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Anxiolytic-Like Effects and Increase in Locomotor Activity Induced by Infusions of NMDA into the Ventral Hippocampus in Rat: Interaction with GABAergic System

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ABSTRACT

Introduction: In this study, we investigated the role of N-Methyl-D-Aspartate (NMDA) receptors in the ventral hippocampus (VH) and their possible interactions with $GABA_A$ system on anxiety-like behaviors.

Methods: We used an elevated-plus maze test (EPM) to assess anxiety-like behaviors and locomotor activity in male Wistar rats.

Results: The results showed that intra-VH infusions of different doses of NMDA (0.25 and 0.5 µg/rat) increased locomotor activity, and also induced anxiolytic-like behaviors, as revealed by a tendency to increase percentage of open arm time (%OAT), and a significant increase in percentage of open arm entries (%OAE). The results also showed that intra-VH infusions of muscimol (0.5 and 1 µg/rat) or bicuculline (0.5 and 1 µg/rat) did not significantly affect anxiety-like behaviors, but bicuculline at dose of 1 µg/rat increased locomotor activity. Intra-VH co-infusions of muscimol (0.5 µg/rat) along with low doses of NMDA (0.0625 and 0.125 µg/rat) showed a tendency to increase %OAT, %OAE and locomotor activity; however, no interaction was observed between the drugs. Interestingly, intra-VH co-infusions of bicuculline (0.5 µg/rat) along with effective doses of NMDA (0.25 and 0.5 µg/rat) decreased %OAT, %OAE and locomotor activity, and a significant interaction between two drugs was observed.

Discussion: It can be concluded that GABAergic system may mediate the anxiolytic-like effects and increase in locomotor activity induced by NMDA in the VH.

1. Introduction

The hippocampus has two sub-regions including dorsal and ventral portions (Hawley & Leasure, 2012). The ventral hippocampus (VH) has been shown to be associated with anxiety-related functions and regulation of locomotor activity, while the dorsal part mainly involved in spatial learning and memory processing (Bannerman et al., 2004; Engin & Treit, 2007; Hawley, Morch, Christie & Leasure, 2012; Zhang, Bast & Feldon, 2002). It has been reported that memories of fearful experiences can lead to pathogenic conditions such as anxiety and phobias (Orsini & Maren, 2012). According to previous researches, stimulation of the hippocampus with NMDA may disrupt particular hippocampal functions (Bast & Feldon, 2003; Berke & Eichenbaum, 2001), and impair processing of sensory stimuli, which is the core deficit

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in neuropsychiatric disorders such as schizophrenia and anxiety disorders (Bast & Feldon, 2003; Benes, 2000; Grace, 2000). Previous reports in animal models have also shown that intra-VH infusions of NMDA agonists increase locomotor activity (Bast, Zhang & Feldon, 2001; Swerdlow et al., 2001), and their antagonists decrease level of anxiety (Rezvanfard, Zarrindast & Bina, 2009). Therefore, NMDA receptors in the VH may have an important role in modulating anxiety-like behaviors (Cortese & Phan, 2005; Harvey & Shahid, 2012; Motevasseli, Rezayof, Zarrindast & Nayer-Nouri, 2010).

Furthermore, it has been reported that the balance between the inhibitory and excitatory systems in the brain is important for both emotional and cognitive health (Femenia, Gomez-Galan, Lindskog & Magara, 2012). Glutamate excitation may regulate the inhibitory tone by activating GABAergic neurotransmission (Olney, Newcomer & Farber, 1999). The $GABA_A$ receptors has been also shown to play a critical role in the control of excitation in the mammalian central nervous system (Bormann, 2000). Previous studies have revealed that intra-VH injection of $GABA_A$ receptor antagonist increases locomotor activity, whereas $GABA_A$ receptor stimulation has an opposite effect (Olney, Newcomer & Farber, 1999).

NMDA subtype of glutamate receptors are key elements underlying formation of certain type of fear memory in the hippocampus, and much recent evidences suggest that glutamatergic neurotransmission, especially through NMDA receptors, plays a key role in the onset of anxiety-related disorders [for review see (Harvey & Shahid, 2012; Riaza Bermudo-Soriano, Perez-Rodriguez, Vaquero-Lorenzo & Baca-Garcia, 2012)]. According to clinical data, the most used drugs to control anxiety disorders are those that target GABA and serotonergic receptors (Garakani, Mathew & Charney, 2006).

Besides some adverse effects, many patients are also non-responders to these treatments. By examining mechanisms involved in fear and anxiety in greater detail, we will be able to develop more effective treatments for many patients (Riaza Bermudo-Soriano, Perez-Rodriguez, Vaquero-Lorenzo & Baca-Garcia, 2012). It has been recently shown that several drugs targeting glutamate receptors have a potential psychiatric application (Krystal et al., 2010; Wieronska & Pilc, 2009). Therefore, the development of compounds that modulate the function of NMDA receptors in the brain is a great of interest (Bergink, van Megen & Westenberg, 2004). A growing body of evidence also suggests that some of the learning mechanisms which regulate fear extinction involve NMDA and GABA receptors [for review see (Kaplan & Moore, 2011)]. The aim of this study was to investigate the effects of NMDA receptors in the VH on anxiety-like behaviors using the elevated plus maze in rats, and their possible interaction with GABAergic system in this effect was also examined.

2. Methods

2.1. Subjects

Male Wistar rats (Pasteur Institute, Tehran, Iran), weighing 220-260 g at the time of surgery, were used. All the animals were housed in standard cages in groups of four. The cages were kept in a room with controlled 12/12 hour of light and dark cycles (lights on at 7:00 a.m.) and temperature maintained at 22 ± 2°C. The animals had free access to food and water except during the test periods. The animals were allowed to adapt to the laboratory conditions for at least 1 week before the surgery, and handled for 3 minutes each day one week before behavioral testing. All of the experiments were performed between 8:00 and 11:00 (a.m.), and each rat was tested only once. Seven to eight animals were used in each experimental group. The study was carried out according to the international guidelines for animal care and use (NIH Publication 80−23, revised in 1996).

2.2. Drugs

The drugs used in this study were muscimol ($GABA_A$ receptor agonist), bicuculline ($GABA_A$ receptor antagonist), which both were purchased from Sigma (St. Louis, CA, USA). NMDA was purchased from Tocris (Bristol, UK).

2.3. Surgery and Microinfusions

The rats were anesthetized intraperitoneally with ketamine (50 mg/kg) and xylazine (4 mg/kg), placed in a stereotaxic apparatus, and bilaterally implanted with stainless steel guide cannulae (22 gauge) directed to the VH. Stereotaxic coordinates for the VH were -4.4 mm posterior to the bregma, ±5.2 mm lateral to the midline, and 7.2 mm ventral from the dura (Paxinos & Watson, 2007).

Damage to the target site was decreased by terminating each guide cannula at 1mm above the center of the VH. Stainless steel stylets (27 gauge) were placed in the guide cannulae to prevent obstruction until the time of microinfusions. All animals were allowed one week of recovery, and then were randomly allocated to treatment conditions and testing.

For drug microinfusions, the stylets were removed and 27-gauge injection needle, which was 1 mm longer than the guide cannulae and connected to a 2.5µl Hamilton syringe via polyethylene tubing, was inserted. The drugs were infused into the VH in a volume of 0.5 µl per each side over a 60 seconds period. The injection cannula was left in the place for an additional 60 seconds to allow the diffusion of the solution, and prevent the possibility of reflux. The intra-VH infusions were made 5 minutes before testing, but in experiments which two drugs were injected, the first drug was injected 10 min before the test, and the second drug injected 5 min after the first one.

2.4. Elevated-Plus Maze

The elevated-plus maze was used to assess anxiety-like behavior and locomotor activity. The method utilized in the present study was basically the same as that described previously (Walf & Frye, 2007). This apparatus consisted of four arms (two open and two closed arms) arranged in the shape of a plus sign, and elevated to a height of 50 cm from the floor. The open arms had no walls (50×10 cm), but to prevent the rats from falling a rim of Plexiglas (0.5 cm high), surrounded the perimeter of the open arms. The closed arms were enclosed by walls with 40 cm height (50×10×40 cm). At the intersection of four arms, there was a square platform of 10×10 cm without any walls. The apparatus was situated in a room that was illuminated with only one 100W electric bulb placed above the apparatus, which produced enough and equal illumination within arms. The animals were left undisturbed in the testing room for 1 hour prior to testing, to adapt the testing environment. After the drug injections and before the initiation of the test, the rats were placed in a separate wooden test arena (50 cm × 50 cm × 35 cm) for 5 minutes, and then moved to the testing apparatus.

Each animal was individually placed in the center of the maze facing an open arm, and allowed 5 minutes of free exploration. The number of entries (with all four paws) into open and closed arms, and the total times that the animal was spent in the open and closed arms were separately recorded. The percentage of open arm time (%OAT) and open arm entries (%OAE) that are used as the standard anxiety indices (Rodgers and Johnson, 1995) were calculated using the following formulae:

- (a) %OAT (the ratio of the total time spent in the open arms to the total time spent in four arms × 100)

- (b) %OAE (the ratio of the total entries into the open arms to the total entries in four arms × 100)

The sum of all the open and closed arms entries was used as the index of general locomotor activity.

2.5. Experimental Design

2.5.1. Experiment 1: Effects of NMDA on Anxiety-Like Behaviors

In this experiment, four groups of rats were used. One group of them received intra-VH infusion of saline (1 µl/rat), and the other three groups received intra-VH infusions of different doses of NMDA (0.125, 0.25, and 0.5 µg/rat).

2.5.2. Experiment 2: Effects of $GABA_A$ Agonist (muscimol) or Antagonist (bicuculline) by themselves on Anxiety-Like Behaviors

Six groups of rats were examined in this experiment. Three groups of the animals received intra-VH infusions of saline (1 µl/rat) or muscimol (0.5 and 1 µg/rat), and another three groups received intra-VH infusions of saline (1 µl/rat) or bicuculline (0.5 and 1 µg/rat).

2.5.3. Experiment 3: Effects of NMDA in Combination with muscimol on Anxiety-Like Behaviors

In this experiment, three groups of rats received intra-VH infusions of saline (1 µl/rat) in combination with saline or NMDA (0.0625 and 0.125 µg/rat). The other three groups received intra-VH infusions of muscimol (0.5 µg/rat) in combination with saline or NMDA (0.0625 and 0.125 µg/rat).

2.5.4. Experiment 3: Effects of NMDA in Combination with Bicuculline on Anxiety-Like Behaviors

Six groups of rats were used. Three groups of the rats received intra-VH infusions of saline (1 µl/rat) in combination with saline or NMDA (0.125 and 0.5 µg/rat). The other three groups received intra-VH infusions of bicuculline (0.5 µg/rat) in combination with saline or NMDA (0.125 and 0.5 µg/rat).

2.6. Histology

At the end of each experiment, the animals were killed and received intra-VH infusions of 1µl/rat of 1% methylene blue solution. Then, the animals were decapitated, the brains removed, and moved into formaldehyde solution (10%) for several days. After slicing of the fixed brains, the cannulae placements were verified according to Paxinos and Watson (2007). Data obtained from the animals with correct injection sites were statistically analyzed.

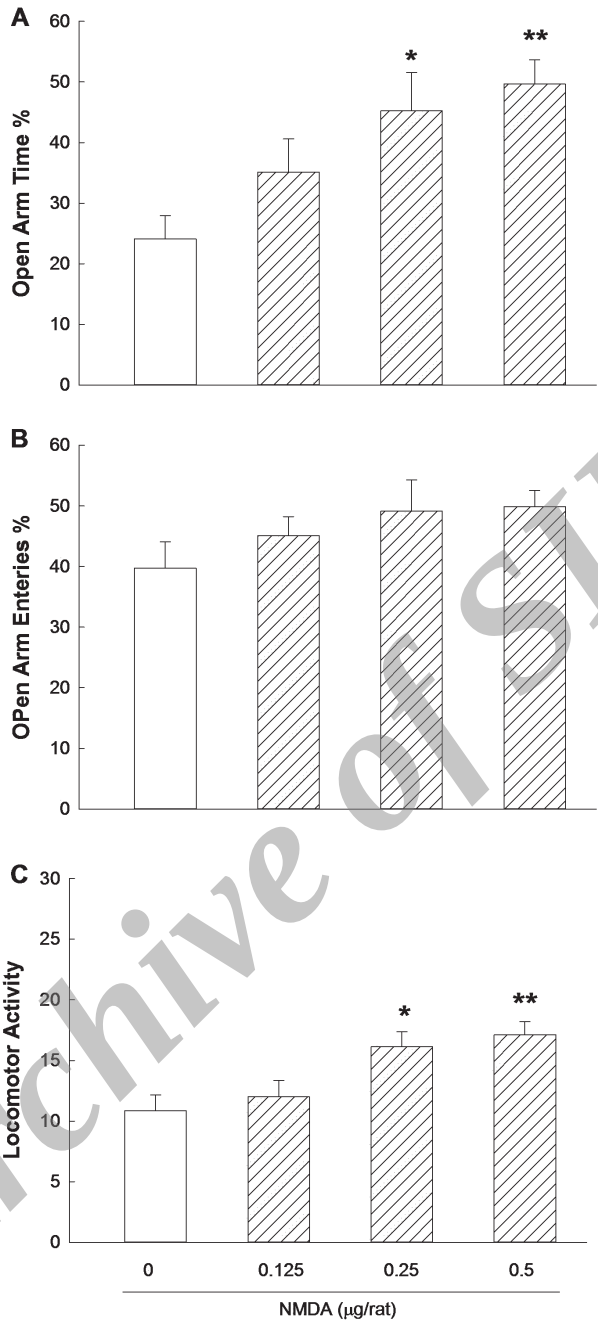

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Figure 1. The effects of the intra-VH infusions of NMDA on anxiety-like parameters of the elevated-plus maze. Four groups of rats received saline (1 µl/rat) or different doses of NMDA (0.125, 0.25 and 0.5 µg/rat). Each bar represents mean ± SEM for %OAT (A), %OAE (B), and locomotor activity (C). * P<0.05 and ** P<0.01 compared to the saline-treated control group.

2.7. Statistical Analysis

The data were analyzed with one- or two-way ANOVA for comparisons between groups. Following a significant F value, post-hoc Tukey's test was performed to assess paired groups comparisons. P<0.05 was statistically considered significant.

3. Results

3.1. Intra-VH Infusions of NMDA Induced Anxiolytic-Like Behaviors and Increased Locomotor Activity

Figure 1 shows the effects of the intra-VH infusions of NMDA (0.125, 0.25, and 0.5 µg/rat) on the anxiety-like

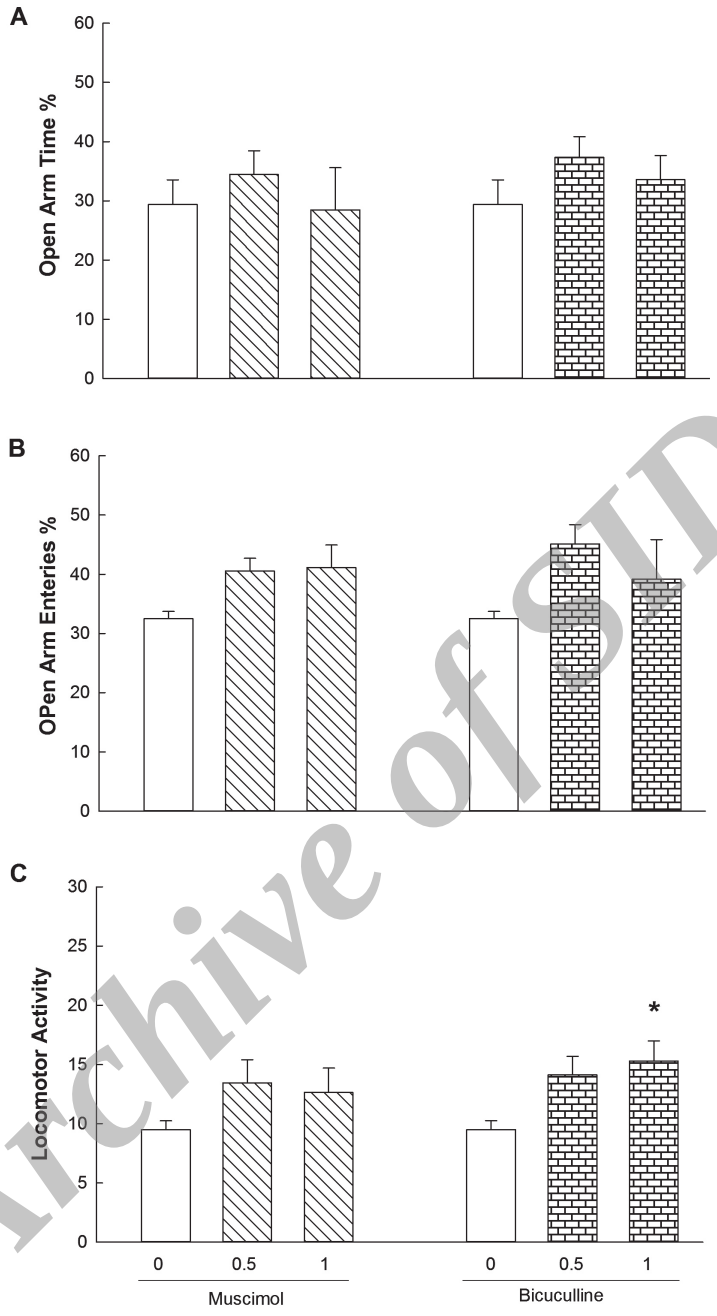

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Figure 2. The effects of intra-VH infusions of muscimol or bicuculline on anxiety-like parameters in the elevated-plus maze. The groups received saline (1 µl/rat), muscimol (0.5 and 1 µg/rat), or bicuculline (0.5 and 1 µg/rat). Each bar represents mean ± SEM for %OAT (A), %OAE (B), and locomotor activity (C).

behaviors in the elevated-plus maze. One-way ANOVA indicated that intra-VH infusions of NMDA significantly increased %OAT [$F(3,28)=5.15$, $P<0.01$] and locomotion [$F(3,28)=6.07$, $P<0.01$], but not %OAE [$F(3,28)=1.37$, $P>0.05$]. Post hoc Tukey's test revealed that NMDA at doses of 0.25 and 0.5 µg/rat increased %OAT and locomotor activity compared to the control saline-treated group. None of the animals revealed any sign of epileptic activity following administration of the drug.

3.2. $GABA_A$ Agonist (muscimol) or Antagonist (bicuculline) by Themselves Had no Significant Effects on Anxiety-Like Behaviors

Figure 2 illustrates the effects of muscimol and bicuculline on the anxiety-related behaviors in the elevated-plus maze. One-way ANOVA revealed that the intra-VH infusions of muscimol (0.5 and 1 µg/rat) did not cause any significant difference in %OAT [$F(2,20)=0.33$,

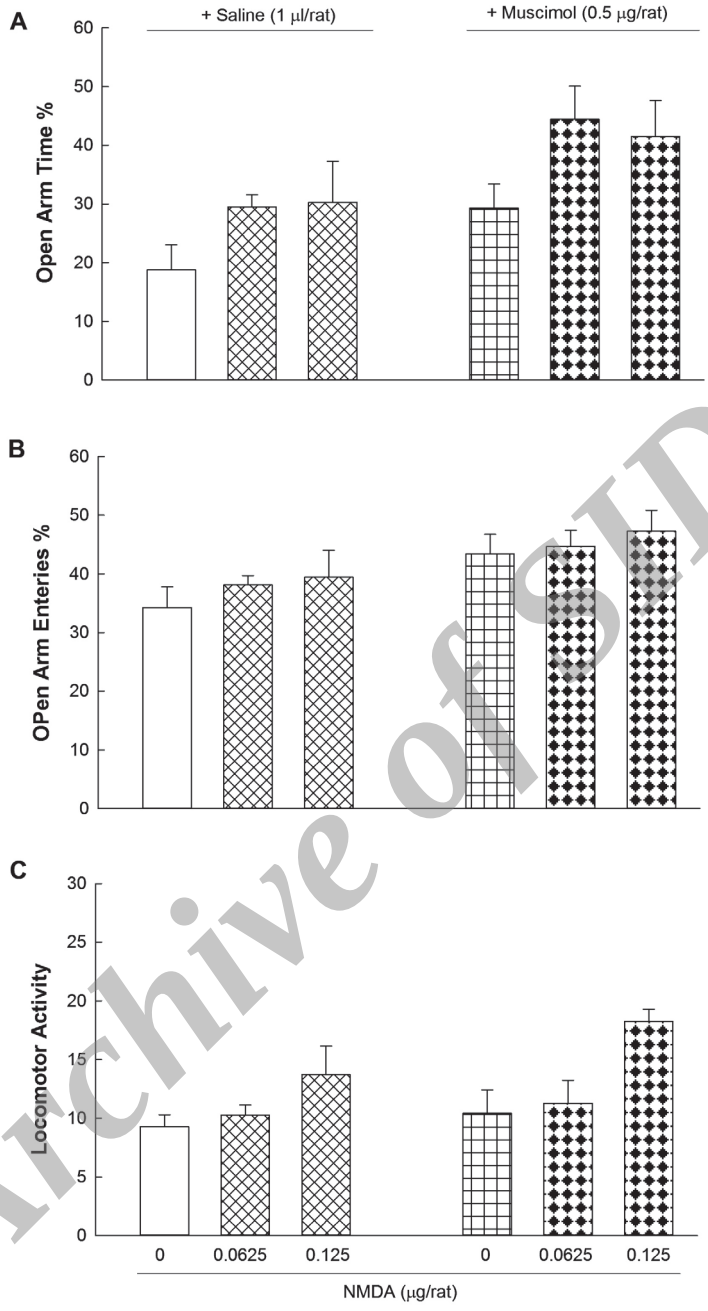

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Figure 3. The effects of intra-VH infusions of muscimol along with low doses of NMDA on anxiety-like parameters. Three groups of rats received intra-VH infusions of saline (1 µl/rat) plus saline (1 µl/rat) or NMDA (0.0625 and 0.125 µg/rat). The other three groups of rats received intra-VH infusions of muscimol (0.5 µg/rat) plus saline (1 µl/rat) or NMDA (0.0625 and 0.125 µg/rat). Testing was performed 5 min after the last intra-VH infusions. Each bar represents mean ± SEM for %OAT (A), %OAE (B), and locomotor activity (C).

P>0.05], %OAE [F(2,20)=3.37, P>0.05], and locomotor activity [F(2,20)=1.54, P>0.05]. The analysis by one-way ANOVA also showed that the intra-VH infusions of bicuculline (0.5 and 1 µg/rat) did not significantly alter %OAT [F(2,20)=1.09, P>0.05], and %OAE [F(2, 20)=2.49, P>0.05]; however, increased locomotor activity [F(2,20)=5.01, P<0.05]. Post-hoc analysis with Tukey's test indicated that bicuculline at dose of 1µg/rat enhanced locomotor activity. The animals also revealed no sign of epileptic activity following intra-VH injections of the drugs.

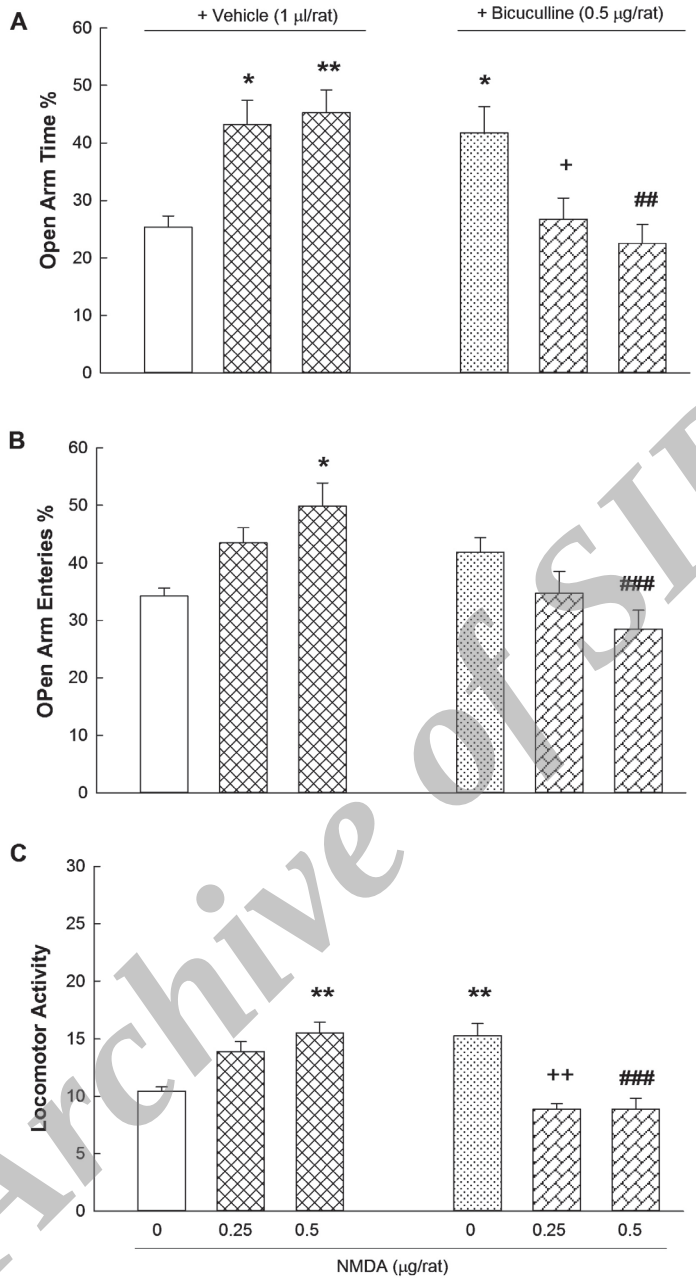

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Figure 4. The effects of intra-VH infusions of bicuculline along with higher doses of NMDA on anxiety-like parameters. Three groups of rats received intra-VH infusions of vehicle (1 µl/rat) plus saline (1 µl/rat) or NMDA (0.25 and 0.5 µg/rat). The other three groups of rats received intra-VH infusions of bicuculline (0.5 µg/rat) plus saline (1 µl/rat) or NMDA (0.25 and 0.5 µg/rat). The elevated-plus maze test was performed 5 min after the last intra-VH infusions. Each bar represents mean ± SEM for %OAT (A), %OAE (B), and locomotor activity (C). * P<0.05 and ** P<0.01 compared to the vehicle+saline control group. + P<0.05, ## P< 0.01 and ###P< 0.001 compared to the respective vehicle+NMDA groups.

3.3. Intra-VH Infusions of Muscimol in Combination with Low Doses of NMDA Showed a Tendency to Increase Anxiety-Like Behaviors and Locomotor Activity

Considering muscimol as factor A (with two levels) and NMDA as factor B (with three levels), two-way ANOVA about %OAT revealed a significant effect of factor A [F (1,38)=8.63, P<0.01], and factor B [F(2,38)=3.89, P<0.05]; however, there was no significant interaction between two factors [F(2,38)=0.11, P>0.05]. Two-way ANOVA about %OAE also revealed a significant effect for factor A [F(1,38)=8.44, P<0.01], but not for factor

B [F(2,38)=0.91, P>0.05], and interaction of two factors [F(2,38)=0.07, P>0.05]. Analysis of the results by two-way ANOVA also showed a significant effect for factor B on locomotor activity [F(2,38)=8.39, P<0.01], but not for factor B [F(1,38)=2.87, P>0.05], and interaction between two factors [F(2,38)=0.77, P>0.05]. In brief, although muscimol along with low doses of NMDA showed a tendency to induce anxiolytic-like effects, but no significant interaction was detected (Figure 3).

3.4. Intra-VH Infusions of Bicuculline in Combination with Effective Doses of NMDA Prevented Anxiolytic-Like Effects and increase in Locomotor Activity that had been Induced by the Later Drug

Considering bicuculline as factor A (with two levels) and NMDA as factor B (with three levels), two-way ANOVA showed no significant effect of NMDA on %OAT [F(2,42)=0.08, P>0.01], but a significant effect of bicuculline on %OAE [F(1,42)=6.29, P<0.05], and an interaction between two factors was observed [F (2, 42)=15.93, P<0.001]. Two-way ANOVA also showed no significant effect of NMDA on %OAE [F(2,42)=0.08, P>0.01], but a significant effect for bicuculline [F(1,42) =8.98, P<0.01], and an interaction between two factors was observed [F(2,42)=12.25, P<0.001]. Analysis of the results by two-way ANOVA also showed a significant effect of bicuculline on locomotor activity [F (2,42) = 11.6, P<0.01], but not for NMDA [F(2,42)=1.66, P>0.05]. No interaction between two factors was observed on locomotor activity [F(2,42)=27.87, P<0.001]. In brief, intra-VH infusions of bicuculline along with effective doses of NMDA prevented anxiolytic-like effects, and increase in locomotor activity induced by NMDA. Post hoc Tukey's test revealed that bicuculline (0.5 µg/rat) prevented increase of %OAT that induced by NMDA at doses of 0.25 and 0.5 µg/rat, increase of %OAE induced by NMDA at dose of 0.5 µg/rat, and increase of locomotor activity induced by NMDA at doses of 0.25 and 0.5 µg/rat (Figure 4).

4. Discussion

Our results showed that the stimulation of the NMDA receptors in the VH induced anxiolytic-like effects, and also increased locomotor activity. Our findings are consistent with previous studies that reported marked locomotor hyperactivity following a pharmacological stimulation of the VH by NMDA (Bast, Zhang, Heidbreder & Feldon, 2001; Zhang, Bast & Feldon, 2002). Although NMDA infusions into the VH increased locomotor activity, but it also induced a significant increase in %OAT, so it can be suggested that NMDA have induced anxiolytic-like effects. According to research, a major role for glutamate receptors in the pathogenesis of anxiety disorders has been proposed (Bergink, van Megen & Westenberg, 2004). There are no exact reports on the effects of the intra-VH infusions of NMDA on anxiety-like behaviors, whereas there are studies underscoring the anxiolytic-like effects of NMDA competitive antagonist when injected in the VH (Motevasseli, Rezayof, Zarrindast & Nayer-Nouri, 2010; Rezvanfard, Zarrindast & Bina, 2009).

Our results also showed that intra-VH infusions of muscimol and bicuculline by themselves had no significant effects on anxiety-like behaviors. Consistent with our findings, previous studies have demonstrated that intra-VH infusions of muscimol had no influence on anxiety-like parameters and locomotion (Motevasseli, Rezayof, Zarrindast & Nayer-Nouri, 2010; Rezvanfard, Zarrindast & Bina, 2009). The results also showed that locomotor activity was increased following intra-VH injection of a high dose of bicuculline. Thus, it may be suggested that there is a basic inhibitory GABAergic control in the VH, which also mediates responses induced by the higher doses of NMDA. In support of this later suggestion, there are some reports that glutamate excitation may regulate the inhibitory tone through activation of GABAergic neurons (Olney, Newcomer & Farber, 1999), and/or increase in GABA level in the VH due to the over-activation of NMDA receptors placed on inhibitory interneurons (Grunze et al., 1996; Schoffelmeer et al., 2000). It has been shown that in fear extinction, there are widespread and important changes in GABA and glutamatergic signaling in key cortical, hippocampal and amygdalar pathways that result in the inhibition of fear outputs (Kaplan & Moore, 2011). According to the present results, it is suggested that $GABA_A$ and NMDA receptors in the VH have a modulatory interaction with anxiety-like behaviors.

Alternatively, NMDA agonists appear to trigger a signaling cascade resulting in AMPA receptor subunit internalization, which may alter synaptic transmission (Mao, Lin & Gean, 2008). It can be suggested that anxiolytic-like effects of NMDA may result in internalization of AMPA receptors on the same target neurons, which subsequently decreases activation of NMDA receptors and excitatory outputs of the VH, and finally result in anxiolytic-like effects.

Taken together, it seems that stimulation of the VH by infusions of NMDA may exert its effect through disturbing hippocampal control on physiological or behavioral processes. Higher doses of NMDA may potentiate GABAergic inhibitory tone in the VH, which indirectly may

result in attenuation of inhibitory control on locomotor activity. Therefore, $GABA_A$ receptor antagonists may oppose the effect of higher doses of NMDA on locomotion through suppression of NMDA effects on the inhibitory projections. Evaluation of anxiety-related parameters in elevated-plus maze task is highly dependent on locomotor activity. Thus, precise evaluation of the effect of hippocampal over-stimulation via NMDA injection on anxiety-like behaviors requires more specific behavioral models to allow examining anxiety-like parameters independent from locomotor activity.

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