Involvement of the nucleus accumbensshell presynaptic NMDA receptors on anxiolytic-like behaviors induced by NMDA in adult male Wistar rat

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ABSTRACT

Glutamatergic system stimulationthe nucleus accumbens shell, may affect anxiety-related behaviors, aversive learning and memory. Glutamate receptors are differentially distributed in pre- and postsynaptic sites contributing to neuronal communications.The present study aimed to examine the possible involvement of the NAc shell presynaptic NMDA receptors on NMDA induced responses, using the elevated-plus maze (EPM) task in maleWistar rats. Bilateral guide cannulae were implanted to allow microinjection of glutamatergic agonist (NMDA) or ca^{+2} channel blocker (SKF96365 hydrochloride) agents. Pretest intra-NAc shell infusion of NMDA induced anxiolytic-like behaviors and impaired the EPM-associated memory upon test and retest, respectively. In addition our findings showed that, the intra-NAc shell infusion of Ca^{+2} channel blocker at applied doses, does not alter the anxiety-like response and aversive memory upon test and retest, respectively. Furthermore, infusing the subthreshold dose SKF prior to the injection of effective doses of NMDA, reduced the anxiolytic-like response and improved the aversive memory impairment which had already been induced by intra-NAc shell NMDA injection. Our study showed that,inhibition of the neurotransmitter exocytosis from pre-synaptic neuron via Ca^{+2} channel blockade bySKF96365 decreases affected induced by NMDA in the NAc shell region, indicating the involvement of the pre-synaptic NMDA receptors in NMDA induced responses.Therefore, NMDA's ability to increase anxiolytic-like behaviors and the aversive memory impairment may be the result of an action on pre-synaptic glutamatergic receptors which in turn decrease the glutamate effect at synaptic terminal level.

Keywords*:* Anxiety; Aversive learning; NMDA; SKF96365 hydrochloride; Nucleus Accumbens; Rat

INTRODUCTION

Glutamate is one of the main excitatory neurotransmitters in central nervous system [1] which is involved in many cognitive and non-cognitiveprocesses including learning and memory as well as thepathogenesis of anxiety-related disorders, fear conditioning [2-7].Glutamate exerts its actions via two different receptor types: ionotropic receptors and metabotropic. NMDA receptor (as an ionotropic glutamate receptor) plays a critical role in the regulation of glutamate-induced behaviors such as learning and memory formation [4, 5] and anxiety-related behaviors [2, 8]. Both ionotropicand metabotropicreceptors are differentially distributed in pre-and postsynaptic sites contributing to neuronal communications and signal processing which underlie functionssuch as learning and memory formation [9, 10].NMDA is shown to exert its effect via pre- and postsynaptic receptors[11, 12].The presynaptic effect is mediated either directly by NMDA receptors localized on axons or synaptic buttons or indirectly through postsynaptic receptors which release retrograde messengers that act on presynaptic elements[12-14]. The postsynaptic effect is mediated either by the synaptic NMDA receptors localized on dendritic spines orextra-synaptic receptors localized on dendritic shafts and the soma [15]. Furthermore, some of investigations have postulated that, the postsynaptic release of glutamate activates the presynaptic NMDA receptors hence regulatedifferentforms of different forms of synaptic plasticity [14, 16].Ca^{+2}entry through presynaptic voltage-gated Ca^{+2}channels initiates

release of neurotransmitters. Neuromodulation affects the ability of voltage-gated Ca^{+2}channels to open, close, or inactivate in response to membrane depolarization and alters their response to repetitive stimuli in an activity-dependent manner[17].

Thenucleus accumbens(a major component of the ventral striatum) is a structure located in the rostrobasal forebrain andcomposed ofthe core and shell parts[18]. The NAc shell is strongly suggested to be a pivotal brain site for modulation of emotion, motor activity [19], motivation-related learning, memory[20, 21]and anxiety [22-24].Evidence has shown that, NAc NMDARs are involved in novel physiologicaland pathophysiological emotionalresponses [25].More recentinvestigations have suggested thatNMDARs may also present atthe presynaptic axonal terminals and potentially affectthe presynaptic neurotransmitter release[12, 21, 26-32].

Emotional states (including, fear and aversion)can be modulated, by enhancing or impairing, memory formation[33]. Due to possible misinterpretations, the available animal models for learning and memory seem to have a limited ability to detect the effectof drugs on anxiety and fear [34]. Therefore, the proposed test-retest paradigm in the elevated plus-maze(EPM) task is an attempt to concomitantly assess the effects of drugs on anxiety, learning and memory in Rat.The present study aimed to examine the possible involvement of the NAc shell presynaptic NMDA receptors onNMDA induced responses, using the elevated-plus maze (EPM) task in adult Wistar rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (Institute of cognitive science; Tehran, Iran) weighting 250 – 280 g at the time of surgery were used. The animals were housed in colony, maintained at (22 ± 2 °C) a 12-h light/12-h dark cycle (lights on at 07:00 h) and allowed free access to food and water within standard polypropylene cages. The animals were permitted to adapt to the laboratory condition for at least 1 week prior to surgery. Eight animals were considered in each experimental group. The experiments were carried out during the light phase of the cycle.

Drugs

Ketamine and xylazine (Alfasan Chemical Co, Woerden, Holland) were used for animal anesthesia.N-methyl-D-Aspartate (NMDA) receptor agonist and SKF96365 hydrochloride(SKF, ca^{+2} channel blocker) were used for microinjections prior to the behavioral tests.All drugs were dissolved in sterile 0.9% saline, just before the experiment. NMDA and SKFwere administered into the NAcshell at a volume of 0.3µl in each side.Control animals received saline.

Surgical procedure and microinjections

Animals were anesthetized intraperitoneally using ketamine hydrochloride (50 mg/kg) and xylazine (4 mg/kg) then placed in a Stoelting stereotaxic instrument (Wood Dale, IL, USA).A stainless steel guide cannula (22 gauge) was implanted in the right and left NAc shell regions according to the atlas of Paxinos and Watson [35]. The stereotaxic coordinates for the NAc regions were as follows: +1.4 mm posterior to bregma, ±0.8 mm lateral to the midline, and− 5.5mm ventral to the dorsal surface of the skull. The cannulae were fixed to the skull using acrylic dental cement. Rats were allowed 5 days before the test to recover from surgery. The left and right NAc were infused by means of an internal cannula (27 gauge), terminating 2 mm below the tip of the guides, connected by polyethylene tubing to a 2µl Hamilton syringe (Bonaduz, GR, Switzerland). A volume of 0.3µl solution was injected over a 60-second period, in each side. The inner cannulae were left in place for an additional 60 seconds to allow diffusion of the solution and to reduce the possibility of reflux. Intra-NAc shell injections were made just five minutes before testing.

Apparatus

We used a wooden elevated plus-maze (EPM) apparatusconsisting of two oppositely positioned open- arms (50×10 cm) and two enclosed arms (50×10×40 cm),surrounded by1cm high Plexiglas ledgeso thatto prevent falls set up 50 cm above the floor. The junction area of the four arms (central platform) measured10×10 cm[36-38].

Behavioral testing

Rats were placed in the experimental room at least 1h before testing. All experiments were done during the light phase of the L/D cycle between 11 a.m. and 2 p.m. Animals' behaviors were tracked and recorded by an observer who quietly sat 1m behind one of the closed arms of the maze, using a chronometer. Five minutes post- drug treatment, rats were individually placed at the center of the plus maze, facing one of the open arms and allowed for 5 min free exploration in EPM (test session) after which were taken back to their home cages. In 24 hours, rats were returned to the test room and placed again in the EPM for a new exploration period of 5 min (retest session). The observer measured: 1- time spent in open arms, 2- time spent in closed arms, 3-number of entries into open arms and 4- number of entries into closed arms during the 5minute period both upon test and retest. An entry was defined as 'all four paws in the arm'. In between EPM sessions and after each rat, the maze was cleaned with distilled water. The obtained data were used to calculate: a- % OAT (the ratio of time spent in open arms to the time spend in all arms ×100); b-%OAE (the ratio of entries into open arms to total entries×100) [39-41] and c- the total closed and open arm entries were considered as a relatively pure index for the locomotor activity [42, 43].

Experimental design

Experiment1: the effects ofintra- NAc shell pretest microinjections of NMDA,SKF,SKFprior to NMDA on open-arms exploratory behaviors

To substantiate whether the intra- NAc shell microinjection of drugsinvolved in anxiety, drug infusion were given before EPM testing. In this experiment11groups of animals were examined.

These were as follow: 1- animals which received intra-NAc shell saline (0.3µl/rat) or NMDA (0.125, 0.25, and 0.5µg/rat), 5 min after saline (0.3µl/rat); 2- animals which received intra-NAc shell saline (0.3µl/rat) or SKF (0.0625, 0.0125, 0.25 and 0. 5µg/rat), 5 min before saline (0.3µl/rat) and 3- animals which received intra-NAc shell saline (0.3µl/rat) or the subthreshold dose SKF (0.0125µg/rat), 5 min before different doses of NMDA (0.25 and 0.5µg/rat).To investigate the possible drugs carryover effects of aversive learning during test day to aversive memory upon retest, treated groups were retested in the EPM 24 h later, undrugged.

Statistical analysis

Data were expressed as mean±S.E.M and analyzed using the repeated measure protocol during test and retest days. In addition to the analysis made to compare test to test or retest to retest, the two-way analysis of variance (ANOVA) was also applied. Where F-value was significant, one-way ANOVA and post-hoc analysis (Tukey-test) was performed. Between-groups differences with $P<0.05$, were considered statistically significant.

RESULTS

The experiment results:

The effects of pretestintra-NAc shell microinjection of NMDA on open-arms exploratory-like behaviors

Repeated measure and Post hoc analyses demonstrated that, NMDA increases the %OAT (at 0.5µg/rat, Fig.1; panel 1A), %OAE (at 0.25 and 0.5µg/rat, Fig.1; panel 1B) while decreases the locomotor activity (at 0.25 and 0.5µg/rat, Fig.2; panel 1C) upon test, indicating an anxiolytic-like response to NMDA.

Adding to the above, data showed that NMDA increases the %OAT (at 0.5µg/rat, Fig.1; panel 2A) while does not alter %OAE (Fig.1; panel 2B) and the locomotor activity (Fig.1; panel 2C) upon retest as compared to the control group, indicating an impairment of the aversive memory acquisition by NMDA. On the other hand, this intervention decreased the %OAE on retest day (Fig.1, panel 2B) as compared to same groups on test day (Fig.1, panel 1B).

According to the above data, NMDA induced anxiolytic-like behavior. Furthermore, the retest data suggest that the NMDA anxiolytic-like effect may also be linked to the impairment in further avoidance acquisition. The corresponding repeated measure results have been demonstrated in table 1.

Table1: repeated measure and two ANOVA results with *P* values for experiments

Experiments	Behaviors	Day		Group		Day and Group interaction		Final results conclusion for each experiment
		$F_{(1,35)}$	*P*	$F_{(4,35)}$	*P*	$F_{(4,35)}$	*P*	NMDA into NAc shell induced anxiolytic-like behavior andimpaired aversive memory acquisition
Repeated measure analysis results for NMDA microinjection into NAc (Panel 1 and 2 of fig.1)	**%OAT**	0.006	0.939	8.495	0.000	0.887	0.482	
	%OAE	12.800	0.001	10.617	0.000	5.972	0.000	
	Locomotion	4.143	0.049	2.245	0.084	0.309	0.870	
Repeated measure analysis results for SKF microinjection into NAc (Panel 3 and 4 of fig.1)		$F_{(1,28)}$	*P*	$F_{(3,28)}$	*P*	$F_{(3,28)}$	*P*	SKFinto NAc shell did not alter anxiolytic-like behavior and aversive memory acquisition
	%OAT	5.672	0.024	1.775	0.175	1.027	0.396	
	%OAE	5.612	0.025	1.651	0.200	1.127	0.355	
	Locomotion	2.594	0.119	11.397	0.000	2.225	0.107	
Two ANVA results between(panel5 and 1of fig. 1)		$F_{(1,48)}$	*P*	$F_{(2,48)}$	*P*	$F_{(1,48)}$	*P*	SKFintoNAcshell reversed anxiolytic-like responseand aversive memory impairment induced by NMDA into NAc shell
	%OAT	35.996	0.000	5.723	0.006	4.172	0.022	
	%OAE	25.408	0.000	11.925	0.000	1.261	0.294	
	Locomotion	0.022	0.883	1.711	0.193	14.319	0.000	
TwoANOVA results between (panel 6 and 2 of fig. 1)		$F_{(1,48)}$	*P*	$F_{(2,48)}$	*P*	$F_{(1,48)}$	*P*	
	%OAT	16.837	0.000	6.848	0.003	9.933	0.000	
	%OAE	1.075	0.306	2.556	0.090	0.436	0.650	
	Locomotion	0.105	0.748	0.938	0.399	1.836	0.172	

The effects of pretest intra-NAc shell microinjection of SKF on open-arms exploratory-like behaviors

Repeated measure and post hoc analyses revealed that, SKF does not alter %OAT (Fig.1; panel 3A), %OAE (Fig.1; panel 3B) while decreases the locomotor activity (at 0.125 and 0.25μg/rat, Fig.1; panel 3C) upon test as compared to the control group, indicating that SKF does not appear to alter anxiety-like behaviors in the EPM task.

Based on our data, SKF elicited no effect on the %OAT (Fig.1; panel 4A), %OAE (Fig.1; panel 4B) while decreases the locomotor activity(at 0.255μg/rat, Fig.1; panel 4CB) upon retest as compared to control group.

To conclude, while SKF did not seem to alter the anxiety-like behavior compared to control groups on test day, it improved the aversive memory on retest day as compared to the same groups on test day. This experiment repeated measure results are demonstrated in table 1.

The effects of pretestintra-NAc shell microinjection of SKF prior to NMDA on open-arms exploratory-like behaviors

According to the two-way ANOVA and post hoc analyses, intra-NAc shell microinjection of SKF prior to NMDA causes a significant decrease in the %OAT (at 0.25 and 0.5μg/rat, Fig.1; panel 5A), %OAE (at 0.25 and 0.5μg/rat, Fig.1; panel 5B) while does not significantly affect the locomotor activity on test day (Fig.1; panel 5C) as compared to the NMDA-treated groups on test day (Fig.1; panel 1A, 1B and 1C). These data indicated that, the intra-NAc shell microinjection of the subthreshold dose of SKF may potentially reverse the NMDA-induced anxiolytic-like response.

Moreover, the above experiment led to significant decrease in %OAT (at 0.5μg/rat, Fig.1; panel 6A), without altering the %OAE (Fig.1; panel 6B) and locomotor activity (fig.1; panel 6C) on retest day as compared to NMDA-treated groups during the same day (Fig.1; panel 1A, 1B and 1C). These data indicated that, intra-NAc shell microinjection of the subthreshold dose SKF reverses the NMDA-induced aversive memory acquisition impairment.

Given the above data, the subthreshold dose SKF seems to decrease the NMDA-induced anxiolytic-like behaviors and the aversive memory acquisition impairment on test and retest days. This experiment ANOVA results are summarized in table 1.

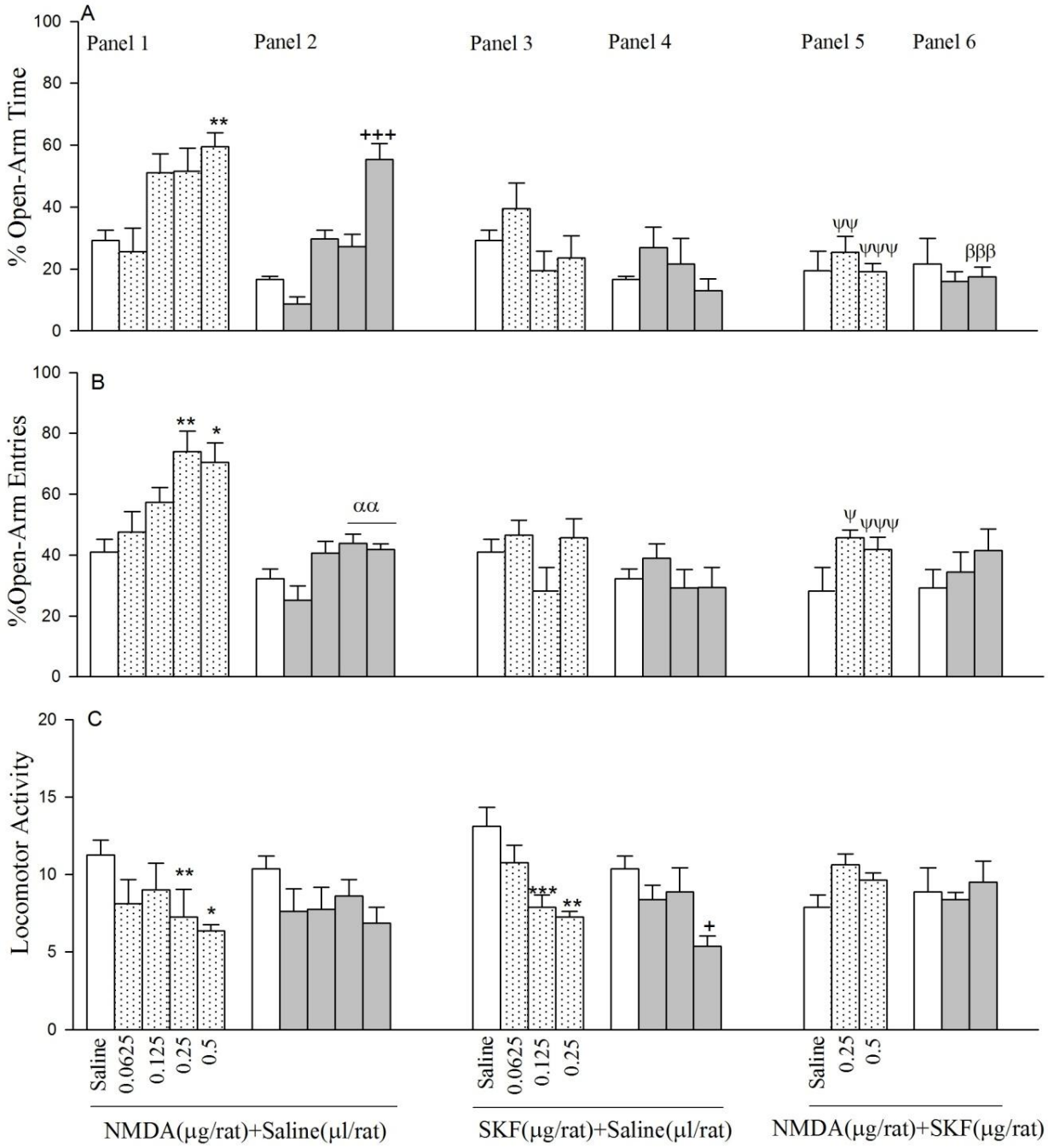

Figure 1: Open-arms exploratory behaviors following the pretest microinjections of NMDA, SKF, SKF prior to intra NAc shell injection of NMDA.After 24 h, all groups were retested in the EPM undrugged. %Open-Arms Time (A); %Open-Arms Entries (B) and Locomotor Activity (C).Values are expressed as mean±S.E.M (n = 8 in each group).*P<0.05, **P<0.01 and ***P<0.001 different from controlsaline group on test day. +P<0.05, ++P<0.01 and +++P<0.001 different from controlsaline groups on retest day. αP<0.05, ααP<0.01 and αααP<0.001 different from the test and retest group. ψP<0.05, ψψP<0.01 and ψψψP<0.001 different from the test groups. βP<0.05, ββP<0.01 and βββP<0.001 different from the retest groups.

DISCUSSION

In our study, animals were given pretest intra-cerebral drugs injection, followed by no injection upon retest, 24 h later. Based on this; drug effects on anxiety-like behaviors and aversive learning with subsequent long-term effects on memory in 24 h, were tested. It has been reported that the prior experience of an undrugged EPM testing session may alter the behavioral responses in an undrugged retest session [44, 45].

Our results indicated that the intra-NAc shell infusion of NMDA receptor agonist at applied doses induces an anxiolytic-like response in EPM. This NMDA- induced anxiolytic-like effect emerges into the retest day. Current findings suggest that NMDA treatment induces impairment in the aversive memory acquisition upon test. There is a body of evidence supporting that NAc shell is an essential brain site regulating emotion, motor activity [19], motivation-related learning, memory[20, 21] and anxiety-like behaviors [22-24]. On the other hand, the NMDA receptor (as an ionotropic glutamate receptor) plays a critical role in the regulation of glutamate-induced behaviors such as learning and memory formation [4, 5] and anxiety-related behaviors [2, 8].

Some studies have displayed that glutamate retains an important role in anxiety and anxious behaviors and elicits its effect through different combinations of metabotropic and ionotropic glutamate receptors and potentially different subunit combinations [3, 46]. Our results are in agreement with the previous investigation showing that NMDA agonist releases behavioral and anxiolytic-like behaviors indicating the role of NMDA receptors in modulation of anxiety-related behaviors [8, 46].Moreover, there is also an investigation showing that the activation of NMDA receptor induces anxiogenic-like effects in EPM and social interaction tasks [47].Based on some genetic insights, the NR2A subunit of the NMDA receptors involves in the anxiolytic-like responses seen in mice [48].The difference in anxiogenic/anxiolytic responses observed between studies can possibly be related to difference in endogenous glutamatergic tone under different laboratory settings [49]. These can partly be attributed to the doses of glutamatergic agents, the method used and the site(s) of drug administration.

Evidence has suggested the critical role of NAc in regulation of several learning functions which require a flexible use of sensory information [50-52]. It has been postulated that NAc manipulations induce spatial memory deficit in the Morris water-maze [52, 53] and radial maze [54, 55]. In agreement with our results, evidence has demonstrated that the systemic administration of NMDA leads to an impaired dark-avoidance learning in rats [56]. On the other hand, some investigations have postulated that the deactivation of the NAc glutamatergic ionotropic receptors disrupts the working memory[57-59] and spatial responses[60] while other contradictory studies have shown that the NMDA receptor blockade in NAc shell does not alter the spatial learning [51, 60].

In addition our findings showed that, the intra-NAc shell infusion of Ca^{+2} channel blocker SKF at applied doses, does not alter the anxiety-like response and aversive memory upon test and retest, respectively. Furthermore, infusing the subthreshold dose SKF prior to the injection of effective doses of NMDA, reduced the anxiolytic-like response and improved the aversive memory impairment which had already been induced by intra-NAc shell NMDA injection. Our study showed that,inhibition of the neurotransmitter exocytosis from pre-synaptic neuron via Ca^{+2} channel blockade bySKF96365 decreases anxiolytic-like behaviors induced by NMDA in the NAc shell region, indicating the involvement of the pre-synaptic NMDA receptors in NMDA induced responses.Therefore, NMDA's ability to increase anxiolytic-like behaviors and the aversive memory impairment may be the result of an action on pre-synaptic glutamatergic receptors which in turn decrease the glutamate effect at synaptic terminal level.

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