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SHORT COMMUNICATION

INVOLVEMENT OF CRF BUT NOT NPY IN THE ANXIETY REGULATION *VIA* NMDA RECEPTORS

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The study attemps to evaluate whether neuropeptide Y (NPY) and corticotropin-releasing factor (CRF) are involved in anxiogenic and anxiolytic reactions induced by NMDA receptor ligands.

The animals were given MK-801 (1 mg/kg, *ip*), a non-competitive NMDA-receptor antagonist, which acts as anxiolytic agent, or NMDA (15 mg/kg, *ip*), which has an anxiogenic effect. The anxiogenic or anxiolytic actions of these compounds were evaluated in the plus-maze test. The animals, which were given MK-801, were administered BIBO 3304 (130 ng/0.5 μl/site) intraamygdalarly and the animals which were given NMDA were administered α-helical CRF (500 ng/0.5 μl/site). BIBO 3304 did not attenuate MK-801-induced anxiolysis and α-helical CRF abolished NMDA-induced anxiogenesis. Our results show that anxiogenic effect of NMDA is mediated *via* CRF1 receptors and anxiolytic action of MK-801 is not dependent on Y1 receptors.

Key words: *glutamate, anxiety, plus-maze test, neuropeptides*

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Abbreviations: NPY – *neuropeptide Y,* CRF – *corticotropin-releasing factor,* NMDA – *N-methyl-D-aspartic acid*

INTRODUCTION

Amygdala is the main structure which mediates all reactions connected with fear and anxiety [5, 6]. Several neuropeptides have been identified in the amygdala, including neuropeptide Y (NPY) and corticotropin-releasing factor (CRF) [2, 14]. NPY is a 41-amino acid peptide, which is present in a number of nerve cell bodies and terminals scattered over the whole nucleus [10]. CRF is a 36 amino acid peptide, and CRF-positive nerve cell bodies and terminals form a cluster in a central nucleus of the amygdala [24]. NPY and CRF have strong and opposite effect on anxiety-related behavior in rodents. NPY produces an anxiolytic effect in rats after intraventricular or intraamygdalar administration [11, 12], while CRF produces anxiogenic effect [1].

It has been shown in a several studies that NPY exerts anxiolytic effect in the amygdala through Y1 receptors, because the blockade of these receptors by specific antagonists abolished this effect of NPY [23]. On the other hand, blockade of CRF1 receptors attenuated the anxiogenic effect of CRF, which means that CRF1 receptors mediate anxiogenic action of CRF [13].

Glutamate is a major excitatory neurotransmitter in the central nervous system. Glutamate acts *via* ionotropic or metabotropic glutamate receptors, which are present on almost all nerve cell bodies. Ionotropic glutamate receptors are divided into three main classes: NMDA, AMPA and kainate [32]. Several lines of evidence indicate that NMDA receptor ligands modulate anxiety in rodents [3]. Antagonists (MK-801, memantine) and functional antagonists (ACPC) of these receptors have anxiolytic activity, while NMDA alone exerts anxiogenic action [21, 22]. In our previous studies, we have shown that NPY and CRF neurons may be regulated through glutamatergic system *via* NMDA receptors [28]. Several other studies performed in different laboratories have also shown the interaction between glutamate and NPY or CRF [15, 17, 27].

In the present studies, we planned to evaluate if the blockade of Y1 receptors by BIBO 3303, a selective Y1 receptor selective antagonist, influences anxiolytic action of MK-801, a non-competitive NMDA receptor antagonist, and if the blockade of CRF1 receptors by α-helical CRF, a selective CRF1 receptor antagonist, influences anxiogenic action of NMDA. We studied the effect of these ligands in the plus-maze test, a well known paradigm to evaluate anxiolytic action of drugs.

MATERIALS and METHODS

Rats

Male Wistar rats weighing 230–250 g were used. The animals were obtained from local breeding farm. The rats were age-matched, fed *ad libitum* and housed 6 to a cage under 12:12 light-dark cycle. The rats after cannulae implantation were housed singly. During the experiment, all efforts were made to minimize animal suffering and to reduce the number of animals used, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

MK-801 (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, a non-competitive NMDA receptor antagonist, was obtained from RBI, BIBO 3304 ((R)-N-[[4-(aminocarbonylaminomethyl)phenyl]methyl]-N2-(diphenylacetyl)-argininamide trifluoroacetate)3304, a Y1 receptor antagonist, was a gift from Dr. Mueller, Boehringer-Ingelheim (Biberach, Germany), NMDA and α-helical CRF were obtained from Sigma. MK-801 and NMDA were dissolved in 0.9% NaCl and pH was adjusted to 7.0, BIBO 3304 and α-helical CRF were dissolved in a distilled water.

Cannulae implantation and histology

The rats, anesthetized with equitesin, were immobilized in a Kopf stereotaxic instrument. The skin was cut and the skull was cleaned for bilateral implantation of guide cannulae made of 23-gauge stainless steel tubing, 2 mm above the sites of injection. The guide tubes, secured by the dental cement, were anchored to three stainless steel screws fixed to the skull. In order to prevent clogging, 30-gauge stainless steel stylets were placed in the guide cannulae and remained there until the animals were given intracerebral injections five days later. The rats were adapted to handling and on the test day the stylets were withdrawn and replaced by

bilateral injection units (30 gauge stainless steel tubing) terminating 2 mm below the tip of the guides.

The coordinates for injection sites were: A –2.56; L ± 5; H –8.6, measured from bregma according to Paxinos and Watson stereotaxic atlas (1986) with the aim to make injections to the basolateral nucleus of the amygdala.

On completion of each experiment, the rats were killed and their brains were removed, fixed in formalin for 24 h and quickly frozen on dry ice. To check the position of the cannulae tracks, the frozen brains were cut in the coronal plane in a Cryocut. Representative drawings of the histological sections, showing the injection sites are shown in Figure 1.

Plus-maze procedure

Plus-maze test was performed according to the method described by Pellow et al. [18]. Briefly, the plus-maze apparatus was made of wood and consisted of two open arms, 50 × 10 cm and two closed arms 50 × 10 × 40 cm. It was painted in black but wooden walls of the closed arms had their natural color, with an open roof. The apparatus was elevated 50 cm above the floor. Two open arms were opposite to each other and were illuminated by a 40 W bulb positioned 20 cm above each open arm.

Each rat was placed individually in a new cage which was similar to home cage for 5 min immediately before the test (this procedure results in an increase in the total number of arm entries during the test). Each rat was then placed in the center of plus-maze facing one of the open arms. During the five-minute test, the number of entries into open arms and time spent in the open arms was measured. The maze was cleaned with a paper towel after each trial. Experiments were performed between 9.00 and 12.00.

MK-801 was given at a dose of 0.1 mg/kg, *ip* 30 min before the test and BIBO 3304 was given bilaterally at a dose of 130 ng/0.5 μl/site, intraamygdalarly, 10 min before MK-801 administration. NMDA was given intraperitoneally at a dose of 15 mg/kg, 30 min before the test and α-helical CRF was given bilaterally intraamygdalarly at a dose of 500 ng/0.5 μl/site, 30 min before NMDA administration.

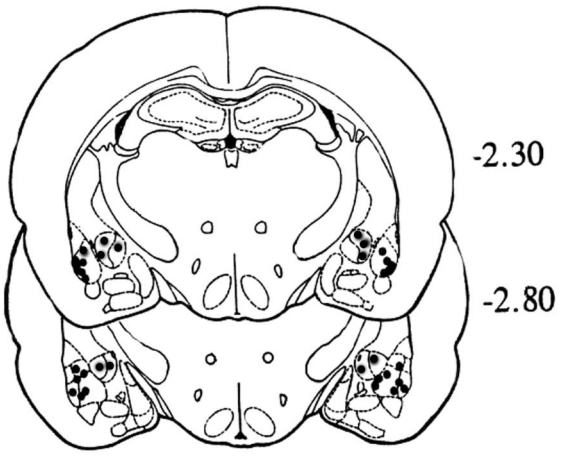

Fig. 1. Schematic drawings of coronal sections of the rat brain showing the localization of the cannula tips in rats in which injections were made into the basolateral/central amygdala. Only data from animals in which the histologically reconstructed sites of microinjections were localized in the indicated area were included in the results of each experiment

RESULTS

Effect of MK-801 and BIBO 3304 in the plus-maze test

The anxiolytic activity of MK-801 (0.1 mg/ml) in the plus-maze procedure was observed only as the increased time that animals spent in the open arms, while a number of the open arms entries re-

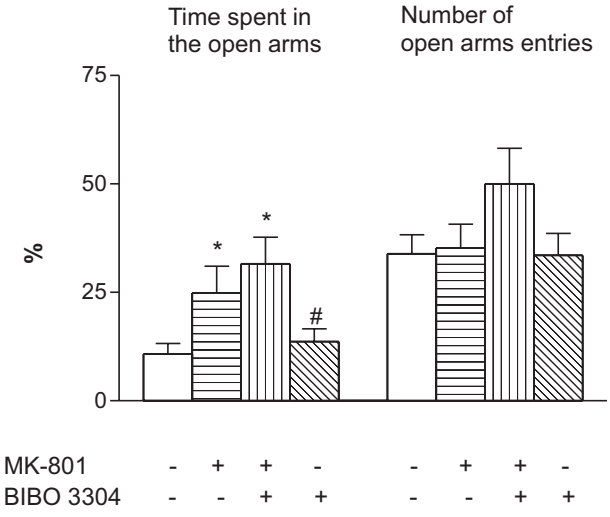

Fig. 2. The effect of BIBO 3304 (130 ng/0.5 μl/site) on the anxiolytic-like activity of MK-801 (0.1 mg/kg, *ip*) in the plus-maze test in rats. MK-801 was injected 30 min before the test and BIBO 3304 was given 10 min before MPEP administration. N = 6 rats per group, * $p < 0.05$ in relation to control group and $^{\#}$ $p < 0.05$ in relation to MK-801- and MK-801 + BIBO 3304-treated group

mained unchanged. BIBO 3304, when given 10 min prior to MK-801 administration, did not change the behavior of animals, and it had no effect on the behavior of animals when given alone (Fig. 2).

Effect of NMDA and α-helical CRF in the plus-maze test

The typical anxiogenic effect of NMDA (15 mg/kg) was demonstrated in the plus-maze test, as the animals spent less time in the open arms than the controls, and they made less open arms entries comparing to control animals.

α-Helical CRF did not influence the behavior of animals when given alone, but it completely reversed effect of NMDA when given prior to NMDA administration (Fig. 3).

Two-way ANOVA revealed that the effect of NMDA was statistically significant ($p < 0.001$). *Post-hoc* analysis performed using Turkey test showed $F(3,40) = 11.31$, $p < 0.001$ when NMDA was compared to vehicle (time spent in the open arms) and $F(3,49) = 6{,}45$ $p < 0.05$ (open arms entries). α-Helical CRF abolished the effect of NMDA, ($p < 0.001$) for time spent in the open arms and $p < 0.001$ for open arms entries.

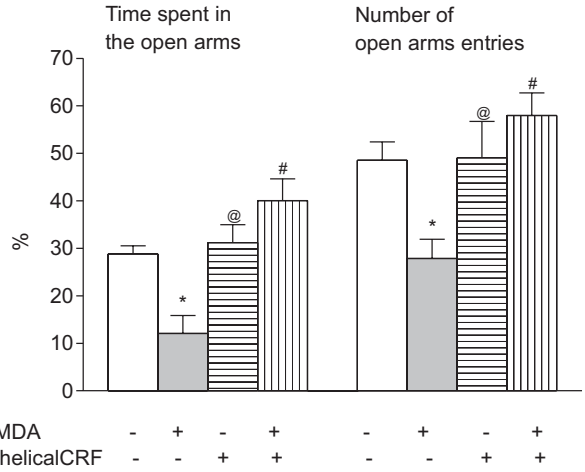

Fig. 3. The effect of α-helical CRF (500 ng/0.5 μl/site) on the anxiogenic-like activity of NMDA (50 mg/kg, *ip*) in the plus-maze test in rats. NMDA was injected 30 min before the test and α-helical CRF 30 min before NMDA administration. N = 6 rats per group, * at least $p < 0.05$ in relation to control group, @ and # at least $p < 0.05$ in relation to NMDA-treated group

DISCUSSION

NMDA receptor antagonists are recently widely tested in animal models detecting anti-anxiety activity of drugs. They all have clear anxiolytic-like activity in plus-maze test, Vogel's conflict test, open field test or social interaction test, and this activity is very similar to that of diazepam and chlordiazepoxide [19, 22, 31]. The most potent is the non-competitive NMDA receptor antagonist, MK-801, which is active at a dose range 0.01–1 mg/kg in the plus-maze test in mice [31]. Unfortunately, MK-801 causes serious side effects in humans, such as psychosis and locomotor disturbances, that is why it cannot be used as the therapeutic agent but only as a tool drug [25]. On the other hand, NMDA alone has anxiogenic effects in animal test [7, 30].

The aim of the present study was to check if the blockade of Y1 or CRF1 receptors in the amygdala influences the MK-801-induced anxiolytic or NMDA-induced anxiogenic behavior. Amygdala was chosen because this structure is responsible for the integration of behavioral and physiological manifestation of defensive reactions against fear [5, 6, 9].

Our behavioral studies showed that MK-801 administration caused an anxiolytic effect, which was similar to previous results obtained by Fraser et al. [8] and was evidenced by spending more time in the open arms of the maze. We did not observe an increase in open arms entries. The agonist, NMDA, was shown earlier to evoke anxiogenic effect in rats. In our studies, we observed a decrease in both time spent in the open arms and in the number of open arms entries, which is typical of anxiogenic response. The NMDA dose, which we used in our studies, did not cause any convulsions in rats.

Earlier studies have revealed that anxiolytic action of NPY was antagonized by specific antagonist of Y1 receptor, BIBO 3304, injected into the amygdala [23, 26]. Anxiogenic action of CRF is mediated by the CRF1 receptors in the amygdala [1, 13]. This anxiogenic action of CRF was antagonized by α-helical CRF, a specific CRF1 antagonist [13].

Moreover, pretreatment with α-helical CRF potentiated anxiolytic-like effect of NPY in Vogel's conflict test and NPY antagonized anxiogenic-like effect of CRF in the elevated plus-maze [1]. α-Helical CRF (9–41) prevented anxiogenic-like effect of NPY Y1 receptor antagonist BIBP 3226 in rats in the plus-maze test [14].

The effect of α-helical CRF on NMDA-induced anxiogenesis found in our studies indicates that CRF mediates the anxiogenic effect of NMDA. It was previously shown in our immunohistochemical studies that NMDA caused a slight decrease in CRF-IR after multiple administrations [29]. It was also shown that glutamate increased CRF release *in vitro* [4], so it is quite possible that this decrease in CRF-IR after NMDA administration induces CRF release and then anxiogenic action.

On the other hand, our results show that the anxiolytic effect of NMDA receptor blockade seems not to be related with NPY activation, at least by Y1 receptors, because Y1 receptor antagonist BIBO 3304 did not antagonize the anxiolytic action of MK-801. In our previous studies, we showed that MPEP, a mGlu5 metabotropic receptor antagonist possessed an anxiolytic activity and this effect was blocked by BIBO 3304 and not by flumazenil [16]. Therefore, we may speculate that, in contrast to anxiolysis induced by metabotropic glutamate receptor ligands, anxiolytic effects of NMDA receptor antagonist do not depend on Y1 receptors.

Therefore, we hypothesize that anxiolytic effects after NMDA receptor blockade are not mediated *via* NPY mechanisms or that the other NPY receptors, not Y1, may be engaged. Our present findings that anxiogenic effect of NMDA is mediated *via* CRF1 receptors may open a new prospect for understanding brain mechanisms engaged in the anxiolysis.

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