; Sal, saline; SGZ, subgranular zone; SI, social isolation; SSRI, selective serotonin reuptake inhibitors.

enhanced locomotor activity under a novel environment (Wilkinson *et al.* 1994), aggressive behaviors (Wongwitdecha and Marsden 1996), and impairment of pre-pulse inhibition (Day-Wilson *et al.* 2006) and spatial learning in a water maze test (Lu *et al.* 2003).

The social environment in early life significantly influences not only the organization of behavior but also neurochemical development of the brain. For instance, dopamine and serotonin systems are affected by social isolation (SI) in the nucleus accumbens (Hall *et al.* 1998), prefrontal cortex (Heidbreder *et al.* 2000) and hippocampus (Muchimapura *et al.* 2003). The neuroanatomic consequences of isolation rearing include decreased spine density of pyramidal neurons in the prefrontal cortex and hippocampus (Silva-Gomez *et al.* 2003) and fewer hippocampal synapses (Varty *et al.* 1999).

Hippocampal development is affected by environmental factors, but the underlying mechanisms are unclear. Accumulating evidence has demonstrated that neurogenesis occurs in adults in certain brain areas such as the hippocampus, in which newly divided neurons play a role in physiological function (Lledo *et al.* 2006). Recent studies suggested that the impairment of adult neurogenesis is involved in the development and expression of neuropsychiatric disorders (Jacobs *et al.* 2000; Reif *et al.* 2006; Maeda *et al.* 2007). For instance, the genesis of stem-like cells in the dentate gyrus (DG) of the hippocampus is decreased in patients with schizophrenia, which may contribute to the pathogenesis of the disorder (Reif *et al.* 2006).

It has been demonstrated that some mood-stabilizing drugs and selective serotonin reuptake inhibitors (SSRI) enhance adult neurogenesis in the hippocampus, and the effect may contribute to their clinical effects (Santarelli *et al.* 2003; Encinas *et al.* 2006). For example, an SSRI fluoxetine (Flx) prescribed for depression and anxiety disorders including obsessive compulsive disorder and panic disorder is reported to enhance neurogenesis in the hippocampus (Santarelli *et al.* 2003; Encinas *et al.* 2006).

It is well known that some genes, such as *reelin* and *brain-derived neurotrophic factor* regulate the development and migration of newly divided cells (Polleux *et al.* 2002; Gong *et al.* 2007). They are supposed to be associated with cognitive deficits in mental disorders (Angelucci *et al.* 2005; Fatemi 2005). It remains to be determined whether gene expression in the hippocampus is affected by environmental stress (e.g., SI) in early life.

In the present study, to investigate the effects of early life stress on neurogenesis in the hippocampus and on cognitive function and emotion related-behaviors, mice after weaning were subjected to SI rearing for 4 weeks. In addition, we investigated the effect of Flx on SI-induced impairment of survival of newly divided cells in the hippocampus, and emotion related-behaviors and memory impairment. Finally, to identify the genes whose expression in the DG of hippocampus is affected by SI, we examined the changes in gene expression in SI mice by using DNA microarray and real-time reverse transcription (RT)-PCR.

Materials and methods

Animals

The Institute for Cancer Research mice (Japan SLC Inc., Hamamatsu, Japan) were purchased when they were 3 and 8 weeks old and used for the experiments. The study was completed exclusively with male mice, because estrogen in female mice affects memory and SI-induced emotion-related behaviors (Li *et al.* 2004; Starkey *et al.* 2007). They were housed under a standard 12-h light/dark cycle (light phase 8:45 AM–20:45 PM) at a constant temperature of 23 ± 1°C with free access to food and water throughout the experiments. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Kanazawa University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolation rearing

After 3 days of acclimatization, mice were randomly divided into two groups: SI rearing and group-housed (GH) rearing. Mice in the SI group were individually housed in wire-topped opaque polypropylene cages (20 cm× 12 cm× 10 cm) while other mice in the GH group continued to be housed under normal conditions (five per cage) in wire-topped clear plastic cages (34 cm× 22 cm× 15 cm). After the 4-week SI, mice were subjected to behavioral and histological analyses as described below. During the behavioral analysis, the housing conditions were maintained.

Drug administration

Both 5-Bromo-2′-deoxyuridine (BrdU) and Flx were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in saline. To label newly divided cells in the DG, BrdU (75 mg/kg) was injected intraperitoneally (i.p.) three times at 2 h intervals. Flx (10 mg/kg) or saline was administrated i.p. once a day for at least 2 weeks. Daily administration was started 2 weeks after SI and continued until the end of the study. During behavioral analysis, Flx was administrated 1 h and 30 min before water maze test and intruder-evoked aggressive test, respectively.

Immunohistochemistry

In histological analysis, mice were used without behavioral analyses. They were deeply anesthetized with diethyl ether at the indicated time and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Their brains were removed, post-fixed in the same fixative and then cryoprotected. Thick coronal brain sections of 30 μm were cut on a cryostat and mounted on slides. Every fifth section was collected between stereotaxic coordinates bregma −1.2 to 3.0 according to the brain atlas (Paxinos and Franklin 2004). Sections were treated overnight with 0.1% nonidet-40/0.01 M PBS (pH 7.2) at 4°C and

denatured in the microwave oven in 0.01 M citrate buffer (pH 6.0). After blocking in 10% goat serum/PBS with 0.1% nonident-40 for 30 min, BrdU-positive cells in the sections were detected using a BrdU labeling and detection kit 2 (Roche Diagnotics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

For double-staining of BrdU/neuronal nuclei (NeuN, neuronal marker) and BrdU/glial fibrillary acidic protein (GFAP, astroglial marker), sections were pre-treated with 1 M HCl for 30 min at 37°C, followed by 10 min in 0.1 M borate buffer and then washed in PBS before blocking. Rat anti-BrdU antibody (1 : 200; Abcam, Cambridge, UK), mouse anti-NeuN antibody (1 : 100; Chemicon, Temecula, CA, USA) and mouse-GFAP antibody (1 : 1500; Sigma-Aldrich) diluted in PBS containing 0.1% Triton X-100 and 5% goat serum was applied to sections which were then incubated overnight at 4°C and for 6 h at around 25°C. After washing in PBS, goat anti-rat Alexa 568 and anti-mouse Alexa 488 (1 : 1000; Invitrogen, Eugene, OR, USA) were added to sections for 2 h at room temperature.

Quantification of immunostaining cells

Every fifth section throughout the hippocampus (total 12 sections from each mouse) was processed for BrdU immunohistochemistry. All BrdU-labeled cells in the subgranular zone (SGZ), hilus and granule cell layer (GCL) were assessed using a light microscope (Axio Imager; Zeiss, Jene, Germany) and counted by an experimenter blinded to the code. To distinguish single cells within clusters, all counts were performed at 400 × magnification (objective; 40 ×). To obtain the total number of cells per DG, we multiplied the counted number of positive cells by five (Maeda *et al.* 2007).

Double-stained cells were quantified using a confocal laser scanning microscope (LSM 510; Zeiss). Each cell was analyzed along the entire '*z*' axis. Approximately 20 BrdU-positive cells in each mouse were randomly identified between five and six sections. Ratios of BrdU-positive cells co-labeled with NeuN or GFAP among BrdU-positive cells were determined.

Water maze test

After the 4-week SI, a water maze test was carried out as described previously (Jhoo *et al.* 2004; Miyamoto *et al.* 2005). Briefly, a pool (120 cm in diameter) was prepared, and the water temperature was maintained at 21–23°C. Swimming paths were analyzed by a computer system with a video camera (TAMRON, Saitama, Japan).

In the training trials, the platform (7 cm in diameter) was submerged 1 cm below the water surface. After reaching the platform, the mouse was allowed to remain on it for 20 s. If the mouse did not find the platform within 60 s, the trial was terminated and the animal was put on the platform for 20 s. After training trials for 6 days, mice were subjected to the probe test on day 7 where in they swam for 60 s in the pool without the platform. We measured the time spent in each quadrant of the pool as a measure of spatial memory. One hour after the probe test, to measure swimming ability or motivation, mice were subjected to the visible test in which the platform was marked with a flag that protruded 12 cm above the water surface to be highly visible, but in a new location. Three starting positions were used pseudo-randomly and each mouse was subjected to three trials per day in the training trials (day 1–6) and visible test (day 7). During training trials and the visible test, we measured both path length (swim distance) and escape latency as measures of performance.

Intruder-evoked aggressive test

We used 8-week-old male Institute for Cancer Research mice as intruders which have not shown aggressive behaviors against their peers. The day after the probe test in the water maze test (e.g., day 8), an intruder-evoked aggressive test was carried out as previously reported (Miczek and O'Donnell 1978). A resident mouse was habituated in a test cage (20 cm× 12 cm× 10 cm) for 10 min, and then an intruder mouse was put in the test cage. The investigating behavior performed by the resident mouse against the intruder was observed for 10 min. The frequency of attacking/biting and duration of aggression including attacking/biting, tail rattling, aggressive grooming, sideways posturing and pushing under were analyzed. The behavioral observation was made by the blinded experimenters.

Total RNA isolation for DNA microarray and real-time RT-PCR

Mice reared under GH and SI conditions for 3 days, 2 weeks, and 4 weeks were decapitated and their brains were removed. These mice had never been used for behavioral experiments. Brain slices including the hippocampus were made using brain matrix and the DG of the hippocampus was isolated using a dissecting microscope (AS ONE Co., Ltd., Osaka, Japan). Tissues from two animals and four animals were pooled as one sample for DNA microarray and real-time RT-PCR, respectively. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

DNA microarray and expression profiling

The purified total RNA was checked for quality using Bioanalyzer 2100 electropherograms (Agilent; Santa Clara, CA, USA) and used for expression profiling with GeneChip mouse genome 430 2.0 arrays (Affymetrix; Santa Clara, CA, USA) containing 45 101 probe sets, according to the protocol provided by the manufacturer. Briefly, 5 μg of total RNA was reverse-transcribed into double-stranded cDNA with a T7-Oligo (dT) primer. Labeled cRNA was synthesized in the presence of T7 RNA polymerase and biotin-labeled nucleotides fragmented by metal-induced hydrolysis and hybridized overnight to the array. Each array was washed, stained with streptavidin-coupled phycoerythrin and scanned by a GCS3000 laser scanner (Affymetrix).

The resulting expression profiles were pre-processed using the robust multi-array average of G+C content (GCRMA) algorithm from Bioconductor (http://www.bioconductor.org/) in the statistical programming language R (http://www.r-project.org/). The changes in gene expression between GH and SI groups were quantified using a general linear model in the limma package from Bioconductor. Scatter plots and hierarchical clustering analysis were carried out in SPOTFIRE 8.2.1 (TIBCO Software Inc., Palo Alto, CA, USA).

Quantitative analyses of *Nurr1* and *Npas4* mRNA by real-time RT-PCR

Total RNA isolated from the DG was converted into cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Levels of mRNA expression were quantified by using a 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The quantitative real-time PCR was performed in a volume of 25 μL with 500 ng of cDNA and 500 nM primers in the Power SYBR Green Master Mix (Applied Biosystems). The primers used were as follows: 5′-ATGACCAGCCTGGACTATTCC-3′ (forward) and 5′-CAGGAGATCGTAGAACTGCTGGA-3′ (reverse) for *Nurr*1 and

5′-AGCATTCCAGGCTCATCTGAA-3′ (forward) and 5′-GGCGA AGTAAGTCTTGGTAGGATT-3′ (reverse) for *Npas*4. The mouse glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (Applied Biosystems).

Statistical analysis

All data were expressed as the mean ± SE. Differences between two groups were analyzed by two-tailed Student's *t*-test or chi-square (χ^2) test. Differences among multigroups were analyzed by ANOVA followed by the Bonferroni's test when F ratios were significant ($p < 0.05$). Differences among multigroups of path length in the water maze test were analyzed by ANOVA with repeated measures, followed by the Bonferroni's test when F ratios were significant ($p < 0.05$).

Results

Effect of SI after weaning on newly divided cell proliferation in the DG of the hippocampus

To examine the effect of SI after weaning on newly divided cell proliferation in the hippocampus, BrdU was injected on the last day of the 4-week isolation and the number of BrdU-labeled cells was counted 24 h after the injection (Fig. 1a). As shown in Fig. 1b, BrdU-positive cells in SI and GH mice were observed as clusters, and there were no apparent differences in the morphology and location. Most of the BrdU-labeled cells were found in the SGZ of the DG in both GH and SI mice (Fig. 1b). There was no significant difference in the number of BrdU-labeled cells in the hippocampus between SI and GH mice (Fig. 1c).

Effect of SI after weaning on the cell survival of newly divided cells in the DG of hippocampus

Next, to investigate the effect of SI after weaning on the survival of newly divided cells, BrdU was injected one day before starting the SI and the number of BrdU-labeled cells in the hippocampus was counted after the 4-week SI (Fig. 2a). As shown in Fig. 2b, there was an apparent difference between SI and GH mice in the number and location of BrdU-labeled cells in the DG of hippocampus. Some of the BrdU-labeled cells in GH mice were found in the GCL of DG whereas in SI mice, fewer cells were detected in the GCL. The total number of BrdU-labeled cells in the hilus + SGZ + GCL of SI mice was 75% of that in GH mice, and the number of BrdU-labeled cells in the GCL of SI mice was 63% of that in GH mice. The number of BrdU-labeled cells in the hilus + SGZ + GCL and GCL of hippocampus in SI mice was significantly decreased compared with that in GH mice (Fig. 2c).

Effect of SI after weaning on the differentiation of newly divided cells in the DG of hippocampus

To examine the effect of SI after weaning on the differentiation of newly divided cells, we counted NeuN- and

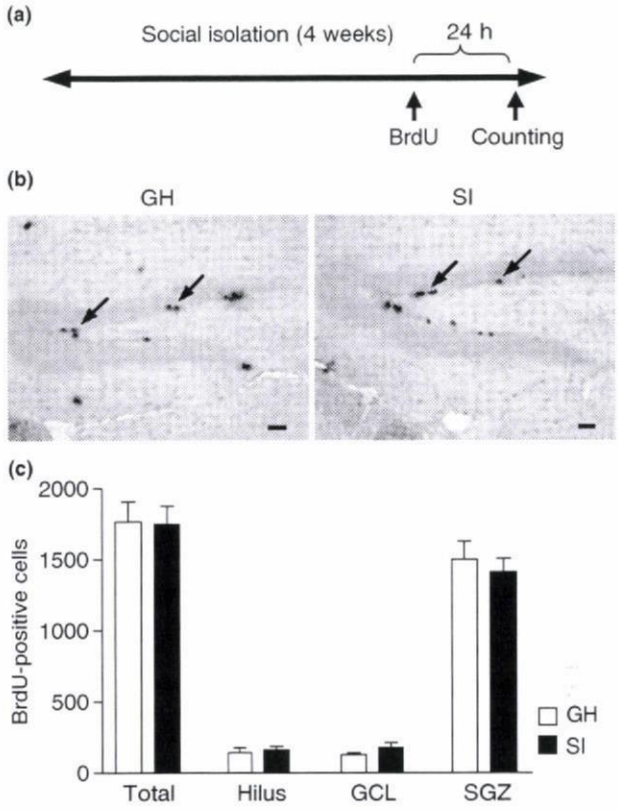
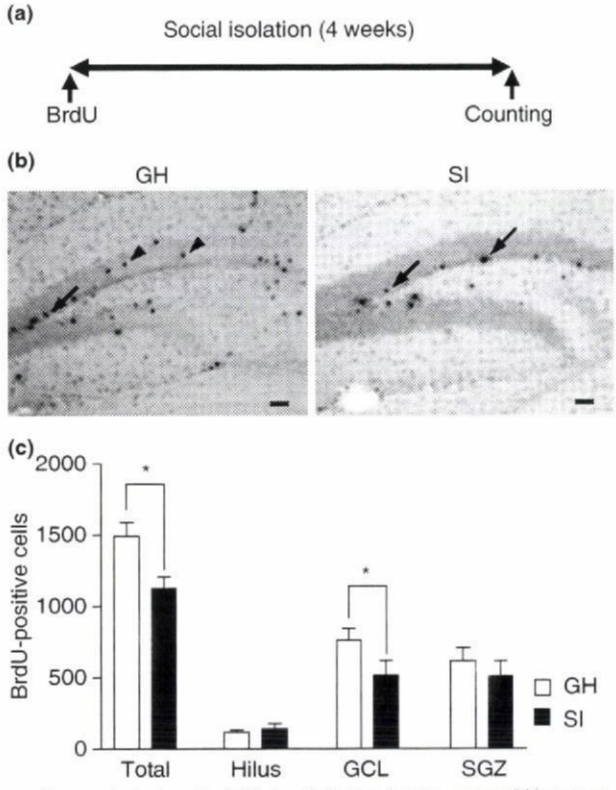


Fig. 1 Effect of social isolation (SI) for 4 weeks from 3-week-old in mice on the proliferation of newly divided cells in the dentate gyrus of hippocampus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals on the last day of 4-week isolation. Animals were killed 24 h after the last injection of BrdU, and BrdU-positive cells in the subgranular zone (SGZ), hilus, and granule cell layer (GCL) were counted as described in *Materials and Methods*. (a) Experimental schedule. (b) Representative photographs showing the distribution of BrdU-positive cells in group-housed (left) and SI (right) mice, respectively. Scale bar: 200 μm. (c) Total numbers of BrdU-positive cells are expressed as the sum of the number in the SGZ (arrows), hilus, and GCL. Values indicate the mean ± SE ($n = 4$).

GFAP-positive cells among BrdU-labeled cells in the hippocampus. Mice were subjected to SI after BrdU-labeling and killed after the 4-week SI for double-immunostaining (Figs 3a and 4a). Most of the BrdU-labeled cells in GH mice were NeuN-positive, but some were NeuN-negative (Fig. 3b). The rate of NeuN-positive cells among BrdU-labeled cells in the hilus + SGZ + GCL of SI mice was significantly lower than that in GH mice (Fig. 3c). The impairment of neural differentiation of BrdU-labeled cells was also evident in the SGZ of SI mice compared with GH mice, while there was no difference in the hilus and GCL. Thus, the rate of NeuN-positive cells among BrdU-labeled newly divided cells in the hilus + SGZ + GCL and SGZ of hippocampus in SI mice was significantly lower than that in GH mice (Fig. 3c). By contrast, a small fraction of



the survival of newly divided cells in the dentate gyrus of hippocampus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals one day before starting 4-week isolation. Animals were killed after SI, and BrdU-positive cells in the subgranular zone (SGZ), hilus, and granule cell layer (GCL) were counted as described in *Materials and methods*. (a) Experimental scedule. (b) Representative photographs showing the distribution of BrdU-positive cells in group-housed (GH, left) and SI (right) mice, respectively. Scale bar: 200 μm. (c) Total numbers of BrdU-positive cells were expressed as the sum of the number in the SGZ (arrows), hilus, and GCL (arrowheads). Values indicate the mean ± SE ($n = 7$). *$p < 0.05$ versus GH (two-tailed *t*-test).

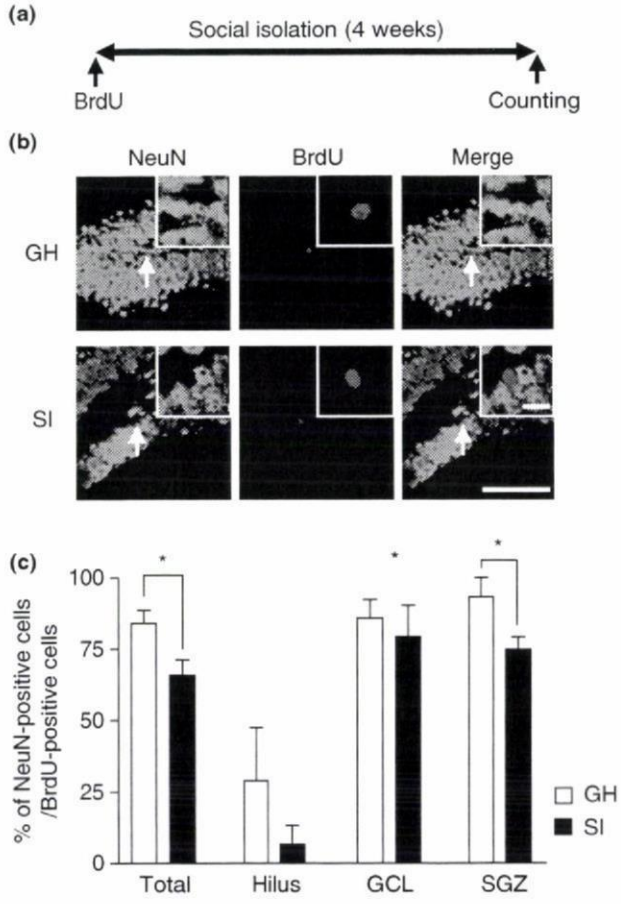


Fig. 3 Effect of social isolation (SI) for 4 weeks in 3-week-old mice on neurogenesis in the dentate gyrus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals before 4-week isolation. Animals were killed after SI, and BrdU-labeled cells in the subgranular zone, hilus, and granule cell layer were counted as described in *Materials and Methods*. (a) Experimental scedule. (b) Representative photographs showing confocal analysis to determine the percentage of neurons [neuronal nuclei (NeuN)-positive cells: green] among the population of newly divided cells (BrdU-positive cells: red) at 4 weeks after BrdU labeling (double-stained cells: yellow). Scale bar: 100 μm (inset, 10 μm). (c) Percentage of neurons (NeuN-positive cells) among BrdU-positive cells. Values indicate the mean ± SE ($n = 5$). *$p < 0.05$ versus group-housed (two-tailed *t*-test).

BrdU-labeled cells was co-labeled with an astrocyte marker, GFAP (Fig. 4b). There was no significant difference between GH and SI mice in the rate of GFAP-positive cells among BrdU-labeled newly divided cells in the hilus + SGZ + GCL, hilus, SGZ or GCL of hippocampus (Fig. 4c).

Effect of repeated Flx treatment on SI-induced spatial learning and memory deficits

To examine the functional significance of SI-induced impairment of neurogenesis in the hippocampus, we compared the performance of GH and SI mice in the water maze test which was used to examine spatial learning and memory associated with the hippocampal function (Jhoo *et al.* 2004; Miyamoto *et al.* 2005). At the same time, we investigated the effect of repeated administration of Flx, an SSRI reported to enhance neurogenesis in the hippocampus (Malberg *et al.* 2000; Santarelli *et al.* 2003) on maze performance in GH and SI mice. Repeated daily administration of Flx at a dose of 10 mg/kg (Encinas *et al.* 2006) was started 2 weeks after starting the SI until the end of the maze test (Fig. 5a), as we confirmed that SI for 2 weeks after weaning had little effect on cell proliferation and survival of BrdU-labeled cells in the hippocampus [data not shown, $p = 0.995$ (hilus + SGZ + GCL)].

There was no difference in the time spent in each quardrant of the pool among four groups of mice in the pre-probe test that was carried out before training trials [data not shown, $F_{(3,38)} = 0.246$; $p = 0.864$], indicating that

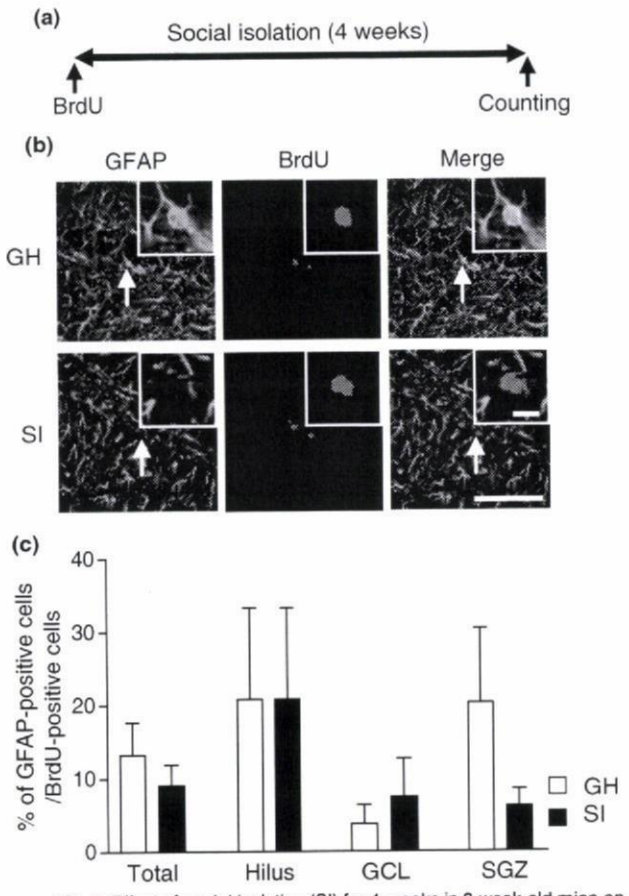


Fig. 4 Effect of social isolation (SI) for 4 weeks in 3-week-old mice on gliogenesis in the dentate gyrus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals before 4-week isolation. Animals were killed after SI, and BrdU-labeled cells in the subgranular zone, hilus, and granule cell layer were counted as described in *Materials and Methods*. (a) Experimental design. (b) Representative photographs showing confocal analysis to determine the percentage of astroglial cells [glial fibrillary acidic protein (GFAP)-positive cells: green] among the population of newly divided cells (BrdU-positive cells: red) at 4 weeks after BrdU labeling (double-stained cells: yellow). Scale bar: 100 μm (inset, 10 μm). (c) Percentage of glial cells (GFAP-positive cells) among BrdU-positive cells. Values indicate the mean ± SE (n = 4).

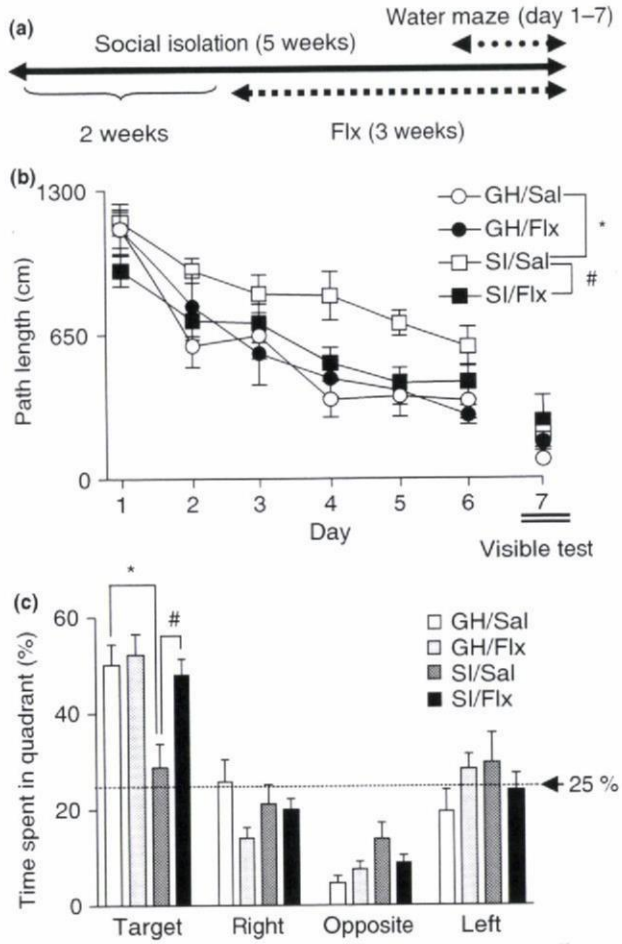


Fig. 5 Effect of repeated fluoxetine (Flx) treatment on social isolation (SI)-induced spatial learning and memory deficits in the water maze test. Mice were subjected to SI for 4 weeks and then to training trials on days 1–6, and visible and probe tests on day 7. The daily administration of Flx (10 mg/kg, i.p.) was started 2 weeks after SI and continued to the end of the water maze test. GH/Sal: saline-treated group-housed mice (n = 7); GH/Flx: fluoxetine-treated GH mice (n = 10), SI/Sal: saline-treated SI mice (n = 12), SI/Flx: fluoxetine-treated SI mice (n = 10). (a) Experimental design. (b) Path length (swim distance) to find the hidden platform during training trials (day 1–6) and visible test (day 7). (c) Time spent in quadrants in the probe test (day 7). Values indicate the mean ± SE. *p < 0.05 versus GH/Sal, #p < 0.05 versus SI/Flx.

neither the housing condition nor Flx treatment had any effect on space preference before maze training.

We preliminarily checked that SI did not affect swim speed [data not shown, $F_{(1,8)} = 0.730$; $p = 0.418$]. Furthermore, previous paper suggested that treatment of repeated Flx (10 mg/kg) did not affect swim speed (Song *et al.* 2006). In addition to results of visible test in this study, these findings indicate that the changes in performance during the training and probe trials were not because of an impairment of swimming ability or motivation.

On the other hand, there was a significant difference in performance (path length) of four different groups of mice [saline-treated GH (GH/Sal) mice, Flx-treated GH (GH/Flx) mice, saline-treated SI (SI/Sal) mice, and Flx-treated SI (SI/Flx) mice] during training trials on days 1 to 6 (Fig. 5b, ANOVA with repeated measures: group, $F_{(3,38)} = 6.134$, $p < 0.05$; day, $F_{(5,185)} = 39.796$, $p < 0.001$; group × day, $F_{(15,185)} = 1.128$, $p = 0.334$). *Post-hoc* analysis indicated that performance by SI/Sal mice was significantly impaired compared with GH/Sal mice, suggesting that SI in juveniles induces the impairment of spatial learning. Furthermore, repeated Flx treatment significantly improved performance in SI mice although it had no effect on

performance in GH mice. When escape latency was analyzed as a measure of performance, the same results were obtained [data not shown, $F_{(3,38)} = 7.217$; $p < 0.001$].

Next day (day 7), the animals were subjected to the probe test and then to the visible test. In the probe test (Fig. 5c), there was a significant difference in time spent in the target quadrant in which the submerged platform had been located during training trials among four groups [$F_{(3,38)} = 6.763$, $p < 0.05$]. *Post-hoc* analysis by Bonferroni's test indicated that SI/Sal mice spent significantly less time than GH/Sal mice, indicating an impairment of spatial memory. Furthermore, repeated Flx treatment significantly improved the impairment of spatial memory in SI mice, although the treatment had no effect on performance in GH mice. In the visible test conducted after the probe test on day 7, there was no significant difference in performance (both path length and escape latency) among the four groups of animals (Fig. 5b).

Effect of Flx on SI-induced aggressive behaviors

The day after the water maze probe test (day 8), the animals were used in the intruder-evoked aggressive test (Fig. 6a). Aggressive biting behavior was observed in all four groups (1/13 in GH/Sal, 0/9 in GH/Flx, 8/12 in SI/Sal, and 2/10 in SI/Flx mice). The difference between GH/Sal and SI/Sal groups was statistically significant ($\chi^2 = 9.420$, $p < 0.01$). Repeated Flx treatment in SI mice significantly reduced the rate of mice exhibiting biting ($\chi^2 = 4.791$, $p < 0.05$). Similarly, the rate of SI/Sal mice showing tail rattling against the intruder (8/12) was significantly increased compared to GH/Sal (2/13, $\chi^2 = 6.838$, $p < 0.01$). Repeated Flx treatment significantly reduced the rate of animals showing tail rattling in the SI group (1/10, $\chi^2 = 7.246$, $p < 0.01$) without affecting behavior in the GH group (0/9).

As shown in Fig. 6(b) and (c), there were significant differences in biting counts [Fig. 6b; $F_{(3,40)} = 3.650$, $p < 0.05$] and total time of aggression [Fig. 6c; $F_{(3,40)} = 16.075$, $p < 0.001$] among the four different groups of mice. *Post-hoc* analysis indicated that biting counts and total time of aggression against the intruder in SI/Sal were increased compared with those in GH/Sal mice. There was no difference in biting counts between SI/Sal and SI/Flx mice (Fig. 6b), but the total time of aggression in SI/Flx mice was significantly reduced compared to SI/Sal mice (Fig. 6c).

Effect of Flx on SI-induced reduction of survival of newly divided cell in the hippocampus

To examine the effect of repeated administration of Flx on SI-induced impairment of cell survival in the DG of the hippocampus, we compared the number of BrdU-positive cells in the DG among the four groups. Repeated daily administration of Flx at a dose of 10 mg/kg was started 2 weeks after starting SI and continued for 2 weeks (Fig. 7a).

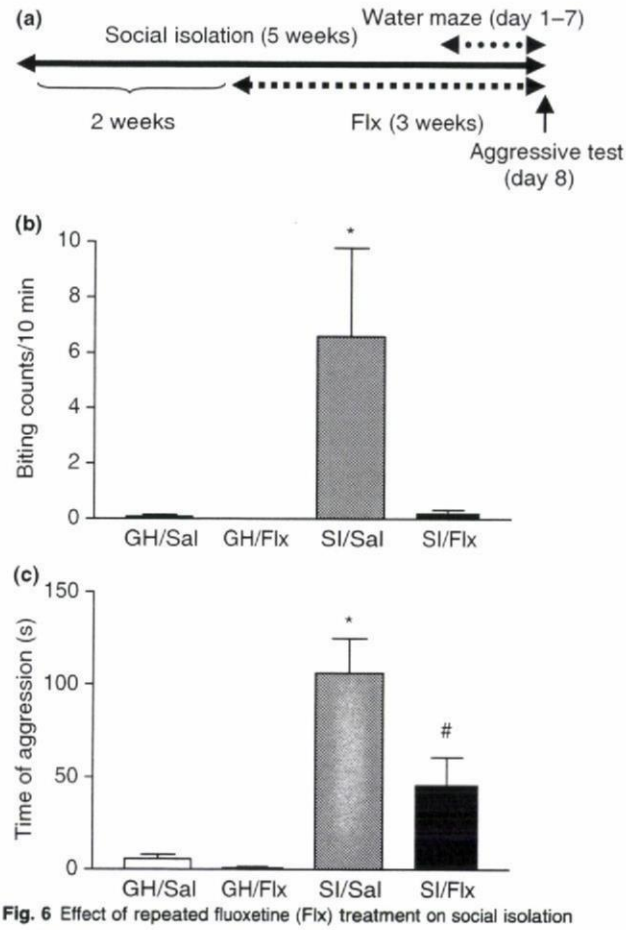


Fig. 6 Effect of repeated fluoxetine (Flx) treatment on social isolation (SI)-induced aggressive behavior in the intruder-evoked aggressive test. Mice that had previously been subjected to the water maze test following SI for 4 weeks were used in the intruder-evoked aggressive test. Daily administration of Flx (10 mg/kg, i.p.) was started 2 weeks after SI and continued to the end of the behavioral test. Frequency of biting and duration of aggressive behavior against the intruder were measured for 10 min. GH/Sal: saline-treated group-housed mice ($n = 13$), GH/Flx: fluoxetine-treated GH mice ($n = 9$), SI/Sal: saline-treated SI mice ($n = 12$), SI/Flx: fluoxetine-treated SI mice ($n = 10$). (a) Experimental schedule. (b) Biting counts. (c) Total time of aggression. Values indicate the mean ± SE. $^*p < 0.05$ versus GH/Sal, $^\#p < 0.05$ versus SI/Sal.

As shown in Fig. 7b, there was an apparent difference in the number and location of BrdU-labeled cells in the DG of the hippocampus in SI/Sal mice compared with GH/Sal, GH/Flx, and SI/Flx groups. ANOVA of the number of BrdU-positive cells in the GCL ($F = 6.183$, $p < 0.01$) and in the DG of hippocampus ($F = 6.536$, $p < 0.01$) revealed a significant effect of treatment. *Post-hoc* analysis with Bonferroni's test indicated that the total number of BrdU-positive cells in the DG of the hippocampus was significantly reduced in SI/Sal mice compared with GH/Sal mice ($p < 0.05$), and this impairment of survival of newly divided cells in the SI/Sal group was significantly ameliorated by

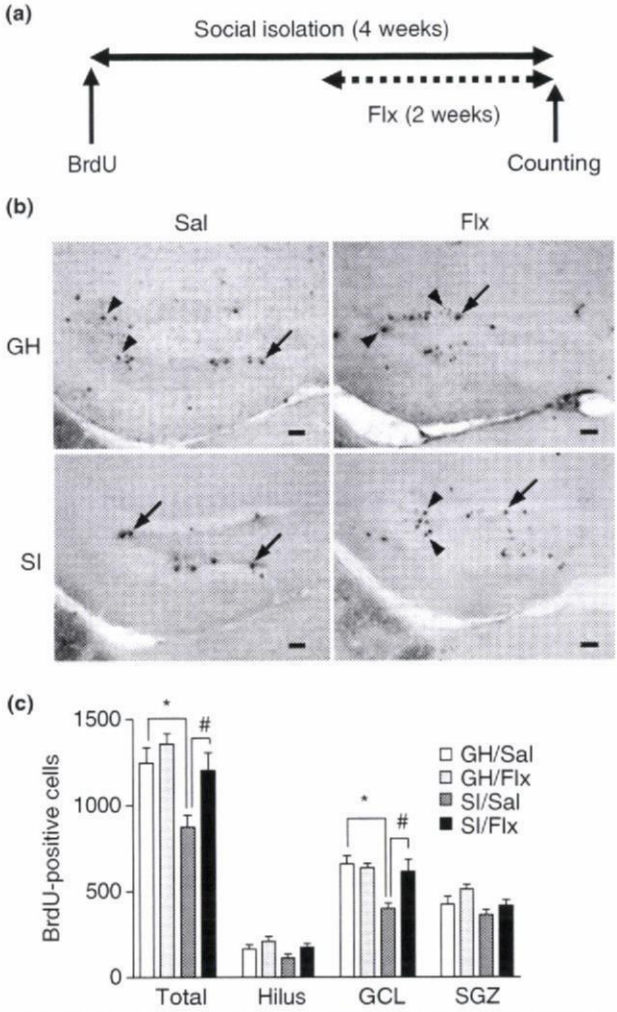


Fig. 7 Effect of repeated fluoxetine (Flx) treatment on social isolation (SI)-induced impairment of the survival of newly divided cells in the dentate gyrus of hippocampus. 5-bromo-2′-deoxyuridine (BrdU; 75 mg/kg, i.p.) was injected three times at 2 h intervals one day before starting 4-week isolation. Daily administration of Flx (10 mg/kg, i.p.) was started 2 weeks after SI and continued for 2 weeks. Animals were killed after SI, and BrdU-positive cells in the subgranular zone (SGZ), hilus, and granule cell layer (GCL) were counted as described in *Materials and methods*. GH/Sal: saline-treated group-housed mice ($n = 6$), GH/Flx: fluoxetine-treated GH mice ($n = 6$), SI/Sal: saline-treated SI mice ($n = 7$), SI/Flx: fluoxetine-treated SI mice ($n = 7$). (a) Experimental schedule. (b) Representative photographs showing the distribution of BrdU-positive cells in GH/Sal, GH/Flx, SI/Sal, and SI/Flx mice, respectively. Scale bar: 200 μm. (c) Total numbers of BrdU-positive cells were expressed as the sum of the number in the SGZ (arrows), hilus, and GCL (arrowheads). Values indicate the mean ± SE. $^*p < 0.05$ versus GH/Sal, $^\#p < 0.05$ versus SI/Flx.

repeated Flx treatment ($p < 0.05$). The same result was observed in the number of BrdU-positive cells in the GCL (Fig. 7c). Furthermore, there was a significant difference in the ratio of NeuN-positive cells among BrdU-labeled newly divided cells in the hilus + SGZ + GCL between SI/Sal (69.4 ± 0.6%, $n = 3$) and SI/Flx (78.0 ± 1.1%, $n = 3$; $p < 0.01$) mice.

Changes in gene expression in the DG of hippocampus by SI

To compare the changes in gene expression in the DG of hippocampus between GH and SI mice, we used Affymetrix GeneChip mouse genome assays. Scatter plots and hierarchical clustering analysis showed no obvious changes in global expression profiles between GH and SI groups. In an attempt to detect gene-specific significant changes in expression between GH and SI groups, a general linear model incorporating the feeding period was used. Among genes whose p-value of change between GH and SI groups was less than 0.01 and whose absolute value of log2-fold change was larger than 0.263 (1.2-fold), one gene (0.0022%) was increased and 21 (0.047%) genes were decreased in the expression levels in SI mice compared to GH mice (Table 1). Three genes, *Nurr1*, *Npas4*, and a gene of unknown function AK003534 had a false discovery rate less than 0.5. We confirmed by the real-time RT-PCR that the expression levels of *Nurr1* and *Npas4* mRNA in the DG of hippocampus were significantly reduced after 4-week SI compared with those in GH controls (Fig. 8).

Discussion

In the present study, we found that long-term SI after weaning in mice had little effect on the proliferation of newly divided cells in the hippocampus as measured by BrdU labeling. In contrast, Lu *et al.* (2003) previously reported that SI in early life reduces cell proliferation in the DG of rats. Apparently, our present findings are inconsistent with their findings, but the discrepancy may be explained by the difference of the administration schedule of BrdU and/or species difference used. We administrated BrdU three times at 2 h intervals and then counted the number of BrdU-labeled cells 24 h after the last BrdU injection, while Lu *et al.* (2003) administrated BrdU twice daily on the last 3 days of the rearing treatment. Furthermore, because neurogenesis is affected by genetic background (Jacobs *et al.* 2000), hereditary differences between rats and mice may result in distinct effects on cell proliferation.

The present study indicates that the survival and differentiation of newly divided cells in the hippocampus are reduced by SI after weaning in mice, which is consistent with previous studies in rats and guinea pigs (Lu *et al.* 2003; Rizzi *et al.* 2007). It is possible that SI may induce the cell death of newly divided cells (Rizzi *et al.* 2007), but we could not detect apoptotic cells labeled by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling in the DG of either GH or SI mice (unpublished observation). Further studies are required to clarify the mechanism by which the survival of newly divided cells was impaired by SI after weaning. The present study also indicates that cell survival of newly divided

Table 1 List of genes that the expression in the DG of the hippocampus was altered by SI

Gene title	Public ID	Gene symbol	Probe	Expression ratio		
				3 days	2 weeks	4 weeks
Euchromatic histone methyltransferase 1	BB409568	*Ehmt1*	1454776_at	1.12	1.17	1.54
Neuronal PAS domain protein 4	AV348246	*Npas4*	1459372_at	0.74	0.36	0.76
Activity regulated cytoskeletal-associated protein	NM_018790	*Arc*	1418687_at	0.67	0.61	0.76
FBJ osteosarcoma oncogene	AV026617	*Fos*	1423100_at	0.76	0.57	0.75
RIKEN cDNA C330006P03 gene	BB398124	*C330006P03Rik*	1436387_at	0.83	0.60	0.78
Nuclear receptor subfamily 4, group A, member 2 (Nr4a2), mRNA	BB703394	*Nr4a2 (Nurr1)*	1455034_at	0.77	0.67	0.78
Nuclear receptor subfamily 4, group A, member 2	BB322941	*Nr4a2*	1447863_s_at	0.72	0.68	0.85
Vacuolar protein sorting 52 (yeast)	BB429200	*Vps52*	1447894_x_at	0.60	0.89	0.85
Nuclear receptor subfamily 4, group A, member 1	NM_010444	*Nr4a1*	1416505_at	0.82	0.65	0.91
Early growth response 1	NM_007913	*Egr1*	1417065_at	0.78	0.76	0.87
Corticotropin releasing hormone binding protein	AI854101	*Crhbp*	1436127_at	0.87	0.82	0.71
Period homolog 2 (Drosophila)	AF035830	*Per2*	1417602_at	0.75	0.84	0.83
16 days embryo head cDNA, RIKEN full-length enriched library, clone: C130019I03 production: unclassifiable, full insert sequence	BB363968	–	1460098_at	0.88	0.83	0.74
Kinesin family member 1B	BE199508	*Kif1b*	1425270_at	0.77	0.91	0.78
ADP-ribosylation factor 4-like	NM_025404	*Arfl4*	1418250_at	0.81	0.77	0.89
Poliovirus receptor	BB049138	*Pvr*	1451160_s_at	0.90	0.71	0.87
Nuclear receptor subfamily 4, group A, member 2	NM_013613	*Nr4a2*	1450750_a_at	0.77	0.80	0.92
Homer homolog 1 (Drosophila)	AF093257	*Homer1*	1425671_at	0.92	0.68	0.89
Dihydrouridine synthase 4-like (*Saccharomyces cerevisiae*)	AK010138	*Dus4l*	1453252_at	0.73	0.83	0.93
Diacylglycerol kinase, iota	BE647270	*Dgki*	1439986_at	0.90	0.72	0.87
PHD finger protein 17	BG065238	*Phf17*	1452180_at	0.91	0.82	0.74
Dual specificity phosphatase 1	NM_013642	*Dusp1*	1448830_at	1.01	0.67	0.85

SI, social isolation; DG, dentate gyrus.
Mice were killed after 3 days, 2 weeks, and 4 week of SI. Brain sample (hippocampal DG) from four mice were pooled and used for the DNA microarray. Values indicated the mean (n = 5–6, each from four mice).

cells is diminished in the GCL, while neuronal differentiation is only diminished in the SGZ. It is possible that development of stem-like cells in the SGZ is repressed by SI. Some of those cells in the SGZ of SI mice could not grow up to NeuN-positive cells. Therefore, SI may decrease differentiation to NeuN-positive cells but not cell number in the SGZ.

In the present study, the duration of Flx treatment in the behavioral experiment (3 weeks, Figs 5 and 6) was different from that in the experiment of BrdU labeling (2 weeks, Fig. 7). Although we did not examine whether the different duration of treatment with Flx affected the enhancing effect on neurogenesis in the hippocampus or not, a previous study demonstrated that the effect of repeated Flx treatment for 2 weeks on neurogenesis was similar to that for 4 weeks (Malberg *et al.* 2000). Therefore, we suggest that Flx treatment for 3 weeks in the behavioral experiment may have effects on the number of BrdU-positive cells in the SGZ, similar to those observed after the repeated treatment for 2 weeks.

Current study showed that Flx administration completely prevented the SI-induced impairment of survival of newly divided cells and spatial learning and memory, but not aggression completely, because spatial learning and memory are more strongly associated with the hippocampal function than aggression (Davidson *et al.* 2000; Jhoo *et al.* 2004; Miyamoto *et al.* 2005). To demonstrate our thoughts as described above, further experiments are required.

A recent study showed that an appropriate activity of NMDA receptors plays a role in the survival of newly divided cells in the DG of the hippocampus (Tashiro *et al.* 2006). SI in rats induces the expression of the NMDA receptor, NR1A mRNA in the DG of the hippocampus (Hall *et al.* 2002). Furthermore, it is reported that SI stress elevates circulating levels of corticosterone (Stranahan *et al.* 2006) and that a high dose of corticosterone induces glutamate release in the hippocampus (Karst *et al.* 2005; Venero and Borrell 1999;. Thus, long-term SI after weaning may induce glutamate release and the expression of NR1A subunit of NMDA receptor in the hippocampus, leading to aberrant NMDA receptor activation. As NMDA receptor activation is reported to induce the cell death of stem-like cells *in vitro* (Tashiro *et al.* 2006; Asahi *et al.* 1998), it is possible that

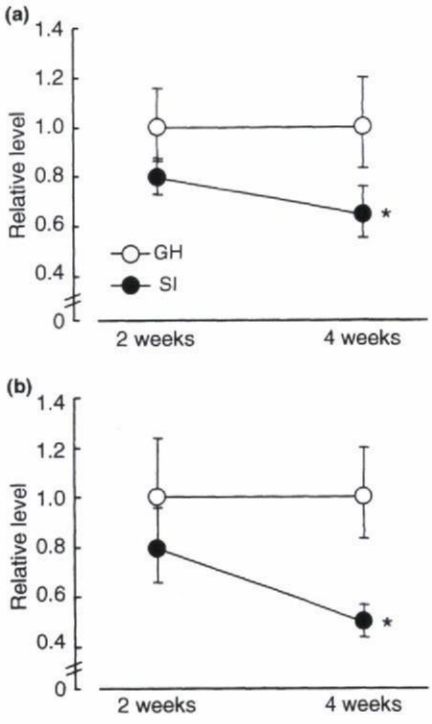


Fig. 8 Changes in the expression of nuclear receptor subfamily 4, group A, member 2 (a) and neuronal PAS domain 4 (b) mRNA in the dentate gyrus of the hippocampus after 2-and 4-week social isolation. Values indicated the mean ± SE (n = 5–10, each from two mice). *$p < 0.05$ versus group-housed (two-tailed t-test).

SI-induced alternation of NMDA receptor signaling may reduce the survival of newly divided cells in the DG of the hippocampus in SI mice.

It has been demonstrated that GABA plays a crucial role in the differentiation of stem-like cells in the SGZ of the hippocampus and that excitatory GABAergic input to stem-like cells in the hippocampus increases the expression of the transcription factor NeuroD, a positive regulator of neuronal differentiation in the DG (Tozuka *et al.* 2005). A further *in vivo* study using $GABA_A$ receptor agonists indicated the promotion of neural differentiation via GABAergic excitation (Li and Pleasure 2005; Tozuka *et al.* 2005). Pinna *et al.* (2006) showed that SI alters the subunit expression of $GABA_A$ receptor leading to dysfunction of the $GABA_A$ receptor agonist-induced sedative effect. Thus, it is also possible that the dysfunction of $GABA_A$ receptors may be involved in the impairment of differentiation of newly divided cells in the DG of the hippocampus induced by long-term SI after weaning in mice.

It is well known that SI induces impairment of emotion-related behaviors and cognition (Wongwitdecha and Marsden 1996; Weiss *et al.* 2004). Consistently, in the present study, we found that long-term SI in mice after weaning impairs spatial learning and memory and induces impulsiveness and aggressive behavior. These behavioral abnormalities in SI mice resemble the symptoms observed in patients suffering from attention-deficit hyperactivity disorder and depression (Castellanos and Tannock 2002; Heim *et al.* 2004). Notably, memory deficit, impulsiveness/aggressive behavior as well as the impairment of survival of newly divided cells in the hippocampus of SI mice were reversed by repeated Flx administration. A single Flx treatment failed to reverse impulsiveness/aggressive behavior and anxiety in SI mice (data not shown). Our findings are in agreement with the previous study that impairment of cell survival induced by experiences in early life (e.g., maternal separation) was reversed by repeated Flx administration in rats (Lee *et al.* 2001). Although the causal relationship between behavioral deficits and impairment of neurogenesis in SI mice is unclear, a previous study suggested that the behavioral effects of chronic Flx may be mediated by the stimulation of neurogenesis in the hippocampus because X-ray irradiation to a restricted region of the mouse brain containing the hippocampus prevented the neurogenic and behavioral effects of Flx (Santarelli *et al.* 2003).

Regarding the mechanism of action of Flx on SI-induced impairment of neurogenesis, it is reasonable to assume the involvement of 5-hydroxytryptamine (5-HT) system because of the selective inhibitory effect of 5-HT reuptake. Previous studies demonstrated that SI induces impairment of the 5-HT system in the hippocampus (Bickerdike *et al.* 1993; Whitaker-Azmitia *et al.* 2000; Muchimapura *et al.* 2002; Preece *et al.* 2004). For instance, previous studies have shown that 5-HT release is reduced in the hippocampus of isolated rats under aversive conditions and following the administration of parachloroamphetamine (a 5-HT-releasing drug) (Bickerdike *et al.* 1993; Muchimapura *et al.* 2002). Regarding changes in 5-HT receptors in the hippocampus, Preece *et al.* (2004) showed that SI from weaning in rats results in alternations of 5-HT_{1A} and 5-HT_{2A} receptor density in the frontal cortex and hippocampus. Gould (1999) suggested that $5HT_{1A}$ receptors are located on the hippocampal stem-like cells. Accordingly, activation of 5-HT_{1A} receptors is required for the effects of Flx on behavior and neurogenesis (Santarelli *et al.* 2003). Furthermore, because NMDA and $GABA_A$ receptors play a role in the survival and differentiation of newly divided cells in the DG of the hippocampus as described above, these receptors may also be involved in the effect of Flx on neurogenesis (Zhong and Yan 2004; Yuen *et al.* 2005).

In the present study, we demonstrated that SI significantly reduced the mRNA levels of *Nurr1* and *Npas4* in the DG of hippocampus. In agreement with our finding, it is reported that both *Nurr1* and *Npas4* mRNA are highly expressed in the hippocampus (Xiao *et al.* 1996; Moser *et al.* 2004). Neuronal PAS domain 4 (Npas4) has constitutive or developmental functions which may be critical for regulating the

transcriptional control of limbic patterning and function (Moser *et al.* 2004). Nuclear receptor subfamily 4, group A, member 2 (Nurr1) is essential for both survival and final differentiation of dopaminergic precursor neurons into a complete dopaminergic phenotype (Saucedo-Cardenas *et al.* 1998). Although we did not examine the alteration of *Nurr1* and *Npas4* mRNA expression in the BrdU-positive cells in the hippocampus, it is possible that Nurr1 and Npas4 may contribute to the impairment of neurogenesis and memory, and aggression in SI mice. In the present study, it is unclear that which regions of the DG *Nurr1* and *Npas4* mRNA expression are altered, because the microarray and RT-PCR in present study were carried out without dissecting multiple regions of the DG, and that whether Flx recovers SI-induced alteration of these genes. Further studies are required to test this assumption and attenuate the limitation. These additional studies will be useful to understand the molecular mechanisms underlying SI-induced impairment of hippocampal neurogenesis.

In conclusion, the present study demonstrated that long-term SI in mice after weaning reduced survival and differentiation but not the proliferation of newly divided cells in the DG of the hippocampus. In parallel, long-term SI in juvenile mice induced hippocampal dysfunction which was manifested by the development of learning and memory impairment as well as impulsiveness/aggressive behavior. Furthermore, we demonstrated that SI-induced impairment of neurogenesis, cognition and emotion-related behaviors were reversed by repeated Flx administration. The DNA microarray and real-time RT-PCR analyses indicated that long-term SI after weaning in mice affects the expression of a very few genes (less than 0.1%) in the DG of hippocampus and that the expression of Nurr1 and Npas4 mRNA was significantly reduced by long-term SI.

Thus, our findings suggested that long-term deprivation of communication with others in juveniles impairs the mechanism of neurogenesis in the hippocampus, which could be involved in the development of psychiatric disorders with impairment of emotion-related behaviors and cognition. Furthermore, Flx may be effective in treating impairment of emotion-related behaviors and memory in which poor environmental conditions and/or social interaction in early life could be involved in the pathogenesis.

Disclosure/Conflicts of interest

The authors declare that there is no conflict of interest in the publication of the present work.

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A novel molecule 'shati' increases dopamine uptake via the induction of tumor necrosis factor-α in pheochromocytoma-12 cells

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Abstract

The psychostimulant properties of methamphetamine (METH) are associated with an increase in extracellular dopamine (DA) levels in the brain, via facilitation of DA's release from pre-synaptic nerve terminals and inhibition of its reuptake through DA transporter. Recently, we have demonstrated that tumor necrosis factor-α (TNF-α) increases DA uptake and inhibits METH dependence. Moreover, we have clarified 'shati' identified in the nucleus accumbens of mice treated with METH is involved in METH dependence. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and established a PC12 cell line transfected with a vector containing shati cDNA to examine the precise mechanism behind the role of shati in DA uptake. Moreover, we examined the relationship between shati and TNF-α. TNF-α increased DA uptake via the mitogen-activated protein kinase kinase pathway and inhibited the METH-induced decrease in DA uptake in PC12 cells. Transfection of the vector containing shati cDNA into PC12 cells, induced the expression of shati and TNF-α mRNA, accelerated DA uptake, and inhibited the METH-induced decrease in DA uptake. These results suggest that the functional roles of shati in METH-regulated behavioral changes are mediated through inhibition of the METH-induced decrease in DA uptake via TNF-α.

Keywords: addiction, dopamine (DA) uptake, methamphetamine, shati, tumor necrosis factor-α (TNF-α).

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The abuse of methamphetamine (METH) has significant psychiatric and medical consequences, including dependence, psychosis, overdose, and even death (Rawson *et al.* 2002). Drugs of abuse, including METH, modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area to the nucleus accumbens

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Abbreviations used: AMPH, amphetamine; ANOVA, analysis of variance; AP, anteroposterior; cDNA, complementary DNA; CNS, central nervous system; CSF, cerebrospinal fluid; D1-R, dopamine D1 receptor; D2-R, dopamine D2 receptor; DA, dopamine; DAT, dopamine transporter; DV, dorsoventral; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GBR 12909, [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate; GFP, green fluorescent protein; GNAT, GCN5-related N-acetyltransferase; JNK, c-Jun N-termial kinase; MAO, monoamine oxidase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; METH, methamphetamine; ML, mediolateral; NAc, nucleus accumbens; NF-κB, nuclear factor-κB; p38, p38 mitogen-activated protein kinase; PC12, pheochromocytoma-12; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methyoxyphenyl)-4H-1-benzopyran-4-one; RIP, serine and threonine protein kinase receptor-interacting protein; RT-PCR, reverse transcription-polymerase chain reaction; shati-AS, shati antisense oligonucleotide; shati-SC, shati-scrambled oligonucleotide; SLC6, solute carrier 6; TH, tyrosine hydroxylase; TNFR I, tumor necrosis factor type I receptor; TNF-α, tumor necrosis factor-α; TRAF2, TNF receptor-associated factor 2; VMAT-2, vesicular monoamine transporter; VTA, ventral tegmental area.

(NAc) (Koob 1992; Wise 1996; Koob *et al.* 1998). The principal target for the action of METH is believed to be the dopamine transporter (DAT), which is a member of the solute carrier 6 (SLC6) gene family of Na^+/Cl^- coupled transporters that also includes the neurotransmitter transporters of norepinephrine, serotonin, glycine, and γ-aminobutyric acid (GABA) (Amara and Kuhar 1993; Torres *et al.* 2003; Chen *et al.* 2004). The DAT controls dopaminergic signaling by the rapid reuptake of dopamine (DA) from synaptic clefts. As a substrate, METH not only competitively inhibits DA uptake and thereby increases synaptic DA but also promotes the reverse transport of nonvesicular DA, resulting in an efflux of DA via the DAT (Sulzer *et al.* 2005; Fog *et al.* 2006). This efflux results in a dramatic increase in extracellular DA and is believed to be of major importance for the psychostimulant properties of METH (Sulzer *et al.* 2005; Fog *et al.* 2006). However, the exact neuronal circuits and molecular cascade essential for drug dependence are still poorly understood. Moreover, the molecules related to the METH-induced increase in DA efflux are unclear.

Tumor necrosis factor-α (TNF-α) plays an important role in a variety of infectious, inflammatory, and autoimmune conditions (Vassalli 1992). TNF-α also affects the CNS directly or indirectly through the stimulation of vagal afferents (Maier and Watkins 1998). Thus, this proinflammatory cytokine is emerging as a modulator of CNS function. Recently, we have demonstrated that TNF-α activates synaptosomal and vesicular DA uptake (Nakajima *et al.* 2004). Moreover, we have reported that TNF-α and its inducer diminish METH and morphine-induced behavioral sensitization and rewarding effects by promoting plasmalemmal and vesicular DA uptake as well as attenuating the METH and morphine-induced increase in overflow of DA in the NAc (Nakajima *et al.* 2004; Niwa *et al.* 2007b,d; Niwa *et al.* 2008). TNF-α modulates cellular responses through the extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor-κB (NF-κB) signaling pathways (van Vliet *et al.* 2005). ERK1/2 regulates the surface expression and capacity of DAT (Morón *et al.* 2003). However, the mechanisms by which TNF-α regulates the uptake of DA are poorly understood.

Recently, we have identified a novel molecule 'shati' in the NAc of mice treated with METH repeatedly using the polymerase chain reaction (PCR)-select complementary DNA (cDNA) substraction method, which is a differential and epochal cloning technique. Further, we have demonstrated that shati, which contains the sequence of GCN5-related *N*-acetyltransferase (GNAT), acetyl-CoA-binding sites, and ATP-binding sites, is involved in METH-induced hyperlocomotion, sensitization, and conditioned place preference (Niwa *et al.* 2007a). Blockage of shati expression by shati antisense oligonucleotide (shati-AS) potentiates not only the increase in extracellular DA levels, but also the decrease in synaptosomal and vesicular DA uptake in the NAc induced by repeated METH treatment, resulting in potentiation of the METH-induced dependence (Niwa *et al.* 2007a).

Pheochromocytoma-12 (PC12) cells are useful as a model of the neuronal system and have DATs. In the present study, we investigated the effects of TNF-α on DA uptake in PC12 cells and the involvement of the mitogen-activated protein kinase kinase (MEK) pathway in the effects of TNF-α on DA uptake. Moreover, we succeeded in the transfection of a vector containing shati cDNA into PC12 cells, investigated the involvement of shati in DA uptake and the METH-induced decrease in DA uptake, and examined the relationship between shati and TNF-α by using these PC12 cells.

Materials and methods

Cell culture and transfection

PC12 cells purchased from the Riken cell bank (No. RCB0009) were cultured on poly-ornithine-coated coverslips in Dulbecco's modified Eagle's medium (Sigma-Aldrich St Louis, MO, USA) supplemented with 10% heat inactivated horse serum and 5% fetal bovine serum (Loder and Melikian 2003). We made the vector containing shati cDNA with the suggested sequence of NM_001001985 using the plasmid pcDNA-DEST53 (Invitrogen, Carlsbad, CA, USA) as an expression vector with green fluorescent protein (GFP), although N-terminal of seven amino acids of shati was missing (CDS 882-1760) in this vector. For transient expression, the cells were transfected with the plasmid expressing shati using Lipofectamine 2000 (Invitrogen).

[^{3}H] DA uptake in PC12 cells

The uptake of [^{3}H] DA in PC12 cells was performed as described before (Melikian and Buckley 1999). The cells were washed in Krebs-Ringers- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer twice before the assay. Uptake was initiated by adding 1 μM [^{3}H] DA (Perkin Elmer, Waltham, MA, USA) containing 10^{-5} M pargyline and 10^{-5} M ascorbic acid. Uptake proceeded for 10 min at 23°C and was terminated with three rapid washes in ice-cold Krebs-Ringers-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The amount of [^{3}H] DA accumulated was determined by liquid scintillation counting (Beckman Coulter, Inc., Fullerton, CA, USA). Non-specific uptake was defined in the presence of 10 μM [1-(2[bis(4-fluorophenyl-) methoxy]ethyl)-4-(3-phenylpropyl)piperazine] bimesylate hydrate (GBR 12909). The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [^{3}H] DA uptake. To neutralize TNF-α in PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd., Minneapolis, MN, USA; Ab; 1, 10, 50, and 100 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sRI; 0.1, 0.5, 1, and 10 ng/mL) 10 min before the treatment with TNF-α (10 ng/mL, 40 min) (Barone *et al.* 1997), and assayed for [^{3}H] DA uptake. The function of TNF-α is mediated through two distinct cell surface receptors, TNF receptor I and TNF receptor II. The majority of TNF functions are mediated primarily through TNF receptor I, whereas TNF receptor II seems to play a role in only a limited number of

TNF responses (Hsu *et al.* 1995). Moreover, it has been reported that immunoreactivity for TNF receptor I is found in cell bodies and process of dopaminergic neurons (Boka *et al.* 1994). Therefore, we have used soluble TNF receptor I for neutralization for TNF-α. To examine the involvement of the MEK pathway in the TNF-α-induced increase in DA uptake in PC12 cells, the cells were pre-treated with a selective MEK inhibitor 2-(2-amino-3-methyoxy-phenyl)-4H-1-benzopyran-4-one (Calbiochem, San Diego, CA, USA; PD98059; 1, 10, 100, and 500 μM) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. PD98059 was dissolved in dimethyl sulfoxide to give a concentration of 50 mM, stored in aliquots at −80°C, and diluted in Dulbecco's modified Eagle's medium to 1–500 μM immediately prior to use. To examine the effects of TNF-α on the METH-induced decrease in DA uptake in PC12 cells, the cells were pre-treated with TNF-α (10 ng/mL) 10 min before being treated with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake, following previous observations (Nakajima *et al.* 2004). Cen *et al.* (2008) have reported that METH (1 μM) decreases plasmmalemmal DAT expression in time-dependent manner (0, 5, 15, 30, 60 min), which is paralleled with the decrease in [^{3}H] DA uptake. Since treatment of METH (1 μM) for 30 min significantly decreases DA uptake compared with control group (Cen *et al.*, 2008), we have selected this time point for treatment of METH before the uptake assay. To examine the involvement of TNF-α in the shati-induced increase in [^{3}H] DA uptake in the shati-over-expressing PC12 cells, the cells were pre-treated with polyclonal goat anti-TNF-α antibody (R&D Systems Ltd, Ab; 50 ng/mL) or soluble TNF receptor I (R&D Systems Ltd, sR I; 1 ng/mL) 10 min before their treatment with METH (1 μM, 30 min), and assayed for [^{3}H] DA uptake.

Immunocytochemistry

Two antibodies against the peptide of the hypothetical protein, CNTAFRGLRQHPRTQLL (S-3) and CMSVDSRFRGKGIAKALG (S-4) unique to shati were generated. These peptides were conjugated to keyhole limpet hemocyanin and injected into rabbits six times at 1-week intervals. Serum was taken from the rabbits 1 week after the final injection. The serum was diluted 200 times for immunostaining (Niwa *et al.* 2007a).

Transfected PC12 cells attached to glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min, and then blocked in 3% normal sera and 0.1% Triton X-100 for 1 h. The coverslips were incubated with primary antibodies at 4°C overnight, washed with phosphate-buffered saline, and then incubated with appropriate secondary antibodies for 2 h. Polyclonal rabbit anti-S-3 or anti-S-4 antibody (1 : 200), monoclonal mouse anti-tyrosine hydroxylase (TH) antibody (1 : 200, Chemicon, Temecula, CA, USA), monoclonal mouse anti-GFP antibody (1 : 500, Chemicon), polyclonal goat anti-rat TNF-α antibody (1 : 100, R&D Systems Ltd), and polyclonal rabbit anti-GFP antibody (1 : 100, Chemicon) served as primary antibodies. Goat anti-mouse Alexa Fluor 546 (1 : 1000, Invitrogen), donkey anti-goat Alexa Fluor 546 (1 : 1000, Invitrogen), rabbit anti-mouse Alexa Fluor 488 (1 : 1000, Invitrogen), and donkey anti-rabbit Alexa Fluor 488 (1 : 1000, Invitrogen) were used as secondary antibodies. After being washed and mounted, stained cells were observed under a fluorescence microscope (Axioskoop 2 plus). Because similar results were obtained using the anti S-3 and anti-S-4 antibodies in the immunohistochemical experiments, only the data obtained with the anti-S-4 antibody is described.

Real time reverse transcription-polymerase chain reaction

Total RNA was isolated using an RNeasy Kit (Qiagen, Hilden, Germany) and converted into cDNA using a SuperScript™ First-Strand System for RT-PCR Kit (Invitrogen). The levels of shati and TNF-α mRNA were determined by real-time RT-PCR using a TaqMan probe. The 18S ribosomal RNA was used as the internal control (Applied Biosystems, CA, USA). The shati primers used for real-time RT-PCR were as follows: 5′-TGTAAACACCCCTAA AGTGCCCT-3′ (forward; bp 2967–2989) and 5′-TCAATCCTGC ATACAAGGAATCAA-3′ (reverse; bp 3022–3045), and the Taq-Man probe was 5′-CACAGTCTGTGAGGCTCAGGTTGCCC-3′ (probe; bp 2995–3020). The amplification consisted of an initial step (95°C for 5 min) and then 40 cycles of denaturation for 30 sec at 95°C, annealing for 40 s at 59°C, and the extension time for 1 min at 72°C in an iCycle iQ Detection System (Bio-Rad Laboratories, Inc., CA, USA) (Niwa *et al.* 2007a). The expression levels were calculated as described previously (Wada *et al.* 2000).

Animals

The male C57BL/6J- wild-type mice were obtained from Slc Japan (Hamamatsu, Japan). Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1°C; 50 ± 5% humidity; 12 : 12 h light/dark cycle starting at 8:00 AM) and had free access to food and water, except during behavioral experiments. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Japanese Pharmaceutical Society.

Shati-antisense oligonucleotide (shati-AS) treatment

Mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity was 90 μL, Alzet 1002; Alza, Palo Alto, CA, USA) filled with shati-antisense oligonucleotide (shati-AS) or -scrambled oligonucleotide (shati-SC). The pump was implanted into the right ventricle [anteroposterior (AP) −0.5 mm, mediolateral +1.0 mm from the bregma, and dorsoventral −2.0 mm from the skull, according to the atlas of Franklin and Paxinos (1997)]. Phosphorothionate oligonucleotides were custom-synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial CSF (147 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.2). The oligonucleotides were phosphorothioated at the first three bases of both the 5′- and 3′-ends, which results in increased stability and less toxicity. The sequences of shati-AS and -SC were 5′-TCTTCGTCTCGCAGAC CATGTCG-3′ and 5′-GGTCTGCTACACTGCTGCTAGTC-3′, respectively. Shati-AS and -SC were continuously infused into the cerebral ventricle at a dose of 1.8 nmol/6 μL/day (flow rate, 0.25 μL/h). Additionally, shati-SC was used as a control. Three days after the start of oligonucleotide infusion, mice were

administered METH (1 mg/kg, s.c.) for 5 days and decapitated 2 h after the final treatment (Niwa *et al.* 2007a).

Statistical analysis

All data were expressed as means ± SE. Statistical differences between two groups were determined with Student's *t*-test. Statistical differences among three groups or more were determined using a one-way analysis of variance (ANOVA), two-way ANOVA, or three-way ANOVA, followed by the Bonferroni multiple comparison test. $p < 0.05$ was regarded as statistically significant.

Nucleotide sequences

The DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory accession number for the primary nucleotide sequence of shati is DQ174094.

Results

Effect of TNF-α on DA uptake in PC12 cells

First, we investigated the effects of TNF on DA uptake in PC12 cells, since we have recently demonstrated that TNF-α activates synaptosomal and vesicular DA uptake in mice (Nakajima *et al.* 2004).

TNF-α (10 ng/mL, 40 min) increased [^{3}H] DA uptake compared with the control group ($F_{3,28} = 4.933$, $p < 0.01$, one-way ANOVA) (Fig. 1a). Moreover, we investigated whether the TNF-α-induced increase was antagonized by the anti-TNF-α antibody and soluble TNF receptor in PC12 cells. Pre-treatment with the antibody (10, 50, and 100 ng/

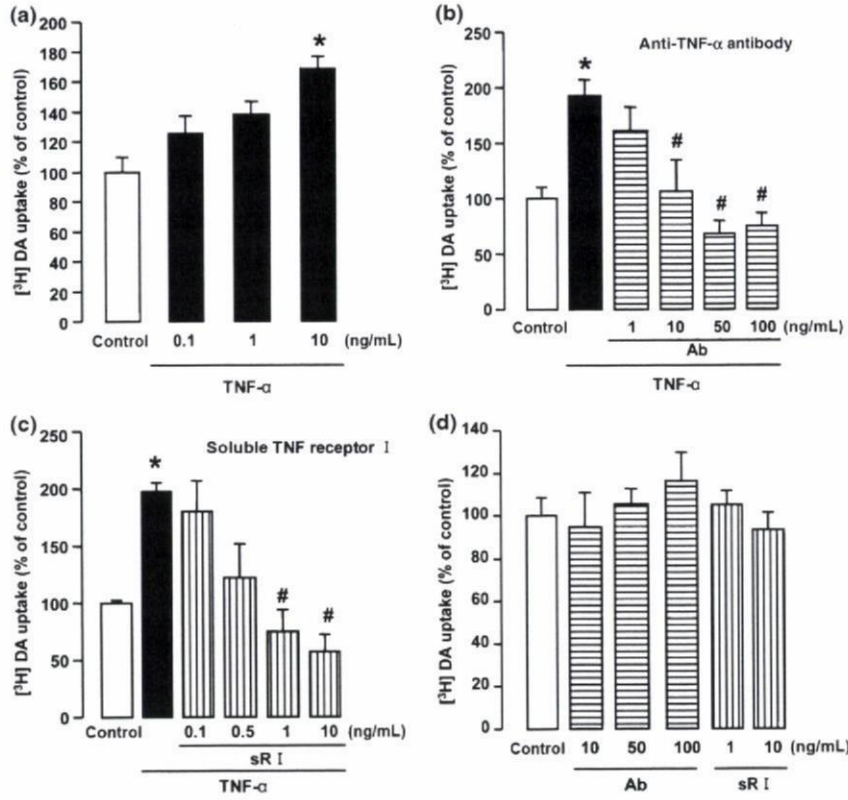

Fig. 1 Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. (a) The cells were pre-treated with TNF-α (0.1, 1, and 10 ng/mL) for 40 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.14 ± 0.01 pmol/10 min for control. The final concentration of [^{3}H] DA was 20 nM. Value are means ± SE ($n = 8$). $^*p < 0.05$ versus control. (b) Effects of anti-TNF-α antibody (Ab) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (1, 10, 50, and 100 ng/mL) 10 min before their treatment with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.10 ± 0.02 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (c) Effects of soluble TNF receptor I (sR I) on TNF-α-induced increase in [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with soluble TNF receptor I (0.1, 0.5, 1, and 10 ng/mL) 10 min before being treated with TNF-α (10 ng/mL, 40 min), and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.06 ± 0.00 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–7). $^*p < 0.05$ versus control. $^\#p < 0.05$ versus TNF-α-treated cells. (d) Effects of anti-TNF-α antibody (Ab) or soluble TNF receptor I (sR I) on [^{3}H] DA uptake in PC12 cells. The cells were pre-treated with anti-TNF-α antibody (10, 50, and 100 ng/mL) or soluble TNF receptor I (1 and 10 ng/mL) for 50 min, and assayed for [^{3}H] DA uptake. The [^{3}H] DA uptake was 0.08 ± 0.01 pmol/10 min for the control. The final concentration of [^{3}H] DA was 20 nM. Values are means ± SE ($n = 6$–8).

