**Synergistic effect between D-AP5 and muscimol in the nucleus accumbens
shell on memory consolidation deficit in adult male Wistar rats: an
isobologram analysis**

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Abstract

The nucleus accumbens (NAc) glutamatergic and GABAergic systems are involved in memory processes. This study was investigated the involvement of NAc shell GABAergic system on D-AP5 induced memory consolidation deficit. The elevated plus-maze (EPM) test-retest paradigm was employed to assess memory in adult male Wistar rats. The results indicated that post-training intra-NAc shell injection of bicuculline (GABA_A receptor antagonist) did not alter emotional memory consolidation. However, post-training intra-NAc shell microinjection of muscimol (GABA_A receptor agonist, 0.1 µg/rat) and D-AP5 (a competitive NMDA receptor antagonist, 4 µg/rat) decreased emotional memory consolidation, suggesting the drugs induced amnesia. Moreover, a sub-threshold dose of muscimol (0.05 µg/rat) potentiated the D-AP5 (2 µg/rat) response on memory consolidation impairment. On the other hand, the middle dose of bicuculline (0.25 µg/rat) reversed memory impairment induced by D-AP5 at the higher dose. Interestingly, there is a synergistic effect between D-AP5 and muscimol on impairment of emotional memory consolidation. None of the above doses changed the locomotor activity. Our results suggest that the glutamatergic and GABAergic neurons of the NAc shell interact with each other for modulation of emotional memory consolidation.

Keywords: GABA_A receptor, D-AP5, NMDA, the NAc-shell, EPM apparatus, emotional memory consolidation

Introduction

The nucleus accumbens (NAc) is a main structure of the ventral striatum [Rasekhi, Oryan, Nasehi, and Zarrindast; 2014], which comprises of two anatomical and functional divisions, the shell and core [Alcaro, Huber, and Panksepp; 2007]. In the NAc, information is conducted from the shell to the core [Sturm, Lenartz, Koulousakis, Treuer, Herholz, Klein, and Klosterkotter; 2003]. The NAc is a vital brain structure involved in motor function, emotional and motivational aspects of behavior, and some aspects of learning and memory [Beiderbeck, Reber, Havasi, Bredewold, Veenema, and Neumann; 2012; Cardinal, Parkinson, Hall, and Everitt; 2002; Groenewegen and Trimble; 2007]. Anatomical evidences have supported the idea that shell division of the NAc is a site of convergence for neural inputs from some brain structures including prefrontal cortex (PFC), amygdala complex and hippocampal formation [Groenewegen, Wright, Beijer, and Voorn; 1999; O'Donnell and Grace; 1995]. Most of the NAc inputs are glutamatergic, which arise from the hippocampal formation, basolateral amygdale (BLA) and PFC [Groenewegen et al.; 1999]. Also, the NAc contains a large number of efferent GABAergic medium spiny neurons (approximately 95% of neurons in the NAc shell) [Heimer, Zahm, Churchill, Kalivas, and Wohltmann; 1991; Shirayama and Chaki; 2006].

N-methyl-d-aspartate (NMDA) subtype of ionotropic glutamate receptors plays a key role in the regulation of synaptic plasticity, learning, and memory processes [Khakpai, Nasehi, Haeri-Rohani, Eidi, and Zarrindast; 2012; Khakpai, Nasehi, and Zarrindast; 2016; Khakpai, Zarrindast, Nasehi, Haeri-Rohani, and Eidi; 2013; Nasehi, Tabatabaie, Khakpai, and Zarrindast; 2015]. It has been revealed that stimulation of both NMDA and AMPA receptors of the NAc are implicated in short-term spatial learning [Ferretti, Sargolini, Oliverio, Mele, and Roullet; 2007]. Furthermore, γ -amino butyric acid (GABA) is the key inhibitory neurotransmitter in the CNS, which influences learning and memory processes [Jafari-Sabet and Jannat-Dastjerdi; 2009; Parsaei, Rangchiyan, Ahmadi, and Zarrindast; 2011]. GABA exerts its action by binding to specific membrane receptors which are subdivided into three main classes: GABA_A, GABA_B and GABA_C receptors [Bormann; 2000; Semyanov and Kullmann; 2002]. The GABA_A and GABA_C receptors are coupled with Cl⁻ channels [Bormann; 2000; McDonald and Moss; 1994], whereas the GABA_B receptors are coupled with G-protein and connected to K⁺ channels [Bowery; 1993; Thompson; 1994]. A wealth of additional evidence has further implicated the existence of synaptic contacts between the glutamatergic and GABAergic neurons in the NAc shell [Heysieattalab, Naghdi, Zarrindast, Haghparast, Mehr, and Khoshbouei; 2015; Rinaldi, Oliverio, and Mele; 2012]. So, the present study builds upon these reports

to test the hypothesis that pharmacological manipulation of NMDA receptors (NMDARs) and GABA_A receptors (GABA_ARs) in the shell region of NAc would improve emotional memory and alleviate D-AP5-induced memory impairment in rats.

2. Experimental Procedures

2.1. Animals

Male Wistar rats (weighing 180–200 g) obtained from the department of pharmacology (Tehran University of Medical Sciences, Iran). They were housed four per cage in a controlled temperature (23 ± 1 °C) room with a 12-h light: 12-h dark cycle (lights on 07:00 h) and with free access to food and water. Rats were allowed to adapt to the laboratory conditions for at least 1 week prior to surgery. Each animal was handled for about 3 min each day before behavioral testing. All experiments were carried out during the light phase of the cycle between 9:00 h and 12:00 h and each rat was tested only once. Eight animals were used in each experimental group. The present study was approved by the Ethics Committee of the Faculty of Science of the University of Tehran which corresponds to the national guidelines for animal care and use.

2.2. Surgery

In the current study intra-peritoneal (i.p.) injections of ketamine hydrochloride (50 mg/kg) plus xylazine (5 mg/kg) used for animal anesthesia. Then, animals positioned in a stereotaxic apparatus (Stoelting Co, Illinois, USA). A midline incision was made in the skin of skull, and the underlying periosteum was cleaned. A stainless steel guide cannulae (22 gauge) was implanted (bilaterally) 2 mm above the NAc Shell region according to the respective coordinates in rats' brain stereotaxic atlas [Paxinos and Watson; 2007]. Stereotaxic coordinates for the NAc shell were +0.8 to + 1.2 mm (depending on body weight) posterior to bregma, ± 0.8 to ± 1.0 mm lateral to the midline and -4 to -5.3 mm ventral to the dorsal surface of the skull. Inserted cannulae were secured to the skull with dental acrylic cement. Two styled (27-gauge) were inserted into the guide cannula to prevent possible obstruction. All rats were allowed about a week to recover from surgery and the effect of the anesthetic agents.

2.3. Drug microinjections

Microinjections were performed via the indwelled cannula (27-gauge, Supa; Iran), terminating 2 mm under the ventral tip of the guide cannulae, connected to a Hamilton microsyringe through polyethylene tubing (2.5 ml). Intra-NAc shell infusions were carried out about 60 s per side, but the infusion cannula was left in the place for an additional 60 s to facilitate diffusion of the drugs from the tip of

the infusion cannula. The interval between two infusions was 5 min [Ahmadi, Nasehi, Rostami, and Zarrindast; 2013; Rasekhi et al.; 2014]. The injection time of drugs and selected drug doses used in the experiments were chosen according to the pilot and published work in scientific literature [Khakpai et al.; 2012; Yousefi, Nasehi, Khakpai, and Zarrindast; 2012].

2.4. Behavioral test (elevated plus-maze)

The elevated plus-maze (EPM) apparatus made of four arms, two of them had no side or end walls (open arms; 50 cm × 10 cm). The other two arms had side walls and end walls, but were open on the top (closed arms; 50 cm × 10 cm × 40 cm). Where the four arms of the apparatus intersected, there was a square platform measuring 10 cm × 10 cm. The experiments were performed in a quiet room with low (40 lux) illumination conditions.

In the EPM apparatus, the memory acquired upon the first exposure associates with an anxiety-related emotional state. The behaviors detected during the test include a tension between motivation to explore the maze and the natural tendency to avoid open spaces [Bertoglio and Carobrez; 2000; Lister; 1987]. After exploration of the EPM apparatus, rats learn, consolidate, and recall the memory related to the exploration of potentially dangerous spaces of the maze [Bertoglio and Carobrez; 2000; Chegini, Nasehi, and Zarrindast; 2014; Galvis-Alonso,

Garcia, Orejarena, Lamprea, Botelho, Conde, Morato, and Garcia-Cairasco; 2010]. The EPM test is proposed to be a simple method for the examination of learning and emotional memory processes.

5-7 days after surgery, the effects of intra-NAc shell injections of the drugs were tested in the EPM apparatus. The training protocol was based on our previous researches [Zarrindast, Piri, Nasehi, and Ebrahimi-Ghiri; 2012]. Animals were allowed to habituate to the experimental room for at least 1 h previous the experiments. Subjects were randomly assigned to treatment conditions and tested in a counter-balanced order. Animals' behaviors were recorded through means of a digital camera with a monitor and a recording system installed in a next room. This allowed the researcher to track the number of entries into the open or closed arms of the EPM apparatus and the total time spent in each arm. In the test sessions, subjects were individually located in the center of the maze facing the open arm and were allowed 5 min to freely explore the EPM apparatus. Then, the rats were taken back to their home cages.

Twenty-four hours after the training, animals were returned to the test room and located again in the EPM apparatus for a new exploration period of 5 min (retest session). The observer recorded the following: (a) time spent in the open arms, (b) time spent in the closed arms, and (c) number of entries into the closed and opened arms during a 5 min period in both test and retest sessions. Entries

were defined as putting “all four paws in an arm”, and measured via a manual counter. Between the EPM sessions and after each experiment, the apparatus was cleaned with a 5% alcohol, distilled water, and a paper towel. The obtained data were used to analyze: (a) %open-arm time (OAT, the ratio of time spent in the open arms to the time spent in all arms $\times 100$); (b) %open-arm entry (OAE, the ratio of entries into the open arms to total entries $\times 100$); and (c) the sum of closed-arm entries which was used as an index of general locomotor activity [Chegini et al.; 2014; Khakpai; 2014; Zarrindast et al.; 2012].

The EPM test is proposed to be a simple method for the examination of learning and emotional memory processes [Khodayar, Oryan, Nasehi, and Zarrindast; 2016]. In memory studies, occurrence of memory consolidation during post-training periods has been well documented [Abel and Lattal; 2001]. Upon this model and previous study, drugs were administrated immediately after training session (test day) and memory consolidation recorded after 24 h (retest day) [Khodayar et al.; 2016]. In the EPM apparatus, the memory acquired upon the first exposure associates with an anxiety-related emotional state. The behaviors detected during the test include a tension between motivation to explore the maze and the natural tendency to avoid open spaces [Bertoglio and Carobrez; 2000; Lister; 1987]. After exploration of the EPM apparatus, rats learn, consolidate, and recall the memory related to the exploration of potentially dangerous spaces of the

maze [Bertoglio and Carobrez; 2000; Chegini et al.; 2014; Galvis-Alonso et al.; 2010].

2.5. Drugs

Drugs used in the present study were D-AP5 (as selective NMDA receptor antagonist; Tocris Cookson, Bristol, UK), muscimol (Tocris, Bristol, UK) and bicuculline (Tocris, Bristol, UK). We administrated ketamine plus xylazine for induction and maintenance of anesthesia during the surgical procedures (Alfasan Chemical Co, Woerden, and Holland). D-AP5 and muscimol were dissolved in the sterile 0.9% saline while bicuculline was dissolved in vehicle (The vehicle was one drop of glacial acetic acid from Hamilton microsyringe, made up to a volume of 5 ml with sterile 0.9% saline, then diluted to the required volume), just before the experiment.

2.6. Statistical analysis

Since the data indicated normality of distribution and homogeneity of variance, the results were statistically evaluated using one-way and two-way analysis of variance (ANOVA). One-way ANOVA was used to analyze the individual effects of post-training intra-NAc injection of D-AP5, muscimol and bicuculline. Two-way ANOVA was used to analyze interaction between the drugs.

Following a significant F value, further analyses for paired-group comparisons were performed using the post-hoc Tukey's test. In all comparisons, P less than 0.05 showed statistical significance.

In addition, isobolographic analysis was done to evaluate the interactions following administration of the two drugs [Naseri, Hesami-Tackallou, Torabi-Nami, Zarrindast, and Nasehi; 2014]. The ED50 of each drug (1 µg/rat for D-AP5 and 0.025 µg/rat for muscimol) was calculated by linear regression analysis and combination of the two drugs was administrated in a constant dose ratio based on ED50 values. For drug combinations, the theoretic ED50 is $D\text{-AP5 ED50}/2 + \text{muscimol ED50}/2$. Moreover, experimental values of drug combinations from fixed ratio-calculated were analyzed by the regression analysis, after which the experimental ED50 value of the drug combinations was determined (%50 OAT). The statistical significance of the difference between the theoretical ED50 and experimental ED50 of the drug combinations was determined by the one-sample t-test. When the experimental ED50 was significantly lower than the theoretical ED50 a synergistic interaction between D-AP5 and muscimol could be inferred, but there was not any difference between them displaying additive interaction rather than synergistic effect [Naseri et al.; 2014]. Differences with $P < 0.05$ between the experimental groups at each point were showed statistically significant. Statistical analysis was performed by SPSS software ver. 19.

2.7. Experimental design

Eight rats were used in each experimental group and each rat was only tested once. Bilateral intra-NAc shell administrations of drugs were in a volume of 0.6 µl/rat (0.3 µl/each side). Immediately after the training session, rats received one or two injections. In experiments that rat received two injections, firstly D-AP5 injected then bicuculline or muscimol administrated. Interval time between two injections was 5 min. The Control groups received saline or vehicle (0.6 µl/rat, bilaterally). The protocol has been explained in Table 1.

[Table 1 here]

2.7.1. Experiment 1

This experiment examined the effect of post-training intra-NAc shell injection of GABA_ARs drugs on emotional memory consolidation and locomotor activity. Animals received post-training administration of saline (0.6 µl/rat), muscimol (0.025, 0.05, and 0.1 µg/rat), vehicle (0.6 µl/rat), or bicuculline (0.125, 0.25, and 0.5 µg/mouse) just after training, and were then retested in the EPM apparatus 24 h later undrugged.

2.7.2. Experiment 2

Experiment 2 evaluated the effects of post-training intra-NAc shell administration of D-AP5 in the presence or absence of muscimol or bicuculline on emotional memory consolidation and locomotor activity. Twelve groups of animals were used in three sets of four groups. They received post-training intra-NAc shell injection of saline (0.6 µl/rat) or different doses of D-AP5 (1, 2 and 4 µl/rat; four groups) just after training. These rats received post-training intra-NAc shell administration of saline (0.6 µl/rat), vehicle (0.6 µl/rat), sub-threshold dose of muscimol (0.05 µg/rat; four groups) or bicuculline (0.25 µg/rat; four groups) just after training. Treated groups were retested in the EPM apparatus 24 h later undrugged.

2.7.3. Experiment 3

To confirm whether D-AP5 and muscimol administration would exert additive or synergistic effects on their induced memory consolidation deficit, the isobolographic analysis was performed to compare the theoretical and experimental ED50 of the drugs when injected together. According to the dose–response curve of D-AP5 and muscimol, animals received administration of D-AP5 1 µl/rat + muscimol 0.025 µl/rat, D-AP5 0.05 µl/rat + muscimol 0.0125 µl/rat and D-AP5 0.025 µl/rat + muscimol 0.00625 µl/rat, immediately after training [Naseri et al.; 2014].

2.8. Histology

After the testing sessions each rat was deeply anesthetized and 0.3 μ l/site of a 4% methylene-blue solution was injected into the NAc shell, as explained in the drug section, then decapitated and its brain removed and placed in formaldehyde solution (10%). After 5-7 days, the brains were sliced and the sites of injections were confirmed according to the atlas of Paxinos and Watson [Paxinos and Watson; 2007] (fig 1). Cannulae were implanted into the NAc shell of a total of 200 animals, but only the data from 184 animals with correct cannulae implants were used in statistical analyses.

[Fig.1 here]

3. Results

3.1. Effects of post-training intra-NAc shell administration of GABA_AR drugs on emotional memory consolidation and locomotor activity

Fig. 2 shows the effects of post-training intra-NAc shell injection of muscimol and bicuculline on emotional memory consolidation and locomotor activity. One-way ANOVA displayed that post-training intra-NAc shell administration of muscimol increased the %OAT [$F(4, 35)=5.234$, $P<0.01$] and %OAE [$F(4, 35)=5.840$, $P<0.01$], but did not alter locomotor activity [$F(4,$

35)=0.326, $P>0.05$] in the retest trial. Post-hoc analyses showed that muscimol at the dose of 0.1 $\mu\text{g}/\text{rat}$ impaired emotional memory consolidation (Fig. 2A, B and C; left panels). On the other hand, bicuculline did not alter the %OAT [$F(4, 35)=0.951$, $P>0.05$], %OAE [$F(4, 35)=1.005$, $P>0.05$] and locomotor activity [$F(4, 35)=0.310$, $P>0.05$] in the retest trial, showing that the drug had no significant effect on consolidation of emotional memory (Fig. 2A, B and C; right panels).

[Fig.2 here]

3.2. Effects of post-training intra-NAc shell administration of GABA_AR drugs on the D-AP5 induced emotional memory consolidation deficit and locomotor activity

In Fig. 3 is seen the effects of muscimol and bicuculline on amnesia induced by D-AP5. One-way ANOVA showed that D-AP5 increased the %OAT [$F(4, 35)=12.104$, $P<0.001$] and %OAE [$F(4, 35)=10.001$, $P<0.001$], but did not change locomotor activity [$F(4, 35)=0.458$, $P>0.05$] compared with the control group in the retest trial, indicating that the drug impaired consolidation of emotional memory (Fig. 3A, B and C; left panels).

Furthermore, two-way ANOVA revealed that a sub-threshold dose of muscimol (0.05 $\mu\text{g}/\text{rat}$) potentiated memory consolidation impairment produced by D-AP5 (2 $\mu\text{g}/\text{rat}$). This intervention increased the %OAT [dose effect: $F(3, 56)=$

11.509, $P < 0.001$; drug effect: $F(1, 56) = 12.978$, $P < 0.001$; dose–drug interaction: $F(3, 56) = 5.206$, $P < 0.01$] but did not change %OAE [dose effect: $F(3, 56) = 0.311$, $P > 0.05$; drug effect: $F(1, 56) = 0.145$, $P > 0.05$; dose–drug interaction: $F(3, 56) = 0.202$, $P > 0.05$] and locomotor activity [dose effect: $F(3, 56) = 0.221$, $P > 0.05$; drug effect: $F(1, 56) = 0.402$, $P > 0.05$; dose–drug interaction: $F(3, 56) = 0.188$, $P > 0.05$] in the retest trial (Fig. 3A, B and C; middle panels).

In addition, two-way ANOVA displayed that non-effect dose of bicuculline (0.25 $\mu\text{g}/\text{rat}$) reversed memory consolidation impairment induced by D-AP5 at the higher dose (4 $\mu\text{g}/\text{rat}$). This intervention decreased the %OAT [dose effect: $F(3, 56) = 19.869$, $P < 0.001$; drug effect: $F(1, 56) = 7.623$, $P < 0.05$; dose–drug interaction: $F(3, 56) = 8.582$, $P < 0.01$] and %OAE [dose effect: $F(3, 56) = 6.102$, $P < 0.01$; drug effect: $F(1, 56) = 5.016$, $P < 0.05$; dose–drug interaction: $F(3, 56) = 1.967$, $P < 0.05$], but not altering locomotor activity [dose effect: $F(3, 56) = 0.381$, $P > 0.05$; drug effect: $F(1, 56) = 0.163$, $P > 0.05$; dose–drug interaction: $F(3, 56) = 0.899$, $P > 0.05$] in the retest trial (Fig. 3A, B and C; right panels).

[Fig.3 here]

3.3. The synergistic effect between D-AP5 and muscimol on emotional memory consolidation deficit

The theoretical additive line revealed that at all points, D-AP5 and muscimol combination produced an effect of theoretical %50 OAT (theoretical ED50) according to an additive interaction (Fig. 4). One sample t-test indicted that there is a significant difference between experimental ED50 and theoretical ED50. Our data suggested a synergistic effect of D-AP5 and muscimol administration upon induction of emotional memory consolidation deficit (Fig. 4).

[Fig.4 here]

4. Discussion

Growing of researches revealed that injection of GABAergic agents affect learning and memory retention [Azizbeigi, Zarrindast, and Ahmadi; 2013; Chapouthier and Venault; 2002; Heysieattalab et al.; 2015; Nakagawa and Iwasaki; 1995; Zarrindast, Shamsi, Azarmina, Rostami, and Shafaghi; 2004]. According to investigation, the GABA_ARs agonist muscimol impairs, whereas their antagonist bicuculline improves memory performance of behavioral tasks [Chapouthier and Venault; 2002; Farr, Flood, and Morley; 2000; Ramirez, Buzzetti, and Savage; 2005; Yousefi et al.; 2012; Zarrindast, Noorbakhshnia, Motamedi, Haeri-Rohani, and Rezayof; 2006]. The result of this study showed that post-training intra-NAc shell administration of muscimol (0.1 µg/rat) decreased emotional memory consolidation but had no significant effect on locomotor activity. It is well-known

that GABA is one of the important inhibitory neurotransmitters in the CNS. In a cell, GABA opens chloride channels via activating its GABA_ARs and so hyperpolarizes the cell [Barnard, Skolnick, Olsen, Mohler, Sieghart, Biggio, Braestrup, Bateson, and Langer; 1998; Costa; 1998]. It appears that muscimol opens chloride channels through activating GABA_ARs and hyperpolarizes the NAc shell neurons, hence impaired emotional memory consolidation. Furthermore, our data indicated that post-training intra-NAc shell injection of bicuculline did not change emotional memory consolidation and locomotor activity. In agreement with our results, some investigations have shown that injection of bicuculline in to the NAc [Azizbeigi et al.; 2013], CA1 [Yousefi, Farjad, Nasehi, and Zarrindast; 2013], BLA [Kangarlu-Haghighi, Oryan, Nasehi, and Zarrindast; 2015], nucleus basalis magnocellularis [Moron, Ramirez-Lugo, Ballesteros, Gutierrez, Miranda, Gallo, and Bermudez-Rattoni; 2002], and posterior striatum [Brioni, Decker, Gamboa, Izquierdo, and McGaugh; 1990] did not change memory. However, other investigators have indicated that GABA_AR blockade in the hippocampus, entorhinal cortex and parietal cortex with bicuculline enhanced memory [Kim, Kim, Park, Cai, Liu, Lee, Shin, and Ryu; 2012; Luft, Pereira, Cammarota, and Izquierdo; 2004; Yousefi et al.; 2012]. On the other hand, Chavez and coworkers (1995) have reported that intra-striatal administration of bicuculline impaired memory consolidation [Chavez, Salado-Castillo, Sanchez-Alavez, Quirarte, and

Prado-Alcala; 1995]. The contradictory reports may result from diverse locations of injections and local functional heterogeneity of GABAergic interneurons in the diverse parts of the striatum (dorsal versus ventral) [Girault, Spampinato, Savaki, Glowinski, and Besson; 1986].

Moreover, the results of current study indicated that post-training intra-NAc shell injection of D-AP5 (4 μ g/rat) decreased emotional memory consolidation without affecting locomotor activity. As mentioned above, the NAc shell is a main brain area mediating emotional and motivational learning [Cardinal and Everitt; 2004; Kelley; 2004]. In rodent models, dynamic variations have been detected in the synaptic NMDARs within the NAc following incentive stimuli, and some of these variations are important for acquiring new emotional/motivational states. Regarding as plasticity triggers, the synaptic NMDARs within the NAc mainly contribute to the acquisition of new physiological and pathophysiological emotional and motivational responses [Huang, Ishikawa, Lee, Nakanishi, Schluter, and Dong; 2011; Kelley, Smith-Roe, and Holahan; 1997]. Recently, Lee and Everitt (2008) reported that the NMDARs are also involved in modulation of emotional memory reconsolidation [Lee and Everitt; 2008]. Akirav and Maroun (2006) also indicated that NMDARs antagonists affect memory reconsolidation differently when they are injected before or after memory reactivation [Akirav and Maroun; 2006]. In line with our results, several studies revealed that blockade of

the glutamate receptors in the NAc impairs spatial learning and memory [Ferretti et al.; 2007; Sargolini, Roullet, Oliverio, and Mele; 2003], short-term and long-term aversive taste memory [Ramirez-Lugo, Zavala-Vega, and Bermudez-Rattoni; 2006], and reconsolidation of drug-associated appetitive memory [Wu, Li, Gao, and Sui; 2012]. As mentioned before, the NAc receives converging input from the PFC, amygdala and hippocampus, which together transmit cognitive information [Rinaldi et al.; 2012; Sesack and Grace; 2010]. These glutamatergic inputs are believed to carry diverse emotional and motivational information and differentially affect the overall functional output of the NAc neurons [Huang et al.; 2011; Luscher and Malenka; 2011]. The NMDARs in the NAc shell are predominately expressed postsynaptically on the medium spiny neurons [Huang et al.; 2011]. Consequently, D-AP5 by inhibiting the postsynaptic NMDARs in the NAc shell impaired emotional memory consolidation. These results show that the NMDARs of the NAc shell have an important role in the emotional memory consolidation, but not locomotor activity.

In the next section of this paper, we found that post-training intra-NAc shell administration of D-AP5 (2 $\mu\text{g}/\text{rat}$) produced stronger impairments in the consolidation of emotional memory, when injected with a sub-threshold dose (0.05 $\mu\text{g}/\text{rat}$) of muscimol rather than alone injection, while its effect on locomotor activity were not significant. We also found significant effect of post-training intra-

NAc shell injection of D-AP5 (4 μ g/rat) before administration of the middle dose (0.25 μ g/rat) of bicuculline, suggesting that it affect the GABA_ARs of the NAc shell for modulation of memory consolidation but not locomotor activity. The activity of GABAergic medium spiny neurons in the NAc is prominently controlled through dopaminergic from the ventral tegmental area and glutamatergic afferents from the PFC, amygdala and hippocampus as well as GABAergic and cholinergic interneurons [Adermark, Clarke, Ericson, and Soderpalm; 2011; Heimer et al.; 1991; Koos and Tepper; 2002; Shirayama and Chaki; 2006]. In this regard, the results of our study support the proposed idea of the existence of synaptic connections between the glutamatergic terminals and GABAergic neurons in the NAc [Heysieattalab et al.; 2015; Rinaldi et al.; 2012]. These synaptic connections implicate that glutamate might exert its effects through medium-sized spiny GABA projection neurons [Heysieattalab et al.; 2015], and suggest that interaction between the glutamatergic and GABAergic neurons of the NAc shell may be involved in modulation of emotional memory consolidation. A possible mechanism may be that D-AP5 by the blockade of NMDARs, muscimol and bicuculline by activation and inhibition of the NAc shell GABA_ARs interact to each other for enhanced and weakened amnesia induced by D-AP5, respectively.

Furthermore, the obtained results displayed that there was an interaction between the NMDARs and GABA_ARs on emotional memory consolidation in the

NAc shell. More interestingly, we found the synergistic effect of D-AP5 and muscimol upon induction of memory impairment, by isobolographic analysis. As mentioned previously, synaptic connections between the glutamatergic and GABAergic neurons of the NAc shell [Heysieattalab et al.; 2015; Rinaldi et al.; 2012] may modulate emotional memory consolidation. A probable mechanism for memory impairment induced by co-administration of D-AP5 and muscimol might be due to the blockade of NMDARs and stimulation of GABA_ARs within the NAc shell which in turn intensely impaired consolidation of emotional memory. The similarity of NMDAR antagonist and GABA_AR agonist behavioral effects are intriguing given that these manipulations likely have similar neurochemical effects, so that isobolographic analysis reveal the synergistic effect of D-AP5 and muscimol on induction of emotional memory consolidation impairment. Consequently, the NAc shell glutamatergic and GABAergic neurons interact with each other for modulation of emotional memory consolidation.

5. Conclusion

These results provide the evidence of the NAc shell participation in the consolidation of emotional memory, but not locomotor activity through their GABA_ARs and NMDARs. Our results showed that the GABA_ARs activity of the NAc shell involved in the consolidation of emotional memory, probably through

their glutamatergic projections coming from the PFC, amygdala and hippocampus [Adermark et al.; 2011; Heimer et al.; 1991; Koos and Tepper; 2002; Shirayama and Chaki; 2006]. An interesting fact about the current results is that the synergistic effect between D-AP5 and muscimol upon induction of memory deficit. However, more experiments are required to determine the exact function of the glutamatergic and GABAergic neurons in the modulation of emotional memory consolidation.

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Legends

Table 1 explains experiments, groups of animals and doses of drugs.

Fig. 1. Approximate location of the injection cannulae tips in the NAc shell for all rats included in the data analyses was taken from the atlas of Paxinos and Watson [Paxinos and Watson; 2007].

Fig. 2. The effects of post-training intra-NAc shell injection of saline, muscimol, vehicle and bicuculline on emotional memory consolidation and locomotor activity. One group received post-training intra-NAc shell administration of saline (0.6 μ l/rat), three groups received muscimol at different doses (0.025, 0.05 and 0.1 μ g/rat, right panel), one group received vehicle (0.6 μ l/rat) and the other three groups received bicuculline at different doses (0.125, 0.25 and 0.5 μ g/rat, left panel), just after training. After 24 h, all groups were retested in the EPM apparatus undrugged. Each bar indicates mean $\pm$ SEM of eight rats per group. *P < 0.05 when compared to the control group in trial 1. ++P < 0.01 when compared to the control group (saline) in trial 2. %OAT (A), %OAE (B) or locomotor activity (C). EPM: elevated plus maze; NAc: nucleus accumbens.

Fig. 3. The effects of post-training intra-NAc shell injection of D-AP5 alone or in combination with muscimol or bicuculline on emotional memory consolidation and locomotor activity. Rats were injected with saline (0.6 μ l/rat), D-AP5 (1, 2 and 4 μ g/rat, left panel) alone or together with muscimol (0.05 μ g/rat, middle panel) or bicuculline (0.25 μ g/rat, right panel), just after training. After 24 h, all groups were retested in the EPM apparatus undrugged. Each bar represents mean $\pm$ SEM of eight rats per group. *P < 0.05 and **P < 0.01 when compared to the control group in trial 1. ++P < 0.01 and +++P < 0.001 when compared to the control group (saline) in trial 2. YYP < 0.01 when compared to the respective group in panel 1. $\Phi\Phi\Phi$ P < 0.001 when compared to the respective group in panel 1. %OAT (A), %OAE (B) or locomotor activity (C). EPM: elevated plus maze; NAc: nucleus accumbens.

Fig. 4. The isobologram analysis of the effects of drugs administration reveals the synergistic effect of intra-NAc shell injection of D-AP5 and muscimol to induce emotional memory consolidation deficit. The foci A and B mean indicate the ED50 of D-AP5 and muscimol, respectively. In Brief, the ED50 of each drug (1 μ g/rat for D-AP5 and 0.025 μ g/rat for muscimol) was calculated by linear regression analysis. The oblique line between A and B is the theoretic additive

effect line of D-AP5 and muscimol administrations. The focus C, in the middle of line, is the theoretical ED50 of the drug combination, which is measured from the individual drug ED50. The focus D, far below the line, is the experimental ED50 of drugs combination, which is actually perceived after drugs administration. Statistical analysis displayed that there is significant difference between experimental ED50 and theoretical ED50 points, suggesting a synergistic effect of the drugs injection (for more details see the statistical analysis section). ED50, effective dose 50.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1

The table explains experiments, groups of animals and doses of drugs.

Experiment	Figure	Post-training treatment (intra-NAc shell)	%OAT (panel A)	%OAE (panel B)	locomotor activity (panel C)
1	Left	Muscimol (Saline, 0.025, 0.05 and 0.1 µg/rat)	Increase	Increase	No effect
	Right	Bicuculline (Vehicle, 0.125, 0.25 and 0.5 µg/rat)	No effect	No effect	No effect
2	Left	D-AP5 (Saline, 1, 2 and 4 µg/rat)	Increase	Increase	No effect
	Middle	Muscimol (0.05 µg/rat)	Increase	No effect	No effect
	Right	Bicuculline (0.25 µg/rat)	Decrease	Decrease	No effect