Although μ-opioids have potential for abuse and/or addiction, clinical studies have shown that abuse and addiction do not usually occur when μ-opioids are used appropriately to control pain (Eisenberg et al., 2005). We have proposed that the abuse potential of morphine is potently suppressed under chronic pain in rodents (Suzuki et al., 1996; Narita et al., 2005; Niikura et al., 2010). However, over the past decade, there has been some concern regarding the abuse of and addiction to μ-opioids caused by the inappropriate use, misuse, or overdose of prescription opioids (Woodcock, 2009). Dopamine receptor antagonists have been commonly used to reduce the adverse reactions to μ-opioids, including delusions and hallucinations (McNicol et al., 2003). Olanzapine is an atypical antipsychotic that is clinically indicated for schizophrenia and mania. It blocks multiple neurotransmitters (Glazer, 1997; Tohen and Grundy, 1999). In the present study, we found that olanzapine at 0.3 mg/kg, which did not induce motor coordination significantly suppressed the hyperlocomotion and rewarding effects induced by morphine. In our preliminary study, treatment with olanzapine at 0.3 mg/kg failed to reduce the antinociceptive effect of morphine. Furthermore, the delay in colonic expulsion induced by morphine was not affected by 0.3 mg/kg of olanzapine, which indicated that olanzapine did not exacerbate morphine-induced constipation. Taken together, these findings support the idea that olanzapine may have a wide margin of safety when used as an adjuvant for μ-opioids.

In a previous binding study in brain tissue, we found that olanzapine exhibited the highest affinity for muscarinic M_1 receptors and also showed affinity toward serotonin 5-$HT_{2A/2C}$, 5-HT_3, histamine H_1, dopamine D_2, dopamine D_4, and 5-HT_4 receptors. In light of this multiple binding property, we previously documented in an *in vivo* study that olanzapine dose-dependently decreased morphine-induced nausea and vomiting that are caused through various mechanisms (Torigoe et al., in press).

Muscarinic M_1 receptors have been suggested to be responsible for the enhancement of opioid-stimulated dopaminergic transmission related to the aggravation of drug addiction (Tanda et al., 2007). Since olanzapine showed the highest affinity toward muscarinic M_1 receptors, it is reasonable to wonder if olanzapine could aggravate the abuse potential of μ-opioids. This contention can be supported by the fact that the selective muscarinic M_1 receptor antagonist trihexyphenidyl significantly enhanced the morphine-induced increase in the release of dopamine in the nucleus accumbens (our preliminary study; data not shown), which indicates that M_1 receptors play an important role in opioid addiction. However, in the present study, olanzapine did not enhance either the hyperlocomotion or place preference produced by morphine. Although the exact mechanism by which olanzapine suppresses morphine's abuse profile remains unclear, this phenomenon may result from the fact that olanzapine acts not only on muscarinic M_1 receptors but also partly on serotonin 5-$HT_{2A/2C}$, 5-HT_3, histamine H_1, dopamine D_2, dopamine D_4, and 5-HT_4 receptors as an antagonist.

Morphine has subjective effects (e.g., "strength of drug effect," "sedated," "heavy or sluggish feeling," and "high") in healthy volunteers (Zacny et al., 1994). Furthermore, morphine can induce drowsiness, hallucination, and delirium, which have been considered to be important cues for the subjective effects of morphine (Adunsky et al., 2002; Maddocks et al., 1996). To assess the subjective effects in humans, animal models for studying the components of drug action that bear on the subjective effects have been developed. A methodology that has considerable potential in this regard is the drug discrimination procedure (Schuster and Johanson, 1988). However, little is known about the mechanism of the discriminative stimulus effects of morphine in animals. In the present study, olanzapine at 0.3 mg/kg significantly attenuated the discriminative stimulus effects of the training dose of morphine in rats. It should be emphasized that neither the D_1 receptor antagonist SCH23390, the D_2 receptor antagonist haloperidol nor their combination affected the discriminative stimulus effects of morphine in rats that had been trained to discriminate between 3.0 mg/kg morphine and saline (Suzuki et al., 1995). Therefore, we hypothesize that the blockade of neurotransmitters other than dopamine receptors by olanzapine at this dose may contribute to attenuate the discriminative stimulus effects of morphine.

In conclusion, we found that olanzapine at a dose that failed to induce motor dysfunction suppresses the hyperlocomotion, place preference, and discriminative stimulus effect induced by morphine. These results further provide evidence that cotreatment with olanzapine may be very useful as an adjuvant for pain control by μ-opioids.

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Usefulness of Olanzapine as an Adjunct to Opioid Treatment and for the Treatment of Neuropathic Pain

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ABSTRACT

Background: The use of opioids for pain management is often associated with nausea and vomiting. Although conventional antipsychotics are often used to counter emesis, they can be associated with extrapyramidal symptoms. However, chronic pain can induce sleep disturbance. The authors investigated the effects of the atypical antipsychotic olanzapine on morphine-induced emesis and the sleep dysregulation associated with chronic pain.

Methods: A receptor binding assay was performed using mouse whole brain tissue. The emetic response in ferrets was evaluated by counting retching and vomiting behaviors. Catalepsy in mice was evaluated by placing both of their forepaws over a horizontal bar. Released dopamine was measured by an *in vivo* microdialysis study. Sleep disturbance in mice in a neuropathic pain-like state was assayed by electroencephalogram and electromyogram recordings.

Results: Olanzapine showed high affinity for muscarinic M_1 receptor in brain tissue. Olanzapine decreased morphine-induced nausea and vomiting in a dose-dependent manner. However, olanzapine at a dose that had an antiemetic effect (0.03 mg/kg) did not induce catalepsy or hyperglycemia. In addition, olanzapine at this dose had no effect on the morphine-induced release of dopamine or inhibition of gastrointestinal transit. Finally, olanzapine inhibited thermal hyperalgesia and completely alleviated the sleep disturbance induced by sciatic nerve ligation.

Conclusion: These findings suggest that olanzapine may be useful for the treatment of morphine-induced emesis and as an adjunct for the treatment of neuropathic pain associated with sleep disturbance.

What We Already Know about This Topic

- Chronic pain is often associated with sleep disturbances
- Severe side effects of opioids given for pain treatment include nausea and vomiting

What This Article Tells Us That Is New

- In ferrets, olanzapine, an atypical thienobenzodiazepine antipsychotic drug, suppressed morphine-induced emesis and improved pain-related sleep disturbances

THE World Health Organization[1] has stated that morphine is the "gold standard" for the treatment of moderate to severe pain caused by cancer. However, the use of morphine for this purpose is often associated with distressing side effects, such as drowsiness, constipation, emesis, and delirium.[2,3] Many clinicians consider that dopamine receptor antagonists, including prochlorperazine, are the preferred drugs for combating opioid-induced nausea and vomiting.[2,3] However, these drugs often produce adverse effects, including extrapyramidal symptoms.[4] Therefore, new approaches are needed to prevent opioid-induced emesis, as is a better understanding of the mechanism of drug action.

Nausea and vomiting are controlled by the "vomiting center" in the medulla oblongata,[5] which receives signals from the chemoreceptor trigger zone (CTZ) in the area postrema, the gastrointestinal tract, the vestibular apparatus in the temporal lobe, and the cerebral cortex.[6] Opioids have emetogenic effects by stimulating the CTZ and the vestibular apparatus and by inhibiting gut motility.[7] Although stimu-

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lation of the CTZ by opioids involves opioid μ and δ receptors,[8] signals from the CTZ to the vomiting center mainly involve dopamine D_2 and serotonin ($5\text{-}HT_3$) receptors in the former. However, opioid-induced stimulation of the vestibular apparatus and subsequent sensory input to the vomiting center have both been suggested to involve histamine H_1 and muscarinic acetylcholine pathways.[9]

Atypical antipsychotic medications treat the positive symptoms of schizophrenia, such as hallucinations and delusions, more effectively than the negative symptoms, such as lack of motivation and social withdrawal. Olanzapine is a newer atypical antipsychotic that blocks dopaminergic, serotonergic, adrenergic, histaminergic, and muscarinic receptors for multiple neurotransmitters. Because it affects neurotransmitters that are associated with nausea, it may have potential as an antiemetic medication.[10]

In addition, patients with chronic pain commonly experience sleep disturbance[11–13] and may benefit from its treatment.[13] Sleep problems and daytime sleepiness seem to be related to depression and the severity of pain.[14] It has been suggested that olanzapine may improve sleep disturbance.[15]

The primary endpoint of the study was to investigate whether olanzapine at doses lower than those that would induce catalepsy could suppress morphine-induced emesis with few side effects. We also examined if olanzapine could improve sleep dysregulation under a neuropathic pain-like state.

Materials and Methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Hoshi University, as adopted by the Committee on Animal Research of Hoshi University (Tokyo, Japan). Every effort was made to minimize the numbers and suffering of animals used in the following experiments.

The observer was not blinded in all of the experiments.

Animals

In the present study, male Institute of Cancer Research mice (20–25 g) (Tokyo Laboratory Animals Science, Tokyo, Japan), male C57BL/6J mice (25–30 g) (CLEA Japan, Tokyo, Japan), and Sprague-Dawley rats (200–300 g) (Tokyo Laboratory Animals Science) were used. Animals were housed in a room maintained at 22° ± 1°C with a 12-h light–dark cycle. Food and water were available *ad libitum*. Each animal was used only once. Male ferrets weighing 1–1.5 kg were obtained from Marshall Research Labs (North Rose, NY) and housed individually in a room kept at 23° ± 1°C under a 12-h light–dark cycle (lights on 8:00 AM-8:00 PM). They were given a standard cat diet (70–80 g/animal, Oriental Yeast Co. Ltd., Chiba, Japan) and allowed free access to water.

Receptor Binding Assay

Mouse whole brain was treated as described previously,[16] and the resulting pellet was resuspended and used as the membrane fraction. The binding assay was performed in triplicate with [^{3}H]clozapine (specific activity, 70–87 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) at 0.2 nM, [^{3}H]ketanserin hydrochloride (specific activity, 67 Ci/mmol; PerkinElmer, Waltham, MA) at 0.5 nM, [^{3}H]BRL-43694 (granisetron) (specific activity, 85.3 Ci/mmol; PerkinElmer) at 0.5 nM, [^{3}H]GR113808 (specific activity, 78.3 Ci/mmol; PerkinElmer) at 0.5 nM, [^{3}H]pyrilamine (specific activity, 30 Ci/mmol; PerkinElmer) at 0.5 nM, and [^{3}H]pirenzepine (specific activity, 72.8 Ci/mmol; PerkinElmer) at 0.5 nM, in a final volume of 500 ml that contained 50 mM Tris-HCl buffer, pH 7.4, and 200 ml homogenized membrane fraction. Ninety to 140 mg membrane proteins were used in each assay. Specific binding was defined as the difference in binding observed in the absence and presence of 1 mM unlabeled clozapine, ketanserin, granisetron, or GR113808, 10 mM unlabeled pyrilamine, or 100 mM unlabeled pirenzepine, respectively. All samples were incubated as described previously,[16] and radioactivity in the samples was determined with a liquid scintillation analyzer. All receptor binding curves were fitted using Prism software (version 5.0a; GraphPad Software, La Jolla, CA).

Evaluation of the Emetic Response

Emesis in ferrets after the administration of morphine (0.6 mg/kg, subcutaneous injection) was evaluated by counting the number of retching or vomiting behaviors as described elsewhere,[17] where retching was defined as a rhythmic abdominal contraction without expulsion and vomiting was the oral expulsion of solid or liquid from the gastrointestinal tract. Emesis was assessed for 30 min after the injection of morphine.[18] To determine the effect of olanzapine on morphine-induced emesis, groups of ferrets were pretreated with olanzapine 30 min before the injection of morphine.

An interval of at least 7 days was allowed between testing for each animal to allow for drug washout and to minimize the development of tolerance.

Horizontal Bar Test for the Evaluation of Catalepsy

Catalepsy[19,20] was evaluated using the horizontal bar test as described previously.[21] Briefly, animals were placed so that both forepaws were over a horizontal bar 5 cm above the floor, and the amount of time (s) the animal maintained this position was recorded for as long as 60 s. Catalepsy was considered to have finished when a forepaw touched the floor or when the mouse climbed on the bar. Scores were assigned based on the duration of the cataleptic posture (score 1: 15 to 29 s, score 2: 30 to 59 s, score 3: 60 s or more).

In vivo *Microdialysis Study and Quantification of Dopamine and Its Metabolites*

After 3 days of habituation to the main animal colony, all of the rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal administration) for surgery as described previously.[21] Briefly, the anesthetized animals were placed in a stereotaxic apparatus, the skull was exposed, and a small hole was made using a dental drill. A guide cannula (AG-8; Eicom, Kyoto, Japan) was implanted into the nucleus accumbens (from the bregma: anterior, +4.0 mm; lateral, −0.8 mm; ventral, −6.8 mm; angle 16 degrees) according to the atlas of Paxinos and Watson[22] and fixed to the skull with cranioplastic cement. Three to 5 days after surgery, microdialysis probes (A-I-8–02; 2 mm membrane length; Eicom) were slowly inserted into the nucleus accumbens through guide cannulas during anesthesia with diethyl ether, and the rats were placed in experimental cages (30 cm wide × 30 cm deep × 30 cm high). The probes were perfused continuously (2 ml/min) with artificial cerebrospinal fluid: 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM $CaCl_2$. Outflow fractions were collected every 20 min. After three baseline fractions were collected from the rat nucleus accumbens, rats were given olanzapine (0.3 mg/kg, intraperitoneal administration), vehicle (5% dimethyl sulfoxide [DMSO]); 1 ml/kg, intraperitoneal administration) or saline (1 ml/kg, intraperitoneal administration) 30 min before treatment with morphine (10 mg/kg, intraperitoneal administration). Dialysis samples were collected for 180 min after treatment and analyzed by high-performance liquid chromatography (Eicom) with electrochemical detection (Eicom). Dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenyl acetic acid, were separated by column chromatography and identified and quantified by the use of standards, as described previously.[21]

Gastrointestinal Transit

In the study of gastrointestinal transit,[23] Institute of Cancer Research mice were fasted for 12 h before the experiments. Groups of mice were pretreated with olanzapine (0.03–1 mg/kg, subcutaneous injection) or vehicle (5% DMSO) 30 min before the administration of morphine (0.7 mg/kg, subcutaneous injection) or saline, and ink (0.3 ml/mouse) was orally administered 20 min after the injection of morphine or saline. Twenty minutes after the administration of ink, the animal was killed by cervical dislocation, and the small intestine was removed. The percentage inhibition of gastrointestinal transit was calculated as follows: (distance traveled by the ink/length from the pylorus to the cecum) × 100.

Blood Glucose Measurement

C57BL/6J mice were administered olanzapine (0.03–1 mg/kg, subcutaneous injection) or vehicle (5% DMSO) once a day for 1 week. At 60 min after the final injection, the tail was cut and blood was collected. Blood glucose was measured using a self-monitoring blood glucose meter (Medisafe-Mini; Terumo, Tokyo, Japan). The Medisafe-Mini system is based on the optoelectric colorimetry method.

Neuropathic Pain Model

C57BL/6J mice were anesthetized with 3% isoflurane. A partial sciatic nerve ligation model was made by tying a tight ligature with 8–0 silk suture around approximately one third to one half the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30; Olympus, Tokyo, Japan). In sham-operated mice, the nerve was exposed without ligation.

Measurement of Thermal Thresholds

The sensitivity to thermal stimulation was measured as described previously.[24] Briefly, the right plantar surface of mice was exposed to a well-focused radiant heat light source (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA) that had been adjusted so that the average baseline latency of paw withdrawal in naive mice was approximately 8–10 s. Only quick movements of the hind paw away from the stimulus were considered to be a withdrawal response: paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between left and right with an interval of more than 3 min between measurements. Before testing, mice were placed in a clear acrylic cylinder (15 cm high and 8 cm in diameter) for at least 30 min. The data represent the average latency of paw withdrawal for the right hind paw.

Electroencephalogram and Electromyogram Recordings

Electroencephalogram and electromyogram recordings were obtained as described previously.[24] Briefly, electroencephalogram signals were monitored with two stainless-steel electroencephalogram recording screws 1 mm anterior to the bregma or λ, both 1.5 mm lateral to the midline, and electromyogram activity was monitored by stainless steel, nonstick-coated wires placed bilaterally into both trapezius muscles. Electroencephalogram and electromyogram signals were amplified, filtered (0.5–30 Hz and 20–200 Hz, respectively), digitized at a sampling rate of 128 Hz, and recorded using SleepSign software (Kissei Comtec, Nagano, Japan), which was also used to automatically classify vigilance over 4-s epochs as wakefulness, rapid eye movement (REM) sleep, or non-REM sleep using standard criteria. Finally, defined sleep–wake stages were examined visually and corrected, if necessary. For each epoch, the electroencephalogram power density in the δ (0.75–4.0 Hz) and θ bands (6.25–9.0 Hz) and the integrated electromyogram value were displayed on a computer monitor. Electroencephalogram and electromyogram activities were monitored for 24 h at 7 days after sciatic nerve ligation. Recordings were started at 8:00 PM. Vehicle

(5% DMSO) or olanzapine (0.06 mg/kg, intraperitoneal administration) was injected every day at 8:00 AM.

Drugs

The drugs used in the current study were morphine hydrochloride (Daiichi-Sankyo, Tokyo, Japan), prochlorperazine maleate (Sigma–Aldrich, St. Louis, MO), clozapine (Wako Pure Chemical Industries, Osaka, Japan), olanzapine (Toronto Research Chemicals, Toronto, Ontario, Canada), telenzepine dihydrochloride hydrate (Sigma–Aldrich), ritanserin (Tocris Biotechnology, Ellisville, CA), pyrilamine maleate salt (Sigma–Aldrich), ketanserin tartrate (Wako Pure Chemical Industries), granisetron (Sigma–Aldrich), GR113808 (Sigma–Aldrich), haloperidol (Sigma–Aldrich), L745870 (Research Biochemicals International, Natick, MA), raclopride (Santa Cruz Biotechnology, Santa Cruz, CA), pirenzepine (Toronto Research Chemicals), and DL-trihexyphenidyl hydrochloride (Sigma–Aldrich).

Statistical Analysis

Data are expressed as the mean with SEM. The statistical significant of differences between the groups was assessed with one-way and two-way ANOVA followed by the Bonferroni multiple comparisons test or Student *t* test (unpaired, two-tailed). The concentration of the test compound that caused 50% inhibition of specific binding (IC_{50} value) was determined from each concentration-response curve. All statistical analyses and IC_{50} values were calculated by Prism software (version 5.0a, GraphPad Software). A P value of <0.05 was considered to reflect significance.

Results

Binding Properties of Clozapine

In mouse brain membranes, we determined the competitive displacement binding of [^{3}H]clozapine with graded concentrations (10^{-11}–10^{-4} M) of unlabeled clozapine, olanzapine, telenzepine, ritanserin, pyrilamine, ketanserin, GR113808, granisetron, haloperidol, L745870, and raclopride. The binding of [^{3}H]clozapine was displaced by olanzapine in a concentration-dependent manner (fig. 1A). In addition, the binding of [^{3}H]clozapine was partially displaced by telenzepine (M_1), ritanserin (5-HT_{2A}), pyrilamine (H_1), ketanserin (5-HT_{2C}), GR113808 (5-HT_4), granisetron (5-HT_3), haloperidol (D_2), L745870 (D_4), and raclopride (D_2) (fig. 1B).

Binding Properties of Olanzapine with 5-$HT_{2A/2C}$, 5-HT_3, 5-HT_4, H_1, and M_1 Receptors

In mouse brain membranes, we determined the competitive displacement binding of [^{3}H]ketanserin, [^{3}H]BRL-43694 (granisetron), [^{3}H]pyrilamine, [^{3}H]GR113808, and [^{3}H]pirenzepine with graded concentrations (10^{-11}–10^{-4} M) of unlabeled ketanserin, granisetron, pyrilamine, GR113808, telenzepine, pirenzepine and olanzapine. The binding of [^{3}H]ketanserin and [^{3}H]pirenzepine was displaced by olanzapine in a concentration-dependent manner (fig. 2, A and B). The binding of [^{3}H]pyrilamine, [^{3}H]BRL-43694, and [^{3}H]GR113808 was partially displaced by olanzapine (fig. 2, C, D, and E).

Suppression of Morphine-induced Emesis by Olanzapine or Prochlorperazine

Pretreatment with either olanzapine (0.03 mg/kg, subcutaneous injection) 30 min before the injection of morphine (0.6 mg/kg, subcutaneous injection) or prochlorperazine (0.3 mg/kg and 1.0 mg/kg, subcutaneous injection) 60 min before the injection of morphine reduced the number of retching and vomiting behaviors induced by morphine (fig. 3).

Effects of Antipsychotics on Catalepsy

The results from the horizontal bar test as a measure of catalepsy were obtained at 15, 30, 45, and 60 min after the subcutaneous injection of vehicle, prochlorperazine (0.3–1 mg/kg), haloperidol (0.03–0.3 mg/kg), risperidone (0.01–0.1 mg/kg), or olanzapine (0.03–0.3 mg/kg). Although the catalepsy scores were not affected by a single subcutaneous injection of olanzapine (0.03–0.3 mg/kg), catalepsy was observed with the other antipsychotics within this dose range (fig. 4).

Effects of Olanzapine on the Morphine-induced Increase in the Concentrations of Dopamine and its Metabolites in Dialysate

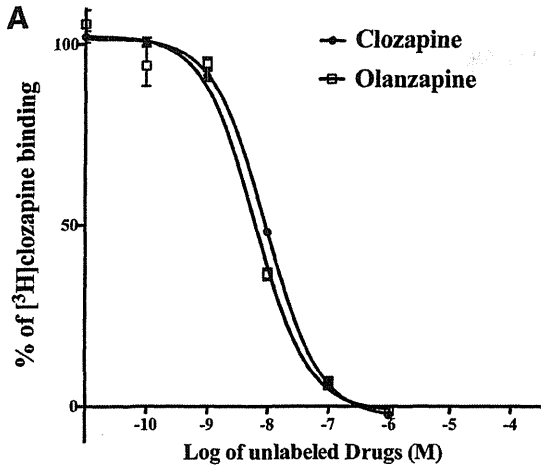
In the microdialysis study, the concentrations of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenyl acetic acid in dialysate from the rat nucleus accumbens were markedly increased by the intraperitoneal administration of morphine at 10 mg/kg compared with those under the administration of saline. The increased concentrations of dopamine, 3,4-dihydroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenyl acetic acid in the nucleus accumbens after the administration of morphine were not affected by olanzapine at 0.3 mg/kg (olanzapine-morphine *vs.* vehicle-morphine: $F_{(1,77)} = 0.1516$, $P = 0.7086$ fig. 5A; $F_{(1,77)} = 0.06326$, $P = 0.8086$ fig. 5B; $F_{(1,77)} = 1.851$, $P = 0.2158$ fig. 5C).

Effect of Olanzapine on the Morphine-induced Inhibition of Gastrointestinal Transit

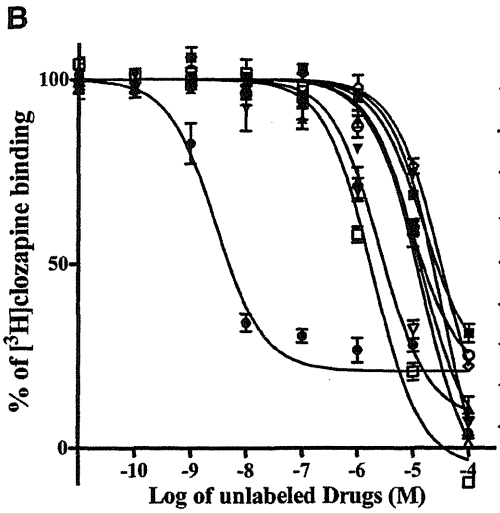
Morphine slowed gastrointestinal transit, and this effect was not significantly altered by the coadministration (subcutaneous injection) of olanzapine at 0.03–1 mg/kg (fig. 6A). Olanzapine itself did not slow gastrointestinal transit at doses of 0.03 and 0.1 mg/kg but significantly inhibited gastrointestinal transit at 0.3 and 1 mg/kg (fig. 6B).

Effects of Olanzapine on Blood Glucose

Blood glucose was measured after long-term treatment with olanzapine, saline, or vehicle (5% DMSO) in mice. Hypergly-



Antagonists	Clozapine	Olanzapine
IC50 (nM) for displacing [^{3}H]clozapine binding	9.56 (8.55-10.63)	6.26 (4.27-9.20)



Antagonists	IC50 (nM)
Telenzepine	2.91 (1.66-5.13)
Ritanserin	1885 (1368-2597)
Pyrilamine	2559 (1949-3339)
Ketanserin	> 10000
GR113808	> 10000
Granisetron	> 10000
Haloperidol	9127 (6329-13160)
L745870	> 10000
Raclopride	> 10000

Fig. 1. Displacement of the binding of [^{3}H]clozapine in membranes of mouse brain without the cerebellum by clozapine, olanzapine, telenzepine, ritanserin, pyrilamine, GR113808, granisetron, ketanserin, haloperidol, L745870, and raclopride. Experiments were performed in the presence of [^{3}H]clozapine (0.2 nM) and increasing concentrations of either clozapine or olanzapine (*A*) or of telenzepine, ritanserin, pyrilamine, GR113808, granisetron, ketanserin, haloperidol, L745870, or raclopride (*B*). The data represent the mean ± SEM of three to four samples. IC_{50} values were determined using an analysis of variance and linear regression techniques. To calculate the IC_{50} values, at least six drug doses were used, and three samples were used for each dose. Values in parentheses indicate the 95% confidence range.

cemia was not observed under treatment with olanzapine at 0.03, 0.1, or 0.3 mg/kg (subcutaneous injection) (fig. 7).

Thermal Hyperalgesia Induced by Sciatic Nerve Ligation in Mice

Sciatic nerve ligation markedly decreased the latency of paw withdrawal in response to a thermal stimulus on the ipsilateral side. This state of persistent pain caused by partial ligation of the sciatic nerve was suppressed by treatment with olanzapine at 0.06 mg/kg (fig. 8).

Changes in Sleep Vigilance in a Neuropathic Pain-like State Using Electroencephalogram and Electromyogram Recordings

We next investigated the changes in sleep patterns in sciatic nerve-ligated mice. Vigilance was classified automatically offline as wakefulness, REM sleep, or non-REM sleep. Mice with sciatic nerve ligation showed a statistically significant increase in wakefulness ($P = 0.0009$ *vs.* sham operated mice with vehicle, fig. 9A) and a decrease in non-REM sleep ($P = 0.0067$ *vs.* sham-operated mice with vehicle, fig. 9C) during the light phase. REM sleep during the light phase was not affected by sciatic nerve ligation ($P = 0.2896$ *vs.* sham-operated mice with vehicle, fig. 9B). On the other hand, there was no statistically significant difference in the sleep conditions during the dark phase between the two groups (wakefulness: $P = 0.6170$ *vs.* sham operated mice with vehicle, fig. 9D; REM: $P = 0.3936$ *vs.* sham operated mice with vehicle, fig. 9E; non-REM: $P = 0.5479$ *vs.* sham operated mice with vehicle, fig. 9F).

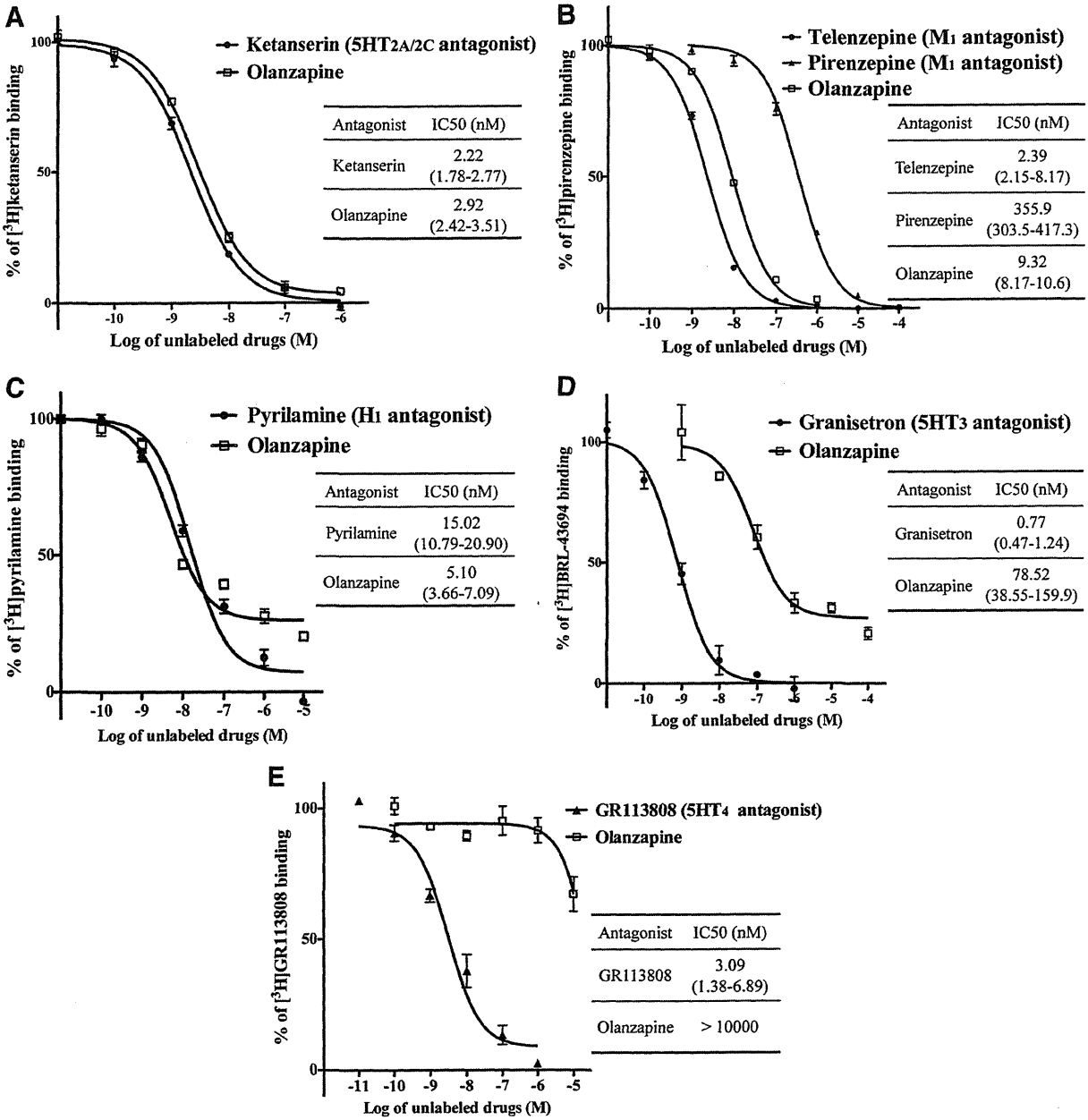


Fig. 2. Displacement of the binding of the serotonin (5-HT)$_{2A/C}$ receptor ligand [^{3}H]ketanserin (*A*), the muscarinic M_1 receptor ligand [^{3}H]pirenzepine (*B*), the H_1 receptor ligand [^{3}H] pyrilamine (*C*), the 5-HT_3 receptor ligand [^{3}H]BRL-43694 (granisetron) (*D*), or the 5-HT_4 receptor ligand [^{3}H]GR113808 (*E*) in membranes of mouse brain without the cerebellum by ketanserin, pirenzepine, telenzepine, pyrilamine, granisetron, GR113808, or olanzapine. Experiments were performed in the presence of [^{3}H]ketanserin (0.5 nM), [^{3}H]BRL-43694 (0.5 nM), [^{3}H]pyrilamine (0.5 nM), [^{3}H]GR113808 (0.5 nM), or [^{3}H]pirenzepine (0.5 nM) and increasing concentrations of ketanserin, granisetron, pyrilamine, GR113808, pirenzepine, telenzepine, or olanzapine. The data represent the mean ± SEM of three to four samples. IC_{50} values were determined using an analysis of variance and linear regression techniques. To calculate the IC_{50} values, at least six drug doses were used, and three samples were used for each dose. Values in parentheses indicate the 95% confidence range.

Changes in the Hypnotic Effects of Olanzapine in a Neuropathic Pain-like State Using Electroencephalogram and Electromyogram Recordings

To ascertain the hypnotic effect of olanzapine in a neuropathic pain-like state, we performed electroencephalogram and electromyogram recordings. The increased wakefulness and decreased non-REM during the light phase in nerve-ligated mice were significantly attenuated compared with those in sham-operated mice by the intraperitoneal administration of olanzapine (wakefulness: $P = 0.0006$ *vs.* nerve-

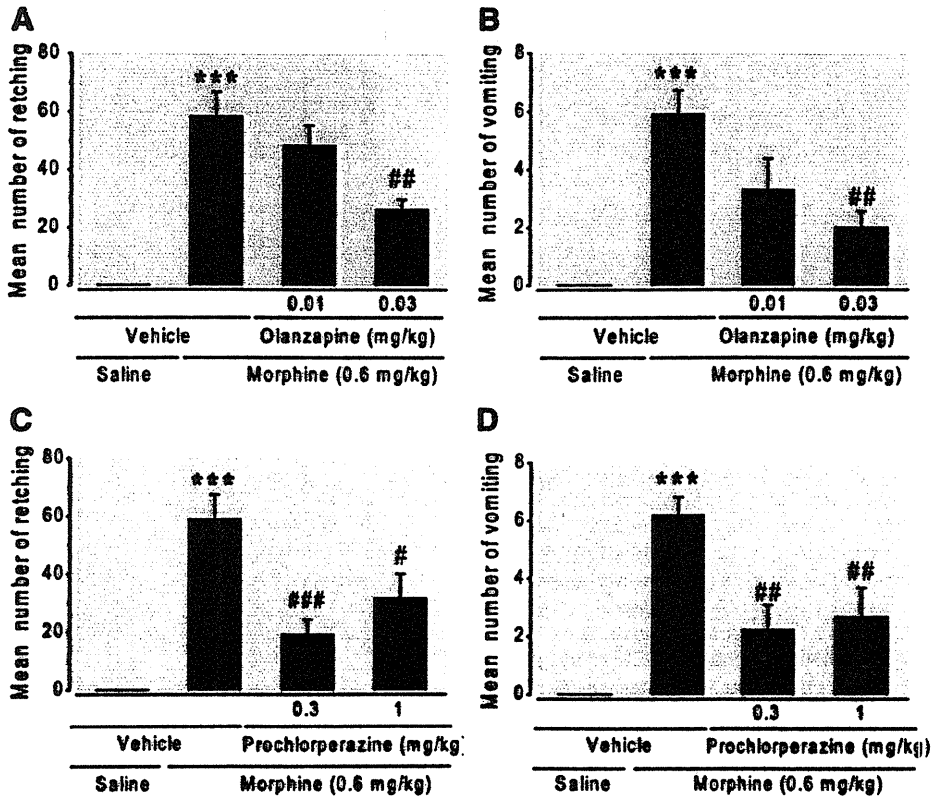


Fig. 3. Effects of olanzapine on subcutaneous injection morphine-induced retching (*A*, *C*) and vomiting (*B*, *D*) in ferrets. Groups of ferrets were pretreated with olanzapine (0.01 and 0.03 mg/kg, subcutaneous injection) (*A*, *B*), prochlorperazine (0.3 and 1.0 mg/kg, subcutaneous injection) (*C*, *D*), or vehicle before the administration of morphine (0.6 mg/kg, subcutaneous injection). Animals were observed for 30 min after subcutaneous injection of morphine. Each column represents the mean ± SEM of 9–10 ferrets. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni multiple comparisons test: $F_{(3,39)} = 20.41$, $P < 0.0001$ (*A*); $F_{(3,39)} = 11.29$, $P < 0.0001$ (*B*); $F_{(3,37)} = 15.13$, $P < 0.0001$ (*C*); $F_{(3,37)} = 13.70$, $P < 0.0001$ (*D*). *** $P < 0.001$ *versus* vehicle-saline; ### $P < 0.001$; ## $P < 0.01$ or # $P < 0.05$ *versus* vehicle-morphine.

ligated mice with vehicle, fig. 9A; non-REM: $P = 0.001$ *vs.* nerve-ligated mice with vehicle, fig. 9C).

Discussion

The use of opioids for pain management is often associated with nausea and vomiting. Opioids induce emesis by stimulating the CTZ in the brainstem and by enhancing vestibular sensitivity.[25,26] Although several compounds are known to act on receptors in the CTZ, opioid-induced nausea and vomiting are attributable primarily to the transmission of dopamine. Many clinicians consider that typical antipsychotics such as prochlorperazine and haloperidol, which mainly act as dopamine D_2 receptor antagonists, are the drugs of choice for preventing the nausea and vomiting induced by morphine.[27–29] However, such compounds often produce extrapyramidal symptoms.[4]

Olanzapine is an atypical thienobenzodiazepine antipsychotic that is clinically indicated for schizophrenia and mania.[30] It blocks multiple neurotransmitters, including dopaminergic, serotonergic, adrenergic, histaminergic, and muscarinic receptors.[31] In the current binding study, olanzapine showed the highest affinity for muscarinic M_1 receptors. To understand its affinity in greater detail, we investigated the affinity of olanzapine toward serotonin 5-$HT_{2A/2C}$, 5-HT_3, histamine H_1, dopamine D_2, dopamine D_4, and 5-HT_4 receptors. Olanzapine also showed affinity for each of these receptors. Because of its effect on several neurotransmitters that are associated with nausea and vomiting, we expected that olanzapine would have potential as an antiemetic medication. In a behavioral study, we found that morphine-induced nausea and vomiting were decreased in a dose-dependent manner by pretreatment with olanzapine at a dose that was almost 1/200 of that at which an antipsychotic effect is observed,[32] whereas olanzapine at a dose that had antiemetic effects failed to induce catalepsy. However, although the dopamine D_2 receptor antagonist prochlorperazine suppressed morphine-induced nausea and vomiting, it did so at a dose that caused a dose-dependent increase in the expression of catalepsy. Furthermore, olanzapine had no effect on the morphine-induced release of dopamine in the nucleus accumbens. Muscarinic M_1 receptors have been suggested to be responsible for the opioid-induced stimula-

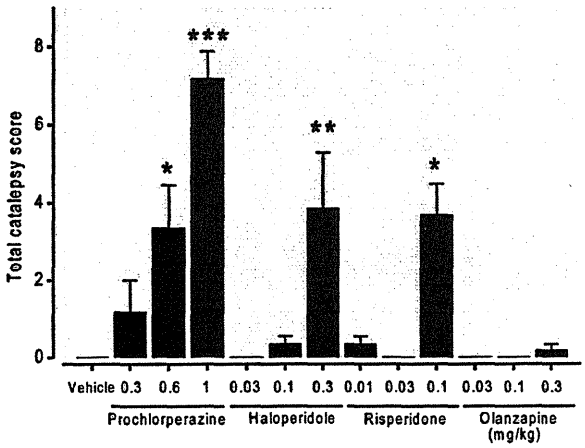


Fig. 4. Expression of catalepsy caused by antipsychotics in mice. Catalepsy values were obtained for 60 min after subcutaneous injection of antipsychotics. The time until the forepaw touched the floor or until the mouse climbed up on the bar was measured for as long as 60 s. A score was assigned to each test based on the duration of the cataleptic posture (score 1, 15 to 29 s; score 2, 30 to 59 s; score 3, 60 s or more). Each column represents the mean ± SEM of six mice. Statistical analyses were performed with one-way ANOVA followed by the Bonferroni multiple comparisons test: $F_{(12,77)} = 12.59$, $P < 0.0001$. * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ *versus* vehicle.

tion of the vestibular apparatus.[6] In addition, sensory input from the vestibular apparatus to the vomiting center follows muscarinic M_1 receptor pathways. Taken together with the fact that olanzapine showed the highest affinity toward muscarinic M_1 receptors, these findings suggest that, although the exact mechanism by which olanzapine suppresses morphine-induced emesis remains unclear, muscarinic M_1 receptors may be a critical target for morphine-induced emesis. To prove our hypothesis, we next investigated whether the selective muscarinic M_1 receptor antagonist trihexyphenidyl could affect morphine-induced nausea and vomiting. Trihexyphenidyl significantly suppressed morphine-induced retching and vomiting in ferrets (data not shown), which indicates that M_1 receptors play an important role in the opioid-sensitive emetic pathway. However, trihexyphenidyl significantly enhanced the morphine-induced increase in the release of dopamine in the nucleus accumbens (data not shown). If we consider the risk of the overexcitation of dopamine transmission with the use of drug combinations, a specific M_1 receptor antagonist might not be a better choice as an adjunct agent in combination with opioids. Because olanzapine acts not only on muscarinic M_1 receptors, but also partly on histamine H_1 and dopamine D_2 receptors as an antagonist,[10] it is likely that olanzapine at a dose lower than that at which it has antipsychotic effects could be useful for strongly preventing opioid-induced emesis without severe side effects.

Constipation is another adverse effect of treatment with morphine. In the current study, olanzapine at doses that had antiemetic effects had no effect on the morphine-induced

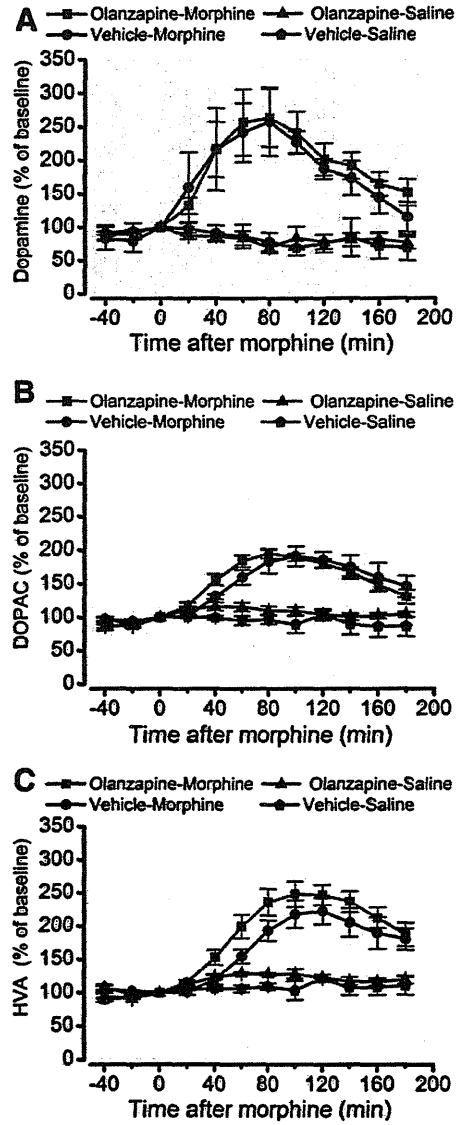


Fig. 5. Effect of olanzapine on the influence of intraperitoneal administration morphine on the dialysate concentrations of dopamine (*A*) and its metabolites (*B*, *C*) in the nucleus accumbens. After baseline fractions were collected, rats were pretreated with olanzapine (0.3 mg/kg, intraperitoneal administration) or vehicle 30 min before the injection of morphine (10 mg/kg, intraperitoneal administration) at time 0 to evoke the release of dopamine. Data are expressed as percentages of the corresponding baseline levels with SEM for three to five rats (number of rats: olanzapine-morphine, n = 5; vehicle-morphine, olanzapine-saline, n = 4; vehicle-saline, n = 3). Statistical analyses were performed with two-way ANOVA followed by the Bonferroni multiple comparisons test: vehicle-saline *versus* vehicle-morphine, $F_{(1,55)} = 19.48$, $P = 0.0069$ vehicle-saline *versus* vehicle-morphine, $F_{(1,77)} = 0.1516$, $P = 0.7086$ olanzapine-morphine *versus* vehicle-morphine (*A*), $F_{(1,55)} = 32.57$, $P = 0.0023$ vehicle-saline *versus* vehicle-morphine, $F_{(1,77)} = 0.06326$, $P = 0.8086$ olanzapine-morphine *versus* vehicle-morphine (*B*), $F_{(1,55)} = 23.42$, $P = 0.0047$ vehicle-saline *versus* vehicle-morphine, $F_{(1,77)} = 1.851$, $P = 0.2158$ olanzapine-morphine *versus* vehicle-morphine (*C*).

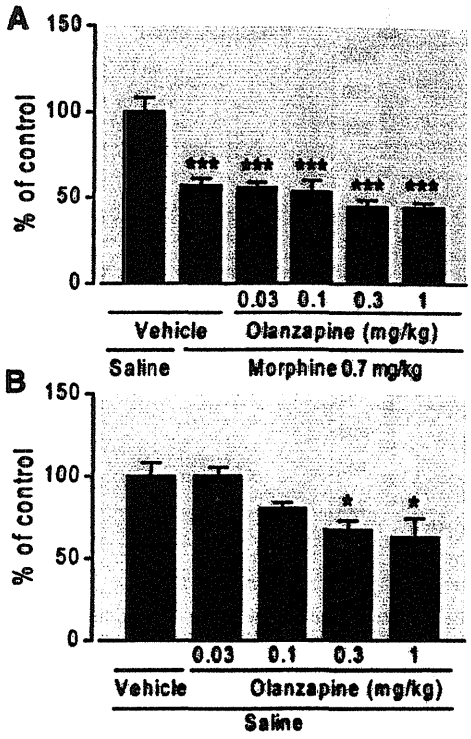


Fig. 6. Effect of pretreatment with olanzapine on the morphine-induced (*A*) inhibition of gastrointestinal transit and the effect of olanzapine itself (*B*). Each column represents the mean ± SEM of six mice. Ink was administered orally 20 min after the injection of morphine (0.7 mg/kg, subcutaneous injection) or saline, respectively. Groups of mice were pretreated with olanzapine (0.03–1 mg/kg, subcutaneous injection) at 30 min before the administration of morphine. Gastrointestinal transit was evaluated at 20 min after the oral administration of ink. Statistical analyses were performed with one-way ANOVA followed by the Bonferroni multiple comparisons test: $F_{(5,35)} = 15.99$, $P < 0.0001$ (*A*); $F_{(4,29)} = 5.778$, $P = 0.0020$ (*B*). * $P < 0.05$, *** $P < 0.001$ *versus* vehicle-saline.

inhibition of gastrointestinal transit. This may be attributable to the high central transitivity of olanzapine.

Long-term treatment with olanzapine is most commonly associated with increased weight gain, obesity, and diabetes mellitus.[33] Therefore, we evaluated the effect of chronic treatment with olanzapine on blood glucose. As a result, hyperglycemia was not observed during treatment with olanzapine at a dose at which it had an antiemetic effect (0.03 mg/kg). However, long-term treatment with olanzapine at a dose of 1 mg/kg was associated with a slight but statistically significant increase in blood glucose concentrations. These findings support the idea that olanzapine may have a wide margin of safety when used as an antiemetic.

With regard to sleep disturbance in severe pain, we confirmed that mice with sciatic nerve ligation showed a statistically significant increase in wakefulness and a decrease in non-REM sleep during the light phase. Under the current conditions, treatment with olanzapine at doses at which the pain threshold was restored improved the sleep disturbance

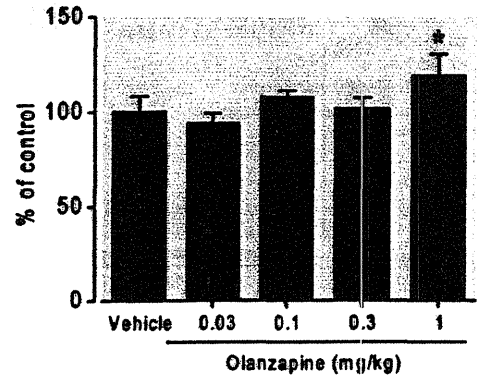


Fig. 7. Blood glucose concentrations after chronic treatment with olanzapine. Hyperglycemia was not observed in treatment with olanzapine (0.03, 0.1, or 0.3 mg/kg subcutaneous injection) for 7 days, whereas the glucose concentration was significantly increased by subcutaneous injection of olanzapine at 1 mg/kg. Values are expressed as a percentage of the control. Each column represents the mean ± SEM of four mice. Statistical analyses were performed with one-way ANOVA followed by the Bonferroni multiple comparisons test. * $P < 0.05$ *versus* vehicle.

after sciatic nerve ligation. Histamine and serotonin are the key neurotransmitters that regulate wakefulness, and their receptors are the ultimate targets of many wakefulness- and sleep-promoting drugs. In particular, histamine H_1 receptor antagonist and serotonin 5-$HT_{2A/2C}$ receptor antagonist are known to shift one's arousal state from hyperactivity to sleep.[34] Therefore, the improvement of sleep disturbance during treatment with olanzapine may result from the

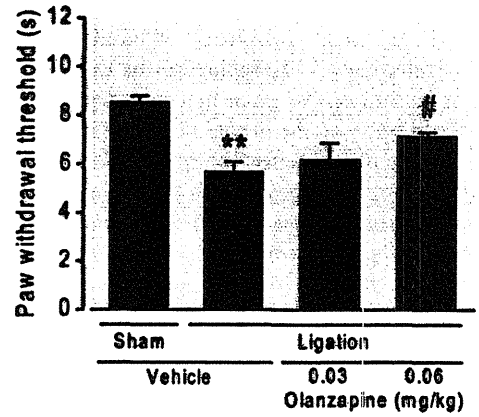


Fig. 8. Effect of olanzapine on thermal hyperalgesia induced by nerve ligation in mice. Groups of mice were injected with olanzapine (0.06 mg/kg, intraperitoneal administration) or vehicle at 7 days after sciatic nerve ligation or sham operation. Thermal hyperalgesia was measured 1 h after a single intraperitoneal administration of olanzapine or vehicle. Each column represents the mean ± SEM of six to eight mice (number of mice: sham-vehicle, n = 6; ligation-vehicle, ligation-olanzapine, n = 8). Statistical analyses were performed with Student *t* test. ** $P = 0.0017$ *versus* sham-vehicle. # $P = 0.031$ *versus* nerve ligation-vehicle.

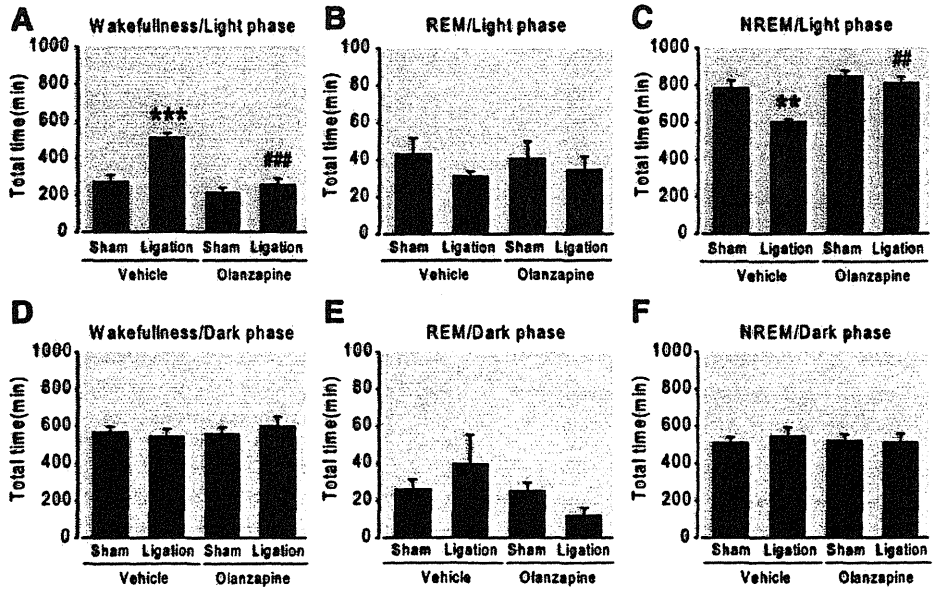


Fig. 9. Changes in sleep vigilance related to the hypnotic effects of olanzapine in a neuropathic pain-like state as determined by electroencephalogram and electromyogram recordings. Sleep–wake states after the injection of vehicle (5% dimethyl sulfoxide) or olanzapine at 7 days after sciatic nerve ligation. Vehicle or olanzapine (0.06 mg/kg, intraperitoneal administration) was injected once at 8:00 AM. The total time spent in wakefulness in the light phase (*A*) and the dark phase (*D*), rapid eye movement (REM) sleep in the light phase (*B*) and in the dark phase (*E*), and non-REM sleep in the light phase (*C*) and in the dark phase (*F*) was determined by electroencephalogram and electromyogram recording. Each column represents the mean ± SEM of five mice. Statistical analyses were performed with Student *t* test. ** $P = 0.0067$, *** $P = 0.0009$ *versus* sham-vehicle; ## $P = 0.001$, ### $P = 0.0006$ *versus* nerve ligation-vehicle.

agent's antagonistic effects toward histamine H_1 and serotonin 5-$HT_{2A/2C}$.

Overall, the current results suggest that olanzapine may be useful for the treatment of morphine-induced emesis, reducing neuropathic pain, and improving pain-related sleep disturbance. Against a background of increasing concern about "polypharmacy," olanzapine can be used as a single adjunct agent and can be given in a state-dependent dose, which should improve the quality of life for patients while greatly reducing the side effects of opioids.

In conclusion, we propose that treatment with olanzapine may lead to a new strategy for controlling emesis when patients are given opioid medications.

In addition, the current study provides evidence that olanzapine may be a useful agent for improving the sleep disturbance caused by neuropathic pain that is observed in some patients with cancer.

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Research report

The hydrophobic dipeptide Leu–Ile inhibits immobility induced by repeated forced swimming *via* the induction of BDNF☆

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Tyrosine kinase receptor B

ABSTRACT

Depression has recently become a serious problem in society worldwide. However, we lack appropriate therapeutic tools, since the causes of depression remain unclear. Degeneration of neuronal cells and a decrease in neurogenesis have been suggested recently as two of the factors responsible for depression-like behavior. Furthermore, brain-derived neurotrophic factor (BDNF) is also suggested to be an important factor in recovering from such behavior. We have previously demonstrated that the hydrophobic dipeptide leucyl–isoleucine (Leu–Ile) induces BDNF in cultured neuronal cells. We therefore investigated possible antidepressant-like effects of Leu–Ile in an animal model using the repeated forced swim test (FST). Mice were forced to swim for 6 min once a day in a cylinder containing water. The mice were treated with Leu–Ile *s.c.* or *p.o.* immediately after each FST. Five-day repeated Leu–Ile treatment significantly increased BDNF mRNA levels and activated the BDNF/Akt/mTOR signaling pathway in the hippocampi of the mice. While 2-week repeated FST increased immobility time, Leu–Ile treatment for 2 weeks offset this increase. In C57BL/6J-BDNF heterozygous knockout (BDNF(+/−)) mice, Leu–Ile failed to reduce the immobility time increased by repeated FST. We next investigated the extent of cell proliferation in the hippocampus as 5-bromo-2′-deoxy-uridine (BrdU) uptake into hippocampal cells. Repeated FST significantly reduced the number of BrdU-positive cells in the hippocampal dentate gyrus, while this deficit was prevented by repeated Leu–Ile treatment. These results suggest that Leu–Ile has an antidepressant-like effect, at least in part by supporting cell proliferation through the BDNF signaling pathway.

1. Introduction

Recently, the pressures and social problems inherent in our stressful lives have become responsible for the development of depression. For successful treatment and prevention of depression, it has been necessary to supply safety medicines or supplements with antidepressant effects.

Previous reports have suggested that the decrease in levels of monoamines such as serotonin in the brain causes depression. However, recent studies have demonstrated that damage to neuronal cells and a decrease in neurogenesis in the hippocampus are contributory factors in the development of depression. For example, reduction of hippocampal volume has been shown in depressed patients, by an *in vivo* magnetic resonance imaging study [1]. Further, it has been shown that adrenal steroids induced by chronic stress inhibit cell proliferation in the dentate gyrus during the early postnatal period and in adulthood [2]. Additionally, irradiation-mediated disruption of proliferating cells in the hippocampus inhibits a behavioral response to antidepressant drugs, suggesting that neurogenesis is an intrinsic requirement for recovery from depression [3]. Blocking hippocampal neurogenesis is also

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sufficient to increase hypothalamo-pituitary-adrenal (HPA) axis activity, which highly related with depressive disorder [4].

Brain-derived neurotrophic factor (BDNF) and other members of the neurotrophic factor family, including nerve growth factor and neurotrophin-3, influence cellular function *via* activation of their respective tyrosine kinase receptors [5]. BDNF binds to tyrosine kinase receptor B (TrkB) and induces the phosphorylation of several mediator kinases, including Akt, mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), and their downstream targets [6,7]. BDNF has important roles in neuronal survival and differentiation in the adult brain [8,9]. In layer II/III of cortex BDNF released from postsynaptic target neurons promotes the formation of proliferation of GABAergic synapses through its local actions [10]. It is suggested that GABA has a prominent role in the brain control of stress, the most important vulnerability factor in mood disorders [11]. Frontcortical and hippocampal deficits in GABAergic inhibition increase HPA axis activity, which one of course of major depression. Moreover, BDNF is highly involved in the regulation of HPA axis hyperactivity [12–14]. A number of studies suggested that relation of BDNF level and depression. Serum BDNF levels were low in antidepressant-free depressed patients relative to controls and to depressed patients who were treated with an antidepressant [15]. BDNF-knock down in the dentate gyrus of hippocampus induced depression-like behavior [9]. Moreover, direct infusion of BDNF into midbrain can reduce the immobility time in depression-like rats [16]. Furthermore, injection of BDNF into the dentate gyrus of rat hippocampus also produces behavioral responses similar to the phenomena induced by antidepressants [17]. Antidepressant treatment increases the expression of BDNF in the limbic structures of the hippocampus [18]. Chen et al. [19] have analyzed postmortem brains and demonstrated that BDNF expression increases in the dentate gyrus, hilus and supragranular regions in subjects treated with antidepressant medications, compared with untreated subjects. These observations suggest important roles for BDNF in the biological response induced by chronic antidepressant treatments, and that BDNF could be a target gene/protein for antidepressant therapy.

The immunosuppressant FK506 reduces the cortical damage induced by ischemia and excitotoxic cortical damage induced by quinolinate in rats [20]. Since FK506 treatment increases BDNF expression in cortical astrocytes from newborn rats, the upregulation of BDNF mRNA and protein levels may underlie the neuroprotective effect of FK506 [21]. However, the immunosuppressive effect of FK506 is a serious problem for neuroprotective treatments. For resolution of the problem, Tanaka et al. [22] have reported that the non-immunosuppressive FK506 derivative GPI1046, shows a protective effect from the oxidative damage induced by hydrogen peroxide in NG108-15 neuroblastoma × glioma hybrid cells. We have further observed that the hydrophobic dipeptide, leucyl-isoleucine (Leu-Ile), which resembles the partial structure of FK506, protects rat primary cultured hippocampal cells from necrosis, *via* the induction of BDNF [23]. Furthermore, Leu-Ile also shows a pharmacological effect on neurological damage in a mouse model of methamphetamine dependence [24]. In the present study, we investigated the antidepressant-like effect of Leu-Ile in relation to BDNF induction in an animal model.

The forced swim test (FST) is a standard test used for evaluation of the antidepressant effects of new therapeutic tools [25,26]. In this test, the extent of depression-like behavior is quantified as the length of immobility time during a swimming task. The antidepressant drugs imipramine and desipramine reduce the length of immobility time in mice. Hitoshi et al. [27] have demonstrated that repeated FST increases immobility time and represses the proliferation of neuronal cells, as observed in depressed patients. For these reasons we modified this repeated FST model and used it for evaluation of the antidepressant-like effect of Leu-Ile.

2. Materials and methods

2.1. Animals and environmental conditions

Mice were housed under a standard 12-h light/dark cycle (lights on at 9:00 am) at a constant temperature of 23 ± 1 °C with free access to food and water throughout experiments. Animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya 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. Inbred ICR male mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). C57BL/6J-BDNF heterozygous knockout (BDNF(+/−)) mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and propagated in our laboratory. Male BDNF(+/−) mice, 8–12 weeks of age, were used in experiments, since BDNF homozygous knockout (BDNF(−/−)) mice die a few days after birth (postnatal 7 days), but BDNF(+/−) mice are viable [28]. Littermate BDNF(+/+) mice were used as controls in our all experiments.

2.2. Materials

The following compounds were purchased from commercial sources; Leu-Ile (Kokusan Kagaku, Tokyo, Japan), 5-bromo-2′-deoxy-uridine (BrdU) (Sigma-Aldrich Japan, Tokyo, Japan), BrdU stain-detection kit (Roche Applied Science, Mannheim, Germany), total RNA extraction kit (Qiagen, Hilden, Germany), reverse transcriptase and reagents for the real time reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen Life Technologies, Carlsbad, CA), rapamycin (Merck KGaA, Darmstadt, Germany). Anti-phospho-Akt (Ser473) rabbit polyclonal antibody, anti-Akt rabbit polyclonal antibody, anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) rabbit polyclonal antibody, anti-p44/42 MAPK rabbit polyclonal antibody, anti-phospho-p70S6 kinase (Thr389) rabbit polyclonal antibody, anti-p70S6 kinase rabbit polyclonal antibody, anti-phospho-S6 ribosomal protein (Ser235/236) (91B2) rabbit monoclonal antibody and anti-S6 ribosomal protein (54D2) mouse monoclonal antibody were obtained from Cell Signaling Technology Japan (Tokyo, Japan). Anti-β-actin goat polyclonal antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

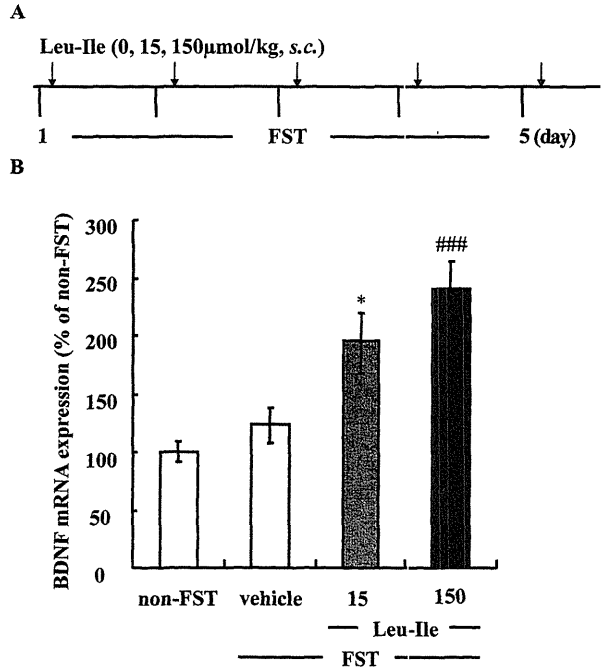

Fig. 1. Effect of repeated *s.c.* Leu-Ile administration on the induction of BDNF mRNA. A: Experimental schedule for the repeated forced swimming test (FST) and Leu-Ile administration. Male mice (8–10-week-old) were subjected to daily FST for 6 min. The experiment was carried out from 13:00 to 17:00 for 5 days continuously. Mice were treated with Leu-Ile (0, 15, 150 μmol/kg, *s.c.*) immediately after daily FST. The control group of mice was administered saline. B: Dose dependent effect of Leu-Ile on the expression of BDNF mRNA in mice undergoing repeated FST. Total RNA was prepared from the hippocampus of mice treated with daily FST and Leu-Ile (0, 15, 150 μmol/kg, *s.c.*) for 5 days, 30 min after the last treatment with Leu-Ile. Values are means ± S.E. ($n = 6$) (one way ANOVA, $F(3) = 8.024$, Tukey's multiple comparison test, $^{*}p < 0.05$, $^{###}p < 0.005$, versus FST plus vehicle-treated mice).

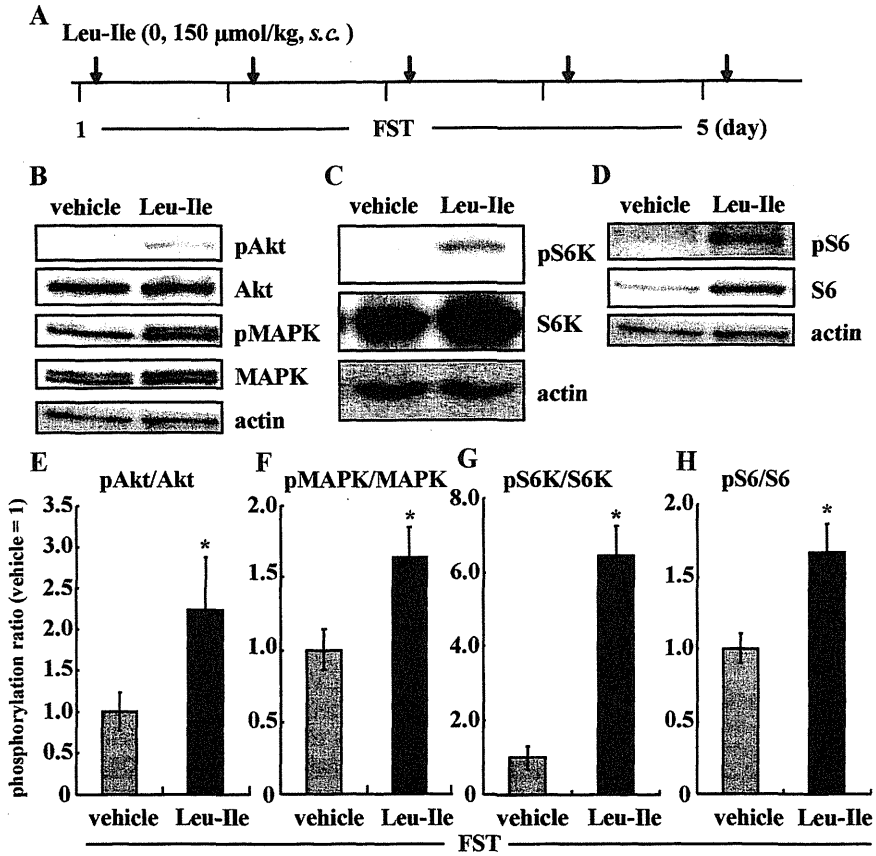

Fig. 2. Activation of the Akt/mTOR signaling pathway by Leu-Ile in the hippocampus of mice that have undergone repeated FST. A: Experimental schedule for the repeated FST and Leu-Ile administration. The experiment was carried out from 13:00 to 17:00 for 5 days continuously. Mice were treated with Leu-Ile (0, 150 μmol/kg, *s.c.*) immediately after daily FST. The control group of mice was administered saline. Phosphorylation of B: Akt and p44/42 MAP kinase, C: S6 kinase and D: S6 were detected by Western blotting in the hippocampus of mice subjected to FST and Leu-Ile (0, 150 μmol/kg, *s.c.*) for 5 days. E–H: Phosphorylation levels were normalized to total protein in each case. Leu-Ile-treated mice were compared to vehicle-treated mice (normalized value of 1). Data represent means ± S.E. ($n = 3$–5) (Student's *t*-test, $^*p < 0.05$ versus vehicle-treated mice).

2.3. Chronic FST and repeated drug administration

In each group, 8–12-week-old male mice were subjected to daily FST ($n = 10$). Mice were placed in a transparent plastic cylinder (19 cm high, 14.5 cm in diameter), containing water (depth of 15 cm, temperature of 24–25 °C) for 6 min. Immediately after mice are placed in the water, they swim and try to escape from the cylinder. They subsequently stop swimming and become immobile. Immobility time was measured during the final 5 min with a SCANET MV-10 AQ apparatus (Toyo Sangyo Co., Toyama, Japan). Mice were administered Leu-Ile immediately after the FST session on each day. Rapamycin was also administered at the time of Leu-Ile treatment. Experimental schedules for FST and the administration of Leu-Ile as well as those for the behavioral, biochemical and histochemical investigations are shown in Figs. 1A–7A.

2.4. Selection of Leu-Ile doses

Leu-Ile solutions were prepared immediately before daily administration. Leu-Ile was dissolved in saline for subcutaneous injection (*s.c.*) in ICR, BDNF(+/−) and BDNF(+/+) mice (Figs. 1–4), or in distilled water for oral administration (*p.o.*) to ICR mice (Figs. 5–8). We previously reported that Leu-Ile (15 μmol/kg, *i.p.*) treatment for 5 days inhibits methamphetamine-induced sensitization [24]. Therefore, we here evaluated the effect of Leu-Ile on BDNF induction at doses of 15 and 150 μmol/kg (*s.c.*) for 5 days (Fig. 1A). Since *p.o.* administration is more convenient than *s.c.* in the clinical context, we also evaluated the effect of Leu-Ile *p.o.* (Fig. 5A). We selected higher doses (450, 750, 1500 μmol/kg) in addition to 150 μmol/kg, *s.c.*, since the effect of *p.o.* administration was lower than that of *s.c.* administration. From the results of dose response measurements, the dose of Leu-Ile treatment was set at 150 μmol/kg for *s.c.* (Fig. 1B) and 750 μmol/kg for *p.o.* administration (Fig. 5B).

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

The level of BDNF mRNA was determined by real-time RT-PCR using a Fast Real-Time PCR System (Applied Biosystems). Total RNA was isolated from the hippocampi of 5-day FST and Leu-Ile-treated mice using an RNeasy Mini Kit. For reverse transcription, 1 μg RNA was converted into cDNA in a standard 20 μl reverse transcriptase reaction using oligo-dT primers and the SuperScript™ First-Strand System. Total cDNA (1 μl) was amplified in a 25 μl reaction mixture using 0.1 μM each of forward and reverse primers and Platinum Quantitative PCR SuperMix-UDG. The primers and dye probes were designed and synthesized by Nippon EGT (Toyama, Japan). The following mouse BDNF primers were used: 5′-GCAAACATGTCTATGAGGGTTCG-3′ (forward), 5′-ACTCGCTAATACTGTCACACACG-3′ (reverse) and FAM-TAMRA labeled Taq-Man probe 5′-ACTCCGACCCTGCCCGCCGT-3′. For the internal control, the following mouse GAPDH primers were used: 5′-CATGGCCTTCCGTGTTCCTA-3′ (forward), 5′-ATGCCTGCTTCACCACCTTCT-3′ (reverse) and FAM-TAMRA labeled Taq-Man probe 5′-CCCAATGTGTCCGTCGTGGATCTGA-3′.

2.6. Western blotting analysis

After repeated FST and repeated Leu-Ile treatment for 5 days, animals were decapitated, the hippocampi were removed on an ice-cold glass plate and stored at −80 °C until use, to reconfirm the increase of BDNF protein following the observed increase in expression of BDNF mRNA with FST and Leu-Ile treatment (Fig. 1B). Hippocampal protein extracts were obtained by homogenization in radioimmunoprecipitation assay (RIPA) buffer [20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, complete mini (Roche Applied Science)]. Homogenates were centrifuged at 13,000 × *g* for 20 min to obtain the desired supernatant from the extracts. Tris-buffered saline (TBS) washing buffer, pH 7.4, contains 10 mM Trisma base, 137 mM NaCl and 0.1% Tween20. Five-microgram protein samples were resolved by 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, MA, USA). Membranes were incubated in 5% skimmed milk in TBS containing 0.1% (v/v) Tween20 for 1 h at room temperature. Anti-phospho-Akt (Ser473) antibody (1:1000), anti-Akt antibody, anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1:1000), anti-p44/42 MAP kinase antibody (1:1000), anti-phospho-p70S6 kinase (Thr389) antibody (1:1000), anti-p70S6 kinase antibody (1:1000), anti-phospho-S6 ribosomal protein (Ser235/236) (91B2) antibody (1:1000), anti-S6 ribosomal protein (54D2) antibody (1:1000) and anti-β-actin antibody (1:1000) were used for detection of each protein.

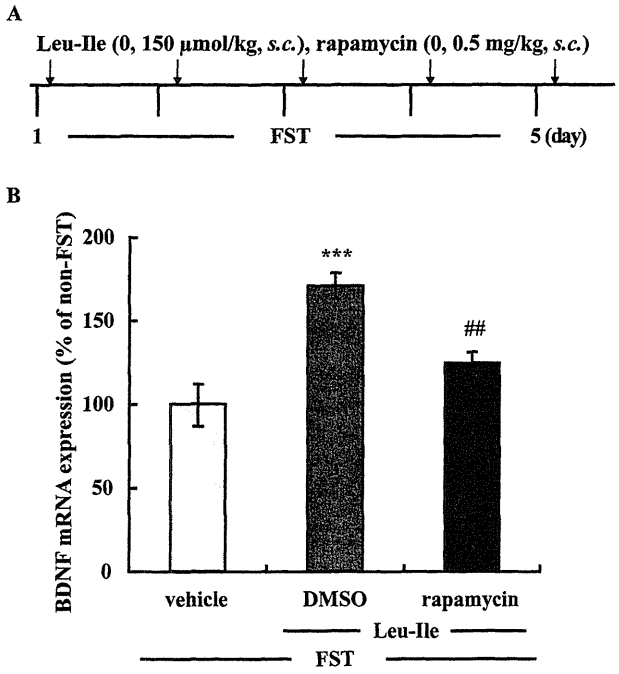

Fig. 3. Effect of mTOR Inhibitor Rapamycin on BDNF Induction by Leu-Ile. A: Experimental schedule for the repeated FST and Leu-Ile administration. The experiment was carried out from 13:00 to 17:00 for 5 days continuously. Mice were treated with Leu-Ile (0, 150 μmol/kg, *s.c.*) and rapamycin (0, 0.5 mg/kg, *s.c.*) immediately after daily FST. The control group of mice was administered saline and DMSO. B: Effect of rapamycin on the expression of BDNF mRNA induced by Leu-Ile in mice that underwent repeated FST. Total RNA was prepared from the hippocampus of mice 30 min after the last treatment with Leu-Ile and rapamycin. Values are means ± S.E. ($n = 8$) (one way ANOVA, $F(3) = 13.95$, Tukey's multiple comparison test, $^{***}p < 0.005$ versus vehicle-treated mice, $^{\#\#}p < 0.01$, versus DMSO-treated mice).

2.7. BrdU labeling and immunohistochemistry

The protocol followed for BrdU labeling and detection was as previously described by us [29]. On the final day of repeated FST and Leu-Ile treatment, mice were injected with BrdU (Sigma; 75 mg/kg, *i.p.* dissolved with saline) three times at 2-h intervals, and their brains fixed 18 h after the last injection. The mice were anesthetized with diethylether, before being transcardially perfused with 4% paraformaldehyde. Their brains were then removed and postfixed with the same fixative overnight, before being cryoprotected with 30% sucrose in 0.01 M PBS at 4 °C. Next, 30 μm-thick coronal brain sections were cut on a cryostat and mounted on slides. Every fifth section was collected at from −1.2 to 3.0 mm of bregma according to the brain atlas [30]. The sections were treated overnight with 0.1% Nonidet-40/0.01 M PBS (pH 7.2) at 4 °C and denatured in a microwave oven in 0.01 M citrate buffer (pH 6.0). After being blocked in 10% goat serum/PBS with 0.1% Nonidet P-40 for 30 min, BrdU-positive cells in sections were detected using a BrdU labeling and detection kit 2 (Roche Diagnostics GmbH) according to the manufacturer's instructions. The number of BrdU positive cells in the dentate gyrus was counted in 8 slices per mouse ($n = 4$).

3. Statistical analysis

All data are expressed as the mean ± S.E. Differences between two groups were analyzed using the two-tailed Student's *t*-test. Differences among multiple groups were analyzed by ANOVA followed by the Tukey's multiple comparison test when *F* ratios were significant ($p < 0.05$).

4. Results

4.1. Leu-Ile increases BDNF transcription level in the hippocampus of mice

In the hippocampus, BDNF is an important factor in cell proliferation, differentiation and maintenance. Furthermore, BDNF is suggested to be a key factor in the therapeutic effect of antidepressant drugs [19]. Our previous data demonstrated that Leu-Ile induces BDNF expression in rat primary cultured hippocampal cells [23]. In this study, we investigated the *in vivo* effect of Leu-Ile on BDNF transcription levels in the hippocampus. We previously reported that Leu-Ile (15 μmol/kg, *i.p.*) treatment for 5 days inhibits methamphetamine-induced sensitization [24]. Accordingly, we evaluated the effect of Leu-Ile (0, 15, 150 μmol/kg, *s.c.*) treatment for 5 days on BDNF mRNA induction. One hour after the final FST and Leu-Ile treatment, total RNA was purified from the hippocampus of mice, and BDNF mRNA levels were determined by real-time RT-PCR (Fig. 1A). In the hippocampus of the mice administered Leu-Ile (15, 150 μmol/kg, *s.c.*) after FST on each day, BDNF mRNA expression was significantly increased 2.4-fold (150 μmol/kg, *s.c.*) over that of the vehicle group (Fig. 1B). On the basis of this result, *s.c.* doses of 150 μmol/kg Leu-Ile were employed in following investigations.

4.2. Leu-Ile activates the BDNF signaling pathway

We next investigated the effect of Leu-Ile treatment on activation of the BDNF/TrkB signaling pathway. Mice were treated with FST and Leu-Ile (0, 150 μmol/kg, *s.c.*) for 5 days (Fig. 2A), their hippocampi removed 30 min after the final Leu-Ile administration and the phosphorylation of Akt, p44/42 MAPK detected by Western blotting. We also measured the phosphorylation of mTOR pathway components, p70S6 kinase and S6 ribosomal protein, since BDNF induces transcription and cell proliferation through activation of this signaling pathway [7]. On Leu-Ile treatment, phosphorylation of all these proteins was increased significantly (Fig. 2B–H).

4.3. Leu-Ile increases BDNF induction via the mTOR pathway

To evaluate the effect of the mTOR signaling pathway on BDNF induction by Leu-Ile, mice were treated with rapamycin (0.5 mg/kg, *s.c.*), an inhibitor of mTOR, at the same time as Leu-Ile treatment. Since rapamycin inhibited BDNF mRNA induction by repeated FST and Leu-Ile (Fig. 3B) treatment, it is suggested that activation of the Akt/mTOR signaling pathway is necessary for the Leu-Ile-mediated induction of BDNF mRNA.

4.4. Leu-Ile reduces the prolongation of immobility time induced by chronic FST

We evaluated the antidepressant-like effect of Leu-Ile using repeated FST. Six-minute FST and Leu-Ile treatment were performed once a day and continued for 2 weeks, since more than 2 weeks of administration are required to show clinical effects of antidepressant drugs. Mice were treated with Leu-Ile (150 μmol/kg) immediately after each daily FST (Fig. 4A). The vehicle group of mice was administrated saline. Immobility time data on days 1–2, 7–8 and 13–14 are shown (Fig. 4C). Repeated FST prolonged the immobility time. In the first week, there was no difference in immobility time between the vehicle- and Leu-Ile-treated groups. However, the immobility was decreased from days 9–10 (data not shown), and 13–14 days of administration of Leu-Ile decreased immobility time in FST mice to the level seen at the start period (vehicle and Leu-Ile = 182.9 ± 16.4 and 130.5 ± 17.8 s, respectively) (two way ANOVA, $F(2, 114) = 4.71$, $^{*}p < 0.05$ versus vehicle Bonferroni posttests; Fig. 4C). This result suggested that Leu-Ile showed an antidepressant-like effect.

4.5. Leu-Ile shows an antidepressant-like effect via BDNF induction

To confirm that an antidepressant-like effect of Leu-Ile depends on induction of BDNF, we examined the effect of Leu-Ile in

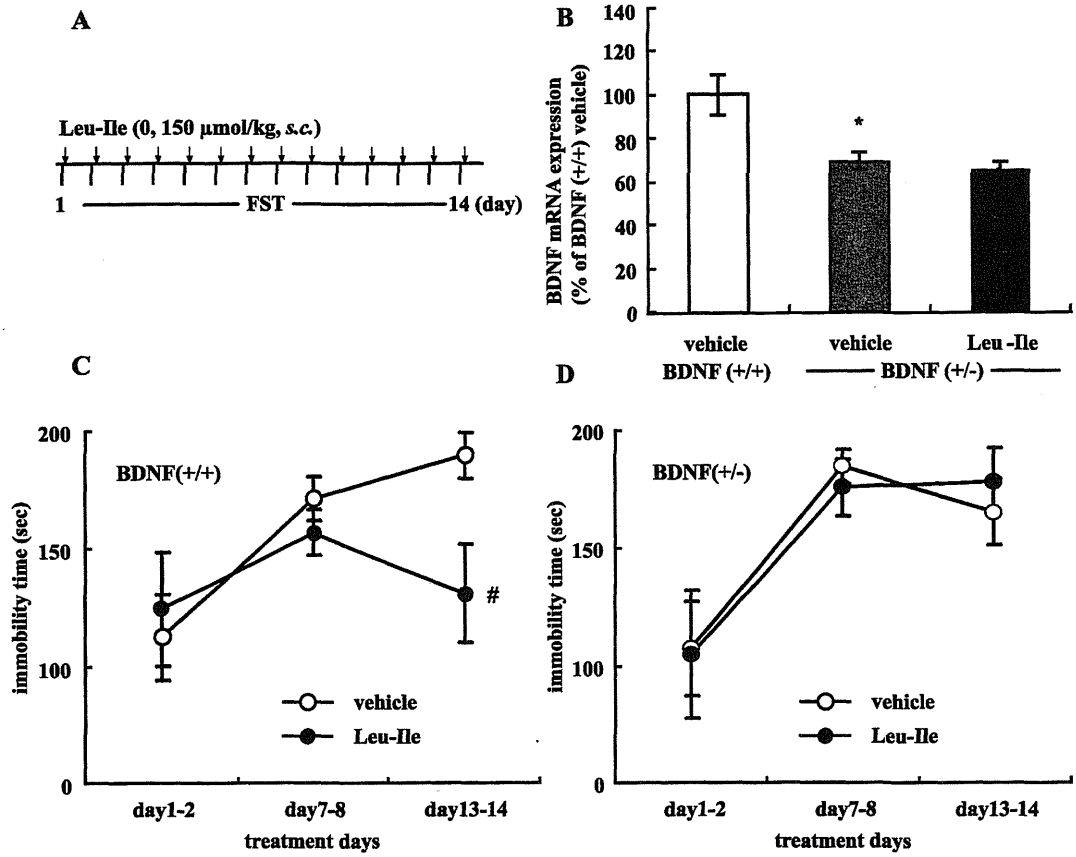

Fig. 4. Effects of Leu-Ile on prolongation of immobility time and BDNF mRNA levels induced by repeated FST in wild-type and BDNF hetero knockout (BDNF(+/−)) mice. A: Experimental schedule for treatment by repeated FST and Leu-Ile. Leu-Ile (150 μmol/kg, *s.c.*) was administered immediately after the FST each day. B: BDNF mRNA levels in the hippocampus of wild type and BDNF(+/−) mice. Total RNA was prepared from the hippocampus of mice 30 min after Leu-Ile administration on the final day of repeated FST and Leu-Ile administration. Values indicate the mean ± S.E. ($n = 4$) (Student's *t*-test, $^{*}p < 0.05$ versus vehicle-treated wild type mice). C, D: Immobility time of Leu-Ile-treated mice was compared to that of vehicle-treated mice. C: Effect of Leu-Ile on the immobility time of wild type mice. The data concerning immobility time were plotted as the sum of days 1–2, 7–8 and 13–14. Data represent means ± S.E. ($n = 20$) (two way ANOVA, $F(2, 114) = 3.91$, Student's *t*-test at days 13–14, $^{\#}p < 0.05$ versus vehicle-treated mice). D: Effect of Leu-Ile on the immobility time of BDNF(+/−) mice. The data concerning immobility time were plotted as the sum of days 1–2, 7–8 and 13–14. Data represent means ± S.E. ($n = 20$).

BDNF(+/−) mice using repeated FST. Because BDNF(−/−) mice die a few days after birth [28], we used BDNF(+/−) mice for this purpose. BDNF expression level in the hippocampus of BDNF(+/−) mice was 68.4% of that in littermate BDNF(+/+) mice (Fig. 4B). Mice were treated daily with 6 min of FST and immediately afterwards with Leu-Ile (150 μmol/kg, *s.c.*), for 2 weeks as shown in Fig. 4A. Immobility time on days 1–2 of the task was not different between wild type and BDNF(+/−) mice (Fig. 4C and D). Daily repeated FST for 2 weeks prolonged the immobility time of BDNF(+/+) and BDNF(+/−) mice (Fig. 4C and D). Although prolongation of immobility time was inhibited significantly by Leu-Ile treatment in BDNF(+/+) mice (Fig. 4C), Leu-Ile had no effect on immobility time in BDNF(+/−) mice (vehicle and Leu-Ile = 182.9 ± 14.6 and 158.9 ± 13.8 s, respectively) (Fig. 4D). Leu-Ile failed to induce BDNF mRNA in BDNF(+/−) mice (vehicle = 67 ± 6, Leu-Ile = 63 ± 6) (Fig. 4B). These results suggest that Leu-Ile shows an antidepressant-like effect through induction of BDNF expression, at least in part.

4.6. Leu-Ile shows an antidepressant effect via p.o. administration

Since *p.o.* administration is more convenient than *s.c.* in the clinical context, we also evaluated the effect of Leu-Ile (*p.o.*). We selected higher doses (450, 750, 1500 μmol/kg) in addition to 150 μmol/kg *s.c.*, since the effect of *p.o.* administration was less than that of *s.c.* In the hippocampus of mice administered Leu-Ile (at the dose of 750 μmol/kg, *p.o.* only) after FST on each day, BDNF mRNA was significantly increased 2.5-fold over that of the vehicle group (Fig. 5B), although repeated Leu-Ile (0, 750 μmol/kg, *p.o.*) treatment without FST did not induce BDNF transcription (data not shown). To evaluate the antidepressant-like effect of Leu-Ile, mice were treated with 750 μmol/kg immediately after each daily FST (Fig. 6A). The vehicle group of mice was administrated distilled water. Immobility time data on days 1–2, 7–8 and 13–14 are shown (Fig. 6B). Repeated FST prolonged immobility time. In the first week, there was no difference in immobility time between the vehicle- and Leu-Ile-treated groups. However, 13–14 days of administration of Leu-Ile decreased immobility time in FST mice to the level seen at the start period (Fig. 6B). In Fig. 6B, the ratio of immobility time at 13–14 days was compared to that at the start period. These results suggest that Leu-Ile has an antidepressant-like effect, but that its metabolite amino acids do not.

4.7. Leu-Ile recovers the decrease of cell proliferation in the dentate gyrus in FST mice

Previous studies have demonstrated that there is a decrease in cell proliferation in the dentate gyrus in animal model of depression, and that antidepressants restore cell proliferation [31,32]. Therefore, we examined the effect of repeated Leu-Ile treatment on cell proliferation by measuring BrdU uptake into proliferating cells. The number of BrdU positive cells in the dentate gyrus was counted. In non-FST mice, repeated Leu-Ile (750 μmol/kg, *p.o.*) treatment did not affect the BrdU positive cell number (vehicle

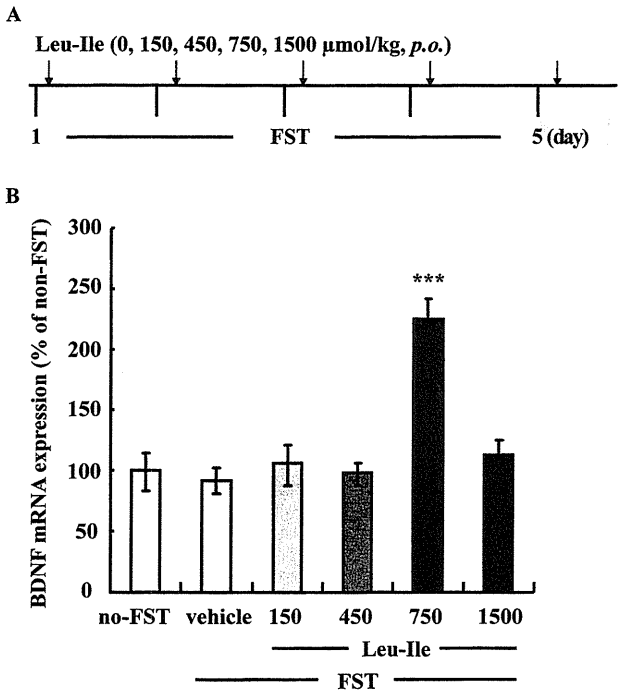

Fig. 5. Induction of BDNF mRNA by oral administration of Leu–Ile. A: Experimental schedule for repeated FST and Leu–Ile administration. Male mice (8–10-week-old) were subjected to daily FST for 6 min. The experiment was carried out from 13:00 to 17:00 for 5 days continuously. Mice were treated daily with Leu–Ile (0, 150, 450, 750, 1500 μmol/kg, *p.o.*) immediately after the FST. The control group of mice was administered distilled water (*p.o.*). B: Dose dependent effect of Leu–Ile on the expression of BDNF mRNA in mice that underwent repeated FST. Total RNA was prepared from the hippocampus of mice undergoing daily FST and Leu–Ile (0, 150, 450, 750, 1500 μmol/kg, *p.o.*) for 5 days, 30 min after the final treatment with Leu–Ile. Values are means ± S.E. ($n = 6$–10) (one way ANOVA, $F(5) = 10.30$) (Tukey's multiple comparison test, $^{***}p < 0.005$ versus FST plus vehicle-treated mice).

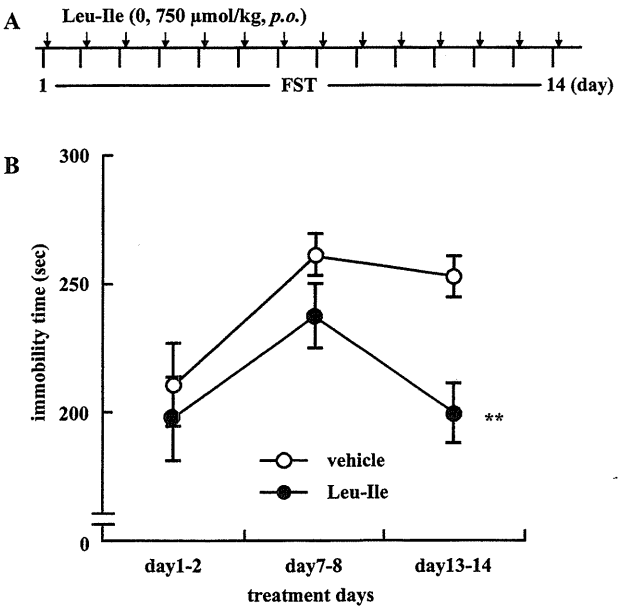

Fig. 6. Inhibition of the increase in immobility time by oral administration of Leu–Ile in mice undergoing repeated FST. A: Experimental schedule for repeated FST and Leu–Ile administration. Male mice (8–10-week-old) were subjected to daily FST for 6 min. The experiment was carried out from 13:00 to 17:00 for 2 weeks continuously. Mice were treated daily with Leu–Ile (0, 750 μmol/kg, *p.o.*) immediately after each FST. The control group of mice was administered distilled water. B: The data concerning immobility time were plotted as the sum of days 1–2, 7–8 and 13–14. Data represent means ± S.E. ($n = 10$) (two way ANOVA, $F(2, 54) = 1.43$, Student's *t*-test at days 13–14, $^{**}p < 0.01$ versus vehicle-treated mice).

and Leu–Ile = 81.65 ± 6.78/8 slices and 87.5 ± 13.5/8 slices, respectively) (Fig. 7B, C and F). On the other hand, daily FST for 2 weeks significantly decreased the number of BrdU positive cells in the dentate gyrus (Fig. 7B, D and F). However, repeated Leu–Ile (750 μmol/kg, *p.o.*) treatment inhibited the decrease in the number of BrdU positive cells (Fig. 7D–F). These results suggest that the antidepressant-like effect of Leu–Ile was due to inhibition of the reduction of cell proliferation in the dentate gyrus induced by chronic FST.

4.8. A mixture of leucine (Leu) and isoleucine (Ile) fails to affect the increase in immobility time in mice undergoing repeated FST

It was unclear whether or not the effect of Leu–Ile was due to its metabolic breakdown products. To evaluate a possible antidepressant-like effect of the Leu–Ile metabolites Leu and Ile, mice were administered a mixture of Leu and Ile at a dose of 750 μmol/kg in place of Leu–Ile, after each FST (Fig. 8A). Daily repeated FST for 2 weeks prolonged immobility time of mice. The mixture of Leu and Ile failed to block the increase in immobility time induced by repeated FST (Fig. 8B). Moreover, repeated treatment with this mixture did not increase BDNF mRNA expression (Fig. 8C). These results suggest that Leu–Ile has an antidepressant-like effect, but that its metabolite amino acids do not.

5. Discussion

There are several factors that induce cell proliferation, such as epidermal growth factor and fibroblast growth factor 2 [33]. BDNF is also a potentially important regulator of neurogenesis in the adult brain [8]. Furthermore, mutation of BDNF is related to depression. A single nucleotide polymorphism of BDNF, in which codon 66 of BDNF is altered from valine to methionine (BDNF Val66Met), is associated with a reduced hippocampal volume in patients with major depression [34]. Moreover, fluoxetine does not affect knock-in mice that are homozygous for the BDNF Val66Met substitution [35]. It has been reported that BDNF signaling and cell proliferation are involved in the mechanisms of action of antidepressant drugs [3,36–39]. In the present study, we demonstrated that repeated Leu–Ile treatment increased the transcription level of BDNF in the hippocampus of mice subjected to repeated FST, compared with vehicle treatment (Figs. 1B and 5B). The induction of BDNF transcription was maintained even on the final day of a 2-week period of repeated FST and Leu–Ile treatment (data not shown). On the other hand, Eisch et al. has suggested that blockage of BDNF activity, blockage of TrkB receptor, in the striatum exerts an antidepressant-like activity [40]. However, there is a report suggests that exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats [41]. Our results suggested that Leu–Ile increased BDNF expression not only in the hippocampus but also in the striatum [23], and reduced the depression-like behavior of the stressed mice. Eisch et al. blocked the action of BDNF only in the striatum, although Leu–Ile treatment or exercise may have effect on the whole brain. The BDNF may have effect on the psychiatric behavior by the interaction of various areas of brain. BDNF promotes the formation or proliferation of GABAergic synapses in the cortex [10], actions modify subsequent synaptic plasticity in the hippocampus [42]. GABA signaling reduces the hyperactivation of HPA axis [11]. Moreover, the effect of antidepressant drugs on activation of CREB and increase of hippocampal neurogenesis were downstream of increased GABA signaling [43]. Since BDNF is a transcriptional target gene of CREB [44], BDNF may able to be a positive feedback loop that upregulates GABAergic signaling. These results show that BDNF has an important role on the antidepressant effect through the reduction of HPA axis. Chronic

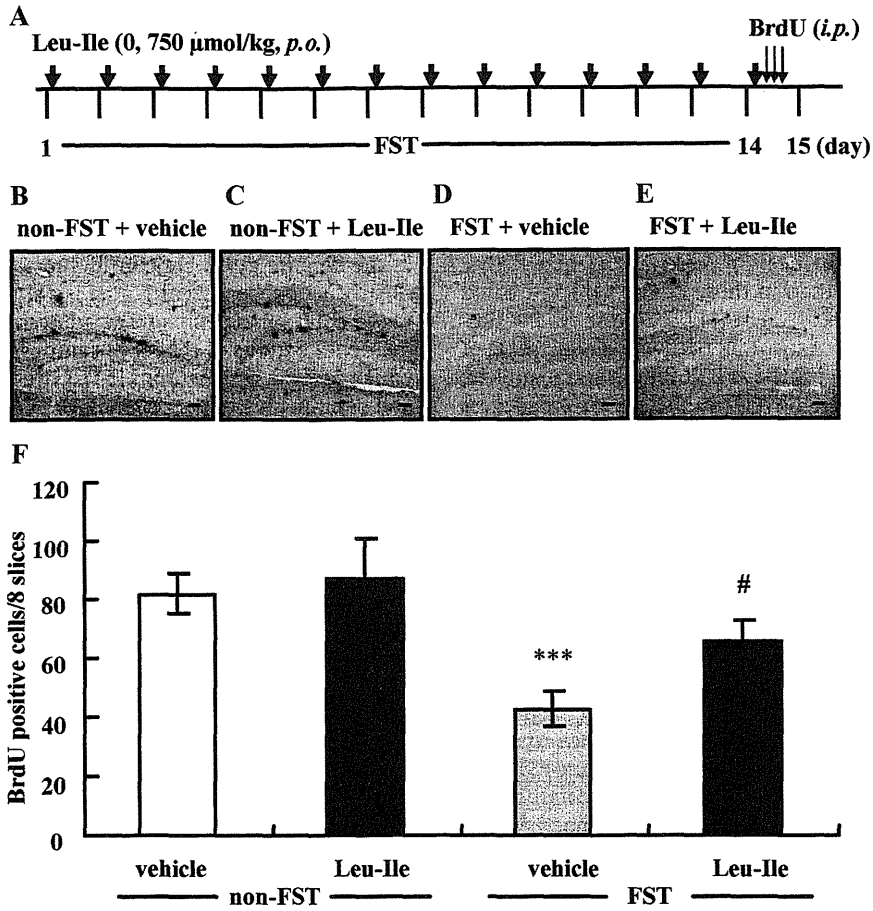

Fig. 7. Effect of repeated Leu–Ile administration on BrdU uptake into the dentate gyrus in mice undergoing repeated FST. A: Experimental schedule for repeated FST and Leu–Ile administration. The experiment was carried out for 14 days continuously. Mice were treated daily with Leu–Ile (0, 750 μmol/kg, *p.o.*) immediately after FST. The control group of mice was administered distilled water. On the last day of repeated FST, mice were injected with BrdU (75 mg/kg, *i.p.*) three times at 2-h intervals, and sacrificed 18 h after the final injection. Representative photographs showing the distribution of BrdU-positive cells are B: non-FST plus vehicle-treated, C: non-FST plus Leu–Ile-treated, D: FST plus vehicle-treated and E: FST plus Leu–Ile-treated mice (B–E; scale bar: 200 μm). F: Total numbers of BrdU positive cells are expressed as the sum of numbers in the dentate gyrus. Values indicate the mean ± S.E. ($n = 4$) (Tukey's multiple comparison test, $F(4) = 10.74$, $^{***}p < 0.005$ versus non-FST plus vehicle-treated mice, $^{\#}p < 0.05$ versus FST plus vehicle-treated mice).

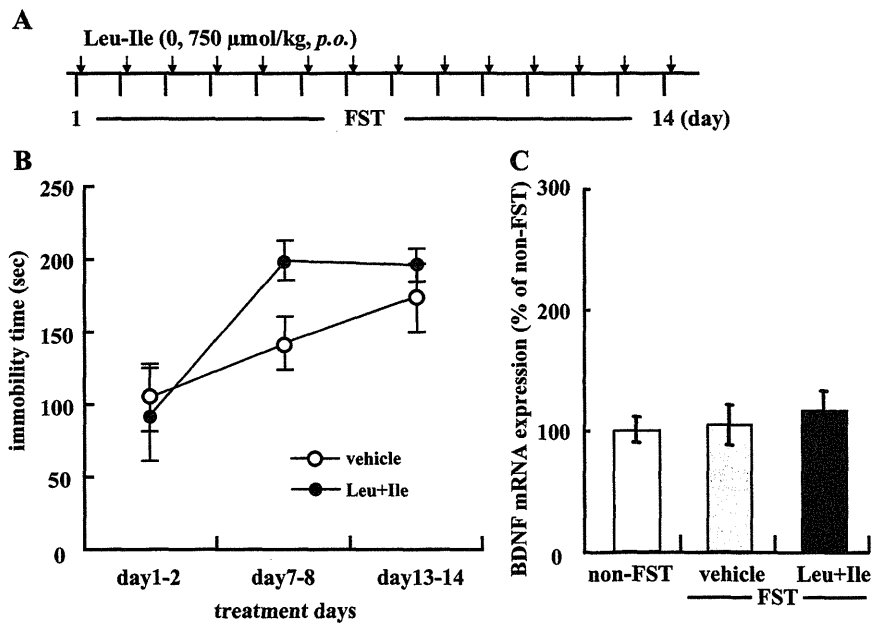

Fig. 8. Lack of effect of a mixture of Leu and Ile on immobility time and BDNF levels in mice undergoing repeated FST. A: Experimental schedule for repeated FST and administration of a mixture of Leu and Ile (each 750 μmol/kg, *p.o.*) immediately after the FST each day. B: Data concerning immobility time were plotted as the sum of values for each 2-day period. Data represent means ± S.E. ($n = 5$). C: Total RNA was prepared from the hippocampi of mice 30 min after the administration of a mixture of Leu and Ile on the final day of repeated FST. Data represent means ± S.E. ($n = 5$).