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Effect of chronic administration of a CRF_1 receptor antagonist, CRA1000, on locomotor activity and endocrine responses to stress

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Abstract

Corticotropin-releasing factor (CRF) is involved in the regulation of stress responses. The actions of CRF in the brain are mediated through two distinct CRF receptor subtypes, CRF_1 and CRF_2 receptors. In the present study, we examined the effects in rat of chronic administration of a nonpeptidic CRF_1 receptor-selective antagonist, CRA1000, 2-[*N*-(2-methylthio-4-isopropylphenyl)-*N*-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine), on locomotor activity, feeding behavior and the hypothalamic–pituitary–adrenal axis. Chronic CRA1000 treatment significantly decreased locomotor activity in the dark phase of the diurnal cycle. However, chronic CRA1000 treatment showed no effect on food and water intake, or on body weight. After a 10-day period of CRA1000 treatment, plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone in basal conditions and under immobilization stress were no different from those in rats treated with vehicle. However, CRA1000 administered 2 h before immobilization stress significantly reduced ACTH and corticosterone responses to stress with no effect on basal ACTH and corticosterone concentrations. These results suggest that CRF_1 receptors are involved in the regulation of locomotor activity during the dark period, but are not involved in the regulation of feeding behavior under non-stressful conditions. Furthermore, the results suggest that a 10-day treatment with CRA1000 does not affect hypothalamic–pituitary–adrenal axis activity either under basal conditions or after acute stress.

Keywords: CRF receptor; CRA1000; Immobilization stress; ACTH (adrenocorticotropic hormone); Corticosterone

1. Introduction

Corticotropin-releasing factor (CRF) is a hypothalamic neuropeptide that regulates the hypothalamic–pituitary–adrenal axis, the autonomic nervous system, the immune system and behavior. In addition to being present in the hypothalamic paraventricular nucleus, CRF neurons and their receptors are also widely distributed in extrahypothalamic regions in the mammalian brain. Two CRF receptor subtypes, CRF_1 and CRF_2 receptors, have been identified (Chen et al., 1993; Lovenberg et al., 1995). CRF_1 receptors are widely distributed in the brain, including the cerebral cortex, cerebellum, amygdala and olfactory bulb. CRF_2 receptors, on the other hand, are mainly expressed in the lateral septum and various hypothalamic nuclei. Their heterogeneous distribution in the central nervous system suggests that these receptors mediate different actions of CRF and its family of peptides (Chalmers et al., 1995). CRF is thought to play an important role in the regulation of locomotor activity and feeding behavior in stress (Dunn and Berridge, 1990). Central administration of CRF increases motor activity in a familiar environment, and inhibits food intake (Sutton et al., 1982), and these CRF-induced changes are reversed by non-selective CRF receptor antagonists (Britton et al., 1986; Krahn et al., 1986; Menzaghi et al., 1994).

Nonpeptidic CRF_1 receptor-selective antagonists have recently been developed to investigate the roles of CRF receptor subtypes. Anxiety-related behavior is reduced by administration of CP-154,526 (butyl-ethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]amine) in rats in the elevated plus maze (Lundkvist et al., 1996), and in mice in a light–dark choice test (Griebel et al., 1998). The induction and expression of conditioned fear is

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attenuated by treatment with antalarmin (*N*-butyl-*N*-ethyl-(2,5,6-trimethyl)-7-[2,4,6-trimethylphenyl]-7H-pyrrolo[2,3-d]pyrimidine-4-yl-amine) (Deak et al., 1999). CRA1000 (2-[*N*-(2-methylthio-4-isopropylphenyl)-*N*-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl]-6-methylpyrimidine) reverses the swim stress-induced reduction of time spent in the light area in a light–dark exploration task (Okuyama et al., 1999), and the inhibition of food intake induced by emotional stress in a communication box (Hotta et al., 1999). Studies using these CRF_1 receptor antagonists suggest that CRF_1 receptors mediate anxiety-related behavior.

We now administered CRA1000 systematically twice a day, and measured locomotor activity, food and water intake and body weight every day to assess the physiological roles of CRF_1 receptors in mediating the functions of endogenous CRF family members under non-stress conditions. After these measurements, plasma adrenocorticotropic hormone (ACTH) and corticosterone responses to restraint stress were examined with and without administration of CRA1000 2 h prior to restraint stress to assess the effect of chronic administration of the CRF_1 receptor antagonist on ACTH and corticosterone secretion.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 170–210 g, were housed in individual cages [30 (L) × 30 (W) × 38 (H) cm] placed within a frame equipped with infrared beams. Throughout the experiment the animal room conditions were kept at a constant temperature (23 °C) with a fixed schedule of illumination (08:00–20:00). Rats were allowed ad libitum access to pellets and fresh water.

All experimental procedures were conducted in accordance with the guidelines on the use and care of laboratory animals approved by the Animal Care Committee in Nippon Medical School.

2.2. Drug treatment

A selective nonpeptidic CRF type 1 receptor antagonist, CRA1000, was supplied by Taisho Pharmaceutical CRA1000 was dissolved in 0.3% polyoxyethylene sorbitan monooleate (Wako, Osaka, Japan) (5 mg/ml) just before injection. The animals were weighed at 9:30 everyday, and 10 mg/kg CRA1000 or vehicle was given intraperitoneally (i.p.) at 10:00 and 17:00 daily for 9 days. Half the rats received another i.p. injection of CRA1000 or vehicle at 10:00 on the 10th day. The others received i.p. injection of CRA1000 or vehicle at 10:00 and 17:00 on the 10th day and at 10:00 on the 11th day. The last dose of CRA1000 was 26 h prior to immobilization stress in the former group of rats and was 2 h prior to stress in the latter group of rats.

2.3. Behavioral measurements

An automatic behavioral measurement system (PAW-2000, MELQEST Toyama, Japan) was used to measure locomotor activity, food and water intake from day 0 to day 7. Locomotor activity was counted whenever a rat crossed a 5 × 5 array of infrared beams. Food intake was assessed with an automatic pellet feeder to count the number of pellets dispensed, and water intake was assessed with a counting device to measure the number of water drops taken. These data were collected and recorded in a computer with Dataquest A.R.T. software (Data Science, St. Paul, MN).

2.4. Jugular vein cannulation

After the behavioral measurement, rats were anesthetized with pentobarbital (50 mg/kg, i.p., Dainabot, Osaka, Japan). The right jugular vein was exposed, and a silicone cannula filled with heparinized saline was inserted into the vein. The tip of the cannula was placed in the entrance of the right atrium. The free end of the cannula was tunneled subcutaneously and externalized on the back of the rat. There was no tether system attached to the cannula and the rat's movement was not restricted until the day before blood sampling. During the recovery period, the cannulae were flushed with heparinized saline.

2.5. Blood sampling and hormone measurements

After the recovery period, the free end of the cannula was connected to an extension tube protected by a concentric spring, which was anchored to the dorsal skin using a snap hook. The rats were then left alone in their cages for at least 2 h prior to blood sampling. Blood samples were taken from the unrestrained rats under resting conditions. Just after the first sampling (time point 0), each rat was moved to another room and was exposed to restraint stress, whereby the whole body of the rat was wrapped in flexible wire mesh, and immobilized in the prone position for 30 min. At 15 and 60 min after the start of the restraint stress, blood samples were taken. Blood samples were centrifuged immediately after sampling, and the supernatant was collected in tubes containing EDTA and stored at −20 °C until assay of ACTH and corticosterone levels. Plasma ACTH was measured using a commercially available immunoradiometric assay kit (Yuka Medias Ibaraki) and the corticosterone level was measured with a radioimmunoassay kit (Amersham LIFE SCIENCE, Tokyo).

2.6. Statistical analysis

Results are expressed as the means ± S.E.M. Locomotor activity, food and water intake, body weight, and the concentrations of plasma ACTH and corticosterone were analyzed by a two-way repeated measures analysis of

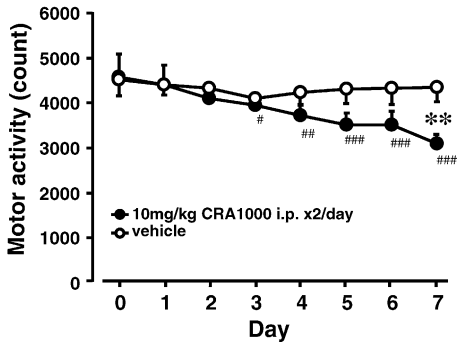

Fig. 1. Changes in mean locomotor activity in the home cage of rats given CRA1000 ($n=9$) or vehicle ($n=11$). Error bars indicate the S.E.M. #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs. day 0. **$P<0.01$ between CRA1000- and vehicle-treated rats on day 7.

variance with CRA1000 treatment as the between-subjects variable and time or day as the within-subjects variable. Significance of group effect was analyzed with Fisher's protected least-significant difference (PLSD) test and simple main effect was analyzed when the interaction was significant.

3. Results

3.1. Effects of chronic administration of CRA1000 on locomotor activity, food and water intake and body weight

Fig. 1 shows the effect of chronic administration of CRA1000 (10 mg/kg, twice a day) on locomotor activity. There was a significant drug treatment × day interaction [$F(7,126)=2.74$, $P<0.05$], suggesting that rats treated chronically with CRA1000 had a significantly decreased locomotor activity compared with that of vehicle-treated rats. Subsequent analysis of simple main effects revealed

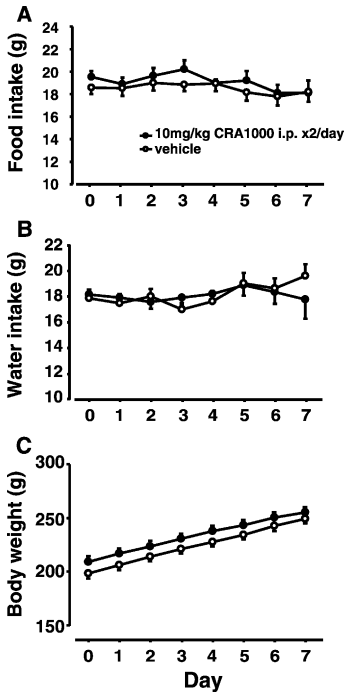

Fig. 3. Changes in mean food intake (A), water intake (B) and body weight (C) of rats given CRA1000 ($n=9$) or vehicle ($n=11$). Error bars indicate the S.E.M. Food and water intake at day 0 is the amount of food and water taken during the 24 h prior to the first drug administration. Body weight on day 0 was measured after the first 24 h measurement of food and water intake and locomotor activity. The first administration of drug was done after the measurement of body weight on day 0.

that locomotor activity of CRA1000-treated rats on day 7 decreased significantly compared with that of vehicle-treated rats ($P<0.01$), and that there was a significant change in locomotor activity of CRA1000-treated rats over the 8 days ($P<0.001$). Fisher's PLSD test revealed that the locomotor activity of CRA1000-treated rats after day 3

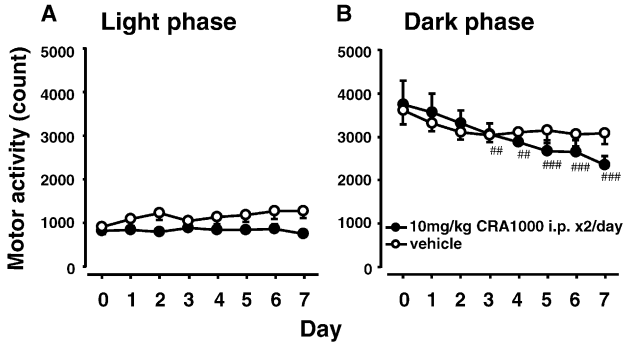

Fig. 2. Changes in mean locomotor activity during the light phase (A) and the dark phase (B) in the home cage of rats given CRA1000 ($n=9$) or vehicle ($n=11$). Error bars indicate the S.E.M. ##$P<0.01$, ###$P<0.001$ vs. day 0.

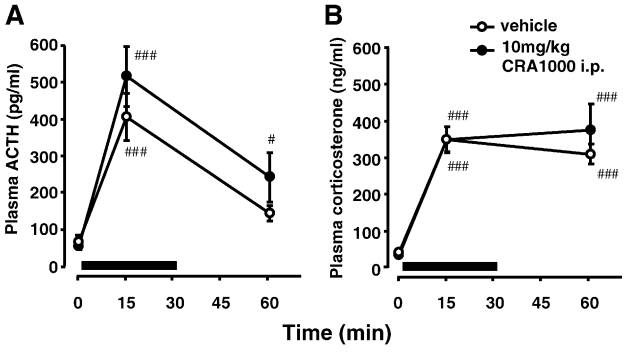

Fig. 4. Plasma ACTH (A) and corticosterone (B) responses to restraint stress in rats that received chronically CRA1000 ($n=3$) or vehicle ($n=7$) until 26 h prior to restraint stress. Error bars indicate the S.E.M. The horizontal bar from 0 to 30 min indicates restraint stress. #$P<0.05$, ###$P<0.001$ vs. time 0.

was significantly reduced compared with that on day 0 ($P<0.05$).

Fig. 2 shows locomotor activity during the light phase (A) and the dark phase (B). There was a tendency for chronic treatment of CRA1000 to decrease locomotor activity during the light phase [$F(1,18)=3.29$, $P=0.87$]. However, there was no significant change in locomotor activity during the light phase in either group. On the other hand, locomotor activity during the dark phase gradually decreased in CRA1000-treated rats [$F(7,56)=7.14$, $P<0.01$], and Fisher's PLSD test revealed that locomotor activity during the dark phase after day 3 was significantly reduced compared with that on day 0 ($P<0.01$).

Fig. 3A and B shows the effect of chronic administration of CRA1000 on food or water intake, respectively. Chronic treatment with CRA1000 induced no significant changes in either food or water intake. Food and water intake during the light phase and the dark phase were not altered in either group. Body weight of both groups increased gradually during the experiment [$F(7,126)=682.30$, $P<0.0001$]. The main effect of drug treatment and the drug treatment × day interaction were not significant (Fig. 3C).

3.2. Effects of CRA1000 on ACTH and corticosterone responses to restraint stress

Basal and stress-induced plasma ACTH and corticosterone concentrations were not changed by chronic CRA1000 treatment 26 h after the last dose (Fig. 4). However, in rats receiving chronic CRA1000 treatment, with the last dose of CRA1000 given 2 h prior to stress exposure, restraint stress did not significantly elevate plasma ACTH at 15 and 60 min. The plasma ACTH concentration of CRA1000-treated rats at 15 min was significantly low compared with that of vehicle-treated rats ($P<0.05$, Fisher's PLSD test). On the other hand, restraint stress significantly elevated the corticosterone concentration at 15 min in both groups ($P<0.05$, Fisher's PLSD test). The plasma corticosterone concentration of CRA1000-treated rats was significantly reduced compared with that of vehicle-treated rats at 60 min ($P<0.05$, Fisher's PLSD test). Basal plasma ACTH and corticosterone concentrations were not different between the CRA1000-treated group and the vehicle-treated group (Fig. 5).

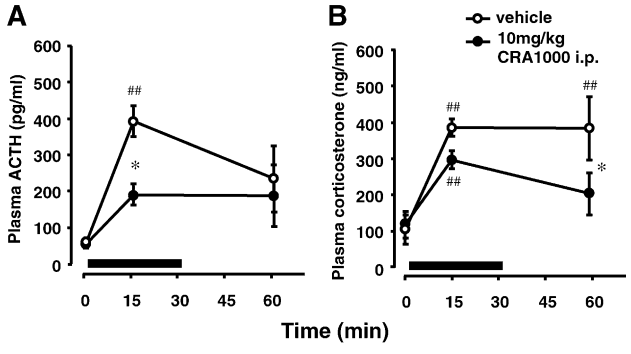

Fig. 5. Plasma ACTH (A) and corticosterone (B) responses to restraint stress in rats that received chronic administration of CRA1000 ($n=6$) or vehicle ($n=4$) until 2 h prior to restraint stress. Error bars indicate the S.E.M. The horizontal bar from 0 to 30 min indicates restraint stress. ##$P<0.01$ vs. time 0. *$P<0.05$ between CRA1000- and vehicle-treated rats.

4. Discussion

Using CRA1000, a nonpeptidic CRF_1 receptor-selective antagonist, we now examined the physiological significance of CRF_1 receptors in the locomotor activity and feeding behavior of rats. Chronic administration of CRA1000 affected neither food and water intake nor weight gain, while locomotor activity in the dark phase was significantly reduced by CRA1000. These results suggest that CRF_1 receptors are involved in the regulation of locomotor activity during the dark period, but are not involved in feeding behavior under non-stressful conditions.

Recently, several studies were performed to investigate the neuroendocrinological and behavioral functions of CRF receptor subtypes. Results from studies using antisense strategies and selective antagonists suggest that a prominent role of CRF_1 receptors is to mediate anxiety-related behavior, but not feeding behavior. Infusion of CRF_1 receptor antisense, but not of CRF_2 receptor antisense, produced an anxiolytic effect in rats in an elevated plus maze following social defeat (Liebsch et al., 1999) and in rats in a light–dark box (Heinrichs et al., 1997). Administration of NBI-27914 (2-methyl-4(*N*-propyl-*N*-cyclopropanemethylamino)-5-chloro-6-(2,4,6-trichloroanilino)pyrimidine), a nonpeptidic CRF_1 receptor antagonist, could not block a CRF-induced anorectic effect (Smagin et al., 1998; Pelleymounter et al., 2000). The function of CRF_1 receptors has also been studied using knockout strategies. Intracerebroventricular (i.c.v.) injection of CRF into CRF_1 receptor-deficient mice does not increase locomotor activity, while i.c.v. injection of CRF into these mutant and wild type mice decreases food and water intake equally (Contarino et al., 2000). The results from this gene manipulating study suggest that CRF_1 receptors play an important role in the CRF-induced increase in locomotor activity, and that the anorectic effect of the CRF peptide family is not mediated by CRF_1 receptors. Furthermore, i.c.v. administration of CRF binding-protein (CRF-BP) inhibitor, which competitively binds CRF-BP but not CRF_1 receptors, increases locomotor activity and facilitates fear conditioning without producing anxiogenic-like behavior (Heinrichs and Joppa, 2001). The authors conclude that the increase in locomotor activity induced by CRF can be attributed to modulation of arousal, and that endogenous brain CRF is involved in arousal-like activation. We have previously reported that a CRF_1 receptor-selective antagonist reverses the restraint stress-induced shortening of pentobarbital-induced sleeping time of rats, suggesting that CRF_1 receptors are involved in the regulation of arousal (Arai et

al., 1998). In the present experiments, chronic administration of the CRF_1 receptor-selective antagonist, CRA1000, decreased locomotor activity. This result could be interpreted as a decrease in arousal-like activation. We should note that acute administration of CRA1000 has been reported to show no effect on locomotor activity at doses up to 100 mg/kg, per os (Okuyama et al., 1999). Thus, the inhibitory effect of CRA1000 on locomotor activity involves a mechanism other than CRF_1 receptor blockade. For example, chronic administration of CP-154,526 dose dependently decreases CRF mRNA expression in the hypothalamic paraventricular nucleus and the Barrington nucleus (Arborelius et al., 2000). A decrease in CRF synthesis in these nuclei might reduce locomotor activity.

Recently, it has been suggested that CRF_1 receptors, as well as CRF_2 receptors, mediate the feeding suppression induced by the CRF family (Bradbury et al., 2000; Coste et al., 2000; Reyes et al., 2001). However, it has been reported that food intake is not altered by CRF_1 receptor antagonists alone, NBI 27914 (Smagin et al., 1998) and CRA1000 (Hotta et al., 1999). In CRF_1 receptor-deficient mice, the basal daily food intake is similar to that of the wild type (Bradbury et al., 2000, Muller et al., 2000; Contarino et al., 2000). In our experiment, chronic administration of the CRF_1 receptor antagonist, CRA1000, failed to affect daily food intake and the circadian pattern of feeding behavior. Therefore, it is unlikely that CRF_1 receptors play a major role in regulating food intake under non-stressful conditions. We previously reported that infusion of anti-rat urocortin γ-globulin into the ventromedial hypothalamic nucleus increased food intake in freely fed rats (Ohata et al., 2000). Chronic infusion of antisauvagine-30 itself increases the basal daily food intake (Cullen et al., 2001). Together, these results suggest that CRF_2 receptors play an important role in modulating food intake under basal conditions.

Body weight of rats that receive a continuous i.c.v. infusion of CRF are significantly lower than those of pair-fed rats, suggesting that CRF-induced inhibition of weight gain cannot be completely explained by suppression of food intake (Hotta et al., 1991; Cullen et al., 2001). I.c.v. injection of CRF stimulates the activity of sympathetic nerve endings in brown adipose tissue (Egawa et al., 1990). These results indicate that CRF increases energy expenditure, and that blockade of CRF_1 receptors would be expected to attenuate energy expenditure and increase body weight gain. However, neither the results from our experiments nor those obtained by others using CRF_1 receptor antagonists support this notion. For example, chronic administration of antalarmin, a nonpeptidic CRF_1 receptor antagonist, to rats is reported to have no effect on body weight (Bornstein et al., 1998). Body weight of CRF_1 receptor-deficient mice is no different from, or even lower than, that of normal control mice (Bradbury et al., 2000; Muller et al., 2000; Cullen et al., 2001). Therefore, it is suggested that CRF_1 receptors are not involved in the energy expenditure mechanism of rats under non-stressful conditions.

Chronic administration of a CRF_1 receptor-selective antagonist for 10 days affected neither basal concentrations of ACTH or corticosterone nor immobilization stress-induced ACTH or corticosterone secretion. Similar results have been reported for immobilization stress after a 1-week period of i.p. injection of antalarmin (20 mg/kg) twice a day (Wong et al., 1999), and for an airpuff startle after a 14-day period of CP-156,526 administration (Arborelius et al., 2000). These results and ours suggest that a hypothalamic–pituitary–adrenal response to stress would not be hindered by repeated administration of CRF_1 receptor antagonists at a dose effective for suppression of the hypothalamic–pituitary–adrenal response to stress after acute treatment. Furthermore, our results show that additional administration of CRF_1 receptor antagonist 2 h before the exposure to stress blunted plasma ACTH and corticosterone responses to stress, suggesting that CRA1000 is effective to inhibit stress-induced increases in plasma ACTH and corticosterone levels even after it is administered repeatedly.

In conclusion, the results of the present study indicate that chronic CRA1000 treatment significantly decreases locomotor activity in the dark phase, but has no effect on food and water intake or body weight, suggesting that CRF_1 receptors are involved in regulating locomotor activity. A 10-day period of CRA1000 treatment did not induce any significant change in the basal and stress-induced activity of the hypothalamic–pituitary–adrenal axis.

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