

Microinjection of Different Doses of Corticotropin-releasing Factor into the Medial Prefrontal Cortex Produces Effects Opposing Anxiety-related Behavior in Rats

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Abstract

Corticotropin-releasing factor (CRF) in the medial prefrontal cortex (mPFC) is suggested to play an important role in mediating fear, anxiety, and depression. The results of the studies of the actions of CRF in the mPFC regarding anxiety-related behavior, however, seem contradictory. In one study, microinjection of CRF into the mPFC produced an increase in anxiety-related behavior on the elevated plus maze, whereas in another study CRF produced an anxiolytic-like effect. To test whether the different doses of CRF used in these experiments are responsible for the differing results, we examined the dose-dependent effects of CRF (0.015, 0.05, 0.15, 0.5, and 1.0 $\mu\text{g}/0.5 \mu\text{L}/\text{site}$) microinjected into the bilateral mPFC of male Wistar rats on anxiety-related behavior in the elevated plus maze. We found that microinjection of 0.05 μg CRF significantly decreased the number of open-arm entries, whereas 1.0 μg CRF significantly increased the time spent on the open arms. The results indicate that CRF has effects opposing anxiety-related behavior in the elevated plus maze: anxiety-related behavior at a lower dose and an anxiolytic-like effect at a higher dose.

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Key words: corticotropin-releasing factor, anxiety, medial prefrontal cortex

Introduction

Corticotropin-releasing factor (CRF) is an important neuropeptide in mediating the behavioral and physiological responses to stress¹. Central administration of CRF induces various responses similar to those observed in stressed animals. The peptide exerts its biological actions through 2 types of receptors, CRF₁ and CRF₂, which have different physiological properties and expression patterns^{2–5}.

The CRF receptors are expressed in the medial prefrontal cortex (mPFC), lateral septum, specific amygdala nuclei, and the hippocampus⁶, and these regions are thought to comprise the limbic stress circuits⁷. Immunohistochemical studies have revealed that CRF is present in the mPFC^{8,9} and that CRF is expressed in glutamate decarboxylase-positive interneurons in the rat cerebral cortex¹⁰. Studies using *in situ* hybridization have shown that CRF₁ receptors are expressed at high levels in the mPFC^{3,11}.

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Human and primate studies suggest that the CRF system in the mPFC plays an important role in mediating fear, anxiety, and depression¹²⁻¹⁴. An electrophysiological study has shown that preincubation of PFC tissue from normal rats with CRF or pre-exposure of rats to stressors alters 5-hydroxytryptamine (5-HT) regulation of gamma aminobutyric acid (GABA) transmission on pyramidal neurons in the PFC¹⁵. These results suggest that CRF alters the actions of 5-HT in mediating stress responses. A recent study of the action of CRF on anxiety-related behavior in the mPFC has found that microinjection of CRF (20 ng) into the mPFC of stressed rats increases anxiety-related behavior in the elevated plus maze¹⁶. On the other hand, another study has shown that microinjection of CRF (200 ng) into the frontal cortex produces anxiolytic-like behavior in unstressed rats¹⁷. A plausible explanation for the discrepancy between these studies is a difference in the CRF doses used. The issue of the CRF doses has often been examined in functional studies of CRF in the brain¹. More detailed investigations are needed to clarify the action of CRF in the mPFC.

In the present study, to clarify the dose-dependent effects of CRF in the mPFC on anxiety-related behavior, we performed behavioral tests with the elevated plus maze after microinjecting different doses of CRF into the mPFC of rats.

Materials and Methods

Subjects

All experimental procedures were approved by the Animal Care and Use Committee of Nippon Medical School (Approval H21-112, H22-098). Male Wistar rats were obtained from Japan Laboratory Animal, Inc (Tokyo, Japan). The body weights at the time of surgery were 250 to 310 g. Animals were housed individually in cages (30 × 20 × 18 cm) under standard laboratory conditions and maintained in a 12-hour light/dark cycle (lights were on from 8 : 00 a.m. to 8 : 00 p.m.) and allowed *ad libitum* access to food pellets and water.

Surgical Procedures

Rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg; Dainippon Sumitomo Pharma, Osaka) and placed in a stereotaxic apparatus (Narishige, Tokyo). A stainless steel guide cannula (26 gauge) was stereotaxically implanted 1 mm above the mPFC bilaterally and secured to the skull using 3 screws and acrylic dental cement. Stereotaxic coordinates, as determined with an atlas of the rat brain¹⁸, were AP=+2.2 mm from bregma; L=±0.5 mm from medial suture; and V=-3.0 from dura. Dummy stylets were inserted into the guide cannula. After surgery, rats were individually housed and allowed to recover for 5 to 7 days.

Infusion Procedures

Ovine CRF (Peptide Institute, Inc., Osaka) was dissolved in sterile saline just before the experiment. After stylets were removed, a sample-filled stainless-steel injection needle (outer diameter, 200 μm) was inserted into the guide cannula so that the injector tip protruded 1 mm beyond the cannula tip. Solutions of ovine CRF (0.015, 0.05, 0.15, 0.5, and 1.0 μg/0.5 μL/site) or sterile saline as a control was bilaterally microinjected through the injection needle, which was connected through polyethylene tubing to a 10-μL microsyringe (701NSR, Hamilton, Reno, NV, USA) mounted on a syringe pump (Nihon Kohden, Tokyo). The solution was delivered at a flow rate of 0.1 μL/minute for 5 minute. During the injection procedure, all rats were permitted to behave freely in the gray box (13 × 25 × 35 cm). After the injection, the injectors were kept in the guide cannula for 1 minute before being removed.

The Elevated Plus Maze Test

Immediately after the injection, rats were moved to the experiment room and kept there for 10 to 15 minute to calm down. Then rats were tested on the plus maze elevated 63 cm above the floor. The plus maze consisted of 4 arms (2 open arms without walls and 2 arms enclosed by 40-cm-long black walls) 50 cm long and 11 cm wide, arranged so that arms of the same type were opposite each other, connected by an open central area (11 × 11 cm). To prevent

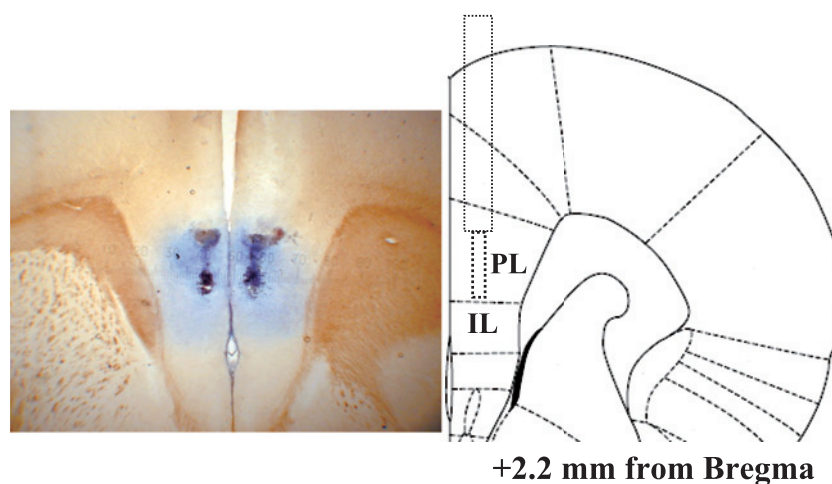


Fig. 1 A representative photomicrograph of a rat coronal section showing the placement of injector tips into the mPFC. Blue dye, 0.5 μ L/site, was injected immediately after experimentation, and the positions of injector tips were verified to be within the prelimbic cortex (PrL) or infralimbic are (IL).

rats from falling off, both sides of the open arms were equipped with rims of white plastic (0.5 cm high). The open arms and enclosed arms were illuminated with 2 small lights fixed on the ceiling at approximately 22 lux and 5 lux, respectively. At the beginning of each session, rats were placed in the center of the maze facing an open arm and allowed to explore the maze for 5 minute. The animals' behavior was recorded with a camera placed above the maze and connected to a computer. After each trial, the maze was thoroughly cleaned with 70% ethanol. Using TopScan software (CleverSys, Inc. Reston, VA, USA), the following data were collected: time spent and number of entries in the open or enclosed arms. An entry was defined only when both the center of the animal's body and the base of the tail entered a designated arm.

Cannula Location

At the end of the experiment, all rats were deeply anesthetized with sodium pentobarbital and received microinjections of 0.5 μ L of Brilliant Blue dye (Tokyo Kasei, Tokyo) through the injector in the same way as in the experiment. Rat's brains were then perfused, stored in 10% formalin, and cut into coronal sections. The location of the injector tip was examined with an atlas of the rat brain¹⁸.

Statistical Analysis

All data are expressed as means \pm SEM. Separate one-way analyses of variance (ANOVAs) were performed to analyze data. Fisher's protected least-significant difference (PLSD) post-hoc tests were performed for significant treatment effects relative to control means. The alpha level for statistical significance was $p < 0.05$. Trends towards significance were noted when $p < 0.10$.

Results

The site of microinjection was considered correct if the tip of the injector had been placed in the mPFC (**Fig. 1**). The area of blue dye diffusion included the prelimbic subregion and infralimbic subregion. The injection site was correct in 72 of the 82 rats, and data from these rats were subjected to statistical analysis.

One-way ANOVA indicated that microinjections of CRF into the bilateral mPFC had significant effects on the percentage of time spent in the open arms [$F(5,66)=3.20$; $p < 0.05$] (**Fig. 2A**) and the enclosed arms [$F(5,66)=2.79$; $p < 0.05$] of the maze (**Fig. 2B**). Comparisons with vehicle-treated rats revealed that microinjection of 0.05 μ g CRF into the bilateral mPFC had a tendency to decrease the time spent in the open arms and that microinjection of 1.0 μ g CRF

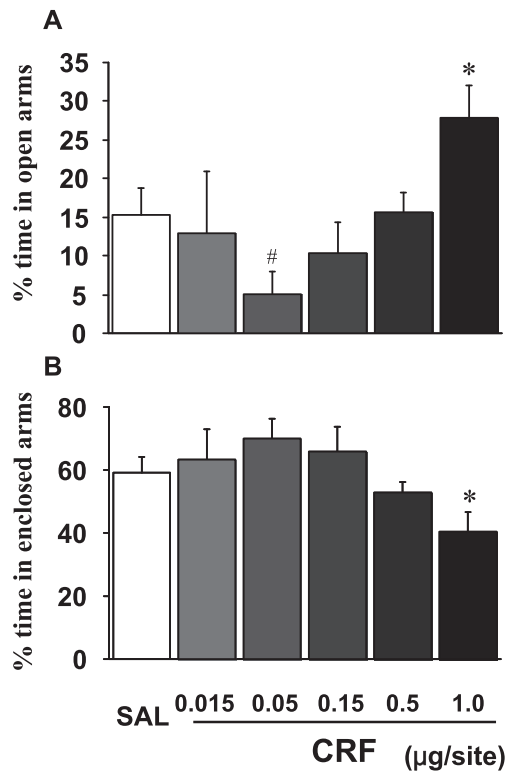


Fig. 2 Effects of microinjection of CRF into the bilateral mPFC on the percentage of time spent in the open and enclosed arms of the elevated plus maze. Each value represents the mean \pm SEM. *Significantly different from vehicle-treated controls ($p < 0.05$). #Trends towards significance from vehicle-treated controls ($p < 0.10$). SAL, saline-injected group.

significantly increased the time spent in the open arms ($p < 0.05$) (Fig. 2A) and significantly decreased the time spent in the enclosed arms ($p < 0.05$) (Fig. 2B).

One-way ANOVA showed that microinjections of CRF into the bilateral mPFC had a significant effect on the number of open-arm entries [$F(5,66) = 2.63$; $p < 0.05$]. Comparisons with vehicle-treated rats revealed that microinjection of 0.05 µg CRF significantly decreased the number of open-arm entries ($p < 0.05$), whereas microinjection of 1.0 µg CRF showed a tendency to increase the number of open-arm entries (Fig. 3A). There was no significant difference in the number of either enclosed-arms entries or total entries (Fig. 3B, C).

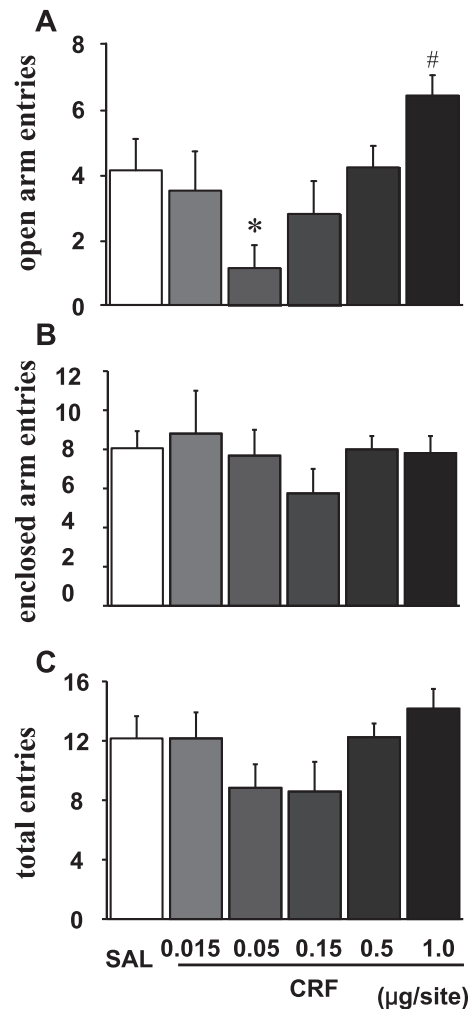


Fig. 3 Effects of microinjection of CRF into the bilateral mPFC on the number of entries into the open and enclosed arms of the elevated plus-maze and their total numbers. Each value represents the mean \pm SEM. *Significantly different from saline-treated controls ($p < 0.05$). #Trends towards significance from vehicle-treated controls ($p < 0.10$). SAL, saline-injected group.

Discussion

The elevated plus maze is a popular behavioral test for assessing the anxiety responses of rodents and to investigate both anxiolytic and anxiogenic effects of drugs under identical conditions¹⁹. In the present study, microinjection of CRF into the mPFC dose-dependently decreased and then increased the number of entries or the percentage of time spent in the open arm of the elevated plus maze without changing the total number of entries, which is an

index of general motor activity²⁰. The results suggest that microinjection of 0.05 µg CRF into the bilateral mPFC increases anxiety, whereas microinjection of 1.0 µg CRF decreases anxiety.

Previous studies showed that intracerebroventricular injection of CRF or of cortagine, a selective agonist of the CRF₁ receptor, significantly decreased general motor activity of rodents and the time spent in the open arm of the elevated plus maze^{21,22}. However, in the present study, microinjection of CRF into the mPFC did not change motor activity, as indicated by the total number of entries, suggesting that the effects of microinjecting CRF into the mPFC are unlikely to be due to the diffusion of CRF into the lateral ventricle.

The present study's finding that injection of low-dose CRF (0.05 µg) into the mPFC increases anxiety-related behavior is consistent with a previous report that the microinjection of CRF (20 ng) into the mPFC of stressed rats increases anxiety-related behavior in the elevated plus maze¹⁶. Jaferi and Bhatnagar¹⁶ also found that rats receiving a higher dose of CRF (200 ng) show no substantial difference in the time spent in open arms or in the number of open-arm entries. Their observations were replicated in our experiment, although we performed the elevated plus maze test with unstressed rats. Another study has found that microinjection of CRF (0.2 µg/site) into the frontal cortex produces anxiolytic-like effects in unstressed rats¹⁷. Our study has also found anxiolytic-like effects of CRF at a higher dose, although we used a higher dose and our injection site was deeper than that of Zieba et al¹⁷. In their study, lower doses of CRF (0.1 and 0.05 µg) had no substantial effects on anxiety-like behavior in the elevated plus maze. Interestingly, their behavioral assessments in the open field test revealed that CRF at a lower dose (0.05 µg) significantly decrease locomotor activity. Although they assessed neither time spent in the central part nor the latency to enter the central part of the open field, the decrease in locomotor activity might be interpreted as an increase in anxiety²³.

Dunn and Berridge¹ have reported that the behavioral effects of CRF at high doses differ from

those at low doses. At higher doses CRF decrease feeding and locomotor activity in a novel environment but at lower doses increases feeding and locomotor activity^{24,25}. The effects on neuronal activity of local infusion of CRF into the raphe nucleus reportedly differ between high doses and low doses^{26,27}. A recent electrophysiological study has revealed that CRF at low doses activates CRF₁ receptors, thereby enhancing GABA activity, which results in the inhibition of 5-HT release, and that CRF at higher doses acts not only on CRF₁ but also on CRF₂ receptors of 5-HT neurons, resulting in the facilitation of 5-HT release²⁸. The finding of the local neuronal mechanism underlying the opposing effect of CRF in the raphe nucleus suggests the presence of an intrinsic system in the mPFC in which CRF has effects opposing anxiety-related behavior.

In the neocortex, CRF is found in GABA interneurons in layers II and III^{10,29}. Such GABA interneurons innervate the pyramidal neurons, in which CRF₁ receptors are expressed. Bath application of CRF to slice preparations of cerebral cortex increases the discharge of action potentials of the pyramidal neurons in the presence of blockers for glutamate and GABA transmission, and the activation of the pyramidal neurons is prevented by the selective CRF₁ antagonist NBI27914²⁹. This result indicates that the action of CRF on pyramidal neurons is excitatory. On the other hand, CRF has also been shown to enhance the inhibitory action of GABA induced by 5-HT on the pyramidal neurons¹⁰. In GABA interneurons of the mPFC, 5-HT_{2A} receptors are expressed³⁰. A recent study has shown that 5-HT dose-dependently elicits a 5-HT_{2A}-mediated increase in the excitability of GABA interneurons³¹. CRF cosynthesized within GABA neurons reportedly enhances GABAergic inhibition of target neurons through CRF receptors in the central nucleus of the amygdala³², the bed nucleus of the stria terminalis³³, and the dorsal raphe²⁸. Therefore, CRF in the mPFC might show a similar effect on the GABAergic inhibition of pyramidal neurons. In the present study, CRF at a dose much higher than that released by 5-HT in the physiological range might enhance GABAergic inhibition on pyramidal neurons. Because enhancement of GABAergic

inhibition by infusion of the benzodiazepine midazolam³⁴ or muscimol³⁵ into the mPFC produces anxiolytic-like effects on the elevated plus maze, CRF at a high dose might induce anxiolytic-like effects through the enhancement of GABAergic inhibition.

In pyramidal neurons of the mPFC, 5-HT_{1A} and 5-HT_{2A} receptors are expressed at high levels, and the electrical stimulation of the raphe nucleus elicits 5-HT_{1A}-mediated inhibition and 5-HT_{2A}-mediated excitation of pyramidal neurons in the mPFC³⁶. Despite of the opposite effect of 5-HT receptors, 5-HT dose-dependently decreases the firing rate of pyramidal neurons³¹. These results suggest that 5-HT_{1A}-mediated inhibition is the predominant effect of 5-HT in pyramidal neurons of the PFC. Recently, activation of CRF₁ receptor in the mPFC has been shown to enhance 5-HT₂ receptor signaling by recruiting 5-HT₂ receptors to the cell surface, and infusion of CRF with an 5-HT agonist into the mPFC has been shown to increase anxiety-related behavior in the open field test and in the elevated plus maze test³⁷. These results indicate that CRF increases 5-HT_{2A}-mediated excitation of pyramidal neurons in the mPFC. To our knowledge, the involvement of CRF in the control of 5-HT_{1A} receptors, which are believed to play important roles in anxiety and depression³⁸, has never been investigated. Furthermore, a new interaction between CRF and dopamine on glutamate transmission in the mPFC has been reported³⁹. Further studies are needed to elucidate the actions of CRF in the mPFC.

We examined the dose-dependent effects of CRF microinjected into the bilateral mPFC on anxiety-related behavior in the elevated plus maze. The results show that CRF has effects opposing anxiety-related behavior in the elevated plus maze: anxiety-related behavior at a lower dose and an anxiolytic-like effect at a higher dose.

Conflict of interest: The authors declare no conflict of interest.

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