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Anxiolytic-Like and Antidepressant-Like Activities of MCL0129 (1-[(*S*)-2-(4-Fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine), a Novel and Potent Nonpeptide Antagonist of the Melanocortin-4 Receptor

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ABSTRACT

We investigated the effects of a novel melanocortin-4 (MC4) receptor antagonist,1-[(*S*)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine (MCL0129) on anxiety and depression in various rodent models. MCL0129 inhibited [^{125}I][Nle^4-D-Phe^7]-α-melanocyte-stimulating hormone (α-MSH) binding to MC4 receptor with a K_i value of 7.9 nM, without showing affinity for MC1 and MC3 receptors. MCL0129 at 1 μM had no apparent affinity for other receptors, transporters, and ion channels related to anxiety and depression except for a moderate affinity for the σ_1 receptor, serotonin transporter, and α_1-adrenoceptor, which means that MCL0129 is selective for the MC4 receptor. MCL0129 attenuated the α-MSH-increased cAMP formation in COS-1 cells expressing the MC4 receptor, whereas MCL0129 did not affect basal cAMP levels, thereby indicating that MCL0129 acts as an antagonist at the MC4 receptor. Swim stress markedly induced anxiogenic-like effects in both the light/dark exploration task in mice and the elevated plus-maze task in rats, and MCL0129 reversed the stress-induced anxiogenic-like effects. Under nonstress conditions, MCL0129 prolonged time spent in the light area in the light/dark exploration task and suppressed marble-burying behavior. MCL0129 shortened immobility time in the forced swim test and reduced the number of escape failures in inescapable shocks in the learned helplessness test, thus indicating an antidepressant potential. In contrast, MCL0129 had negligible effects on spontaneous locomotor activity, Rotarod performance, and hexobarbital-induced anesthesia. These observations indicate that MCL0129 is a potent and selective MC4 antagonist with anxiolytic- and antidepressant-like activities in various rodent models. MC4 receptor antagonists may prove effective for treating subjects with stress-related disorders such as depression and/or anxiety.

Stress initiates a complex cascade of responses that include endocrine, biochemical, and behavioral events. Many of these responses are initiated by release of corticotropin-releasing factor (CRF) (Owen and Nemeroff, 1991). In addition to activation of the brain CRF system, there are several lines of evidence that melanocortins (MCs), which stem from proopiomelanocortin by enzymatic processing, mediate important behavioral and biochemical responses to stress and, consequently, stress-induced disorders. Among MCs, it was reported that α-melanocyte-stimulating hormone (α-MSH) acts as a neurotransmitter or neuromodulator in the brain (Blasquez et al., 1991), and the relationship between α-MSH and adrenocorticotropic hormone (ACTH) and stress has been well documented. α-MSH and ACTH induce excessive grooming behavior in rats, a rodent behavioral response to stressful situations (De Barioglio et al., 1991; Adan et al., 1999), and antiserum to ACTH reduces novelty-induced grooming (Dunn et al., 1979). α-MSH and ACTH have been shown to inhibit the punish response in the Vogel conflict test in rats (Corda et al., 1990), and microinjection of α-MSH into the medial preoptic area and ventromedial nucleus increases anxiety and aggressive behavior (Gonzalez et al., 1996). Moreover, ACTH inhibits social contacts in the social interaction test in rats, an effect indicative of anxiogenic properties (File and Clarke, 1980), and increases isolation-induced distress vocalization in domestic chicks (Panksepp and Nor-

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ABBREVIATIONS: CRF, corticotropin-releasing factor; MC, melanocortin; α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropin; MCL0129, 1-[(*S*)-2-(4-fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine; NDP, [Nle^4-D-Phe^7]; PBS, phosphate-buffered saline; SET, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; MCH, melanin-concentrating hormone.

mansell, 1990). ACTH also has been reported to activate the hypothalamic-pituitary-adrenal axis (Von Frijtag et al., 1998).

To date, five types of receptor subtype for MC (MC1 to MC5) have been reported. In the brain, mainly MC3 and MC4 are expressed, with little expression of MC5 (Gantz et al., 1993, 1994; Roselli-Rehfuss et al., 1993). The MC3 receptor is predominately located in the hypothalamus, whereas the MC4 receptor is ubiquitously distributed in the brain, including the limbic system (Gantz et al., 1993; Mountjoy et al., 1994). MC4 and MC5 receptors have been studied in knockout mice, and the former were shown to be involved in weight homeostasis (Huszar et al., 1997), whereas the MC5 receptor was found to have a role in functions of the exocrine glands (Chen et al., 1997). Among MC receptor subtypes, the MC4 receptor is of interest in terms of the relationship to stress and the regulation of emotional behavior, as based on the following findings. MC4 receptor agonists induce grooming behavior in rats, and the MC4 receptor antagonist, SHU9119, attenuates MC4 receptor agonist-induced grooming as well as novelty-induced grooming (Adan et al., 1999). The selective MC4 receptor antagonist, HS014, blocks immobilization stress-induced anorexia in rats (Vergoni et al., 1999), and the MC4 receptor was reported to be involved in activation of the hypothalamic-pituitary-adrenal axis (Von Frijtag et al., 1998); however, it was also found that MC4 receptor-selective antagonists did not elicit anxiolytic-like effects in the elevated plus-maze task in rats (Kask et al., 1998). Thus, the MC4 receptor might be involved in stress-induced changes in neurochemical and behavior-related responses. However, only recently have selective MC4 receptor antagonists been available, and the relationship between the MC4 receptor and stress-related behavior needs to be addressed using these pharmacological tools.

1-[(*S*)-2-(4-Fluorophenyl)-2-(4-isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine (MCL0129), a nonpeptide-selective MC4 receptor antagonist, was synthesized at our laboratories. We now report involvement of the MC4 receptor in stress-induced behavior such as depression and anxiety, using MCL0129 as a pharmacological tool.

Materials and Methods

Animals. Male ICR mice (20–30 g; Charles River, Yokohama, Japan) were housed 10 per cage. Male Sprague-Dawley rats (220–240 g; Charles River) were housed 3 per cage and used to assess stress-induced anxiogenic-like behavior in the elevated plus-maze task and antidepressant-like effects in the forced swimming test. For other behavioral studies, male Wistar rats (220–240 g; Charles River) were used. Animals were maintained under a 12-h light/dark cycle (light on at 7:00 AM) in a temperature- and humidity-controlled holding room. Food and water were available ad libitum. Behavioral studies were carried out between 9:00 AM and 4:00 PM. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in the *Guidelines for Animal Experiments* (1987).

Chemicals. MCL0129 (Fig. 1) and fluvoxamine were synthesized in Taisho Pharmaceutical Laboratories. [^{125}I][Nle^4-D-Phe^7]α-Melanocyte-stimulating hormone (NDP-α-MSH) (specific radioactivity 81.4 TBq/mmol) and the cAMP assay system were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). COS-1 cells were purchased from American Type Culture Collection (Manassas, VA). α-MSH and NDP-α-MSH were purchased from Peninsula Laboratories (Belmont, CA). Diazepam and buspirone were purchased from Wako Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. All other chemicals used in this study were obtained commercially and were of the highest purity available. For the in vitro study, MCL0129 was dissolved in 0.1% dimethyl sulfoxide, and dimethyl sulfoxide (0.1%) did not affect binding assays and cAMP levels. For behavioral studies, MCL0129, diazepam, buspirone, and fluvoxamine were dissolved in 0.3% Tween 80/saline solution.

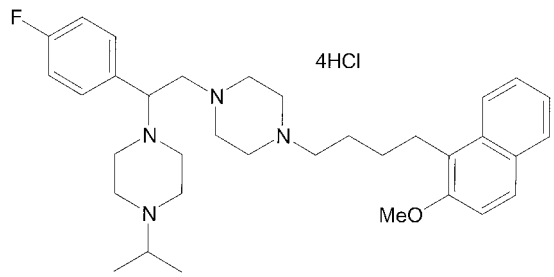

Fig. 1. Chemical structure of MCL0129

MC Receptor Expression Constructs, Cell Cultures, and Transfection. MC4, MC1, and MC3 receptor cDNAs were isolated using reverse transcriptase-polymerase chain reaction from the human hippocampus, WM-266–4 cells, and rat hypothalamus, respectively. MC4 and MC3 receptor cDNAs were cloned into expression vector pcDLΔPE and MC1 receptor cDNA into pTARGET. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C. The MC receptor cDNAs inserted into expression vectors were separately transfected into COS-1 cells using Lipofectin (Invitrogen, Carlsbad, CA), according to the protocol provided by the manufacturer (Felgner et al., 1987). At 72 h after transfection, COS-1 cells expressing MC1, MC3, or MC4 were used for pharmacological experiments.

[^{125}I]NDP-α-MSH Binding. COS-1 cells expressing the MC receptor were washed with PBS, scraped, and pelleted by centrifugation. Cell pellets were homogenized with 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, and 100 μM phenylmethylsulfonyl fluoride, and centrifuged at 48,000*g* for 20 min at 4°C. The pellet was washed twice with the buffer, and the final pellet was suspended in assay buffer [50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, 100 μM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin] and used as the crude membrane preparation for binding studies. Protein concentration was determined according to the method reported by Bradford (1976). Binding assays of [^{125}I]NDP-α-MSH were done according to the method of Schioth et al. (1998). Membranes were incubated with [^{125}I]NDP-α-MSH (0.2 nM) for 120 min at 25°C. The reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.5% bovine serum albumin, after which the filters were washed three times with the buffer. The radioactivity was quantified in a gamma-counter. Nonspecific binding was determined in the presence of 1 μM NDP-α-MSH. Specific binding was determined by subtracting nonspecific from total binding. In the competition assay, the concentration of the test compound that caused 50% inhibition of specific binding (IC_{50} value) was determined from each concentration-response curve. IC_{50} values were determined by the Marquardt-Levenberg nonlinear least-squares curve-fitting procedure, using the ORIGIN program (Origin LabCorp, Northampton, MA) running on Microsoft Windows 3.1.

Determination of cAMP. The effect of MCL0129 on cAMP formation was measured as we reported previously (Chaki et al., 1999), but with modification. COS-1 cells transiently expressing the MC4 receptor and grown in a six-well plate were used. The culture medium was removed, the cells were washed with PBS, and 1 ml of Dulbecco's modified Eagle's medium containing 1 mM isobutylmeth-

ylxanthine, a phosphodiesterase inhibitor, was added. The cells were incubated with α-MSH and/or MCL0129 for 15 min at 37°C; the culture medium was then aspirated and the cells were washed with PBS. Two milliliters of ice-cold 65% EtOH were added, and the cells were scraped from the wells. The supernatant was collected by centrifugation at 15,000 rpm for 15 min at 4°C. cAMP formed in the cells was determined, using a commercially available cAMP enzyme immunoassay system.

Stress-Induced Anxiogenic-Like Behavior in Mice. The swim stress consists of placing mice in a 20-cm-tall, 13-cm-wide cylindrical plastic container containing 10 cm of water maintained at 25 ± 1°C. Duration of the swim stress was 10 min, and the light/dark exploration test was done 10 min after the swim stress. The light/dark exploration test was carried out according to the method reported by Okuyama et al. (1999). The apparatus consisted of two polyvinylchloride boxes (20 × 20 × 14 cm) covered with Plexiglas; one of these boxes was darkened with cardboard. The light compartment was illuminated by a desk lamp (400 lux) placed 17 cm above the box, and the dark compartment provided the only room illumination. An opaque plastic tunnel (5 × 7 × 10 cm) separated the dark and the light compartments. During the observation, the experimenter always sat in the same place, next to the apparatus. The subjects were individually tested in 5-min sessions in the apparatus described above. Each mouse was placed in the center of the light area at the start of the test session. The amount of time spent in the light area was recorded for 5 min after the first entry into the dark area. A mouse whose four paws were in the next box was considered as having changed boxes. Mice were naive to the apparatus. MCL0129 was administered s.c. or p.o. 30 min before application of the swim stress. For nonstress control, vehicle was administered s.c. or p.o. 50 min before the test. Ten mice for vehicle and each for three dosages of compounds were used to generate dose-response reactions. When the effect of MCL0129 in nonstress conditions was investigated, the light/dark exploration test was run 30 min after the subcutaneous administration of MCL0129.

Marble-Burying Behavior in Mice. Marble-burying behavior was determined according to the method reported by Millan et al. (2000). Mice were individually placed in transparent, polycarbonate cages (22 × 32 × 13.5 cm) containing a 5-cm layer of sawdust and 24 glass marbles (1.5 cm in diameter) evenly spaced against the wall of the cage. Thirty minutes later, the animals were removed from the cages and the number of marbles at least two-thirds buried in the sawdust was recorded. The mice were treated s.c. 30 min before the test with either drug or vehicle.

Stress-Induced Anxiogenic-Like Behavior in Rats. The swim stress consists of placing rats in a 40-cm-tall, 20-cm-wide cylindrical plastic container containing 25 cm of water maintained at 25 ± 1°C. Duration of the swim stress was 2 min, and the elevated plus-maze test was done 5 min after the swim stress. The elevated plus-maze test was based on that validated for the rat by Guimaraes et al. (1991). The apparatus consisted of a plus-maze elevated 50 cm high from the floor. The apparatus consists of a plus-shaped maze elevated 50 cm from the floor and two opposite open arms, 50 × 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 40 cm-high walls with an open roof. In addition, a 1-cm-high edge made of Plexiglas surrounded the open arms to avoid falls. Luminosity measured at the center of the maze was 80 lux. During the observation, the experimenter always sat in the same place, next to the apparatus. Each rat was placed in the center of the plus-maze facing one enclosed arm. The amount of time spent in open arms of the maze was recorded. Rats were naive to the apparatus. MCL0129 was administered p.o. 30 min before the swim stress.

Learned Helplessness Test in Rats. The learned helplessness test, using the shuttle box test, was carried out according to the method reported by Takamori et al. (2001a) as a model of behavioral despair. The two-way shuttle box (56 × 21 × 25 cm; Muromachi-Kikai, Tokyo, Japan) was divided into equal-sized chambers with use of a steel divider. Floors of the chambers in the shuttle box consisted of stainless steel rods. Scrambled shocks were delivered through a shock generator (SGS-001; Muromachi-Kikai). Rats were given MCL0129 s.c. 60 min before the inescapable shocks, and on day 1, the rats were individually placed in the chamber and given 90 inescapable shocks (1.8 mA) of 10 s duration at 2-s intervals. Control rats were not given shocks. On day 2, the rats were subjected to the 40-trial escape test. The animals were individually placed in the shuttle box and given a 5-min adaptation period; a tone signal was given during the first 5 s of each trial. If there was no avoidance response within this period, the tone signal remained on and a 1.8-mA shock (10-s duration) was delivered through the grid floor. If no escape response was made within this period, both the tone signal and the shock were automatically terminated. The intertrial interval was 5 s. The number of escape failures, which refers to a noncrossing response during the shock delivery, was recorded.

Forced Swimming Test in Rats. The effect of the compound was evaluated by both the method described by Porsolt (1978) and by a time-sampling technique. A time-sampling technique was used to score several types of behavior (immobility, swimming, climbing) as described by Detke et al. (1995). The swimming sessions were carried out according to the method described by Detke et al. (1995) and was similar to that described by Porsolt et al. (1978), except that the water was deeper. Swimming sessions were conducted by placing rats in cylinders containing 25°C water, 30 cm deep, so that rats could not support themselves by touching the bottom with their feet. Two swimming sessions were conducted between 10:00 AM and 4:00 PM: an initial 15-min pretest followed 24 h later by a 5-min test. MCL0129 was administered s.c. during the period between these two sessions (24 and 0.5 h before the test). Following both swimming sessions, the rats were removed from the cylinders, placed in a heated cage for 15 min, and then returned to their home cages. Test sessions were videotaped from the front of the cylinders for later scoring. The water in the cylinders was changed after every trial. A time-sampling technique was used to score behavior during a single viewing. This method has previously been described by Detke et al. (1995) and was shown to be reliable and valid for detecting effects of different antidepressant drugs. At the end of each 5-s period during the test session, the scorer rated the rat's behavior as one of the following three behaviors: 1) immobility, floating in the water without struggling, and making only movements necessary to keep its head above water; 2) swimming, making active swimming motions between quadrants of the cylinder, more than necessary to merely keep the head above water, moving around in the cylinder; and 3) climbing movements with forepaws in and out of the water, usually directed against the walls.

Spontaneous Locomotor Activity in Rats. Spontaneous locomotor activity was determined as reported (Okuyama et al., 1999). Animals were housed individually in transparent acrylic cages (47 × 28.5 × 29.5 cm), and spontaneous locomotor activity was recorded every 10 min for 60 min, using a SCANET apparatus (Neuroscience Inc., Tokyo, Japan) placed in a sound-proof box. MCL0129 was administered s.c. 30 min before the start of measurements.

Rotarod Performance in Rats. Rotarod performance was carried out as reported previously (Okuyama et al., 1999). The Rotarod (Campden Instruments, Leicestershire, UK), consisted of a gritted plastic roller (3 cm in diameter, 9 cm long) flanked by two large round plates to prevent the animal from escaping, and was run at 10 rpm. All animals were given control trials before the test. A rat was placed on the roller, and the length of time it remained on the rod was measured. A maximum of 2 min was allowed for each animal. MCL0129 was administered s.c. 30 min before the test.

Potentiation of Hexobarbital-Induced Anesthesia in Rats. Hexobarbital-induced anesthesia was given as described (Okuyama et al., 1999). Hexobarbital-induced anesthesia was estimated based on duration of the righting reflex loss. Hexobarbital (100 mg/kg i.p.) was administered 30 min after the subcutaneous administration of MCL0129.

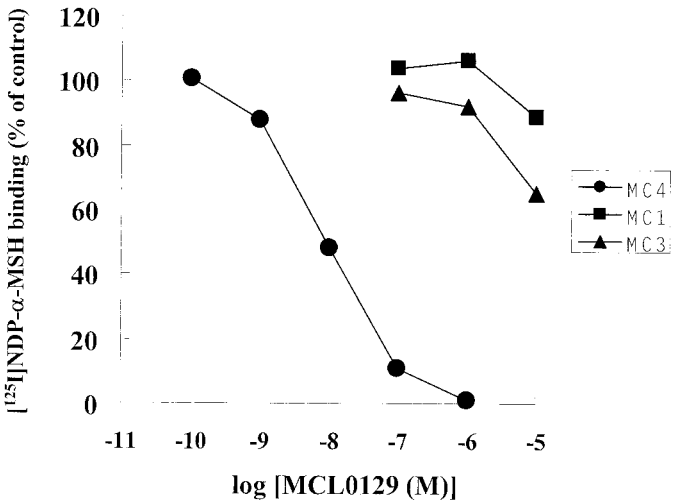

Fig. 2. Inhibition of NDP-α-MSH binding to recombinant MC4 (●), MC1 (■), and MC3 (▲) by MCL0129. Receptor binding assay of MC receptors was done as described under *Materials and Methods*. Results are the mean value of three separate experiments, each done in duplicate.

Statistical Analysis. Data from in vivo experiments were analyzed by one-way analysis of variance, and significant differences between groups were determined using Dunnett's test. In the case of the learned helplessness test, a comparison between two groups was made using the Mann-Whitney *U* test. Between-group comparisons were assessed using the Steel test.

Results

In Vitro Receptor Profiles of MCL0129. MCL0129 inhibited [^{125}I]NDP-α-MSH binding to membranes of COS-1 cells expressing the human MC4 receptor with a K_i value of 7.9 ± 0.35 nM (Fig. 2). By contrast, MCL0129 showed no affinity for the human MC1 receptor and the rat MC3 receptor, even at 10 μM, when assessed by [^{125}I]NDP-α-MSH to membranes of COS-1 cells expressing each receptor (Fig. 2). MCL0129 dose dependently inhibited the cAMP formation induced by α-melanin-concentrating hormone (α-MCH) in COS-1 cells transiently expressing the MC4 receptor, without affecting the basal cAMP level per se (Fig. 3), thereby indicating that MCL0129 acts as an antagonist at the MC4 receptor. MCL0129 showed moderate affinity for the σ1 receptor (IC_{50} = 68.9 nM), the serotonin transporter (SET) (IC_{50} = 383 nM) and the α_1-adrenoceptor (IC_{50} = 630 nM) (Table 1). MCL0129 did not show affinity for other receptors and transporters, including σ2, CRF1, opiates δ and μ, opioid receptor-like-1 (ORL-1), D_2, *N*-methyl-D-aspartate receptors, α_{2A}- and α_{2C}-adrenoceptors, norepinephrine transporter, glutamate transporter, and the Ca^{2+} channel even at 1 μM (Table 1).

Effect on Stress-Induced Anxiogenic-Like Behavior in Mice and Rats. We reported that time spent in the light area in the light/dark exploration task (mice) and time spent on the open arms in the elevated plus-maze task (rats) was significantly reduced by swim stress, and the anxiogenic-like behavior was attenuated by CRF1 receptor antagonists as did diazepam (Okuyama et al., 1999). In the present study, swim stress significantly [$F(1,18) = 40.2$, $p < 0.01$ for s.c.; $F(1,18) = 10.8$, $p < 0.01$ for p.o.] reduced the time in the light area in mice, and MCL0129 significantly and dose dependently attenuated the decrease in time spent in the light area when administered either orally [$F(3,36) = 2.95$, $p < 0.05$] or subcutaneously [$F(3,36) = 3.60$, $p < 0.05$] (Fig. 4). Likewise, the 2-min swim stress significantly [$F(1,28) = 32.8$, $p < 0.01$] decreased the time spent on the open arms in the elevated plus-maze task in rats. MCL0129, when administered p.o., dose dependently and significantly [$F(4,70) = 7.73$, $p < 0.01$] (10 and 30 mg/kg) ameliorated anxiogenic-like behavior caused by swim stress (Fig. 5). Practically the same result was obtained when assessed by the ratio of open arm entries/total arm entries (data not shown).

Effect of MCL0129 in the Light/Dark Exploration Test in Naive Mice. MCL0129 prolonged the time spent in the light area in the light/dark exploration task in mice in a

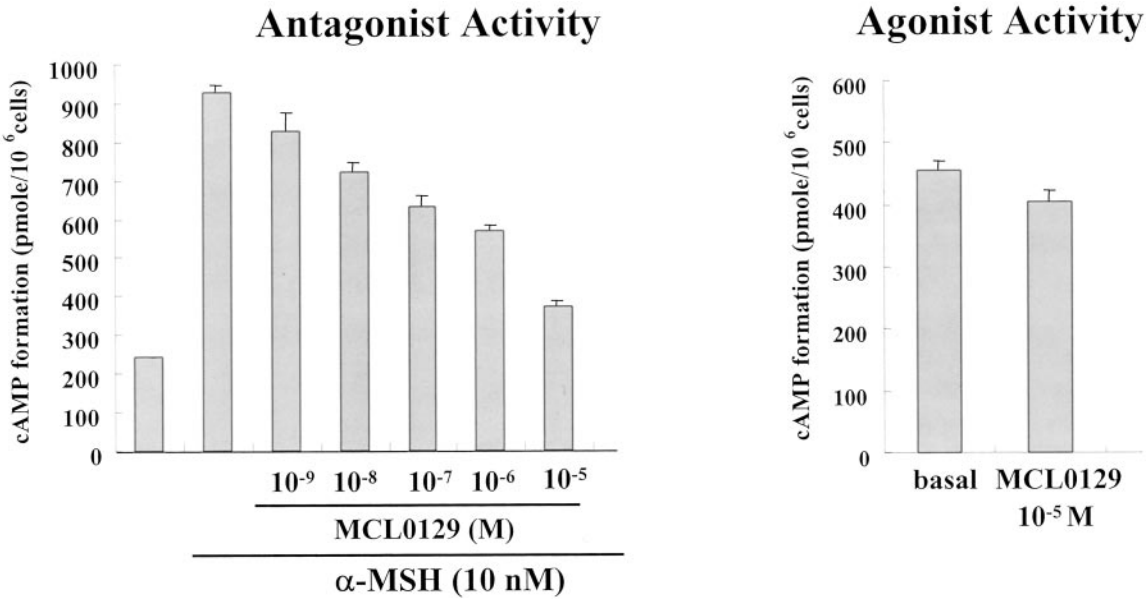

Fig. 3. Effect of MCL0129 on basal and α-MSH-induced increase in cAMP accumulation in COS-1 cells expressing the MC4 receptor. MCL0129 was incubated with or without 10 nM α-MSH for 15 min; then, cAMP formed in the cells was measured as described under *Materials and Methods*. Results are mean ± S.E. obtained from three experiments.

TABLE 1
Receptor selectivity of MCL0129
Effect on EAAT2 was evaluated by [^{3}H]glutamate uptake by Y79 cells, and effects on other receptors, transporters and channels were evaluated by receptor binding.

Receptors/ Transporters	Source	Radioligand	IC50
			nM
SET	Rat cortex	[^{3}H]Paroxetine	383
NET	Rat cortex	[^{3}H]Nisoxetine	>1000
α_1	Rat cortex	[^{3}H]MK912	630
α_{2A}	Human recombinant	[^{3}H]MK912	>1000
α_{2C}	Human recombinant	[^{3}H]MK912	1000
D2	Rat striatum	[^{3}H]Raclopride	>1000
σ_1	Guinea pig brain	[^{3}H](+)-Pentazocine	68.9
σ_2	Guinea pig brain	[^{3}H]DTG	>1000
CRF1	Monkey amygdala	^{125}I-Ovine CRF	>1000
opiate δ	Rat brain	[^{3}H]DPDPE	>1000
opiate μ	Rat brain	[^{3}H]DAMGO	>1000
ORL1	Human recombinant	[^{125}I]Nociceptine	>1000
NMDA	Rat brain	[^{3}H]CGP39653	>1000
EAAT2	Y79 cells	[^{3}H]Glutamate	>1000
Ca^{2+} channel	Rat brain	[^{3}H](+)-PN200-110	>1000

NET, norepinephrine transporter; [^{3}H]DTG, 1,3-di-*o*-tolyguanidine; [^{3}H]DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; [^{3}H]DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin; ORL1, opioid receptor-like-1; NMDA, *N*-methyl-D-aspartate; EAAT2, excitatory amino acid transporter 2.

dose-dependent and significant manner [$F(3,36) = 3.44$, $p < 0.05$] (Fig. 6).

Effect of MCL0129 in Marble-Burying Behavior in Mice. Anxiolytics such as diazepam [$F(3,36) = 8.24$, $p < 0.01$] and buspirone [$F(3,36) = 15.8$, $p < 0.01$] significantly reduced the marble-burying behavior (Fig. 7c and d). Likewise, fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), potently and significantly blocked this activity [$F(3,36) = 9.02$, $p < 0.01$] (Fig. 7b). MCL0129, at 10 mg/kg, also significantly reduced the marble-burying behavior [$F(3,36) = 5.2$, $p < 0.01$] (Fig. 7a).

Effect of MCL0129 in Forced Swimming Test in Rats. MCL0129 at 10 and 30 mg/kg s.c. significantly and dose dependently reduced the immobility time [$F(3,36) = 6.98$, $p < 0.01$] (Fig. 8a). When assessed according to the behavioral scoring method reported by Detke et al. (1995), MCL0129 selectively and significantly increased the swimming behavior without affecting the climbing behavior (Fig. 8b).

Effect of MCL0129 in the Learned Helplessness Test in Rats. As compared with control animals, nonstressed animals exposed to inescapable shock (helpless) manifested a significantly higher number of escape failures ($p < 0.01$). Acute administration of MCL0129, when administered before an inescapable shock (acquisition phase), dose dependently and at 3 mg/kg ($p < 0.01$) and 10 mg/kg ($p < 0.05$) s.c. significantly decreased the number of escape failures (Fig. 9).

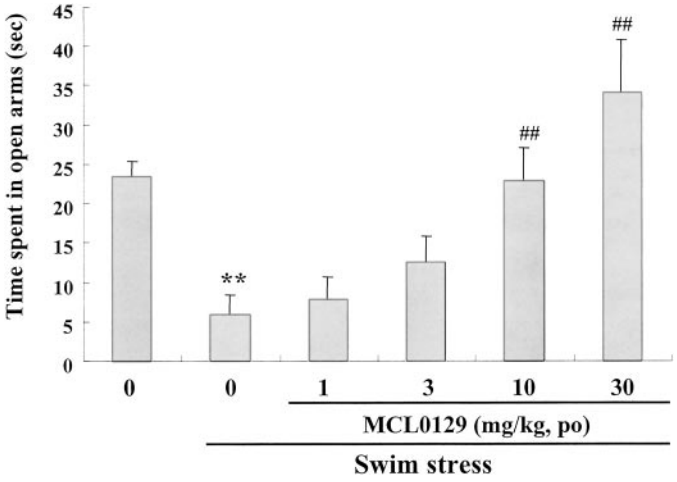

Fig. 5. Effect of MCL0129 on swim stress-induced reduction of time spent in open arms in the elevated plus-maze task in rats. Data represent mean ± S.E. ($n = 15$). ⋆⋆, $p < 0.01$ versus nonstress group (Dunnett's test); ##, $p < 0.01$ versus swim stress group (Dunnett's test).

Effect of MCL0129 on General Behavior. MCL0129 significantly inhibited spontaneous locomotor activity at a relatively higher dose of 100 mg/kg s.c. [$F(3,28) = 6.86$, $p < 0.01$], compared with pharmacologically effective dosages (Fig. 10a). MCL0129 did not affect Rotarod performance [$F(3,36) = 0.63$, $p = 0.76$;0.26, n.s.] and hexobarbital-induced sleeping time [$F(3,36) = 1.16$, $p = 0.63$;0.24, n.s.] (1, 10, and 100 mg/kg s.c.), whereas diazepam potently and significantly affected these functions in the previous study (Okuyama et al., 1999) (Fig. 10, b and c).

Discussion

We obtained evidence that MCL0129 is a potent and selective MC4 receptor antagonist and that MCL0129 showed

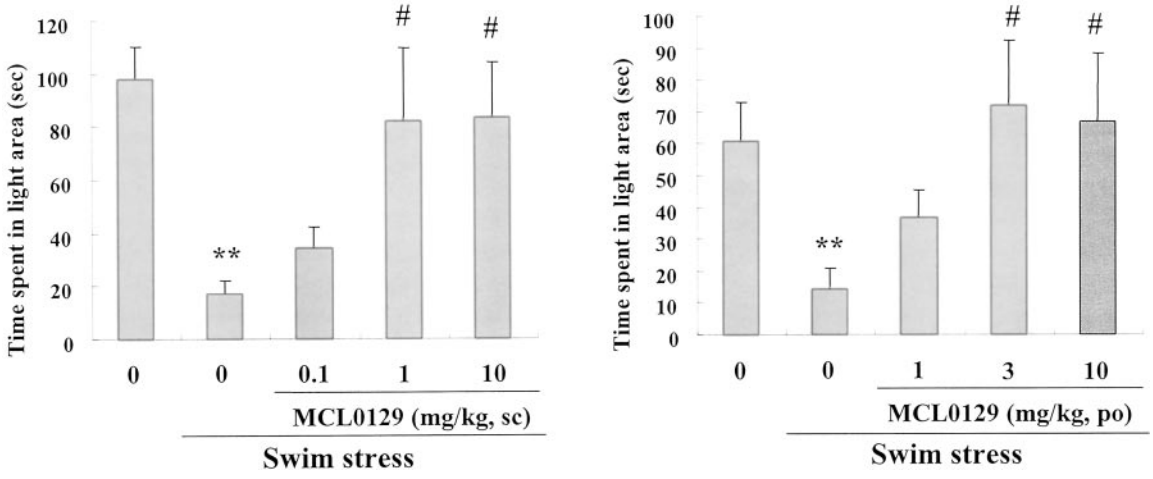

Fig. 4. Effect of MCL0129 on swim stress-induced reduction of time spent in the light area in the light/dark exploration test in mice. MCL0129 was administered either orally or subcutaneously. Data represent mean ± S.E. ($n = 10$). ⋆⋆, $p < 0.01$ versus nonstress group (Dunnett's test); #, $p < 0.05$ versus swim stress group (Dunnett's test).

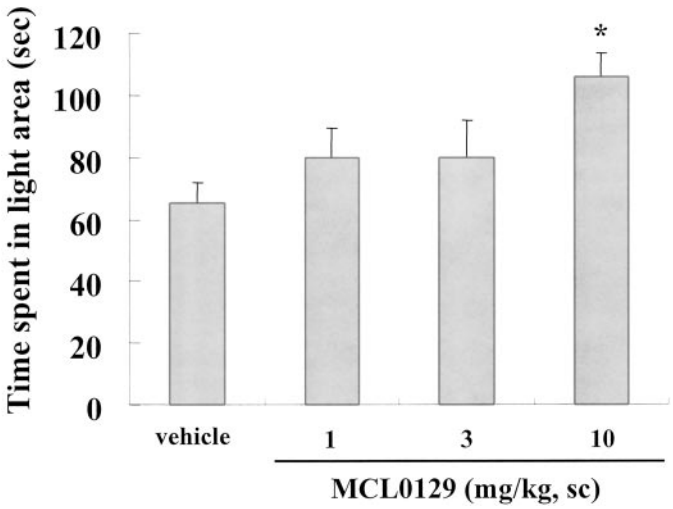

Fig. 6. Effect of MCL0129 on time spent in the light area in the light/dark exploration test in mice (nonstress condition). Data represent mean ± S.E. ($n = 10$). ⋆, $p < 0.05$ versus vehicle-treated group (Dunnett's test).

antidepressant-like and anxiolytic-like activities in various rodent models.

MCL0129 inhibited [^{125}I]NDP-α-MSH binding to the recombinant human MC4 receptor without affecting [^{125}I]NDP-α-MSH binding to other MC receptor subtypes such as MC1 and MC3. By contrast, MCL0129 showed moderate to negligible affinities for other stress- and anxiety/depression-related receptors and transporters. Moreover, MCL0129 attenuated the α-MSH-induced increase in cAMP formation in COS-1 cells expressing the MC4 receptor, whereas MCL0129 itself had no effect on basal levels of cAMP. These in vitro data clearly show that MCL0129 is a selective MC4 receptor antagonist. Because MCL0129 is the most potent and selective nonpeptide MC4 receptor antagonist reported hitherto and is highly selective among MC receptors, MCL0129 should prove to be a useful pharmacological tool for investigation of physiological roles of MC4 receptor.

Stress-induced anxiogenic-like behavior has been used as a model of anxiety, and the swim stress markedly reduced the time spent in the light area in light/dark exploration tests in mice and in open arms in the elevated plus-maze task in rats, both of which were ameliorated previously by the administration of diazepam as well as CRF1 receptor antagonists (Okuyama et al., 1999). In the present study, MCL0129 dose dependently and significantly attenuated the swim stress-induced anxiogenic-like effect in both paradigms. Moreover, MCL0129 significantly prolonged the time spent in the light area in light/dark exploration tests on naive mice. In a previous study, CRF1 receptor antagonists did not show significant effects in this model (Okuyama et al., 1999). Therefore, MC4 receptor antagonists may have different pharmacological profiles from those of CRF1 receptor antagonists in terms of anxiolytic-like activity.

SSRIs, as well as benzodiazepine anxiolytics, have been reported to suppress marble burying without disrupting general behavior (Millan et al., 2001). Although it remains to be established whether blockade of marble-burying behavior in

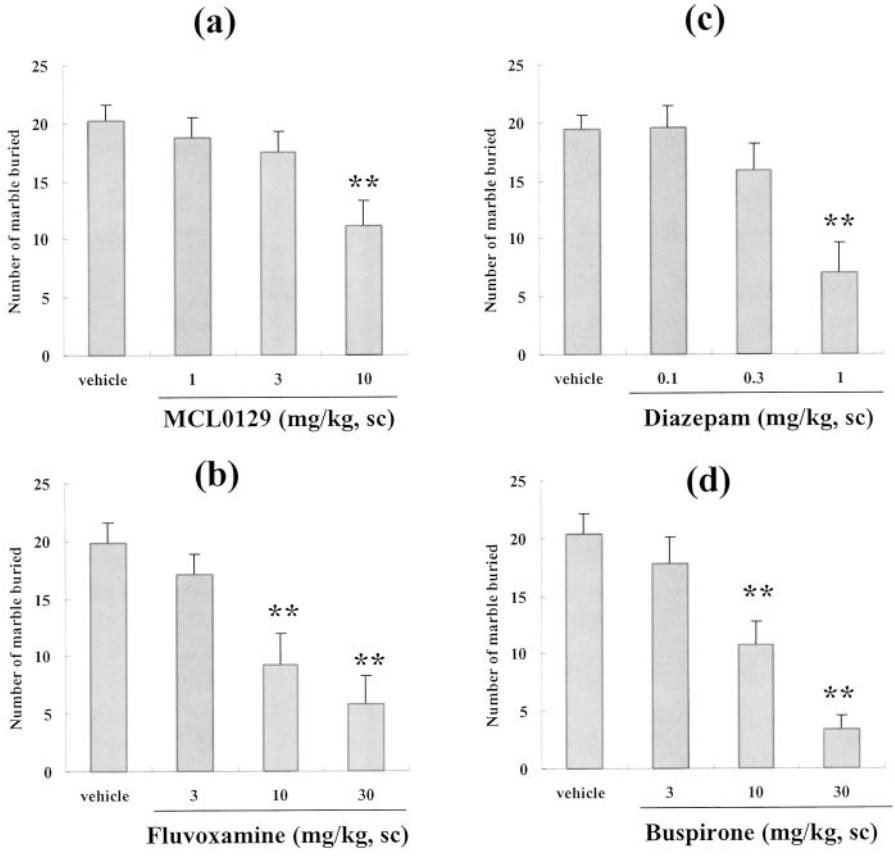

Fig. 7. Effect of MCL0129 (a), fluvoxamine (b), diazepam (c), and buspirone (d) on marble-burying behavior in mice. Data represent mean ± S.E. ($n = 10$). ⋆⋆, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

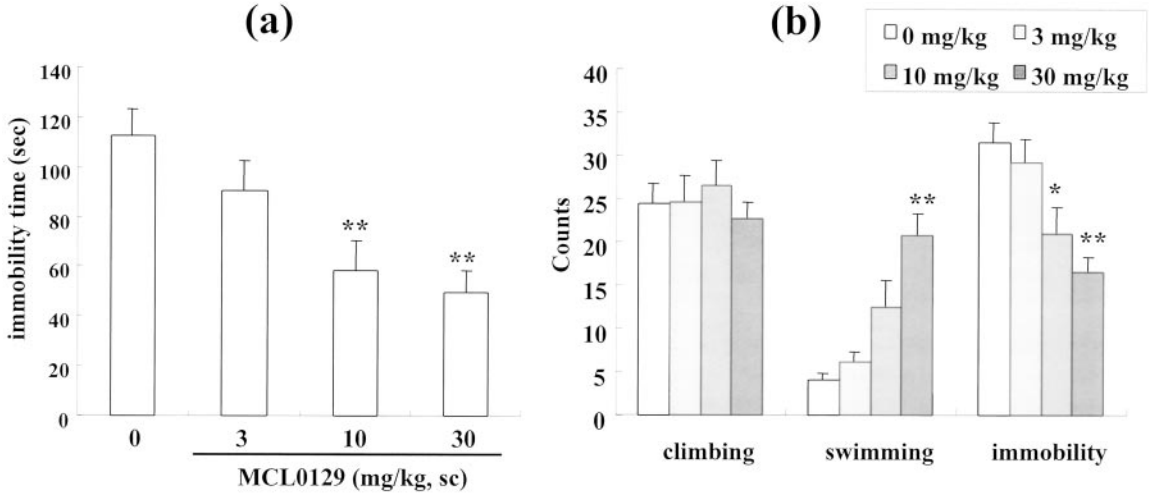

Fig. 8. Effect of MCL0129 in forced swimming tests in rats. The effect of MCL0129 was evaluated by both the method (a) described by Porsolt et al. (1978) and the time-sampling technique (b) described by Detke et al. (1995). Data represent mean ± S.E. (n = 10). ⋆, $p < 0.05$, ⋆⋆, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

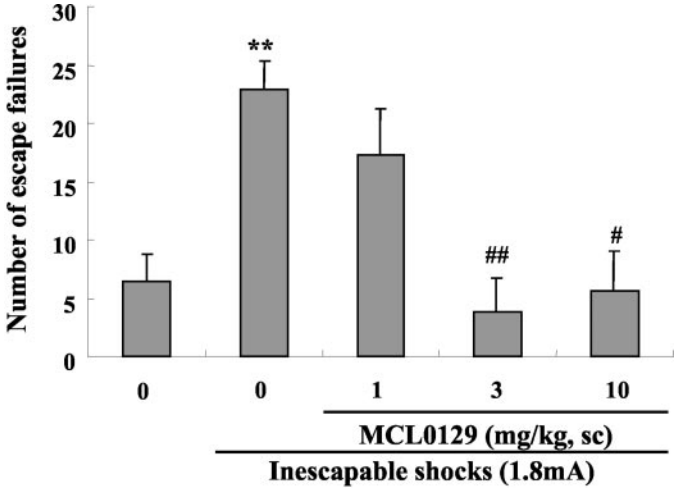

Fig. 9. Effect of MCL0129 in the learned helplessness test in rats. Data represent mean ± S.E. (n = 10). ⋆⋆, $p < 0.01$ versus noninescapable shock group (Mann-Whitney U test). #, $p < 0.05$, ##, $p < 0.01$ versus inescapable shock group (Steel test).

mice is predictive of clinically relevant anti-impulsive properties, this action is of interest in view of the increasing utility of SSRIs in the treatment of subjects with obsessive-compulsive disorders (Pigott and Seay, 1999). In this study, anxiolytics such as diazepam and buspirone as well as the SSRI, fluvoxamine, blocked marble-burying behavior, as reported previously (Millan et al., 2001). In the marble-burying test, MCL0129 was effective in significantly reducing this compulsive burying of novel objects.

MCL0129 significantly shortened immobility time in forced swimming tests in rats. When assessed by a behavioral sampling method, MCL0129 increased swimming behavior without affecting climbing behavior. In this test, SSRIs such as fluvoxamine and fluoxetine specifically increased the swimming score, whereas noradrenaline reuptake inhibitors such as reboxetine increased climbing behavior without affecting swimming (Detke et al., 1995, 1996; Cryan et al., 2002). Thus, it is presumed that compounds that potentiate serotonin transmission may increase the swimming score, whereas drugs with actions on norepinephrine transmission may increase climbing scores. In light of this hypothesis, it is likely that MCL0129 exerted antidepressant-like activity in the forced swimming test by acting on serotonin transmission. This result is consistent with the observation regarding marble-burying behavior in which both MCL0129 and SSRIs were effective. Whether or not the MC4 receptor antagonist influences serotonin transmission remains to be investigated.

We further evaluated the antidepressant potential of the MC4 receptor antagonist in the learned helplessness test in rats. Under the same conditions used in this test, we reported that fluvoxamine and imipramine showed antidepressant-like effects only when administered subchronically for 8 days, whereas CRF1 receptor antagonists showed activity even in cases of acute administration (Takamori et al., 2001a,b). MCL0129, when administered under the same schedule as those compounds, in acquisition phase rather than in consolidation and retention phases, exhibited antidepressant-like effects even with an acute administration. Therefore, MC4 receptor antagonists, like CRF1 receptor antagonists, may exert antidepressant-like activity with an early onset. It was reported that the MC4 receptor is involved in pain, and MC4 receptor agonists increased the sensitivity to mechanical and cold stimulation, whereas MC4 receptor antagonists alleviated cold and mechanical allodynia in a rat model of neuropathic pain (Vrinten et al., 2000, 2001). Therefore, involvement of decrease in pain threshold in this test by MC4 receptor agonists needs to be ruled out.

Many drugs that act on the central nervous system often cause unwanted side effects such as prolongation of sleeping, sleep sedation (Okuyama et al., 1999), and impaired motor coordination (Bristow et al., 1996). In the present study, MCL0129 did not affect hexobarbital-induced sleeping time and Rotarod performance in rats, even at the highest doses given. MCL0129 inhibited spontaneous locomotor activity, but this effect occurred at the highest doses of 100 mg/kg MCL0129. Therefore, MC4 receptor antagonists are expected to be without the unwanted central nervous system side effects sometimes seen in patients on antidepressants and/or anxiolytics, although concern for body weight gain with

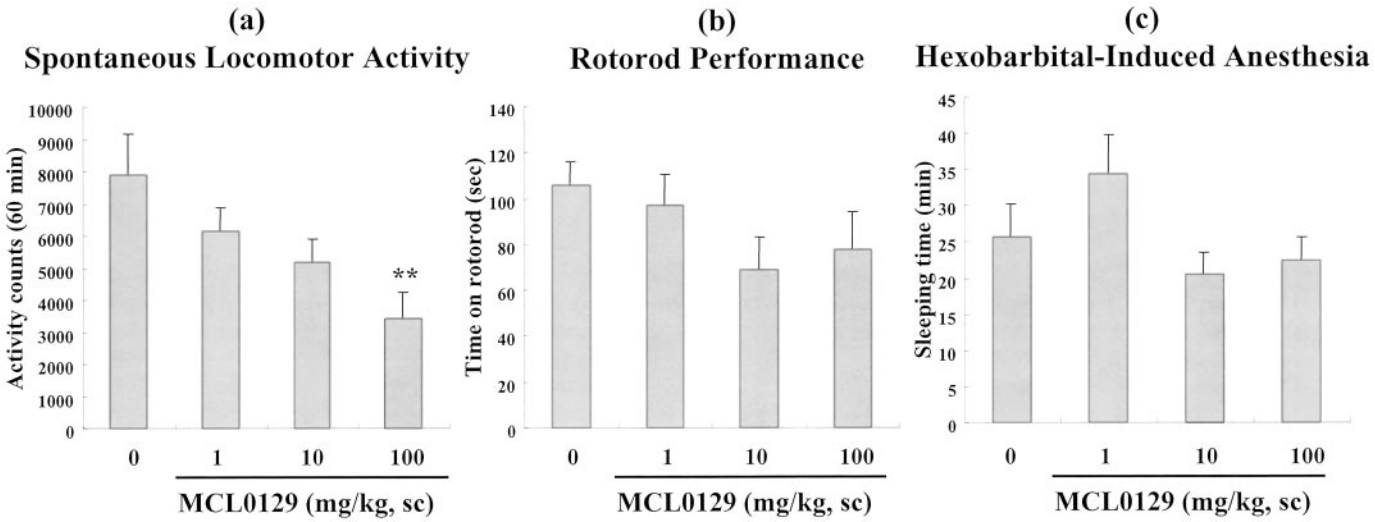

Fig. 10. General behavioral profiles of MCL0129: effect on spontaneous locomotor activity (a), Rotarod performance (b), and hexobarbital-induced anesthesia (c). Data represent mean ± S.E. ($n = 6$ for locomotor activity, $n = 8$ for Rotarod and hexobarbital-induced anesthesia). ⋆⋆, $p < 0.01$ versus vehicle-treated group (Dunnett's test).

chronic administration remains, as MC4 receptor antagonism was reported to lead to obesity (Huszar et al., 1997).

MCL0129 showed moderate affinity for SET in receptor binding studies. With regard to possible involvement of blockade of SET in the pharmacological actions of MCL0129, fluvoxamine, a SSRI, did not show effects on swim stress-induced anxiogenic-like behavior in rats or mice and did not show anxiolytic-like activity in the light/dark exploration test in naive mice (unpublished data). Moreover, fluvoxamine did not have antidepressant activity in the learned helplessness test in the case of an acute administration, whereas it was effective in repeated administration in the present experimental condition (Takamori et al., 2001b). These results clearly show that the effects of MCL0129 are mediated through the MC4 receptor, although involvement of the σ1 receptor needs to be ruled out.

There are reports which suggest that the brain melanocortinergic system might be involved in stress-related behaviors (Corda et al., 1990; de Barioglio et al., 1991; Gonzalez et al., 1996; Adan et al., 1999), and blockade of the MC receptor may lead to anxiolytic-like activity (Dunn et al., 1979; Adan et al., 1999; Vergoni et al., 1999). In a preliminary study, we observed that the peptide MC4 receptor agonist, Ac-[NIle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH-4-10-NH_2, showed anxiogenic-like effects in the Vogel test on rats, and peptidomimetic MC4 receptor antagonists exhibited anti-stress activities in both rats and mice, which means that the MC4 receptor may be involved in stress-related behavior (S. Chaki, S. Ogawa, Y. Toda, T. Funakoshi, and S. Okoyama, unpublished data). These observations are consistent with the present results that MC4 receptor-selective antagonist had antidepressant-like and anxiolytic-like activity in various rodent models. Recently, it was reported that MCH receptor antagonist showed anxiolytic-like and antidepressant-like activities as well as anorectic activity in rodents (Borowsky et al., 2002). It is interesting that MCH and MC act differently in food intake, yet show the same effects in emotional behaviors.

In conclusion, the brain melanocortin system may be involved in stress-related disorders such as anxiety and depression, and MC4 receptor blockade may be a useful approach to treat subjects with anxiety and depression, and without the side effects sometimes seen with medication with anxiolytics and antidepressants. Moreover, MCL0129 is a useful pharmacological tool to investigate involvement of the MC4 receptor in such disorders and to elucidate mechanisms mediated through the MC4 receptor.

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