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Agmatine reverses ethanol consumption in rats: Evidences for an interaction with imidazoline receptors

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ABSTRACT

Alcohol is one of the most widely abused recreational drugs, largely linked with serious health and social concerns. However, the treatment options for alcohol-use disorders have limited efficacy and exhibit a range of adverse drug reactions. Large numbers of preclinical studies have projected a biogenic amine, agmatine as a promising potential treatment option for drug addiction, including alcoholism. In the present study, administration of agmatine (20–40 mg/kg, i.p.) resulted in significant inhibition of ethanol self-administration in the right p-VTA in operant conditioning paradigm. Further, acute intracranial administration of agmatine (20 and 40 μg/rat) significantly reduced the ethanol consumption in the two bottle choice paradigm. Agmatine is degraded to putrescine and guanido-butanoic acid by the enzyme agmatinase and diamine oxidase respectively and inhibition of these enzymes results in augmentation of endogenous agmatine. In the present study, diamine oxidase inhibitor, aminoguanidine and agmatinase inhibitor, arcaine were used to block the agmatine metabolic pathways to increase brain agmatine levels. Drugs that augment endogenous agmatine levels like L-arginine (80 μg/rat, i.c.v.) or arcaine (50 μg/rat, i.c.v.) and aminoguanidine (25 μg/rat, i.c.v.) also reduced the ethanol consumption following their central administration. The pharmacological effect of agmatine on ethanol consumption was potentiated by imidazoline receptor agonists, I_1 agonist moxonidine (25 μg/rat, i.c.v.), and imidazoline I_2 agonist, 2-BFI (10 μg/rat, i.c.v.) and was blocked by imidazoline I_1 antagonist, efaroxan (10 μg/rat, i.c.v.), and I_2 antagonist, idazoxan (4 μg/rat, i.c.v.) at their ineffective doses per se. Thus, our result suggests the involvement of imidazoline I_1 and I_2 receptors in agmatine induced inhibition of ethanol consumption in rats.

1. Introduction

Chronic ethanol consumption leads to serious health and social consequences. A huge mortality worldwide is attributed to alcoholism, even greater than deaths caused by infections or violence (World Health Organization, 2014). Unfortunately, there are only three medications approved by the Food and Drug Administration for the treatment of alcohol abuse and alcoholism: disulfiram, naltrexone and acamprosate (Liang and Olsen, 2014). Medication compliance issues, adverse side effects and the modest efficacy of these compounds reveal the need for better targets of alcoholism in order to develop newer effective medications.

Although, there are different pathways underlying alcohol seeking behavior, the biological process that builds and reinforces alcohol addiction is not yet fully understood. Although alcohol can affect multiple neurotransmitter receptors, including GABA, NMDA, 5-HT3 etc. (Trudell et al., 2014; Morrow et al., 2001) the studies are inconclusive in finding their direct correlation with alcohol intake. In recent years, several studies were executed to identify exactly how does the endogenous systems like neuropeptides, β-endorphins, endocannabinoids mediates reinforcing the effects of alcohol (Kokare et al., 2008; Ron and Messing, 2013; Henderson-Redmond et al., 2015).

Agmatine, an endogenous biogenic amine, has been implicated in the process of drug addiction. It attenuates ethanol, nicotine as well as morphine withdrawal symptoms (Aricioglu-Kartal and Uzbay, 1997; Li et al., 1999; Uzbay et al., 2000; Kotagale et al., 2015, 2018). Further, it reduces impaired performance on a cerebellar-dependent balance tested in a rat model of third trimester binge-like ethanol exposure (Lewis et al., 2007) and ultrasonic vocalization deficits in female rat pups exposed neonatally to ethanol (Wellmann et al., 2010). Agmatine decreases the morphine, cocaine, fentanyl self-administration (Morgan et al., 2002), inhibits the ethanol induced locomotor sensitization

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(Ozden et al., 2011) and the acquisition but not the expression of ethanol conditioned place preference in mice (Sameer et al., 2013). Importantly, several brain nuclei like ventral tegmental area (VTA), nucleus accumbens (NAc), amygdala, etc., which are involved in alcohol seeking behaviour express high agmatine immunoreactivity (Otake et al., 1998; Reis and Regunathan, 2000).

Agmatine is biosynthesized following decarboxylation of L-arginine by arginine decarboxylase (ADC) in the mammalian brain (Reis and Regunathan, 2000). It is a pleiotropic molecule with many central and peripheral functions. It exhibits anxiolytic (Taksande et al., 2014), antidepressant (Taksande et al., 2009; Kotagale et al., 2013), anti-nociceptive (Aglawe et al., 2014), anti-convulsive (Bence et al., 2003; Feng et al., 2005), anti-inflammatory (Satriano et al., 2001; Taksande et al., 2017) and neuroprotective effects (Dixit et al., 2018).

Agmatine is an endogenous ligand for imidazoline receptors (Reis and Regunathan, 1998; Raasch et al., 2001) and its anatomical distribution within the brain areas supports this correlation (De Vos et al., 1994; Raasch et al., 1995). Imidazoline receptors modulate several actions of ethanol, its intake and the development of dependence or withdrawal syndrome (Uzbay et al., 2000; Dobrydnjov et al., 2004; Lewis et al., 2007; Mao and Abdel-Rahman, 1996; Rommelspacher et al., 1991, 1996; Spies et al., 1996). Importantly, the effects of agmatine on ethanol withdrawal induced anxiety and behavioural sensitization were mediated by imidazoline receptors (Taksande et al., 2010, 2019). In view of this background, it was hypothesized that agmatine may alter ethanol consumption in rats through activation of imidazoline receptors. In the present study, we investigated the effects of systemic agmatine injection (10–40 mg/kg, i.p.) on ethanol self-administration in pVTA using operant conditioning procedures. pVTA has been selected as an anatomical target for ethanol self-administration due to its key role in ethanol-induced reinforcement (Rodd et al., 2004). In separate set of experiments we have examined the influence of intracranial agmatine injections (10–40 μg/rat) on ethanol consumption in a two-bottle choice paradigm and its modulation by imidazoline receptors.

2. Materials and methods

2.1. Subjects

Adult Wistar rats of either sex (220–250 g) procured from National institute of Nutrition, Hyderabad, India were used in the study. Animals were group housed (4 per cage) or individually under specific experimental conditions in a temperature (22 + 1 °C) and humidity-controlled (50 + 5%) environment with free access to food and water. All the experimental procedures were carried out under compliance with Institutional Animal Ethics Committee (IAEC) according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, New Delhi.

2.2. Drugs

Agmatine sulphate, L-arginine monohydrochloride, amino-guanidine hemisulphate, arcaine sulphate, moxonidine hydrochloride, efaroxan hydrochloride, idazoxan hydrochloride (Sigma-Aldrich Co., USA) and 2-(2-Benzofuranyl)-2-imidazoline hydrochloride (2-BFI) (Tocris Biosciences, Bristol, UK) were dissolved in saline (0.9%) and administered by intraperitoneal (i.p.) route. For intra-cerebroventricular (i.c.v.) administration drugs were dissolved in artificial cerebrospinal fluid (aCSF composition 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.4). Doses and timings of injections employed in the protocols were selected on the basis of previous and pilot experiments carried out in our laboratory (Taksande et al., 2009, 2010, 2019; Kotagale et al., 2010; Aglawe et al., 2014).

2.3. Apparatus for operant conditioning

The ethanol was self-administrated by rats in the right posterior-VTA using operant conditioning chamber (Panlab, Barcelona, Spain) having the following dimensions: 27 × 25 × 27 cm, with a stainless steel grid floor. Two operant levers (active and inactive) were located 17 cm apart on the same wall, 10 cm above the grid floor. On a reinforced response, a small cue light mounted above the active lever was illuminated during each 5-second infusion. Following each active lever press, a microinfusion pump (Panlab, Barcelona, Spain) connected to the apparatus delivered 100 nl of solution; the inactive lever press was ineffective. The infusion cannula was connected by a polyethylene tube through swivel to a computer equipped microinfusion pump (Panlab, Barcelona, Spain). Computer equipped with an operant control system adjusts the speed of the motor and plunger carrier. In response to the motor speed, plunger carrier pushes syringe piston and proportionally inject the infusion solution. The number of active and inactive lever presses for a period of 30 min was recorded automatically by the software. The infusion was regulated by a pump at a steady rate of 100 nl infusate/5 s, followed by 5 s timeout periods [fixed ratio 1 (FR1) of reinforcement schedule].

2.4. Ethanol self-administration in operant chamber

The animals were implanted with 22-gauge stainless steel guide cannula in the right p-VTA (anteroposterior (AP): −5.6 mm, mediolateral (ML): +2.1 mm, dorsoventral (DV): −8.5 mm at 10°) (Paxinos and Watson, 1998). Guide cannulae were then fixed to the skull with dental cement and secured with three stainless steel screws. A 28-gauge stainless steel dummy cannula was used to occlude the guide cannula when not in use. Following surgery, the rats were placed individually in a cage and allowed to recover for at least 7 days. During this period, from the day 4 onwards, the rats were habituated to gentle handling for about 5 min each day. The cannulated rats were divided in different groups (n = 7–9) and trained in operant chamber for ethanol self-administration. The rats that showed consistent lever pressings (~16–19) for three consecutive sessions were considered as trained. Cannulated rats that showed neurological and motor deficits like impairment in locomotion, aggressiveness, hyper-excitability and stereotype behaviour, were excluded from the study. Animals were not acclimatized to the experimental condition or trained in the operant chamber before surgery.

The protocol implemented in the present study is in line with Shelkar et al. (2015).

In the first experiment, pVTA cannulated rats (n = 31) were assigned to three different groups and trained to press the lever and self-administer ethanol (100 and 200 mg%) into the pVTA. These three groups were subjected to daily session (30 min) of ethanol self-infusion (100 and 200 mg%) and the lever press activity was recorded for 7 days. The data from each group was represented as a number of presses/30 min session. The third group of rats was subjected to aCSF self-administration and the number of lever presses in each session (30 min) was recorded over seven sessions. This group did not show any significant increase in the lever presses and served as non-ethanol control.

In second experiment, a separate group of rats (n = 9) was allowed to self-administer 200 mg% ethanol in pVTA. To confirm the rewarding effect of 200 mg%, separate groups of rats were allowed access to 200 mg% of ethanol for the first four sessions; however, during fifth and sixth sessions, ethanol was replaced by aCSF. Eventually, these rats were restored to 200 mg% of ethanol self-administration at the seventh session and the number of lever presses in each session was recorded, averaged and represented as number of lever presses/30 min.

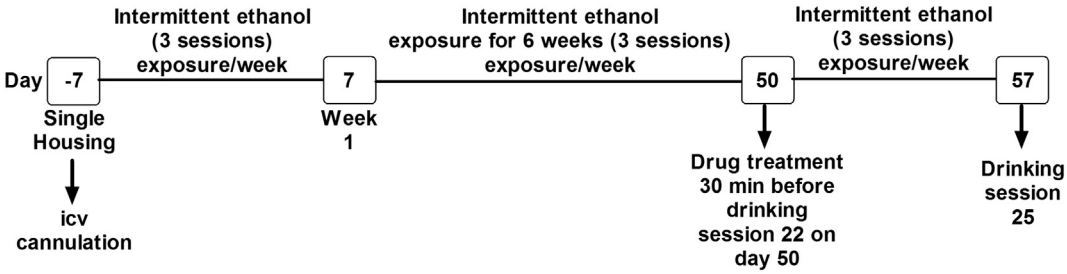

Fig. 1. Schematic illustration of the protocol adopted for ethanol consumption protocol. Ethanol intake was monitored using intermittent access to two bottle choice drinking paradigm. Rats adapted to two water bottles were exposed Monday, Wednesday and Friday, 2 h after the onset of the dark cycle, one water bottle was replaced with a bottle containing an ethanol. Ethanol and water bottle were weighed. A total of 25 acquisition sessions of 24 h ethanol exposure were conducted (57 days) with 31 water drinking days interspersed among the ethanol drinking days. Drugs were injected before beginning of 22nd cycle of ethanol consumption (Day 50).

2.5. Effect of agmatine on ethanol (200 mg%) self-administration in operant chamber

In third experiment, separate groups of animals were trained to self-administer ethanol (200 mg%) for the first four sessions. During sessions 5 and 6, rats were treated with either saline (1 ml/kg, i.p.) or agmatine (10, 20, 40 mg/kg, i.p.) 30 min prior to self-administration of 200 mg% ethanol and change in the number of lever pressings was recorded. During the seventh session, the rats were subjected only to 200 mg% ethanol self-administration.

2.6. Ethanol consumption - two-bottle choice paradigm

Rats were stereotaxically implanted with 22-gauge stainless steel guide cannula (C313G/Spc, Plastics, UK) in right lateral ventricle under ketamine and xylazine anaesthesia using coordinates −0.8 mm posterior +1.2 mm lateral to the midline and −3.5 mm ventral to bregma (Paxinos and Watson, 1998). After 7 days of recovery, rats were randomly assigned to different groups and exposed to two bottle choice ethanol consumption up to 25 repeated cycles (57 days). Schematic illustration of the experimental procedure is given in Fig. 1.

Ethanol intake was monitored using intermittent access to two bottle choice drinking paradigm. Rats were adapted to two water bottles in their home cage continuously for one week prior to the first ethanol exposure. Every Monday, Wednesday and Friday, 2 h after the onset of the dark cycle, one water bottle was replaced with a bottle containing an ethanol and the rats were allowed to drink freely. After 24-h of exposure, the ethanol bottle and water bottle were weighed and the ethanol bottle was replaced with a bottle containing drinking water. The average total fluid intake was ~20–26 ml per 24 h. During the first week, rats were exposed to increasing concentrations of ethanol (3%, 6% and 10% ethanol (v/v) on each day respectively). For subsequent weeks, rats received 20% ethanol (v/v) on these days. A total of 25 acquisition sessions of 24 h ethanol exposure were conducted (57 days) with 32 water drinking days interspersed among the ethanol drinking days. The development of a side preference or bias was minimized by exchanging water and ethanol bottle positions on alternate days. Rats were weighed daily for calculating ethanol consumption (g/kg).

Drugs were injected before beginning of 22nd cycle of ethanol consumption (Day 50), into the right ventricle over a 1 min period with a microliter syringe (Hamilton, Reno, NV, USA) via PE-10 polyethylene tubing connected to a 28-gauge internal cannula (C313I/Spc, Plastics One, UK, internal diameter 0.18 mm, outer diameter 0.20 mm) that extended 0.5 mm beyond the guide cannula. The internal cannula was held in a position for another 1 min after each injection before being slowly withdrawn to promote diffusion of drugs and prevent backflow.

2.7. Effect of agmatine and its modulators on ethanol consumption

On testing day- 22, animals were injected (i.c.v.) with agmatine (10, 20 or 40 μg/rat) or drugs known to enhance its endogenous content like L-arginine (80 μg/rat), arcaine (50 μg/rat), amino-guanidine (25 μg/rat) and aCSF (5 μl/rat). Agmatine and its modulators were injected (i.c.v.) before 30 min of 22nd drinking and testing session.

2.8. Effect of imidazoline receptor agonists and antagonists on ethanol consumption

To investigate the effect of imidazoline receptor ligands on ethanol consumption, separate groups of rats (n = 6–9) were treated with imidazoline I_1 receptor agonist, moxonidine (25–50 μg/rat, i.c.v.) or imidazoline I_2 receptor agonist, 2-BFI (10–20 μg/rat, i.c.v.) or aCSF (5 μl/rat, i.c.v.) alone or in combination with agmatine (10 μg/rat, i.c.v.) 30 min before testing on 22nd session. In Separate experiments, rats were treated with imidazoline I_1 receptor antagonist, efaroxan (10 μg/rat, i.c.v.) or I_2 receptor antagonist, idazoxan (4 μg/rat, i.c.v.) or aCSF (5 μl/rat, i.c.v.) alone or 10 min before administration of agmatine (20 μg/rat, i.c.v.).

2.9. Statistical analysis

The results have been presented as mean ± S.E.M. The effects of different drug treatments were statistically analysed by one or two way Analysis of Variance (ANOVA). Post hoc mean comparisons were done by Bonferroni's test. A value of $P < 0.05$ was considered significant in all cases.

3. Results

3.1. Agmatine inhibits ethanol self-administration in operant conditioning

Ethanol concentrations in the range of 100–200 mg% were employed to determine the dose–response behaviours of rats with guide cannula directed at the pVTA. Rats that were allowed to self-administer ethanol at concentrations 200 mg% showed a significant increase in lever presses. A group of trained rats was allowed to self-administer 200 mg% ethanol in p-VTA for all the seven sessions. Rat consistently showed an increase (16–22) in the number of active lever presses as compared with the inactive lever presses. Two way ANOVA revealed that interaction between sessions and the lever presses [$F(12, 168) = 1.05$, $P = 0.41$]. Post hoc mean comparisons with Bonferroni's test demonstrated the main effect of ethanol concentration on lever presses [$F(2, 168) = 100.12$, $P < 0.0001$] whereas differences in the number of lever presses between sessions were statistically insignificant [$F(6, 168) = 0.27$, $P < 0.95$] (Fig. 2A) ensuring the stable lever press activity. The lever presses in the animals allowed to self-administer

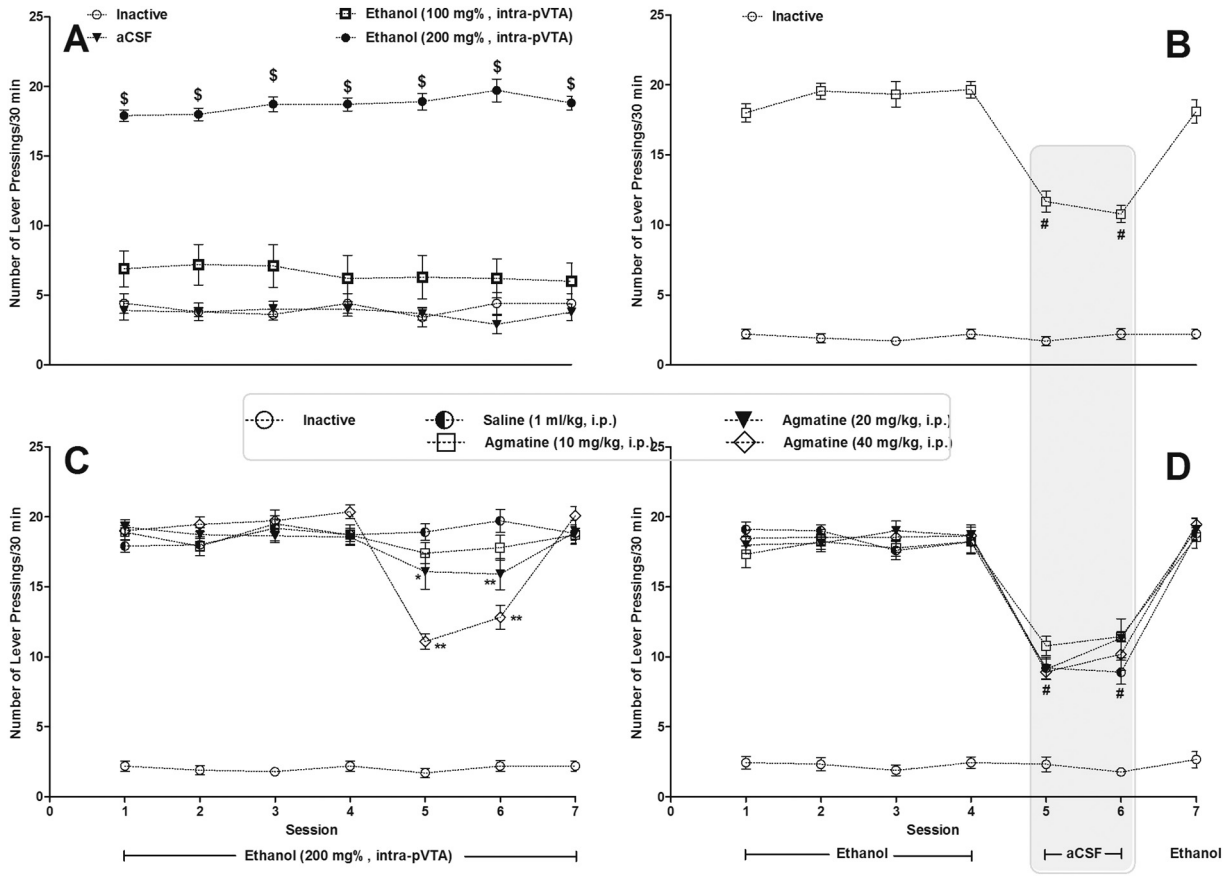

Fig. 2. (A) Consistent lever press activity of rats for seven sessions following training to self-administer ethanol (200 mg%) (n = 10–11). (B) Higher number of lever presses to self-infuse ethanol during first four sessions. Reduction in the lever press activity after replacement of ethanol by artificial cerebrospinal fluid (aCSF) during sessions 5 and 6 reinstated by ethanol in session 7 (n = 9–11). (C) Higher lever press activity of rats to self-infuse ethanol in the first four sessions for all the animals. Rats were injected with saline (1 ml/kg, i.p., n = 10), agmatine (10–40 g/kg, i.p., n = 10–11/group) and allowed to self-administer ethanol after 30 min. (D) lever press activity in separate groups of rats to self-infuse ethanol during first four sessions. In sessions 5 and 6, the animals were injected with saline (1 ml/kg, i.p.) or agmatine (10–40 mg/kg, i.p.) and after 30 min allowed to self-administer aCSF. Data represent mean lever presses ± S.E.M. $P < 0.001 versus inactive lever presses; #P < 0.001 versus ethanol 200 mg% during respective session; *P < 0.05, **P < 0.001 versus saline treated rats (Two way ANOVA followed by post hoc Bonferroni test).

ethanol at concentrations 100 mg% were not significantly different from aCSF control.

Separate group of rats were allowed to self administer ethanol [200 mg% (intra-pVTA)] during first four sessions. Animals demonstrated significantly higher number of lever presses (~15–22) as compared to aCSF control. These animals were then administered (intra-pVTA) with aCSF by substituting ethanol during session 5 and 6 which resulted into significant reduction in the number of active lever pressing during session 5 and 6. Two way ANOVA suggested the significant interaction between lever presses and sessions [$F(6, 102) = 29.53$, $P < 0.0001$]. Statistically significant effect of treatment on lever presses [$F(1, 102) = 1335.58$, $P < 0.0001$] and sessions [$F(6, 102) = 30.04$, $P < 0.0001$] were also determined. Post hoc Bonferroni mean comparisons demonstrated significant decrease in the number of active lever pressing during session 5 ($P < 0.001$) and 6 ($P < 0.001$) as compared to lever pressing during respective sessions in ethanol group (Fig. 2B). Re-pairing ethanol with active lever during session 7 increased the number of active lever presses (~18) demonstrating re-instatement.

Agmatine (10, 20, 40 mg/kg, i.p.) treatment 30 min prior to the fifth and sixth sessions significantly reduced the number of lever presses for ethanol as compared with that of the saline administered rats (Fig. 2C). Application of two-way ANOVA showed significant interaction between treatment and sessions [$F(24, 288) = 7.40$, $P < 0.001$]. Statistical analysis showed the main effect of agmatine treatment [$F(4, 288) = 708.91$, $P < 0.001$] and sessions [$F(6, 288) = 15.40$, $P < 0.001$]. Post-hoc Bonferroni's multiple comparison test revealed that agmatine treatment [20 mg/kg ($P < 0.05$ and $P < 0.001$) and 40 mg/kg ($P < 0.001$ and $P < 0.001$)] resulted in significant reduction in lever presses for ethanol self-administration during session 5 and 6 respectively as compared with that in control rats. However, its lower dose (10 mg/kg) had no effect on the lever pressings and therefore considered sub-effective (Fig. 2C). Administration of agmatine (10–40 mg/kg, i.p.) prior to the presentation of aCSF (intra-pVTA) in place of ethanol (200 mg%, intra-pVTA) did not produce any significant change in lever presses as compared to the saline treated control animals (Fig. 2D).

3.2. Agmatine inhibits ethanol consumption in two bottle choice paradigm

As shown in Fig. 3, the administration of agmatine (20–40 μg/rat, i.c.v.) decreased ethanol intake without significantly altering total fluid intake under continuous access conditions. Two way ANOVA showed the statistically insignificant interaction between ethanol consumption during sessions and treatment [$F(72, 792) = 0.56$, $P = 0.99$]. However, statistical data revealed the main effect of ethanol consumption during session [$F(24, 792) = 7.22$, $P < 0.001$] and treatment [$F(3, 792) = 0.73$, $P = 0.05$] (Fig. 3A). Post-hoc Bonferrani mean comparisons showed that treatment (i.c.v.) of animals with agmatine (20 μg/rat, $P < 0.05$) and (40 μg/rat, $P < 0.01$) prior 22nd drinking session

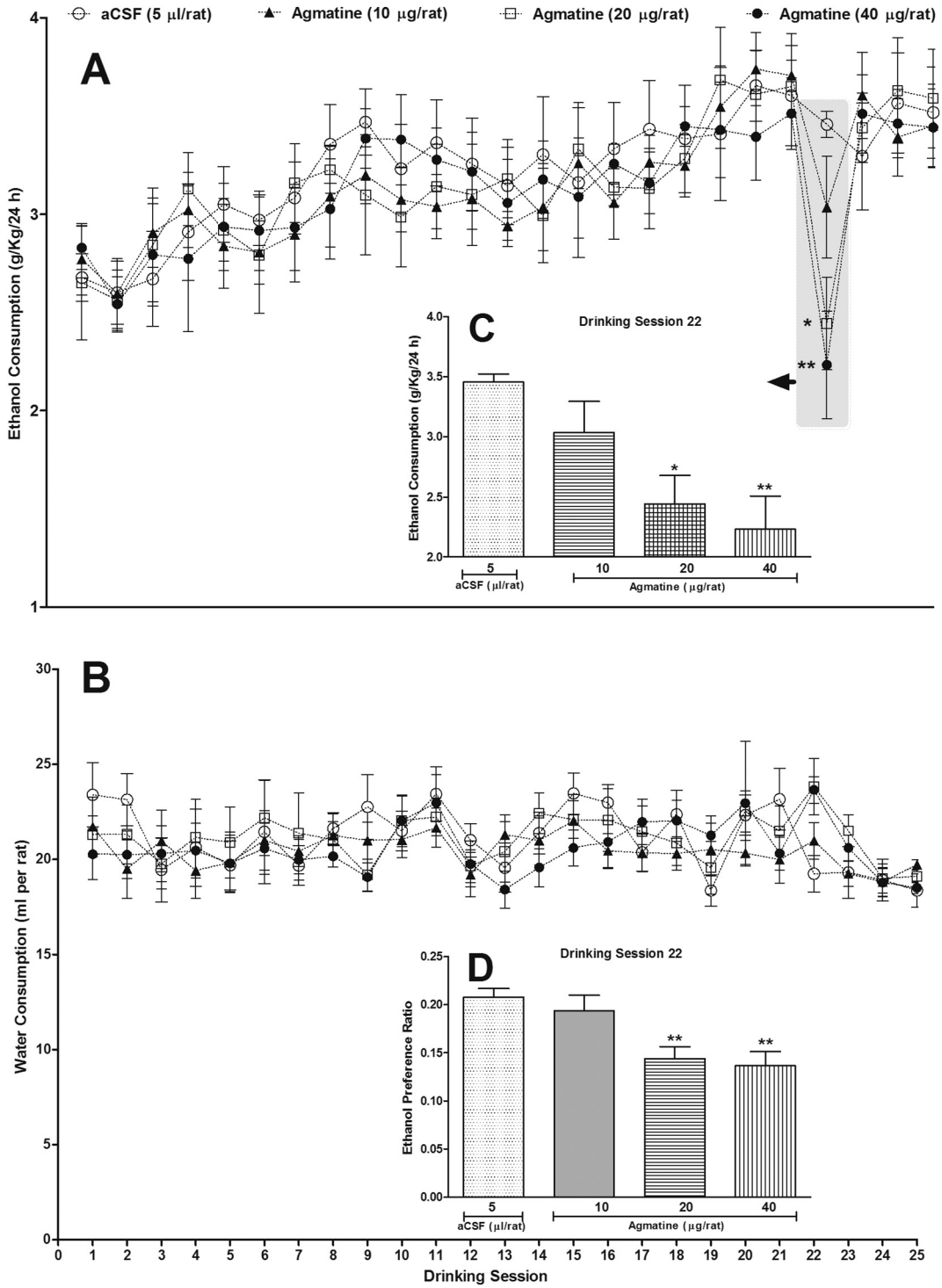

Fig. 3. Effects of agmatine (10–40 µg/rat, i.c.v.) or aCSF (5 µl/rat, i.c.v.) on (A) ethanol consumption (B) water intake during 25 drinking sessions of voluntary ethanol drinking as well as on (C) ethanol consumption (g/kg/24 h) and (D) ethanol preference in two bottle choice paradigm. Data represent mean ethanol consumption (g/kg/24 h)/water consumption (ml/rat)/ethanol preference ratio ± S.E.M. (n = 8–10/group). *P < 0.05, **P < 0.01 versus aCSF treated control animals (one or Two way ANOVA followed by post hoc Bonferroni test).

significantly decrease ethanol consumption (Fig. 3C). No significant difference in the water intake was associated with decreased ethanol consumption in agmatine treated animals (Fig. 3B). As depicted in Fig. 3D, ethanol preference calculated as [ethanol consumed (ml)/ ethanol consumed (ml) + water consumed (ml)] for session 22, was also significantly decreased in the animals treated with agmatine (i.c.v.) in the dose of 20 µg/rat (P < 0.01) as well as 40 µg/rat (P < 0.01) 30 min prior to 22nd drinking session [F (3, 36) = 6.81, P = 0.001].

3.3. Agmatine modulators inhibit ethanol consumption

Although Statistical analysis suggested insignificant interaction between drinking sessions and L-arginine (80 µg/rat) treatment [F (24, 400) = 1.03, P = 0.43], arcaine (50 µg/rat) treatment [F (24, 360) = 0.99, P = 0.47] as well as aminoguanidine (25 µg/rat) treatment [F (24, 384) = 0.94, P = 0.54]; two way ANOVA revealed main effect of modulator treatment [L-arginine - F (1, 400) = 5.92,

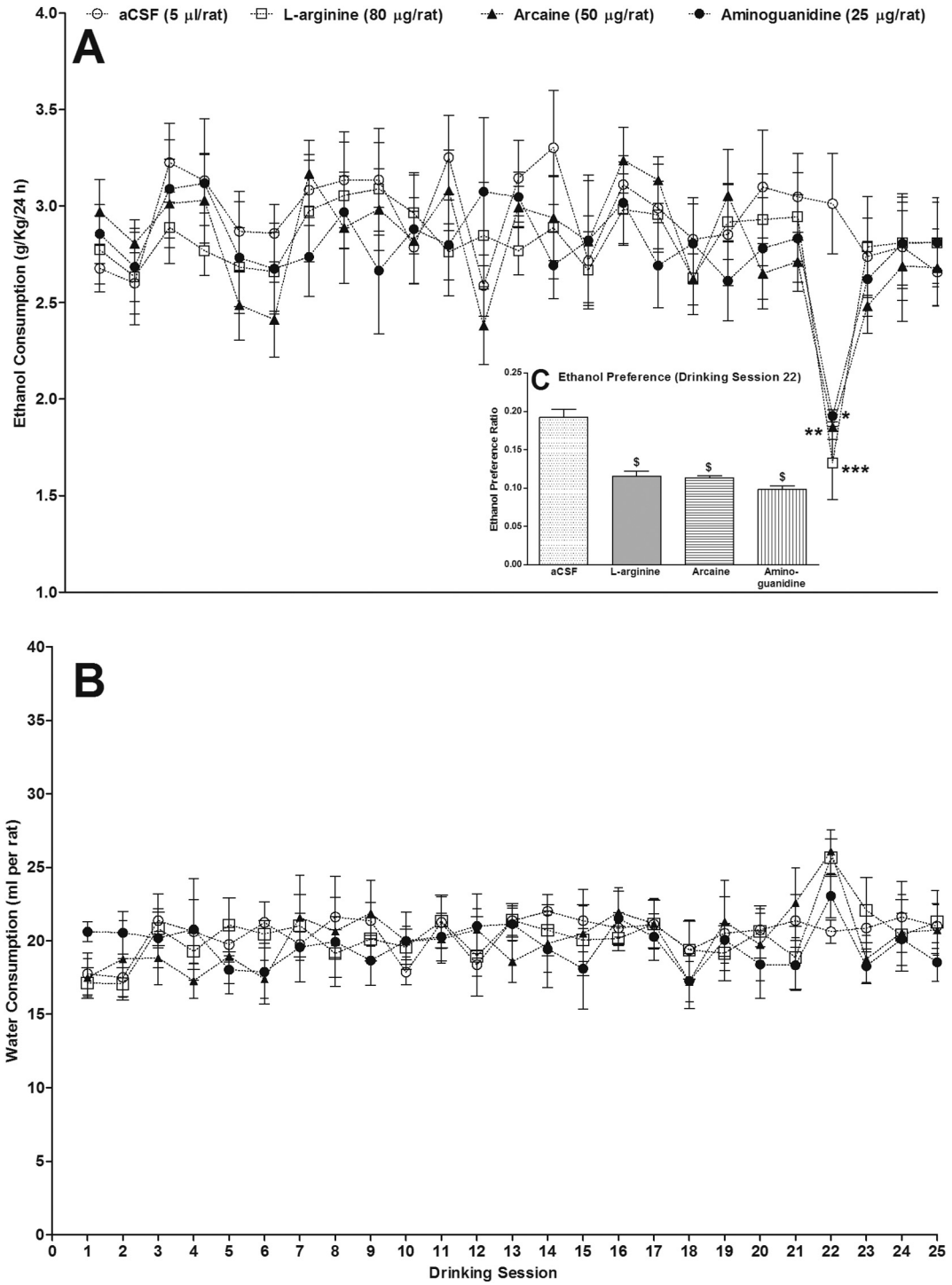

Fig. 4. Effects of modulators of endogenous agmatine, L-arginine (80 µg/rat, i.c.v.), arcaine (50 µg/rat, i.c.v.), aminoguanidine (25 µg/rat, i.c.v.) or aCSF (5 µl/rat, i.c.v.) on (A) ethanol consumption and (B) water intake during 25 drinking sessions of voluntary ethanol drinking and (C) ethanol preference in two bottle choice paradigm. Data represent mean ethanol consumption (g/kg/24 h)/water consumption (ml/rat)/ethanol preference ratio ± S.E.M. (n = 8–9/group). *P < 0.05, **P < 0.01, ***P < 0.001 versus aCSF treated control animals (Two way ANOVA followed by post hoc Bonferroni test). $P < 0.001 versus aCSF treated control animals (One way ANOVA followed by post hoc Bonferroni test).

P < 0.05; arcaine – F (1, 360) = 1.74, P = 0.52; aminoguanidine - F (1, 384) = 2.89, P = 0.11] and sessions [L-arginine - F (24, 400) = 1.44, P = 0.085; arcaine - F (24, 360) = 2.15, P < 0.01; aminoguanidine - F (24, 384) = 1.10, P = 0.34] on ethanol intake in rats (Fig. 4A). Post-hoc Bonferroni mean comparisons demonstrated that L-arginine (80 µg/rat) (P < 0.001) or arcaine (50 µg/rat) (P < 0.01) or amino-guanidine (25 µg/rat) (P < 0.05) treatment 30 min prior to 22nd drinking session decreased the ethanol intake by 45%, 39% and 36% respectively as compared aCSF treated rats. Moreover, two way ANOVA followed by post hoc Bonferroni mean comparisons indicated that compensatory increased water intake in the modulator treated group was statistically non-significant as compared with aCSF treated animals (Fig. 4B). Pretreatment with modulators prior to 22nd drinking session, L-arginine (P < 0.001), arcaine (P < 0.001) as well as

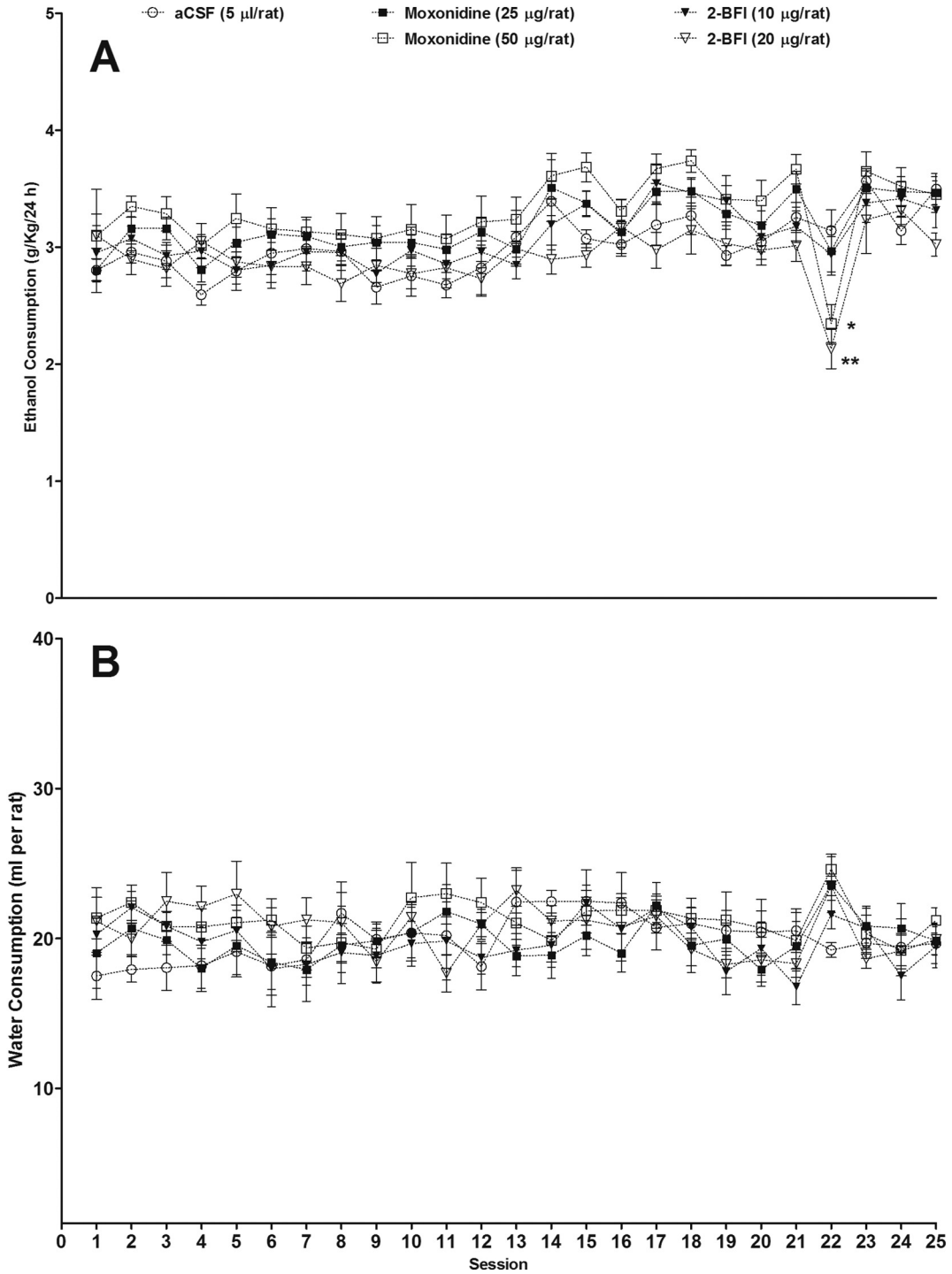

Fig. 5. Effects of imidazoline I_1 receptor agonist, moxonidine (25–50 µg/rat, i.c.v.), imidazoline I_2 receptor agonist, 2-BFI (10–20 µg/rat, i.c.v.) or aCSF (5 µl/rat, i.c.v.) on (A) ethanol consumption and (B) water intake during 25 drinking sessions of voluntary ethanol drinking in two bottle choice paradigm. Data represent mean ethanol consumption (g/kg/24 h) ± S.E.M. (n = 9–10/group). *P < 0.01, **P < 0.001 versus aCSF treated control animals (Two way ANOVA followed by post hoc Bonferroni test).

aminoguanidine (P < 0.001) also decreased preference to ethanol as compare to water (Fig. 4C).

3.4. Imidazoline receptor agonists attenuate the ethanol intake and potentiate the effect of agmatine

As shown in Fig. 5A, two way ANOVA indicated the interaction between ethanol consumption during 25 drinking sessions and treatment with I_1 receptor agonist, moxonidine (50 µg/rat, i.c.v.) [F (48, 624) = 1.19, P = 0.187] as well as imidazoline I_2 receptor agonist, 2-BFI (20 µg/rat, i.c.v.) [F (48, 600) = 1.28, P = 0.104]. Statistical analysis indicated the main effect of moxonidine [F (2, 624) = 3.75, P = 0.037] or 2-BFI [F (2, 600) = 2.41, P = 0.11] treatment and drinking sessions [moxonidine - F (24, 624) = 9.20, P < 0.001; 2-BFI - F (24, 600) = 4.66, P < 0.001]. Treatment of rats with I_1 receptor agonist, moxonidine (50 µg/rat, i.c.v.) as well as imidazoline I_2 receptor agonist, 2-BFI (20 µg/rat, i.c.v.) prior to 22nd drinking session reduced the ethanol consumption in two bottle choice paradigm. Post-hoc

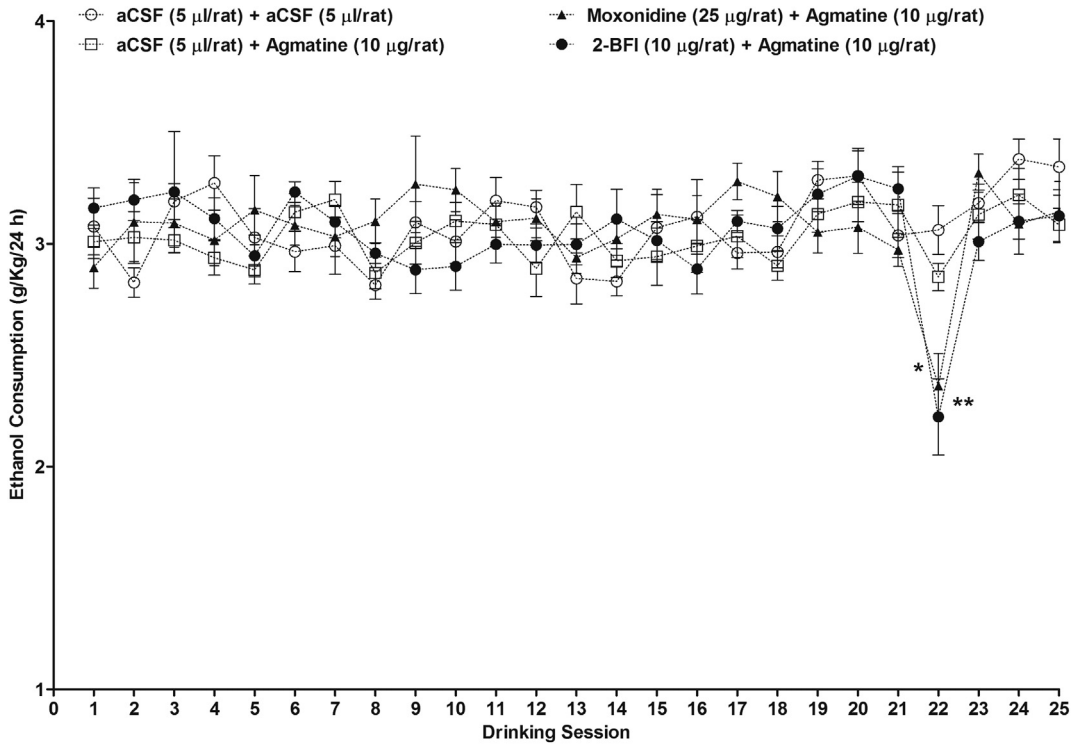

Fig. 6. Influence of moxonidine (25 µg/rat, i.c.v.) or 2-BFI (10 µg/rat, i.c.v.) pretreatment on the effect of agmatine (10 µg/rat, i.c.v.) on ethanol consumption during 25 drinking sessions of voluntary ethanol drinking in two bottle choice paradigm. Data represent mean ethanol consumption (g/kg/24 h) ± S.E.M. (n = 10/group). *P < 0.05, **P < 0.001 versus agmatine (10 µg/rat, i.c.v.) treated control animals (Two way ANOVA followed by post hoc Bonferroni test).

Bonferroni mean comparisons showed a significant reduction in ethanol intake with moxonidine (50 µg/rat, i.c.v.) (P < 0.01) and 2-BFI (20 µg/rat, i.c.v.) (P < 0.001) when compared against control animals receiving aCSF. The lower doses of moxonidine (25 µg) and 2-BFI (10 µg) were ineffective. However, as indicated in Fig. 5B, change in the water intake compensatory to the reduced ethanol consumption was statistically insignificant in moxonidine (50 µg/rat) and 2-BFI (20 µg/rat) treated animals.

As depicted in Fig. 6, there was significant interaction between sub-effective dose combinations of imidazoline I_1 receptor agonist, moxonidine (25 µg/rat, i.c.v.) [F (48, 648) = 1.89, P < 0.001] or 2-BFI (10 µg/rat, i.c.v.) [F (48, 648) = 1.98, P < 0.01] with agmatine (10 µg/rat, i.c.v.) and sessions. Two way ANOVA demonstrated the main effect of agmatine treatment (10 µg/rat, i.c.v.) in combination with moxonidine (25 µg/rat, i.c.v.) [F (2, 648) = 0.75, P = 0.48] or 2-BFI (10 µg/rat, i.c.v.) [F (2, 648) = 0.62, P = 0.544] as well as drinking sessions [moxonidine - F (24, 648) = 2.98, P < 0.001 or 2-BFI - F (24, 648) = 4.16, P < 0.001]. Imidazoline I_1 receptor agonist, moxonidine (25 µg/rat, i.c.v.) or imidazoline I_2 receptor agonist, 2-BFI (10 µg/rat, i.c.v.) with agmatine (10 µg/rat, i.c.v.) administered prior to 22nd drinking session produced a synergistic reduction in ethanol intake. Post-hoc analysis by Bonferroni mean comparisons showed a significant augmentation of agmatine effect by moxonidine (25 µg/rat) (P < 0.05) or 2-BFI (10 µg/rat) (P < 0.001) pretreatment respectively. However, the doses of moxonidine, 2-BFI or agmatine used alone did not influence the ethanol consumption or water intake (data not shown).

3.5. Imidazoline receptor antagonists reverse the effect of agmatine on ethanol intake

As shown in Fig. 7, two way ANOVA demonstrated the interaction between pretreatment of imidazoline I_1 receptor antagonist, efaroxan (10 µg/rat, i.c.v.) [F (72, 744) = 1.22, P = 0.115] or imidazoline I_2 receptor antagonist, idazoxan (4 µg/rat, i.c.v.) [F (72, 792) = 2.10, P < 0.001] 10 min before agmatine (20 µg/rat, i.c.v.) and sessions. Statistical analysis suggested the main effect of efaroxan [F (3, 744) = 1.87, P = 0.156] or idazoxan [F (3, 792) = 1.76, P = 0.1736] pretreatment and drinking sessions [efaroxan - F (24, 744) = 3.18, P < 0.001; idazoxan - F (24, 792) = 5.92, P < 0.001]. Prior administration of imidazoline I_1 receptor antagonist, efaroxan (10 µg/rat, i.c.v.) or imidazoline I_2 receptor antagonist, idazoxan (4 µg/rat, i.c.v.) significantly abolished the effect of agmatine (20 µg/rat, i.c.v.) on the ethanol intake during drinking session 22. Post hoc Bonferroni mean comparison showed significant increase in the ethanol consumption in efaroxan (10 µg/rat) + agmatine (20 µg/rat) (P < 0.01) and idazoxan (4 µg/rat) + agmatine (20 µg/rat) (P < 0.001) as compared to aCSF (5 µl/rat) + agmatine (20 µg/rat) treated animals. However, administration of efaroxan (10 µg/rat) or idazoxan (4 µg/rat) alone in the doses used here did not influence the ethanol consumption or water consumption in two bottle choice paradigm. Moreover, administration of efaroxan (10 µg/rat) (P < 0.05) or idazoxan (4 µg/rat) (P < 0.05) before agmatine (20 µg/rat) also decreased the preference to ethanol consumption.

4. Discussion

This study demonstrated the role of agmatinergic system on ethanol self-administration in operant chamber and ethanol consumption in two bottle choice paradigm.

In the operant conditioning paradigm, the rats were trained on the FR1 schedule of reinforcement to self-administer 200 mg % ethanol according to reported literature (Rodd-Henricks et al., 2000; Shelkar et al., 2015). While rats shown consistent lever presses for ethanol during first four sessions, replacement with aCSF extinguished the response. The high lever press activity was found when ethanol was reinfused on the seventh session, which confirmed that the animals responded to the reinforcing value of ethanol. We used a single 30-minute session every day, for 7 consecutive days of the study as ethanol self-

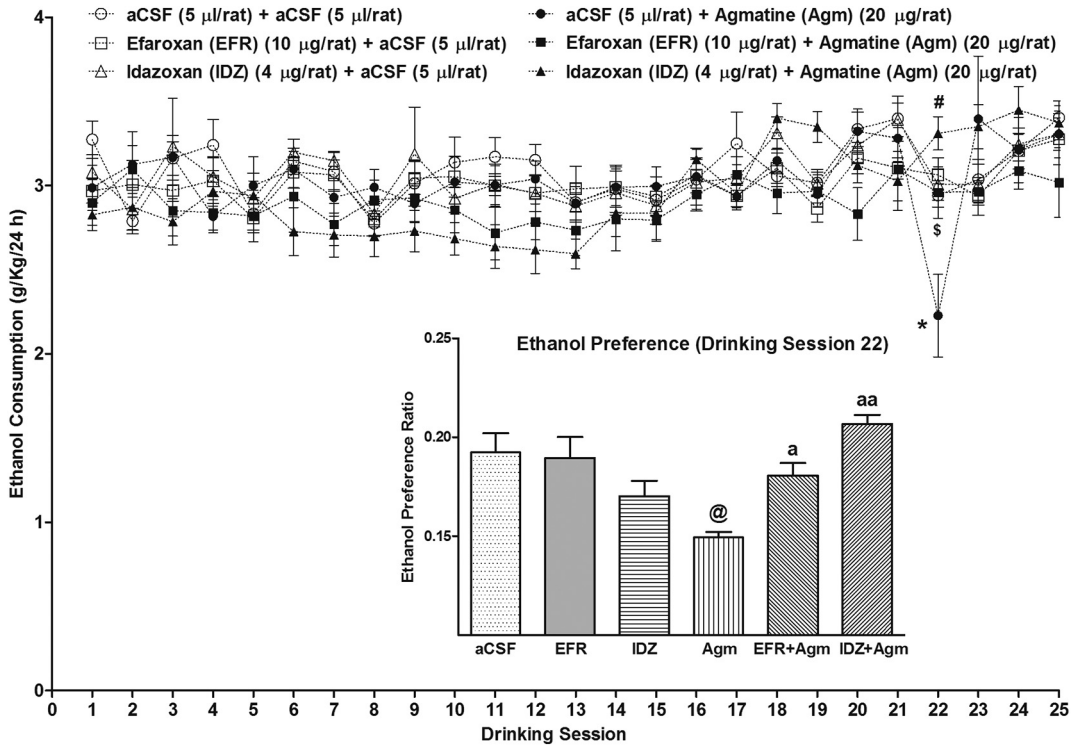

Fig. 7. Influence of imidazoline I_1 receptor antagonist, efaroxan (10 µg/rat, i.c.v.) or imidazoline I_2 receptor antagonist, idazoxan (4 µg/rat, i.c.v.) pretreatment on the effect of agmatine (20 µg/rat, i.c.v.) on ethanol consumption during 25 drinking sessions of voluntary ethanol drinking in two bottle choice paradigm. Data represent mean ethanol consumption (g/kg/24 h)/water consumption (ml/rat)/ethanol preference ratio ± S.E.M. (n = 8–10/group). *P < 0.01 versus aCSF treated control animals; $P < 0.001, #P < 0.001 versus agmatine (20 µg/rat, i.c.v.) treated animals (Two way ANOVA followed by post hoc Bonferroni test). @ P < 0.01 versus aCSF treated control animals; ^{a}P < 0.05, ^{aa}P < 0.001 versus agmatine (20 µg/rat, i.c.v.) treated animals (One way ANOVA followed by post hoc Bonferroni test).

administration at high-frequency was encountered during the first 30 min of total session (Rodd-Henricks et al., 2000). The rewarding effects of centrally administered drugs of abuse depend on the regional heterogeneity and local brain circuitry, and the intracranial self-administration provides a tool for determining the exact regional circuitry (Rodd-Henricks et al., 2000; Ikemoto, 2010). In this study, we observed a significant increase in lever presses when ethanol was being directly delivered in right p-VTA. This area is known to be highly sensitive for the ethanol reward, and the action is reportedly mediated via mesolimbic dopaminergic pathway (Rodd-Henricks et al., 2000; Rodd et al., 2004). Ethanol self-administration, via oral or intravenous routes, has been reported to increase dopamine release in the nucleus accumbens and VTA (Campbell et al., 1996; Ding et al., 2015; Kohl et al., 1998). In this background, the increase in ethanol self-administration observed in the present study, might reflect the rewarding effect via mesolimbic DA system as suggested by earlier authors (Gilpin and Koob, 2008; Rodd et al., 2004). Although the direct interaction of agmatine and dopaminergic system is yet to be reported, it diminished the dopamine levels in ventral tegmental area evoked by morphine withdrawal (Wei et al., 2007) and modulated drug addictive processes (Kotagale et al., 2010; Taksande et al., 2010). The data of present study therefore suggest that regulation of brain dopaminergic signalling in VTA may be critical for inhibition of ethanol self-administration of rats by agmatine.

In two bottle choice paradigm, we found that treatment of agmatine given before 22nd cycle of two bottle choice paradigm significantly reduces ethanol consumption in rats over a 24 h time period. Agmatine decreased ethanol consumption in a dose-related manner, and the ability of agmatine to reduce ethanol consumption did not relate to any sedative or antidipsogenic action. Our results are supported by earlier findings that agmatine decreases the morphine, cocaine, fentanyl self-administration (Morgan et al., 2002), inhibits the ethanol induced locomotor sensitization (Ozden et al., 2011) and blocked the acquisition of ethanol conditioned place preference in mice (Sameer et al., 2013).

Agmatine is metabolized to putrescine and guanido-butanoic acid by enzyme agmatinase and diamine oxidase (DAO) respectively (Reis and Regunathan, 2000) and inhibition of these enzymes result in augmentation of endogenous agmatine levels (Regunathan, 2006; Lu et al., 2003; Huang et al., 2003). Here, we used a DAO inhibitor, amino-guanidine (Lu et al., 2003) and an agmatinase inhibitor, arcaine (Huang et al., 2003; Regunathan, 2006). In fact, results of our previous study clearly indicate that these drugs substantially increase the levels of agmatine brain (Taksande et al., 2009). Although all these drugs inhibited ethanol consumption in rats, the maximum reductions was achieved by L-arginine at comparatively high dose. High dose of L-arginine (1000 mg/kg, i.p.) is reported to inhibit ethanol withdrawal syndrome in rats (Uzbay and Erden, 2003). However, high doses of L-arginine supplement could be associated with activation of NO and ornithine pathways leading to enhanced polyamine formation. The concentration of polyamines has been positively correlated with the severity of withdrawal-induced tremors and seizures in ethanol dependent animals (Davidson and Wilce, 1998) and in the pathogenesis of fatal alcohol syndrome (Littleton et al., 2001). Therefore, selective activation of agmatinergic pathway is highly desirable. This can be achieved by stimulating ADC, an enzyme which synthesized agmatine from L-arginine. However, till date no ADC activator is available. Thus, targeting agmatinase through arcaine would be ideal way to enhance agmatine levels in brain and to reduce the ethanol consumption. Overall, it is possible that ethanol intake may be directly linked with reduced agmatine levels in brain and its elevation may have therapeutic implications in alcoholism. Amino-guanidine augments the endogenous agmatine levels by inhibiting enzyme DAO that metabolizes agmatine into guanido-butanoic acid (Reis and Regunathan, 2000). The elevation of agmatine levels promotes inhibition of NOS in brain. Several NOS inhibitors like NG-nitro arginine-methyl-ester and 7-nitroindazole

exhibit potent inhibitory effects on ethanol intake (Naassila et al., 2000). This indicates that NO production may also be critically involved in ethanol seeking behaviour in rodents. Similarly, in addition to agmatinase inhibitor, arcaine also blocks the NMDA receptor subtypes at polyamine site. Since agmatine selectively blocks NMDA receptor and NO formation (Yang and Reis, 1999; Askalany et al., 2005), it is likely that this property may have some relevance to the beneficial effect of agmatine in ethanol intake.

The pharmacological actions of agmatine are commonly linked to its interaction with imidazoline receptors. Brain regions regulating endocrine and affective functions have abundant imidazoline binding sites and co-localized with its endogenous ligands like agmatine (De Vos et al., 1994; Raasch et al., 1995; Otake et al., 1998). In the present study, the effect of agmatine was potentiated by imidazoline receptor agonists (moxonidine and 2 BFI) and inhibited by its antagonists (Efaroxan and Idazoxan). The results are in line with our earlier observation that agmatine inhibits the ethanol sensitization and withdrawal anxiety through imidazoline receptors (Taksande et al., 2010, 2019). A number of preclinical studies have demonstrated the complex interaction between imidazoline receptors system and ethanol (Aglawe et al., 2014; Taksande et al., 2009). Chronic ethanol administration tends to counteract the hypotensive effect of imidazoline I_1 receptor agonists, clonidine and rilmenidine (El-Mas and Abdel-Rahman, 2001) which was completely reversed by efaroxan, an imidazoline I_1 receptor antagonist. Clonidine, an imidazoline $I_1/\alpha 2$ agonist reduced alcohol intake and reduced the signs of alcohol abstinence (Mondavio and Ghiazza, 1989; Mao and Abdel-Rahman, 1996; Opitz, 1990; Parale and Kulkarni, 1986) and moxonidine, an imidazoline I_1 receptor agonist inhibited ethanol withdrawal-induced elevation of acoustic startle response in rodents (Vandergriff et al., 2000). On the other hand, imidazoline I_2 receptor antagonist idazoxan, stimulated ethanol intake in rats and blocked locomotor stimulant, anxiolytic and hypothermic effect of acute ethanol injections (Durcan et al., 1989; Grupp et al., 1997). These findings suggest that agmatine and its interaction with imidazoline receptors may be responsible to reduce ethanol intake in rats. However, possibility exists for pharmacokinetic interaction between ethanol and imidazoline agents employed in the study.

Nevertheless, we cannot rule out the possibility of α_2 adrenergic receptors as these agents demonstrated significant affinity towards α_2 receptors. In addition, more studies are required to explore the effect of agmatine on ethanol reinforcement and consumption in sex specific manner and absence of intake in presence of reward like conditioned place preference.

In conclusion, this study demonstrated that the ethanol consumption was significantly attenuated by agmatine as well as the drugs known to elevate its endogenous levels like L-arginine, amino-guanidine and arcaine. The effect of agmatine on ethanol intake was potentiated by imidazoline receptor I_1 agonist, moxonidine, imidazoline receptor I_2 agonist 2-BFI and completely blocked by imidazoline receptor I_1 antagonist, efaroxan and imidazoline receptor I_2 antagonist, idazoxan. In conclusion, the present studies suggest the importance of agmatine and imidazoline receptors system to reduce ethanol consumption and may project agmatine as a potential therapeutic target for alcoholism and associated complications.

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