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Pharmacology, Biochemistry and Behavior 82 (2005) 621 – 626
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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MCL0042: A nonpeptidic MC4 receptor antagonist and serotonin reuptake inhibitor with anxiolytic- and antidepressant-like activity

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Received 29 July 2005; received in revised form 21 October 2005; accepted 2 November 2005
Available online 6 December 2005

Abstract

In the present study, we examined the anxiolytic and antidepressant effects of MCL0042, a novel compound showing activity in both MC4 receptor antagonism and serotonin transporter inhibition. MCL0042 showed relatively high affinity for the MC4 receptor and serotonin reuptake site, as determined by receptor binding assays. MCL0042 attenuated [Nle^4,D-Phe^7]α-MSH-increased cAMP formation in MC4 receptor expressing cells, and it inhibited [^{3}H]serotonin uptake by rat brain synaptosomes; thus, MCL0042 is an MC4 receptor antagonist and serotonin transporter inhibitor.

Subcutaneous administration of MCL0042 significantly increased the number of licks in a Vogel punished drinking test in rats, and it also significantly attenuated swim stress-induced reduction in time spent in open arms in an elevated plus-maze task in rats, showing the anxiolytic-like potential of MCL0042. Moreover, repeated administration of MCL0042 for 14 days attenuated olfactory bulbectomy-induced locomotor hyperactivity in rats, indicating antidepressant-like potential.

These data show that MCL0042 has unique properties of both the MC4 receptor antagonist and serotonin transporter inhibitor, and produces anxiolytic and antidepressant activity in rats. Moreover, blockade of both the MC4 receptor and serotonin reuptake sites might represent a useful approach in the treatment of anxiety and depression.

Keywords: MCL0042; MC4 receptor; Serotonin transporter; Antidepressant; Anxiolytic

1. Introduction

Major depressive disorder and anxiety disorders are among the most prevalent forms of mental illness. In clinics, selective serotonin reuptake inhibitors (SSRIs) have been widely prescribed not only to patients with major depressive disorder but also to those with anxiety disorders. Although most patients are successfully treated with SSRIs, drawbacks remain with SSRI medications due to the existence of non-responders and slow onset of action. Thus, antidepressants with different mechanism are required.

Recently, it has been considered that stress may play a pivotal role in both anxiety and depression. Indeed, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which is caused by continuous exposure to stress, has been reported in patients with major depressive disorder, anorexia nervosa and post-traumatic stress disorders (Holsboer et al., 1984; Taylor and Fishman, 1988). Stress responses, including dysfunction of the HPA axis, are initiated by release of corticotropin-releasing factor (CRF) (Owen and Nemeroff, 1991). In addition to activation of the brain CRF system, several lines of evidence indicate that melanocortines (MCs), which stem from pro-opiomelanocortin by enzymatic processing, mediate important behavioral and biochemical responses to stress, and consequently stress-induced disorders. To date, five types of receptor

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doi:10.1016/j.pbb.2005.11.001

subtype for MCs (MC1–MC5) have been reported. Among them, the MC4 receptor is of particular interest in terms of its relationship to stress-related disorders. It has been reported that MC4 receptor agonists induce grooming behavior in rats, while an 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 mediates activation of the HPA axis (Von Frijtag et al., 1998). Thus, the MC4 receptor may mediate stress responses. These events are considered to be partly mediated through the release of CRF in the paraventricular nucleus (PVN), since interaction between MC4 receptor and CRF in the PVN has been reported (Sarkar et al., 2002; Lu et al., 2003). In addition to its involvement in stress responses through the HPA axis, it has also been suggested that the MC4 receptor in the amygdala mediates expression of anxiety, as injection of α-MSH into the amygdala results in anxiety-like behavior, while injection of an MC4 receptor antagonist has the opposite effect (Kokare et al., 2005). Involvement of the MC4 receptor in anxiety and depression has been indicated by studies using selective MC4 receptor antagonists. We previously reported that both the nonpeptidic MC4 receptor antagonist MCL0129 and peptide-mimetic MC4 receptor antagonist MCL0020 exhibited antidepressant, anxiolytic, and anti-stress effects in a variety of animal models (Chaki et al., 2003a,b), suggesting that blockade of the MC4 receptor may be a useful strategy in treating subjects with depressive and anxiety disorders.

In a course of synthesizing the MC4 receptor antagonists, we serendipitously discovered the compound, MCL0042, also showed serotonin transporter inhibitory activity. It has been well recognized that blockade of serotonin transporter is an effective treatment for depression, and SSRIs have become the first line treatment for not only depression but also anxiety disorders (Zohar and Westenberg, 2000). Therefore, this compound, with actions both on the MC4 receptor and on the serotonin transporter, should prove beneficial in the treatment of depression and anxiety disorders. In the present study, we report on the anxiolytic and antidepressant effects of MCL0042, a newly synthesized MC4 receptor antagonist and serotonin transporter inhibitor in some animal models of anxiety and depression.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (220–240 g, Charles River, Yokohama, Japan) were housed 3/cage and used for all studies. All animals were maintained under a 12-h light/dark cycle (lights on at 7:00 AM) in a temperature- and humidity-controlled holding room with food and water available ad libitum. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research Association standard, as defined in the *Guidelines for Animal Experiments* (1987).

2.2. Drugs

1-[2-(4-fluorophenyl)-2-(4-methylpiperazin-1-yl)ethyl]-4-[4-(1-naphthyl)butyl]piperazine (MCL0042) (Fig. 1) was synthesized at Taisho Medicinal Research Laboratories (Saitama, Japan). For in vivo studies, hydrochloric acid salt of MCL0042 (MCL0042·HCl) dissolved in 0.3% Tween 80 was used. [^{125}I][Nle^4,D-Phe^7]α-Melanocyte stimulating hormone ([Nle^4,D-Phe^7]α-MSH) (specific radioactivity: 81.4 TBq/mmol), [^{3}H]serotonin (specific radioactivity: 581 GBq/mmol), and a cAMP assay system were purchased from Amersham International (Buckinghamshire, England). [^{3}H]paroxetine (specific radioactivity: 728.9 GBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). COS-1 cells were purchased from American Type Culture Collection (Rocksville, MD, USA). [Nle^4,D-Phe^7]α-MSH was purchased from Peninsula Laboratories (Belmont, CA, USA). All other chemicals used in this study were obtained commercially, and all were of the highest purity available. For the in vitro study, MCL0042 was dissolved in 0.1% dimethylsulfoxide, and dimethylsulfoxide (0.1%) itself did not affect the binding assay, [^{3}H]serotonin uptake, and cAMP levels.

2.3. [^{125}I][Nle^4,D-Phe^7]α-MSH binding to recombinant MC4 receptor

COS-1 cells expressing the MC receptor, prepared according to the method reported previously (Chaki et al., 2003a), were washed with phosphate buffered saline, 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 phenylmethylsulfonylfluoride, 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 an assay buffer (50 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, 100 μM phenylmethylsulfonylfluoride and 0.1% bovine serum albumin (BSA)), and served as crude membrane preparation for binding studies. Binding assays of [^{125}I][Nle^4,D-Phe^7]α-MSH were performed according to Chaki et al. (2003a). Membranes were incubated with [^{125}I][Nle^4,D-Phe^7]α-MSH (0.2 nM) for 120 min at 25 °C, and the reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.5% BSA, after which the filters were washed three times with the buffer. Radioactivity was quantified in a γ-counter. Nonspecific binding was determined in the presence of 1 μM [Nle^4,D-Phe^7]α-MSH. Specific binding was determined by subtracting

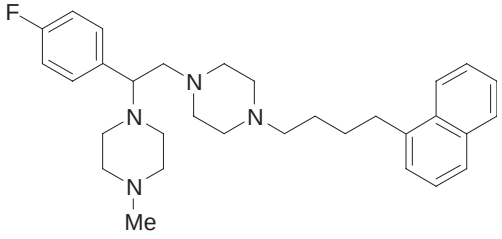

Fig. 1. Chemical structure of MCL0042.

nonspecific from total binding. In the competition assay, concentration of the test compound that caused 50% inhibition of the specific binding (IC_{50} value) was determined from each concentration–response curve.

2.4. Determination of cAMP

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 phosphate buffered saline, and 1 ml of DMEM containing 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, was added. The cells were incubated with [Nle^4,D-Phe^7]α-MSH (1 nM) and/or various concentrations of MCL0042 for 15 min at 37 °C. The culture medium was then aspirated and the cells were washed with phosphate buffered saline. Two milliliters of ice-cold 65% ethanol was 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 EIA system.

2.5. [^{3}H]Paroxetine binding to membranes of rat brain

Each rat was sacrificed, and the brain was rapidly removed. The cerebral cortex was dissected and used for [^{3}H]paroxetine binding. The brain tissue was homogenized with 50 mM Tris–HCl buffer (pH 7.4) and the homogenate was centrifuged at 1000×*g* for 10 min at 4 °C. The supernatant was then 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 the assay buffer (50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl and 0.2% BSA), and served as crude membrane preparation for binding studies. Membranes were incubated with [^{3}H]paroxetine (0.04 nM) for 90 min at 22 °C, and the reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.3% polyethyleneimine, after which the filters were washed three times with the assay buffer (without BSA). The radioactivity was quantified in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM fluoxetine.

2.6. [^{3}H]Serotonin uptake by rat brain synaptosomes

Each rats was sacrificed and the brain was rapidly removed. The frontal cortex was dissected and homogenized with Krebs–Henseleit buffer (pH 7.4)/0.32 M sucrose using Teflon homogenizer. The homogenate was centrifuged at 1500×*g* for 10 min at 4 °C, and the supernatant was centrifuged at 18,000×*g* for 10 min to obtain a P2 fraction. The pellet was then suspended in Krebs–Henseleit buffer (pH 7.4) containing 10 μM pargyline and incubated with various concentrations of MCL0042 for 5 min at 37 °C, after which the homogenate was incubated with [^{3}H]serotonin for an additional 5 min at 37 °C. The reaction was terminated by rapid filtration over a GF/B filter presoaked with 0.3% polyethyleneimine, after which the filters were washed three times with phosphate buffered saline. Radioactivity was quantified in a liquid scintillation counter. The result obtained from the assay performed on ice was used for nonspecific uptake.

2.7. Vogel test in rats

A modification of the method of Vogel et al. (1971) was used. Briefly, groups of 7–12 rats were deprived of drinking water but not of food for 48 h prior to the conflict session and then placed in a Plexiglas conflict test box (Neuroscience Inc., Tokyo) with a stainless steel grid floor. Each box was placed in a sound-attenuated ventilated chamber. Water was provided through a stainless steel drinking tube extending 1 cm into the box, 3 cm above the floor. The drinking tube and the grid floor were connected to a constant-current shock generator and to a licking time-counter. Drinking attempts were punished with an electric shock (0.4 mA), and the impulses were released for every 2 s of cumulative licking time-counter output. Each shock lasted for 0.5 s and if the rat was drinking when an impulse was released, it received a shock. Unpunished drinking was measured in a separate group of animals with the current intensity set to 0 mA. The number of shocks was recorded automatically during a 3-min period. MCL0042·HCl was administered s.c. 30 min prior to the test.

2.8. Stress-induced anxiety in an elevated plus-maze task in rats

Rats were placed in a 40-cm-tall, 20-cm-wide cylindrical plastic container containing 25 cm of water maintained at 25±1 °C for 2 min. Subjects were then removed from the tank and allowed to recover for a further 5 min before the elevated plus-maze test was performed. The elevated plus-maze was conducted according to the method reported previously (Pellow and File, 1986) with modification. The apparatus consisted of a plus-shaped maze, elevated 50 cm from the floor, with two opposite open arms, 50×10 cm, crossed at right angles by two arms of the same dimensions, but 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 prevent falls. Illumination measured at the center of the maze was 40 lx. Each rat was placed in the center of the plus-maze facing one enclosed arm. The amount of time spent in the open arms of the maze for 5 min test session was measured. During observation, the experimenter always sat in the same place, next to the apparatus. Rats were naïve to the apparatus. MCL0042·HCl was administered s.c. 30 min prior to exposure to swim stress.

2.9. Olfactory bulbectomy (OBX)-induced hyperactivity in rats

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). Olfactory bulbs were removed by suction after

which the void following OBX was filled with gel foam, and the animals were then housed one per cage. After a 10–14-day post-surgical period, locomotor hyperactivity was measured. Only rats that exhibited locomotor hyperactivity were selected for the study. Locomotor hyperactivity of olfactory bulbectomized rats was measured in an open field apparatus (75 cm diameter (25 blocks) × 50 cm wall height), and the number of crossings among blocks during a period of 3 min was counted. MCL0042·HCl was administered s.c. daily for 14 days, and locomotor activity was measured 24 h after the last administration.

2.10. Spontaneous locomotor activity in rats

Rats were housed individually in transparent acrylic cages (47 × 28.5 × 29.5 cm), and spontaneous locomotor activity was recorded every 5 min for 60 min, using a SCANET apparatus (Neuroscience Inc, Japan) placed in a sound-proof box. MCL0042·HCl were administered s.c. 30 min prior to the test.

2.11. Statistical analysis

Data from in vivo experiments were analyzed by one-way analysis of variance (ANOVA); significant differences between groups were determined using Dunnett's test.

3. Results

3.1. In vitro profile of MCL0042

MCL0042 showed relatively high affinity for the MC4 receptor with an IC_{50} value of 124 ± 21.4 nM, while it did not bind to other MC receptors such as the MC1 and MC3 receptors up to 10 μM (Table 1). MCL0042 attenuated [Nle^4,D-Phe^7]α-MSH-increased cAMP formation in MC4 receptor expressing cells, while it did not affect basal cAMP formation up to 10 μM (Table 1); thus MCL0042 acts as an antagonist at the MC4 receptor. In addition to its affinity for the MC4 receptor, MCL0042 displayed high affinity for serotonin reuptake sites, as determined by [^{3}H]paroxetine binding to rat brain membranes (Table 1). MCL0042 also inhibited [^{3}H]serotonin uptake by synaptosomes prepared from rat brain (Table 1), indicating that MCL0042 is an inhibitor of serotonin transporter.

Table 1
In vitro profile of MCL0042

	IC_{50} (nM)
Affinity	
MC4	124 ± 21.4
MC1	>10,000
MC3	>10,000
Serotonin transporter	42.3 ± 2.45
MC4 antagonist	5100 ± 2300
MC4 agonist	>10,000*
Serotonin transport	264 ± 60.9

Affinity for each receptor was assessed by receptor binding assays. MC4 receptor agonist/antagonist activity was evaluated by cAMP assay, and effect on 5-HT transport was evaluated by [^{3}H]5-HT uptake assay.
* EC50 value for enhancement of cAMP formation (nM).

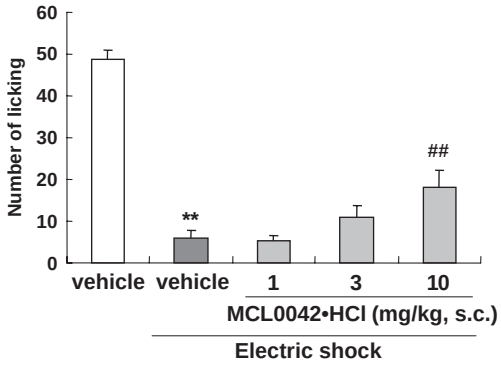

Fig. 2. Effect of MCL0042·HCl on number of licks in the Vogel test in rats. Data represent mean ± SE (n = 10). **p < 0.01 versus non-shock group (Dunnett's test). ##p < 0.01 versus vehicle group (Dunnett's test).

3.2. Effect of MCL0042·HCl in the Vogel conflict drinking test in rats

The number of licks was markedly reduced by exposure to electrical shocks [$F(1,18)$ = 230, p < 0.01]. Subcutaneous administration of MCL0042·HCl significantly and dose-dependently increased the number of licks, with a lowest active dose of 10 mg/kg [$F(3,36)$ = 4.40, p < 0.01] (Fig. 2).

3.3. Effect of MCL0042·HCl on stress-induced anxiety behavior in the elevated plus-maze task in rats

Exposure to swim stress for 2 min markedly and significantly reduced the time spent in the open arms of the maze, indicating an increased level of anxiety [$F(1,18)$ = 30.2, p < 0.01] (Fig. 3). MCL0042·HCl, when administered subcutaneously at 3 mg/kg, significantly reversed stress-induced reduction in time spent in the open arms of the maze [$F(3,36)$ = 5.65, p < 0.01] (Fig. 3).

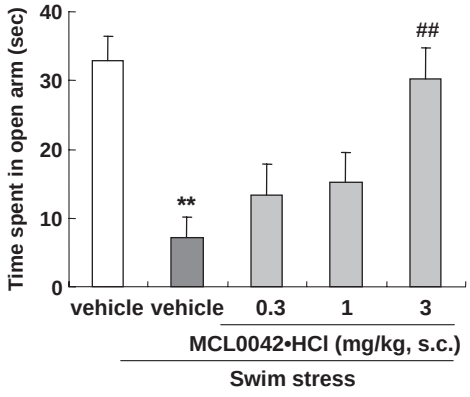

Fig. 3. Effect of MCL0042·HCl on swim stress-induced reduction of time spent in open arms of maze in the elevated plus-maze task in rats. Data represent mean ± SE (n = 10). **p < 0.01 versus nonstress group (Dunnett's test). ##p < 0.01 versus swim stress group (Dunnett's test).

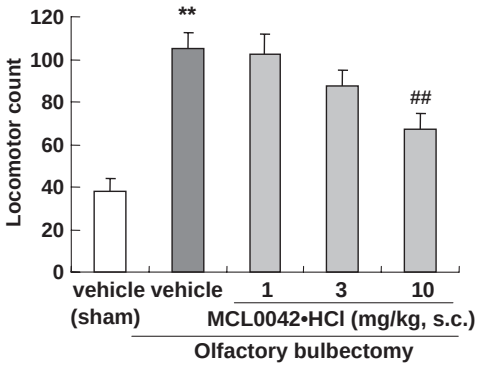

Fig. 4. Effect of MCL0042·HCl on OBX-induced locomotor hyperactivity in rats. Data represent mean ± SE ($n = 10–11$). **$p < 0.01$ versus sham group (Dunnett's test). ##$p < 0.01$ versus vehicle group (Dunnett's test).

3.4. Effect of MCL0042·HCl on OBX-induced hyperlocomotion in rats

Locomotor activity was significantly increased in OBX rats compared with sham-operated rats [$F(1,19) = 49.9$, $p < 0.01$] (Fig. 4). Locomotor hyperactivity was significantly reduced in cases of chronic administration of MCL0042·HCl for 14 days [$F(3,37) = 4.59$, $p < 0.01$] (Fig. 4).

3.5. Effect of MCL0042·HCl on spontaneous locomotor activity in rats

Subcutaneous administration of MCL0042·HCl did not affect spontaneous locomotor activity in rats at pharmacologically effective doses of 1, 3 and 10 mg/kg (Table 2).

4. Discussion

In the present study, we found that MCL0042 showed a relatively high affinity for the MC4 receptor, and that it inhibited [Nle^4,D-Phe^7]α-MSH-increased cAMP formation in MC4 receptor-expressing cells, although MCL0042 did not display affinities for the MC1 and MC3 receptors. In addition, MCL0042 exhibited a high affinity for serotonin transporter, and inhibited [^{3}H]serotonin uptake by brain synaptosomes. In contrast, MCL0042 did not show appreciable affinities for other receptors and transporters including norepinephrine transporter (data not shown). Thus, MCL0042 has unique in vitro properties of both MC4 receptor antagonism and serotonin transport inhibition.

The anxiolytic-like potential of MCL0042 was assessed in two animal models of anxiety; the conflict drinking test and the elevated plus-maze task following exposure to swim stress. In both models, the involvement of activation of the MC4 receptor in expression of anxiety has been suggested (Chaki et al., 2003a,b). In the present study, MCL0042 exhibited significant anxiolytic effects in both models of anxiety. Our previous results have indicated that stress-induced anxiety-like behavior in the elevated plus-maze task, which is represented as a reduction in time in the open arms of the maze, was attenuated by benzodiazepine anxiolytics as well as by stress-related peptide receptor antagonists such as CRF1 receptor antagonists (Chaki et al., 2004). Moreover, we have previously reported that MCL0129, a selective MC4 receptor antagonist, significantly reverses stress-induced anxiety behavior in the same paradigm (Chaki et al., 2003a). Further, in a preliminary study, results showed that fluvoxamine was without effect in stress-induced anxiety behavior in the elevated plus-maze task (data not shown). Likewise, it has been reported that fluvoxamine does not exhibit anxiolytic effects in the elevated plus-maze task in acute administration (Borsini et al., 2002). Therefore, blockade of the MC4 receptor, but not inhibition of serotonin transport, is involved in the anxiolytic-like action of MCL0042 in this model. It has also been reported that antidepressants, including SSRIs, do not show significant effects in the conflict test in acute administration (Borsini et al., 2002), indicating that Vogel punished drinking is not a model for detection of the anxiolytic-like activity of antidepressants including SSRIs. In contrast, involvement of the MC4 receptor in the conflict drinking test is suggested. It has been reported that intracerebroventricular administration of α-MSH and MT II reduces the number of licking periods in the Vogel test on rats (Corda et al., 1990; Chaki et al., 2003b); hence the MC4 receptor is involved in the expression of anxiogenic-like behavior in this model. Based on these findings, it is likely that blockade of the MC4 receptor is responsible for the exhibited anxiolytic-like activity of MCL0042 in the conflict drinking model of anxiety.

The removal of bilateral olfactory bulbs in rats shows drastic behavioral changes including increased irritability (Okuyama et al., 1999), mouse killing behavior (Shibata et al., 1984), poor performance in passive avoidance tasks (Tiffany et al., 1979) and hyperactivity in the open field apparatus (Redmond et al., 1999), all of which behaviors have been reported to be treated by chronic administration of an antidepressant (Okuyama et al., 1999; Redmond et al., 1999; Song and Leonard, 2005). Moreover, we reported that blockade of stress-related peptides, such as CRF was effective in this model (Okuyama et al., 1999); thus, the OBX model is a suitable model to evaluate antidepressant effects of compounds with activity of both serotonin transporter inhibition and MC4 receptor blockade. In the present study, chronic administration with MCL0042 for 14 days significantly reversed OBX-induced locomotor hyperactivity, indicating the antidepressant effects of MCL0042. It has been reported that an SSRI such as paroxetine significantly reversed OBX-induced hyperactivity in the open field following 14 days of treatment (Cryan et al., 1999). Therefore, the

Table 2
Effect of MCL0042·HCl on spontaneous locomotor activity in rats

MCL0042·HCl (mg/kg, s.c.)	Locomotor count
0	4177 ± 468.3
1	5615 ± 804.2
3	5056 ± 444.2
10	4013 ± 409.4

MCL0042·HCl was administered s.c. 30 min prior to the test. Data represent mean ± SE ($n = 6$).

inhibitory activity of MCL0042 against serotonin transport is deeply involved in antidepressant-like activity in this model. It has also been reported that abnormalities of the HPA axis are observed in the OBX model, and that normalizing dysfunction of the HPA axis is involved in the antidepressant effects in this model (Marcilhac et al., 1999). Moreover, an increase in CRF concentration has been observed in the brain of OBX rats (Bissette, 2001), and we have previously found that CRF1 receptor antagonists exhibit antidepressant effects in the OBX model (Okuyama et al., 1999). These observations point to hyperactivity of the brain CRF system, and indicate that the subsequent hyperactivation of the HPA axis might be involved in the behavioral changes seen in OBX rats. Given that MC4 receptor has been reported to regulate activity of the HPA axis through release of CRF in the PVN of the hypothalamus (Sarkar et al., 2002; Lu et al., 2003) and that the selective MC4 receptor antagonist MCL0129 exhibited antidepressant effects in animal models of depression such as the forced swimming test and learned helplessness test (Chaki et al., 2003a), it is conceivable that MC4 receptor antagonist activity might be involved, at least in part, in the antidepressant effects of MCL0042 in the OBX model. However, this hypothesis has yet to be proven using selective MC4 receptor antagonists.

MCL0042 did not affect spontaneous locomotor activity in rats at pharmacologically effective doses. Thus, pharmacological effects of MCL0042 in animal models of anxiety and depression may not be ascribed to altered locomotor activity.

In conclusion, we serendipitously obtained MCL0042, which displays the unique activity of both MC4 receptor antagonism and serotonin transport inhibition, and we found that MCL0042 exhibited anxiolytic and antidepressant effects in animal models. With our previous findings, the present study supports the presumption that blockade of the MC4 receptor may produce anxiolytic-like activity and may be beneficial in treating subjects with anxiety disorders. Considering that SSRIs are widely and successfully prescribed in clinical settings for the treatment of both depressive and anxiety disorders, compounds displaying both MC4 receptor antagonism and serotonin reuptake inhibition should prove useful in the treatment of these disorders.

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