Activation of the ventral tegmental area nitric oxide system potentiates nicotine reversal effects on ethanol amnesia

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Abstract

The possible involvement of nitric oxide (NO) systems in the ventral tegmental area (VTA) in nicotine's reversal effect on ethanol-induced amnesia and ethanol state-dependent memory in adult male Wistar rats was investigated. The animals were bilaterally implanted with chronic cannulae in the VTA. Animals were trained in a step-through type inhibitory avoidance task, and tested 24 h after training to measure step-through latency as memory retrieval. We found that pre-training or pre-test intraperitoneal (i.p.) injection of ethanol (0.8 g/kg) induced amnesia. The pre-test administration of ethanol (0.4 and 0.8 g/kg) reversed the amnesia induced by pre-training ethanol (0.8 g/kg), indicating a state-dependent effect. Similar to ethanol, pre-test intra-VTA injection of nicotine (0.3 and 0.6 µg/rat) alone or nicotine (0.1, 0.3 and 0.6 µg/rat, intra-VTA) plus an ineffective dose of ethanol (0.2 g/kg) also significantly reversed the ethanol amnesia. Ethanol induced amnesia was also reversed by pre-test intra-VTA microinjection of L-arginine (0.4 µg/rat), a nitric oxide precursor. Interestingly, co-administration of L-arginine (0.2 and 0.4 µg/rat, intra-VTA) with an ineffective dose of nicotine (0.1 µg/rat, intra-VTA) significantly potentiated the memory-improving effect of nicotine on ethanol amnesia. In contrast, pre-test intra-VTA administration of L-NAME, a nitric oxide synthase inhibitor blocked the reversal effect of nicotine or nicotine plus L-arginine on ethanol amnesia. These results suggest that the VTA NO system(s) may potentially play an important role in reversal effect of nicotine on ethanol amnesia via dopamine release in the target areas of mesolimbic dopamine pathway originating from the VTA.

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Introduction
Tobacco smoking is often accompanied by the use of alcohol. The vast majority of alcoholics are heavy smokers (Batel et al., 1995). Both ethanol and nicotine has an important role in reward and reward related learning and can be activated mesocorticolimbic dopaminergic pathway (Larsson and Engel, 2004). The involvement of dopaminergic and glutamatergic systems in the improving effect of nicotine on amnesia induced by ethanol has also been shown in previous studies (Rezayof et al., 2007; Rezayof et al., 2008b). Yet, the involvement of other neurotransmitters in the effect of nicotine on amnesia induced by ethanol cannot be excluded. Considering that nicotine increases the glutamatergic synaptic transmission in the ventral tegmental area (VTA) by activation of pre-synaptic nicotinic receptors (Wonnacott, 1997) and nitric oxide (NO) is generated after the activation of the NMDA receptor (Moncada et al., 1991), the aim of the present research was to investigate the role of NO system of the VTA in nicotine’s effects on memory impairment induced by ethanol administration.

Modulation of learning and memory processes by ethanol has been demonstrated in previous studies (Raoufi et al., 2012; Rezayof et al., 2008a; Rezayof et al., 2010a; Rezayof et al., 2010b). The effects of ethanol on memory depend on the timing of drug administration (Rezayof et al., 2008a; Rezayof et al., 2008b; Rezayof et al., 2010b). Pre-training ethanol impairs memory in different models of learning such as spatial memory (Berry and Matthews, 2004; Chin et al., 2011), working memory (Melchior et al., 1993), and inhibitory avoidance memory (Melchior et al., 1993; Raoufi et al., 2012; Rezayof et al., 2007), while pre-test ethanol facilitates memory retrieval in amnesia induced by pre-training administration of ethanol (Melchior et al., 1993; Raoufi et al., 2012; Rezayof et al., 2008b). This phenomenon is known as ethanol state-dependent learning and it has been proposed that it is time- and dose-dependent (Raoufi et al., 2012; Rezayof et al., 2008b; Rezayof et al., 2010b). “Drug-induced state-dependent learning” is used to describe the finding that behavior learned in one drug state is better remembered when retention is tested in the same drug state (Carlezon et al., 1995; Colpaert, 1990).

The ethanol-nicotine interactions on learning and memory have also been demonstrated by some investigators (Gould and Lommock, 2003; Raoufi et al., 2012; Rezayof et al., 2008a; Rezani and Levin, 2002). Both ethanol and nicotine can activate mesocorticolimbic dopamine system which projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), hippocampus and prefrontal cortex. The system has been shown to be involved in reward, attention, motivation and learning (Ikemoto and Panksepp, 1999). There is also evidence, indicating that activation of the functional loop between the dopaminergic neurons of VTA and hippocampus, facilitates the induction of long-term potentiation (LTP) and enhances learning (Lisman and Grace, 2005). We have also recently shown that pre-test administration of nicotine was able to reverse memory impairment induced by pre-training ethanol in inhibitory avoidance task (Raoufi et al., 2012; Rezayof et al., 2008a). However, pre-test nicotine similar to ethanol reverses memory impairment induced by pre-training ethanol, but the drugs have some opposite effects on other cognitive functions (Dawson, 2000; Raoufi et al., 2012; Rezayof et al., 2010a). Nicotine, unlike ethanol enhances learning through a direct effect on attention or through interacting with pre-synaptic nicotinic acetylcholine receptors (nAChR). Nicotine facilitate the release of many neurotransmitters such as acetylcholine, glutamate, dopamine, norepinephrine, serotonin and y-aminobutryic acid (GABA), all of which are critical to normal learning and memory function (Piri and Zarrindast, 2011; Raoufi et al., 2012; Rezayof et al., 2007; Rezayof et al., 2008b). Since ethanol and nicotine have some opposite effects on cognitive functions (Dawson, 2000; Raoufi et al., 2012; Rezayof et al., 2008a; Rezayof et al., 2007; Rezayof et al., 2008b), the interaction between them is complex and not fully understood yet.

On the other hand, Nitric oxide synthase (NOS)
immunoreactivity has been detected in the ventral tegmental area (Piri and Zarrindast, 2011). Nitric oxide (NO) system has also been shown to play a central role in the regulation of learning and memory. The importance of NO in synaptic plasticity and long-term potentiation has been postulated (Kantor et al., 1996; O’Dell et al., 1994; Wilson et al., 1997). It has been shown that the dopaminergic behavior of ethanol and nicotine may be mediated partially via activation of the NO system (Piri and Zarrindast, 2011; Raoufi et al., 2012; Zarrindast et al., 2012). Moreover, evidence suggests that NO is a retrograde messenger that signals to pre-synaptic neurons, causing an increase in the release of dopamine (Pudiak and Bozarth, 1993). Therefore, NO may be a neuronal messenger mediating the release of dopamine in the target area of the mesolimbic dopamine pathways such as NAc and hippocampus. Furthermore, several lines of evidence indicate that some of the behavioral effects of nicotine and ethanol, such as memory are mediated by NO (Piri and Zarrindast, 2011; Raoufi et al., 2012; Rezayof et al., 2010b; Sahraei et al., 2004; Zarrindast et al., 2012). Rezayof and coworkers (Rezayof et al., 2010b) reported that ethanol state-dependent memory can be modulated through the dorsal hippocampal nitric oxide system. Our recent work shows that nicotine improves ethanol-induced memory impairment, at least partly via the NO system(s) in the dorsal hippocampus (Raoufi et al., 2012). Our results have shown that intra-CA1 microinjection of L-arginine potentiated the improving effect of nicotine on ethanol-induced amnesia while injection of L-NAME in to the CA1 regions of the dorsal hippocampus prevented the improving effect of nicotine on memory impairment by pretreatment ethanol (Raoufi et al., 2012).

It seems that the nicotinic acetylcholine receptor and nitric oxide within the VTA have an important role in the acquisition and expression of ethanol reward and also reward-related learning (McGehee et al., 1995; Narita et al., 2001). Considering the ethanol-nicotine interactions and NO-related modulation of ethanol and nicotine effects, the aim of the present study was to therefore evaluate the role of NO in the VTA on nicotine’s effects on memory impairment induced by ethanol in the inhibitory avoidance task.

Materials and methods

Animals

Adult male Wistar rats (Pasteur institute, Tehran, Iran) weighing 220–270 g at the time of surgery were used. They had free access to food and water, were housed four in a cage and kept at (22 ± 2) °C under a 12/12 h light-dark cycle (light beginning at 7:00 a.m). All experiments were carried out during the light phase between 8:00 and 14:00. Experimental groups consisted of eight animals and each animal was tested only once. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (NIH, publication no. 85–23, revised 1985; European Communities Directive 86/609/EEC) and approved by the local ethical committee.

Surgery

Animals were anesthetized intraperitoneally with a ketamine/ xylazine mixture (50 mg/kg ketamine, 5 mg/kg xylazine) and placed in a stereotaxic frame (David Kopf Instruments) in a flat-skin position (incisor bar -3.3 mm relative to the interaural line (Paxinos, 1997b). A midline incision was made and the skin and underlying periosteum retracted. Bilateral stainless steel guide cannulae (22 gauge) were implanted 2 mm above the VTA according to stereotaxic coordinates AP, -4.8 mm forward of bregma; L, ± 0.9 mm from midline; V, -6.8 mm relative to dura (Paxinos, 1997b) and anchored to the skull with dental cement Stainless steel stylets (27 gauge) were inserted into the guide cannulae to maintain patency. All animals were allowed 1week to recover from surgery and clear anesthetic.

Drugs and microinjections

The drugs included ethanol (Merck, Germany), L-NAME (NG-nitro-l-arginine methyl ester, a non-specific inhibitor of nitric oxide synthase), l-arginine (Sigma, St Louis, CA, USA) and nicotine hydrogen tartrate (Sigma, Poole, Dorset, UK). All drugs were dissolved in sterile saline except nicotine which was
dissolved in sterile saline and then the pH of the solution was adjusted to 7.2 with NaOH (0.1 normal solution). 0.8 g/kg dose of ethanol was made from a 12.6% ethanol in 0.9% saline (vol/vol) stock solution which was made freshly for each experiment and then diluted to the required concentration. Ethanol was injected intraperitoneally (i.p.) at a volume of 1 ml/kg. Bilateral microinfusions of L-arginine and L-NAME into the VTA (Intra-VTA) were in a volume of 0.6 µl (0.3 µl/side). Intra-VTA infusions were given by lowering a 27-gauge infusion cannula to extend 2 mm beyond the tip of the guide cannulae to the site of infusion. The infusion cannula was attached with a polyethylene tube to a 1 µl Hamilton syringe. Infusion (0.3 µl/side) were carried out over 60 s, first on one side then the other; the infusion cannula was left in place for an additional 30 s to facilitate diffusion of the drugs from the tip of the infusion cannula. Control animals received either saline or vehicle. In experiments where the animals received one or two injections, the control groups also received one or two saline or vehicle injections. The timing between injections and sequence of injection were selected on the basis of a pilot study and our previous studies (Azami et al., 2010; Nasehi et al., 2010; Piri and Zarrindast, 2011; Raoufi et al., 2012; Zarrindast et al., 2012).

Inhibitory avoidance apparatus
The step-through inhibitory avoidance apparatus consisted of two compartments of the same size (20 × 20 × 30 cm3). In the middle of a dividing wall, a guillotine door (7.9 cm2) could be lifted manually. The walls and the floor of one compartment consisted of white opaque resin and the walls of the other compartment were dark. Stainless steel bars (0.3 mm in diameter and 1 cm intervals) constituted the floor of the dark compartment. Intermittent electric shocks (50 Hz, 3 s, 1 mA intensity) were delivered to the grid floor of the dark compartment by an isolated stimulator.

Behavioral procedures
Training was based on our previous studies (Zarrindast et al., 2002; Zarrindast et al., 2005). All animals were allowed to habituate in the experimental room for at least 30 min prior to the experiments. Then, each animal was gently placed in the brightly lit compartment of the apparatus; after 5 s the guillotine door was opened and the animal was allowed to enter the dark compartment. The latency with which the animal crossed into the dark compartment was recorded. Animals that waited more than 100 s to cross to the dark compartment were eliminated from the experiments. Once the animal crossed with all four paws to the next compartment, the guillotine door was closed and the rat was taken into its home cage. The trial was repeated after 30 min as in the acquisition trial where after 5 s the guillotine door was opened and as soon as the animal crossed to the dark (shock) compartment the door was closed and a foot shock (50 Hz, 1 mA and 3 s) was immediately delivered to the grid floor of the dark room. After 20 s, the rat was removed from the apparatus and placed temporarily into its home cage. Two minutes later, the procedure was repeated; if the rat did not enter the dark compartment during 120 s, a successful acquisition of inhibitory avoidance response was recorded. Otherwise, when the rat entered the dark compartment (before 120 s) a second time, the door was closed and the animal received the same shock again. After retesting, if the rat acquired inhibitory avoidance successfully, it was removed from the apparatus and received post-training injection of morphine immediately.

Twenty-four hours after training, each animal was gently placed in the light compartment for 20 s, the door was opened, and the step through latency was measured for entering into the dark compartment. The testing session ended when the animal entered the dark compartment or remained in the light compartment for 300 s (criterion for retrieval). During testing sessions, no electric shock was applied.

Experimental design
Experiment 1: In this experiment, the effect of pre-training and pre-test administration of ethanol on inhibitory avoidance response was examined using
eight groups (n=8/group). Two groups received saline (1 ml/kg) before training (pre-training) and received either saline or ethanol (0.8 g/kg) before testing (pre-test) as control groups. The other three groups of animals received different doses (0.2, 0.4 and 0.8 g/kg, i.p.) of ethanol 30 min before training. On the test day, all of them received saline (1 ml/kg) 30 min before the test (pre-test). The other three groups of animals received pre-training (0.8 g/kg of ethanol) and pre-test injections of ethanol (0.2, 0.4 and 0.8 g/kg, i.p.) (Fig.1).

Experiment 2: In this experiment, the effects of pre-test administration of nicotine alone or in combination with ethanol on ethanol-induced amnesia was examined using twelve groups (n=8/group). Four groups of animals received pre-training saline (1 ml/kg, i.p.). On the test day, these groups of animals received saline (1 ml/kg) 30 min before testing plus different doses of nicotine (0, 0.1, 0.2 and 0.4 μg/rat, intra-VTA) 5 min prior to the test. Another eight groups received pre-training ethanol (0.8 g/kg). On the test day, four groups of these animals received saline (1 ml/kg) 30 min before testing plus different doses of nicotine (0, 0.1, 0.2 and 0.4 μg/rat, intra-VTA) 5 min prior to the test. The other four groups of these animals received ethanol (0.2 g/kg) 30 min before testing plus different doses of nicotine (0, 0.1, 0.2 and 0.4 μg/rat, intra-VTA) 5 min prior to the test (Fig. 2).

Experiment 3: In this experiment, the effects of pre-test administration of L-arginine alone or in combination with ethanol on ethanol-induced amnesia was examined using twelve groups (n=8/group). Four groups of animals received pre-training saline (1 ml/kg) and on the test day they received L-arginine (0, 0.1, 0.2 and 0.4 μg/rat, intra-VTA) and after 5 min, were injected with vehicle (0.6 μl/rat, intra-VTA). The other four groups of animals received L-arginine (0, 0.1, 0.2 and 0.4 μg/rat, intra-VTA) and after 5 min, were injected with nicotine (0.1 μg/rat, intra-VTA). Step-through latency was measured 5 min after last injections (Fig. 3).

Experiment 4: In this experiment, the effects of pre-test administration of L-NAME alone or in combination with nicotine on ethanol-induced amnesia was examined using eight groups (n=8/group). Four groups of animals received pre-training saline (1 ml/kg) and on the test day they received L-NAME (0, 0.05, 0.1 and 0.2 μg/rat, intra-VTA) and after 5 min, were injected with vehicle (0.6 μl/rat, intra-VTA). The other four groups of animals received a pre-training effective dose of ethanol (0.8 g/kg). On the test day, the animals received L-NAME (0, 0.05, 0.1 and 0.2 μg/rat, intra-VTA) and after 5 min, were injected with effective dose of nicotine (0.4 μg/rat, intra-VTA). Step-through latency was measured 5 min after last injection (Fig. 4).

Experiment 5: In this experiment, the effect of L-NAME on the L-arginine-induced potentiation of the nicotine response was assessed. In this experiment, on the training day, all animals (n=8/group) received pre-training administration of ethanol (0.8 g/kg, i.p.). On the testing day, four groups of the animals received a pre-test intra-VTA injection of L-NAME (0, 0.05, 0.1 and 0.2 μg/rat, intra-VTA). After 5 min, they were injected with L-arginine (0.2 μg/rat, intra-VTA) and 5 min later, they received nicotine (0.1 μg/rat, intra-VTA). Two control groups were also used in this experiment. The first group received three pre-test intra-VTA microinjections of saline (1 μl/rat) with 5 min interval. The second group received two pretest intra-VTA microinjections of saline (1 μl/rat) with 5 min interval, and after 5 min they were injected with nicotine (0.1 μg/rat, intra-VTA). The step-through latency was measured 5 min after the last injection (Fig. 5).

Histology
After the testing sessions, each rat was deeply anesthetized and 0.6 µl of a 4% methylene-blue...
solution was bilaterally infused into the VTA (0.3 µl/side), as described in the drug section. The rat was then decapitated and its brain was removed and placed in formaldehyde (10%). After several days, the brains were sliced and the sites of injections were verified according to Paxinos & Watson, 1997 (Paxinos, 1997a). Cannulae were implanted into the VTA of a total of 400 animals, but only the data from 376 animals with correct cannulae implants were included in the statistical analyses.

Data analysis

The data are expressed as mean ± S.E.M. The statistical analysis was performed using one-way and two-way analysis of variance (ANOVA). Post-hoc comparison of means was carried out using Tukey test for multiple comparisons, when appropriate. The level of statistical significance was set at P<0.05. Calculations were performed using SPSS statistical package.

Results

Effect of ethanol on inhibitory avoidance memory

Fig. 1 shows the effects of pre-training or pre-test administration of ethanol on step-through latency. One-way ANOVA revealed that pre-training or pre-test ethanol (0.8 g/kg, i.p.) impaired inhibitory avoidance memory on the test day, compared with saline-treated animals [F(4, 35) = 10.29, P<0.001]. In the other groups, pre-test ethanol (0.4 and 0.8 g/kg, i.p.) reversed amnesia induced by pre-training ethanol (0.8 g/kg, i.p.) [F(3, 28) = 7.58, P<0.001]. ***P<0.001, when compared to the pre-training saline/pre-test saline. +++P<0.001, +P<0.05, when compared to the pre-training ethanol (0 mg/kg)/pre-test ethanol (0.8 mg/kg).

Effects of pre-test nicotine administration with or without ethanol on inhibitory avoidance memory

Fig. 2 indicates the effects of pre-test injection of nicotine in the presence or absence of ethanol on inhibitory avoidance memory. Two-way ANOVA indicated an interaction between the groups of animals which received pre-training saline (1 ml/kg) and pre-test nicotine (0.1, 0.2 and 0.4 µg/rat, intra-VTA) and those which received pre-training ethanol (0.8 g/kg, i.p.) and pre-test nicotine (for Treatment, F(1,56) = 41.36, P<0.001; Dose, F(3,56) = 4.46, P<0.01; and Treatment × Dose interaction, F(3,56) = 2.81, P<0.05) on inhibitory avoidance memory. Two-way ANOVA also revealed a significant difference between the groups of animals which received pre-training saline (1 ml/kg) and pre-test nicotine and those which received pre-training ethanol (0.8 g/kg, i.p.), followed by pre-test nicotine plus a lower dose of ethanol (0.2 g/kg, i.p.) (for Treatment, F(1,56) = 17.34, P<0.001; Dose, F(3,56) = 7.12, P<0.01; and Treatment × Dose interaction, F(3,56) = 4.48, P<0.01) on inhibitory avoidance memory. Furthermore, two-way ANOVA revealed a significant difference between the groups of animals which received pre-training ethanol (0.8 g/kg, i.p.) and pre-test nicotine and those which received pre-training ethanol (0.8 g/kg, i.p.), followed by pre-test nicotine plus a lower dose of ethanol (0.2 g/kg, i.p.) (for Treatment, F(1,56) = 7.19, P<0.01; Dose, F(3,56) = 18.56, P<0.001; and Treatment × Dose interaction, F(3,56) = 1.28, P>0.05) on inhibitory avoidance memory. In addition, post hoc analysis revealed that in the animals trained after saline treatment and tested following intra-VTA administration of nicotine (0.1, 0.2 and 0.4 µg/rat), no significant change was observed in the retention latencies as compared with saline/saline control group [F(3,28) = 0.15, P>0.05] (Fig. 2, left panel). Furthermore, in the animals that pre-training administration of ethanol (0.8 g/kg) impaired inhibitory avoidance memory, intra-VTA
administration of nicotine (0.4 μg/rat), on the test day, significantly reversed ethanol-induced impairment of memory [F (3,28) = 7.22, P<0.001] (Fig. 2, middle panel). Moreover, the lower dose of pre-test ethanol (0.2 g/kg) alone did not induce a significant ethanol state-dependent memory. However, co-administration of nicotine (0.2 and 0.4 μg/rat, intra-VTA) with 0.2 g/kg of ethanol significantly improved the memory retrieval and mimicked the effects of pre-test administration of a higher dose of ethanol treatment [F(3,28)= 13.39, P<0.001] (Fig. 2, right panel).

**Fig. 2.** Effects of pre-test nicotine administration with or without ethanol on inhibitory avoidance memory.

***P<0.001, when compared to the pre-training saline/pre-test saline. ++P<0.001, when compared to the pre-training ethanol (0.8 mg/kg)/pre-test nicotine (0 μg/rat). $\Psi\Psi\Psi$ P<0.001, $\Psi\Psi$ P<0.01, when compared to the pre-training ethanol (0.8 mg/kg)/pre-test nicotine (0 μg/rat) plus ethanol (0.8 mg/kg).

Effects of pre-test administration of L-arginine alone or in combination with nicotine on ethanol-induced amnesia Fig. 3 indicates the effects of pre-test intra-VTA microinjection of L-arginine in the presence or absence of ethanol and/or nicotine on memory retrieval. Two-way ANOVA indicated an interaction between the groups of animals which received pre-training saline (1 ml/kg) and pre-test L-arginine (0, 0.1, 0.2, and 0.4 μg/rat, intra-VTA) and those which received pre-training ethanol (0.8 g/kg, i.p.) and pre-test L-arginine [for Treatment, F(1,56)= 24.12, P<0.001; Dose, F(3,56)= 2.03, P>0.05; and Treatment ×Dose interaction, F(3,56)= 2.79, P<0.05] on inhibitory avoidance memory. Two-way ANOVA also revealed a significant difference between the groups of animals which received pre-training saline (1 ml/kg) and pre-test L-arginine and those which received pre-training ethanol (0.8 g/kg, i.p.), followed by pre-test a lower dose of nicotine (0.1 μg/rat, intra-VTA) plus L-arginine [for Treatment, F(1,56)= 5.78, P<0.05; Dose, F(3,56)= 3.01, P<0.05; and Treatment ×Dose interaction, F(3,56)= 4.54, P<0.01] on inhibitory avoidance memory. Furthermore, two-way ANOVA revealed a significant difference between the groups of animals which received pre-training ethanol (0.8 g/kg, i.p.) and pre-test L-arginine and those which received pre-training ethanol (0.8 g/kg, i.p.), followed by pre-test a lower dose of nicotine (0.1 μg/rat, intra-VTA) plus L-arginine [for Treatment, F(1,56)= 6.16, P<0.05; Dose, F(3,56)= 10.86, P<0.001; and Treatment ×Dose interaction, F(3,56)= 0.99, P>0.05] on inhibitory avoidance memory. In addition, post hoc analysis revealed that in the animals trained after saline treatment and tested following intra-VTA administration of L-arginine (0.1, 0.2 and 0.4 μg/rat), no significant change was observed in the retention latencies as compared with saline/saline control group [F (3,28) = 0.11, P>0.05] (Fig. 3, left panel). Furthermore, in the animals that pre-training administration of ethanol (0.8 g/kg) impaired memory, administration of higher dose of L-arginine (0.4 μg/rat, intra-VTA), on the test day, significantly reversed ethanol-induced impairment of memory [F (3, 28) = 4.58, P<0.01] (Fig. 3, middle panel). Moreover, co-administration of L-arginine (0.2 and 0.4 μg/rat, intra-VTA) with 0.1 μg/rat of nicotine significantly potentiated the nicotine effect on ethanol-induced amnesia and mimicked the effects of pre-test administration of a higher dose of ethanol or nicotine treatment [F (3, 28) = 7.28, P<0.001], (Fig. 3, right panel).

Effects of pre-test administration of L-NAME alone or
in combination with nicotine on ethanol-induced amnesia. Fig. 4 shows the effect of pre-test intra-VTA administration of L-NAME in the presence or absence of ethanol and nicotine on inhibitory avoidance memory. Two-way ANOVA indicated a significant difference between the effects of L-NAME (0, 0.05, 0.1 and 0.2 μg/rat, intra-VTA) alone and L-NAME plus nicotine (0.4 mg/kg) on inhibitory avoidance memory [for Treatment, F (1, 56) = 0.60, P>0.05; Dose, F (3,56)= 15.38, P <0.001; and Treatment ×Dose interaction, F(3,56) = 1.04, P>0.05]. One-way ANOVA also revealed that pre-test intra-VTA administration of L-NAME (0, 0.05, 0.1 and 0.2 μg/rat, intra-VTA) reduced the step-through latency in the inhibitory avoidance task and induced amnesia [F(3, 28)= 7.54, P<0.001] (Fig. 3, left panel). Furthermore in the animals that pre-training administration of ethanol (0.8 g/kg) impaired inhibitory avoidance memory, pre-test intra-VTA microinjection of L-NAME (0.1 and 0.2 μg/rat) prevented the reversal effect of nicotine (0.4 μg/rat) on memory impairment induced by ethanol [F (3, 28) = 8.89, P<0.001] (Fig. 4, right panel).

Discussion

The present study points to a possible role of the VTA NO system in nicotine's reversal effect on ethanol-induced amnesia in inhibitory avoidance task. In agreement with other investigations, our results showed that pre-training systemic injection of ethanol is known to impair inhibitory avoidance memory in a state-dependent manner (Melchior et al., 1993; Raoufi et al., 2012; Rezayof et al., 2008a; Rezayof et al., 2007; Rezayof et al., 2010a; Rezayof et al., 2010b). Ethanol-induced state-dependent learning leads to impaired inhibitory avoidance memory in a drug-free state while pre-test administration of the same dose of ethanol restores the memory (Rezayof et al., 2008b; Rezayof et al., 2010a; Rezayof et al., 2010b). It has been proposed that cholinergic dysfunctions might be involved in ethanol induced amnesia (Nabeshima et al., 1991). Other evidence also suggests that the amnesic effect of ethanol may be due to decrease in NMDA-evoked neuronal activity or NO production (Lovinger et al., 1989; Rezayof et al., 2010b; Shimizu et al., 1998; White et al., 2000). Considering that the activity of VTA dopaminergic system is highly influenced by ethanol treatment (White et al., 2000) and the synthesis of NO in the VTA is influenced by this factor. It is possible that amnesic effects of ethanol mediate, at least partly, via nicotinic cholinergic mechanisms or NO system of VTA.
In our investigations, we also evaluated the possible role of the VTA nicotinic receptors on inhibitory avoidance memory and ethanol amnesia. Based on our findings, the pre-test intra-VTA microinjection of nicotine by itself does not significantly alter the inhibitory avoidance memory. It however can restore the amnesia induced by the pre-training administration of ethanol. As such, the activation of VTA nicotinic receptors appeared to abrogate the ethanol-induced amnesia. Interestingly, pre-test co-administration of an ineffective dose of ethanol with a lower dose of nicotine significantly restored ethanol amnesia and mimicked the reversal effects of pre-test injection of a higher dose of ethanol or nicotine. This is in agreement with other evidence indicating that amnesic effects of ethanol on both working and reference memory were prevented by nicotine pretreatment (Tracy et al., 1999). Several investigations, indicating an interaction between central nicotinic receptors and ethanol, including in vivo (Larsson and Engel, 2004) and in neuronal cultures (Manzanedo et al., 2004). It seems that nicotine attenuates or opposes the cognitive effects of ethanol (Abreu-Villacca et al., 2007; Gould and Lommock, 2003; Rezvani and Levin, 2002; Tracy et al., 1999). Evidence indicated that the nicotinic receptors of VTA play an important role in reward and reward related learning (Piri and Zarrindast, 2011). Since both ethanol and nicotine activated the mesocorticolimbic dopaminergic pathway that is originated from the VTA, one may expect that pre-test intra-VTA nicotine or systemic ethanol injections may influence ethanol amnesia via activation of mesocorticolimbic dopaminergic pathway. Thus, by such a mechanism ethanol in combination with nicotine may increase learning, which in turn elicits a potentiated state-dependent memory. However, other neurotransmitter systems seem to be involved in reversal effect of nicotine on ethanol amnesia. Stimulation of VTA nicotinic receptors by nicotine may increase the release of glutamate and acetylcholine (McGehee et al., 1995). It has been widely accepted that glutamate and acetylcholine play a critical role in learning and memory (Raoufi et al., 2012; Rezayof et al., 2008a; Rezayof et al., 2008b). Thus the effects of nicotine may be mediated through these neurotransmitters acting in concert (Izquierdo et al., 2006; Izquierdo and Medina, 1997).
High density of NOS immunoreactivity has been detected in VTA and may mediate some behavioral effect of ethanol and nicotine (Manzanedo et al., 2004; Shim et al., 2002; Tayfun Uzbay and Oglesby, 2001). Considering that ethanol and nicotine have functional interactions with NO (Raoufi et al., 2012) and the VTA is a key structure in learning and memory (Piri and Zarrindast, 2011), the aim of the present study was to examine the role of VTA NO system(s) on nicotine’s reversal effects on memory impairment induced by ethanol. Our results showed that pre-test microinjection of L-arginine into the VTA by itself did not significantly alter inhibitory avoidance memory, but can reverse ethanol induced amnesia. Moreover, the data indicated that pre-test co-administration of an ineffective dose of nicotine in combination with lower doses of L-arginine, which had no effect alone, synergistically reversed ethanol amnesia. The results may propose a functional interrelationship between NO system and nicotine’s effects on restoration of memory impairment by ethanol in the VTA. Cumulative evidence indicate that memory formation in the hippocampus is affected by several limbic structures such as VTA (Cammarota et al., 2004; Lisman and Grace, 2005). It has been shown that activation of VTA dopaminergic neurons that project to the hippocampus facilitates the induction of LTP in the hippocampus and improves learning and memory (Lisman and Grace, 2005). The VTA dopaminergic neurons control by excitatory glutamatergic inputs from the prefrontal cortex and pedunculopontine (Carr and Sesack, 2000). It has been shown that nicotinic receptor activation increases the release of glutamate and stimulates the NMDA receptor (Ahmadi et al., 2007a; Ahmadi et al., 2007b; McGeehee et al., 1995). Since nicotine plays a critical role as a NMDA receptor agonist (Ahmadi et al., 2007a; Ahmadi et al., 2007b) and the stimulation of NMDA receptors increases the NO generation (Ledo et al., 2004; Zarrindast et al., 2012), therefore the increase in NO levels by nicotine through such a mechanism may account for reversal of memory by pre-test nicotine plus L-arginine in this study. On the other hand, NO like ethanol and nicotine can activate mesolimbic dopaminergic pathway and it is possible that this similarity is involved in the reversal effect of NO and in the potentiating nicotine’s reversal effect. Therefore, our data may be in agreement with a previous report that NO may be involved in some other behavioral effects of nicotine such as dependence (Tayfun Uzbay and Oglesby, 2001), reward (Martin and Itzhak, 2000), withdrawal (Loh et al., 1998), and the sensitization (Shim et al., 2002). In agreement with these data, our previous studies have shown that NMDA receptors and NO system in the dorsal hippocampus involve in nicotine’s reversal effect on ethanol amnesia (Raoufi et al., 2012; Rezayof et al., 2010a; Rezayof et al., 2010b). Our results also indicated that NO system and NMDA receptors of VTA involve in nicotine’s reversal effect on morphine amnesia (Ahmadi et al., 2007b; Raoufi et al., 2012).

Previous studies indicate that systemic administration of NO precursor, facilitate memory formation (Baratti and Boccia, 1999). However, systemic injection of NOS inhibitors can impair memory consolidation in different paradigms (Baratti and Kopf, 1996; Prast and Philippu, 2001). In agreement with these reports, our data indicated that pre-test intra-VTA injection of L-NAME impairs inhibitory avoidance memory in rats. Our results also indicate that pre-test intra-VTA injection of L-NAME prior to effective dose of nicotine blocked nicotine’s reversal effect on ethanol amnesia. Reversal effect inhibition with NOS inhibitor may provide additional support for the NO influence in pre-testing response of nicotine in the VTA, which may be due to the influence on dopaminergic projections from the VTA to the NAc and hippocampus, ultimately ends up favoring dopamine release. Our finding was in agreement with previous studies reporting that NOS inhibitors decrease nicotine induced DA release and nicotine sensitization of DA release (Hong et al., 2006). In support of this hypothesis, our data also revealed that intra-VTA injection of L-NAME blocked reversal effect of ineffective dose of nicotine plus lower doses of L-arginine on ethanol-induced amnesia. Since intra-VTA injection of L-NAME blocked the L-arginine induced potentiation of the
nicotine response, the reversal effect of L-arginine and nicotine may be mediated through a NO-dependent pathway in the VTA. In some other studies, the inhibitory effects of NOS inhibitors on the behavioral effects of nicotine have been shown. For example, pharmacological evidences have shown that NOS inhibitors could block the development of behavioral sensitization and conditioned place preference induced by nicotine (Martin and Itzhak, 2000) and suppress signs of nicotine withdrawal (Tayfun Uzbay and Oglesby, 2001). The findings of the present study are consistent with previous ones that showed inhibition of NO production or NMDA receptors in the CA1 region of dorsal hippocampus block nicotine's reversal effect on ethanol amnesia (Raoufi et al., 2012; Rezayof et al., 2010a; Rezayof et al., 2010b). Our previous results also indicated that NOS inhibitors or NMDA receptors antagonists in the VTA could block nicotine’s reversal effect on morphine amnesia (Ahmadi et al., 2007b; Piri and Zarrindast, 2011). In conclusion, considering the effects of intra-VTA injection of L-arginine and L-NAME when co-administered with nicotine, it is possible that nicotine improves ethanol-induced memory impairment, at least partly via the NO system(s) in the VTA and subsequent changes in dopamine release in the terminal fields of VTA dopamine neurons such as hippocampus which are involved in mediating learning and memory.

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