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TO STUDY THE EFFECT OF 5-HT$_{1A}$ ANALOGUES, 8-OH-DPAT AND WAY 100635 IN ANXIETY

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ABSTRACT

present work constitutes an evaluatory role of serotonin receptors using its analogues by administering to CeA in ethanol-induced anxiolysis, tolerance to ethanol anxiolysis using elevated plus maze (EPM) paradigm in rats. Ethanol was injected intraperitoneally (i.p.) with exception of ketamine and xylazine that was given by intramuscular (i.m.) route. Ethanol was diluted with 0.9% saline to a concentration of 8% w/v for i.p. injection or to 6% v/v in a liquid diet for oral chronic consumption. 5-HT1A serotonin receptor analogues will be injected by intra central nucleus of amygdala (i.CeA.) Prolonged ethanol (alcohol) consumption leads to the development of tolerance and dependence.

KEYWORDS: Ethanol, 5-HT$_{1A}$ Serotonin analogue, EPM, Tolerance.

1. MATERIALS

1.1. Subjects

Adult male Sprague Dawley rats weighing between 200-250g was used respectively. All animals were on a 12:12-h light/dark cycle (lights on 0700 h) in a temperature-controlled (24±1°C) environment and behavioral assessment was conducted during the light cycle between 0900 h and 1400 h to minimize diurnal steroid fluctuations. Each experimental group had a separate set of animals (n=6), and an individual animal was tested once only to avoid 'one-trial tolerance' to anxiolytic efficiency of drugs including ethanol (Bertoglio and Carobrez, 2002) in EPM test. Animals were brought to the experimental room 12 h prior to the start of the experiment to minimize nonspecific stress-induced steroid increase.

All procedures were carried out under strict compliance with ethical principles and guidelines of the Committee (IAEC/Pharmacol/05/2010-11) for the Purpose of Control and Supervision

of Experimental Animals, Ministry of Environment and Forests, Government of India, New Delhi, and approved by Institutions animal ethics committee. Every effort was made to reduce the suffering of animals during experiments.

1.2. Drugs

Table 1: List of drugs used.

Sr. no	Drugs	Category	Solvent	Route
1	Ethanol	Drug of abuse	0.9% saline	i.p
4	8-OH –DPAT	$5HT_{1A}$ Agonist	0.9% saline	i.cea
5	WAY 1000365	$5HT_{1A}$ Antagonist	0.9% saline	i.cea

1.3. Drugs Solutions and Administrations

Ethanol was injected intraperitoneally (i.p.) with exception of ketamine and xylazine that was given by intramuscular (i.m.) route. Ethanol was diluted with 0.9% saline to a concentration of 8% w/v for i.p. injection or to 6% v/v in a liquid diet for oral chronic consumption. 5-HT1A serotonin receptor analogues will be injected by intra central nucleus of amygdala (i.CeA.). The serotoninergic drugs will be diluted with artificial cerebrospinal fluid (aCSF) of following composition; 0.2 M NaCl, 0.02 M NaH_2Co_3, 2 mM KCl, 0.5 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.8 mM $MgCl_2$, 0.5 mM Na_2SO_4, 5.8 mM D- glucose (Dissolved in double distilled water).

2. METHODS

Elevated plus maze apparatus

The plus maze consisted of opposite facing two open (50×10 cm) and two enclosed arms (50×10×40 cm) connected by a central platform (10×10 cm), thus making a plus sign. The whole maze is raised 60 cm above the floor. rat were tested on the plus maze in a room with low, indirect incandescent lighting (100-W lamp, fixed 2 m above the maze floor) and very low noise levels On the day of testing, rat were placed singly at the center of the maze, head facing an open arm and allowed to explore for 5 min. The number of entries into the open arms, the time spent in open arms as well as the number of closed-arm entries were recorded for 5 min by a digital video tracking device (V. J. instruments, India), connected to computer outside in order to reduce the disturbance to animals behavior during test session. Moreover, closed-arm entries were considered as an index of general activity levels than the total arm entries (Dawson et al., 1995; Pellow et al., 1985; Lister, 1987; Rao et al., 2003; Rodgers and Johnson, 1995). An entry was registered only when all four paws of the animal were placed on the arm. The maze was wiped clean with damp cotton and dried after testing each rat.

Anxiogenic or anxiolytic effects were assessed based on the frequency of open arms entries as well as time spent in the open arms (Pellow et al., 1985). Decrease in time spent on the open arms and a low frequency of open arms entries relative to control animals were considered as an increase in anxious behavior. Separate groups of animals were used for each treatment and each subject tested were given a single 5 min trial. All animals were tested between 0900–1400 h to minimize circadian influences.

2.1. Calculation of Behavioral parameters on EPM

a. % open arms time spent (% OAT): (seconds spent on the open arms of the maze/300) × 100;
b. % open arm entries (% OAE): (the number of entries into the open arms of the maze/number of entries into open + closed arms) × 100;
c. Number of closed arms entries were recorded (Motor activity was assessed by recording the distance travelled by animal in cm).

2.2. Chronic ethanol administration

Ethanol was administered to rats for prolonged period as described previously with some modifications (Miller et al., 1980; Lal et al., 1988; Jung et al., 2000; Hirani et al., 2002; Kokare et al., 2006). Briefly, rats were assigned to different treatment groups and housed individually in polypropylene cages. Initially they received nutritionally balanced liquid diet (PROTINEX, Novartis, India) for two days to allow adaptation to novel food. Water was available ad libitum. From third day onwards, ethanol was added to the liquid diet of some groups (final concentration 6% v/v, ethanol-fed), while isocaloric amount of ethanol was substituted with dextrose in the liquid diet of remaining groups (pair-fed control) and had free access to it for 1, 3, 5, 7 or 10 days. Fresh aliquot (100 ml/rat) of ethanol containing liquid diet was introduced in the respective cages each morning at 0800 h. Diet of pair-fed groups was unchanged but was restricted so that consumption matched the mean amount of the ethanol-containing diet consumed. The dietary consumption and body weight of each animal was monitored daily (0800 h) for all the groups. The average daily ethanol consumption was found to be 10.56 g/kg. Body weights of ethanol-fed rats were not different in comparison to pair-feds during initial drinking phase or throughout the course of the experiment. The animals were subjected for 5 min to elevated plus maze test at different time intervals, but individual animal was tested only once to avoid ‘one trial tolerance’ to drug effect (Bertoglio and Carobrez, 2002).

2.3. Intra-central nucleus of amygdala (i.CeA) drug administration

The procedures for bilateral implantation of cannulae into the CeA and intra-CeA administration of drugs have already been standardized (Kokare et al., 2005; Finn et al., 2007; Dandekar et al., 2008, 2009). Briefly, a stainless steel guide cannula (Plastics One, Roanoke) was implanted into the CeA using the stereotaxic coordinates, −2.4 mm posterior, ± 4.0 mm lateral to midline and 8.0 mm ventral relative to bregma (Paxinos and Watson, 1998). After cannulation, animals were housed individually (until the completion of the study) and allowed to recover for 7 days. The animals with any neurological/motor deficits were excluded from the study. During recovery and chronic treatment period, each rat was habituated to experimental room, intra-CeA injection procedure and handled daily to minimize isolation induced neurochemical and behavioral changes (Kokare et al., 2005). Intra-CeA injections were given using microliter syringe (Hamilton Company, Nevada) connected by polyethylene tubing to a 31-gauge internal cannula for rats (Plastics One), which projected 0.1 mm below the guide cannula.

2.3. Statistical analysis

The data were analyzed by parametric tests (Sharma et al., 2007; Umathe et al., 2008), significant differences between treatment groups were determined by using one-way ANOVA followed by the Newman-Keuls test or two-way ANOVA followed by Bonferroni test for multiple comparisons. All values are expressed as mean ± SEM of 6 rats in each group. P value less than 0.05 was considered statistically significant in all of the cases.

2.4. Tolerance to ethanol anxiolysis

This experiment was designed to examine the development of tolerance to anxiolytic effect of ethanol. Animals were randomly assigned to different treatment group and during chronic ethanol studies, ethanol-fed as well as pair-fed rats were challenged on days 1, 3, 5, 7 or 10 (09:00 h), with anxiolytic dose of ethanol (2 g/kg, i.p., 8% w/v) or saline (10 ml/kg, i.p.). Thirty minutes thereafter, individual rat was subjected to EPM test for 5 min session to check the development of tolerance to ethanol induced anxiety related triats. Separate groups of animals were employed for different experimental days and the group once challenged with ethanol and tested for anxiety parameters was discontinued from further studies to avoid one trail tolerance.

3. Experimental design

3.1. Effect of 8-OH-DPAT or WAY-100635 on ethanol-induced anxiolysis

To assess the role of $5HT_{1A}R$ on ethanol-induced anxiolysis present in amygdala, separate groups of rats were administered with aCSF (intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) or 8-OH-DPAT (0.05 &,0.1, µg/rat; intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) or WAY100635 (0.05 & 0.1, µg /rat; intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) 15 min prior to sub-anxiolytic dose (1 g/kg, i.p.) or anxiolytic dose (2 g/kg, i.p.) of ethanol (8% w/v) or saline (10 ml/kg, i.p.) treatment, respectively. 30 min after ethanol injection, individual rat was subjected to EPM test and anxiety related indices were measured as described above for 5 min.

3.2. Effect of chronic treatment of 5-HT_{1A} analogues, 8-OH-DPAT and WAY 100635 on tolerance to ethanol anxiolysis

These experiments were designed to examine the role of serotonin receptors (5-HT_{1A}Rs) in the development of tolerance to anxiolytic effect of ethanol. Animals were randomly assigned to different treatment groups. During chronic ethanol studies, ethanol-fed as well as pair-fed rats were administered bilaterally twice daily (10:00 h and 22:00 h) either with aCSF (intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) or 8-OH DPAT (0.1 µg/rat; intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) or WAY 100635 (0.1 µg /rat; intra-CeA; 1 µl/rat; 0.5 µl/rat in each side) for 10 days. On days 1, 3, 5, 7 or 10 (09:00 h), some of these rats were challenged with ethanol (2 g/kg, i.p., 8% w/v) or saline (10 ml/kg, i.p.). Thirty minutes thereafter, individual rat was subjected to EPM test for 5 min session. The doses of 5-HT_{1A} agonist/ antagonist, used for this protocol were selected from the results of acute studies. Separate groups of animals were employed for different experimental days and the group once challenged with ethanol and tested for anxiety parameters was discontinued from further studies.

4. RESULTS

4.1. Effects of acute ethanol in EPM test

One way ANOVA revealed that acute treatment of ethanol have significantly affected the open arm behavior of rats after comparing with respective control groups on EPM [ethanol (%OAT, $F(5,30)=349.5$, $P<0.0001$; %OAE, $F(5,30)=178.0$, $P<0.0001$)]. Post hoc test indicates that ethanol (1, 1.5 & 2 g/kg, i.p.) significantly enhances the preference of the rat for open arm as evident from increase in both the indices i.e, %OAT ($P<0.001$) and %OAE ($P<0.001$), without affecting the number of closed arm entries [ethanol $F(5,30)=1.430$,

P>0.05]. However, treatment of higher dose of ethanol (2.5 g/kg, i.p.) shows reduced %OAT and %OAE (P<0.001) along with decrease in closed arm entries (P<0.05), as compared to saline treated rats. Ethanol (0.5 g/kg, i.p.) treatment in this dose was devoid of any effect on anxiety indices on EPM (P>0.05). These results are depicted in Fig. 2.

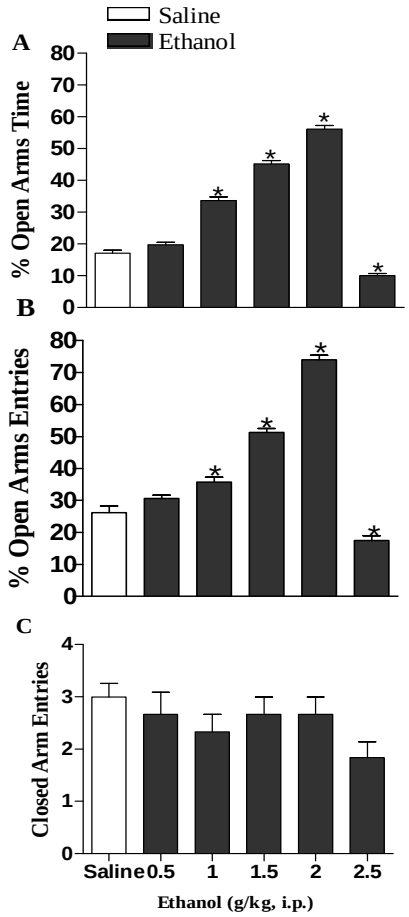

Fig. 2: Effect of acute ethanol (g/kg, i.p.; 8% w/v in saline), anxiety related indices were measured in EPM. Each bar represents mean ±SEM of data from 6 rats. *P<0.001, vs respective control (One way ANOVA followed by Newman-Keuls Test).

4.2. Development of tolerance to ethanol induced anxiolytic effect

Fig. 3. shows the development of tolerance to anxiolytic effect of ethanol in ethanol fed (6% v/v) rats for 10 days, challenged with ethanol (2 g/kg, i.p.; 8% w/v) on days 1, 3, 5, 7 & 10. Two-way ANOVA followed by Bonferroni test revealed significant decrease in %OAT [factor 'treatment' F(1,50)=56.42, P<0.0001, factor 'day' F(4,50)=77.27, P<0.0001 and interaction 'treatment×day' F(4,50)=76.43, P<0.0001] and %OAE [factor 'treatment' F(1,50)=368.4, P<0.0001, factor 'day' F(4,50)=362.3, P<0.0001 and interaction

'treatment×day' F(4,50)=384.9, P<0.0001] with acute ethanol challenge (2 g/kg, i.p.) in aCSF administered ethanol-fed rats as compared to pair-fed rats on days 7 and 10. Consumption of ethanol did not affect the closed arm entries [factor 'treatment' F(1,50)=3.265, P=0.0768, factor 'day' F(4,50)=0.5867, P=0.6737 and interaction 'treatment×day' F(4,50)=1.862, P=0.1317 NS].

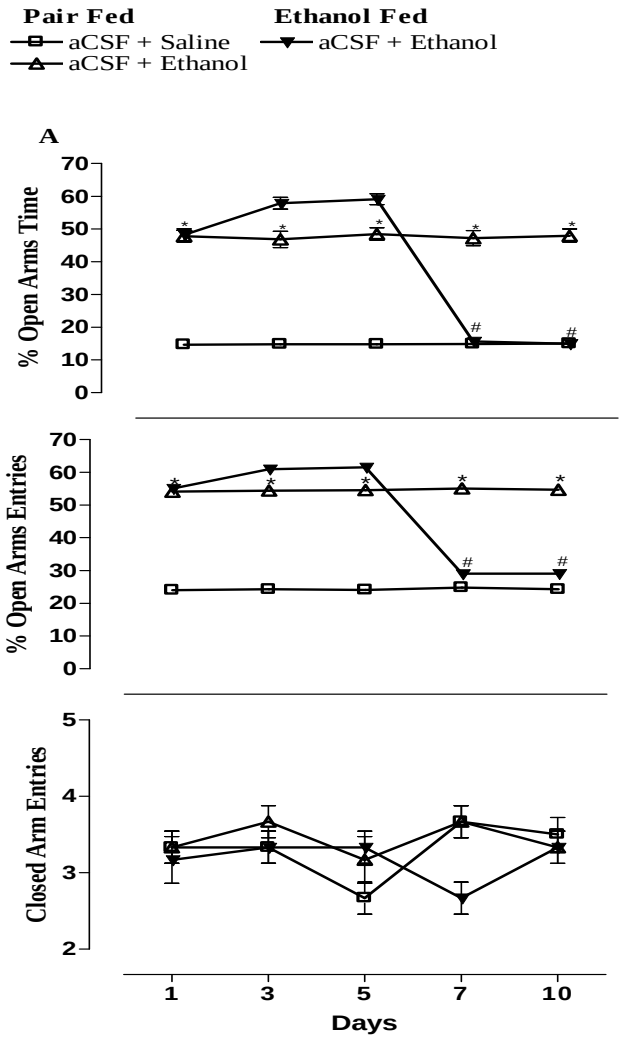

Fig. 3: Development of tolerance to ethanol-induced anxiolysis on EPM. Different groups of rats were treated with vehicle (aCSF) control (1 μl/rat, intra-CeA) twice daily 10:00 and 22:00 h respectively, consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. Rats were challenged on days 1, 3, 5, 7 or 10 with ethanol (2 g/kg, i.p., 8% w/v) or saline (10 ml/kg, i.p.) and 30 min thereafter, individual rat was placed on the central platform to explore the EPM for 5 min. Each bar represents means±S.E.M. of data from 6 rats per group.

***P<0.001 vs. aCSF+saline (pair-fed group); #P<0.001 vs. aCSF+ethanol (pair-fed group) (Two-way ANOVA followed by Bonferroni test).**

4.3 Chronic 8-OH-DPAT administration prevented development of tolerance to ethanol-induced anxiolytic effect

As shown in Fig.4 A, concomitant 8-OH-DPAT administrations (0.1 µg/rat, intraCeA, 1 µl/rat; 0.5 µl/rat in each side 10:00 and 22:00 h) to ethanol-fed rats (6% v/v) prevented tolerance to ethanol (2 g/kg, i.p., 8% w/v)-induced anxiolysis. Two-way ANOVA followed by Bonferroni test revealed reversal of decrease in %OAT [factor 'treatment' $F(1,50)=412.4$, $P<0.0001$, factor 'day' $F(4,50)=125.9$, $P<0.0001$ and interaction 'treatment×day' $F(4,50)=59.12$, $P<0.0001$] and %OAE [factor 'treatment' $F(1,50)=998.1$, $P<0.0001$, factor 'day' $F(4,50)=244.5$, $P<0.001$ and interaction 'treatment×day' $F(4,50)=143.4$, $P<0.001$] with acute ethanol challenge (2 g/kg, i.p.) in 8-OH-DPAT administered ethanol-fed rats as compared to ethanol-fed rats on days 7 and 10. Consumption of ethanol and chronic 8-OH-DPAT treatment did not affect the closed arm entries [factor 'treatment' $F(1,50)=20.00$, $P<0.0001$, factor 'day' $F(4,50)=3.594$, $P=0.0119$ and interaction 'treatment×day' $F(4,50)=0.4688$, $P=0.7584$ NS] throughout the chronic treatment.

4.4. Chronic WAY-100635 administration prevented development of tolerance to ethanol-induced anxiolytic effect

As depicted in Fig.4 B, daily WAY-100635 administrations (0.1 µg/rat, intraCeA, 1 µl/rat; 0.5 µl/rat in each side 10:00 and 22:00 h) to ethanol-fed rats (6% v/v) prevented tolerance to ethanol (2 g/kg, i.p., 8% w/v)-induced anxiolysis. Two-way ANOVA followed by Bonferroni test revealed significant increase in %OAT [factor 'treatment' $F(1,50)=223.1$, $P<0.0001$, factor 'day' $F(4,50)=47.72$, $P<0.0001$ and interaction 'treatment×day' $F(4,50)=77.12$, $P<0.0001$] and %OAE [factor 'treatment' $F(1,50)=233.1$, $P<0.0001$, factor 'day' $F(4,50)=35.05$, $P<0.0001$ and interaction 'treatment×day' $F(4,50)=110.6$, $P<0.0001$] with acute ethanol challenge (2 g/kg, i.p.) in WAY-100635 administered ethanol-fed rats as compared to ethanol-fed rats on days 7 and 10. Consumption of ethanol and chronic WAY 100635 treatment did not affect the closed arm entries [factor 'treatment' $F(1,50)=2.832$, $P=0.0986$, factor 'day' $F(4,50)=0.6643$, $P=0.6197$ and interaction 'treatment×day' $F(4,50)=0.2098$, $P=0.9318$ NS] throughout the chronic treatment.

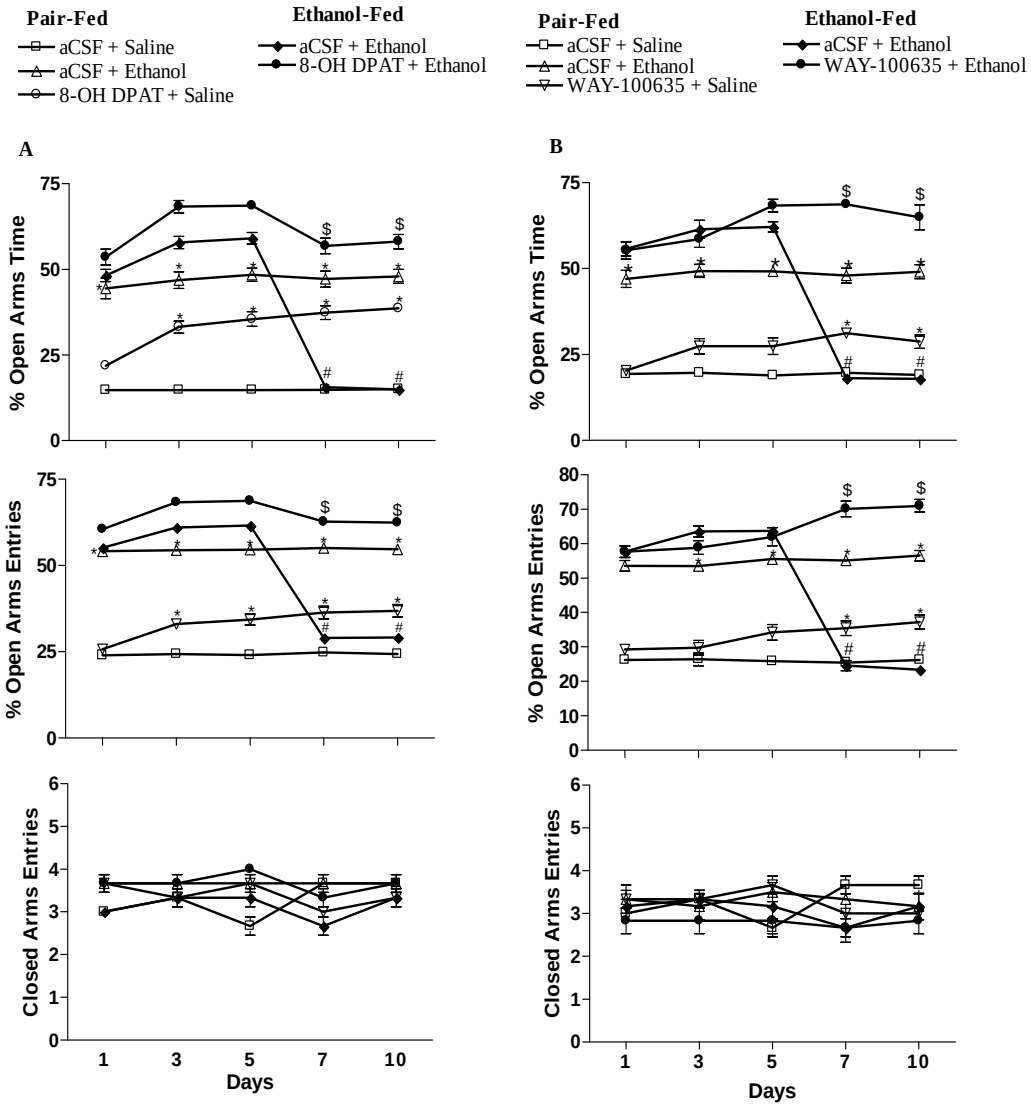

Fig. 4: Effect of daily administration of 8-OH DPAT (A) or WAY 100635 (B) on the development of tolerance to ethanol-induced anxiolysis showing in EPM. Different groups of rats were treated with vehicle (aCSF) control (1 μl/rat, intra-CeA) or 8-OH DPAT (0.1 μg/rat, intra-CeA) or WAY 100635 (0.1 μg/rat, intra-CeA) twice daily 10:00 and 22:00 h respectively, consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. Rats were challenged on days 1, 3, 5, 7 or 10 with ethanol (2 g/kg, i.p., 8% w/v) or saline (10 ml/kg, i.p.) and 30 min thereafter, individual rat was placed on the central platform to explore the EPM for 5 min. Each bar represents means±S.E.M. of data from 6 rats per group. *P<0.001 vs. aCSF+saline (pair-fed group); $^{\#}$P<0.001 vs. aCSF+ethanol (pair-fed group); $^{\$}$P<0.001 vs. aCSF+ethanol (ethanol-fed group) (Two-way ANOVA followed by Bonferroni test).

DISCUSSION

The study examined the differential role of serotonergic receptors in CeA on development of tolerance to anxiolytic effect of ethanol and withdrawal anxiety. Acute i.p administration of ethanol (1.5 or 2g/kg) exhibited antianxiety effect by increase in open arm indices of rats in EPM, whereas higher dose (2.5 kg) exerts sedation by decrease in open and closed arms exploration. These results supporting their classical anxiolytic profile (Gallate et al., 2003; Hirani et al., 2005; Kameda et al., 2007; Kokare et al., 2006; Polivy and Herman, 1976). Ethanol is already suggested to interact with different neurotransmitters such as GABA, NMDA, 5-HT_3, certain peptides and GABAergic neurosteroids (Besheer et al., 2009; et al., 1987; Hirani et al., 2005; Linnoila Nagy, 2008; Mukherjee et al., 2008).

Various reports suggest that alcohol interacts with serotonergic synaptic transmission in brain in several ways (Lovinger, 1999; Overstreet et al., 1994). In addition, reports suggest acute administration of ethanol increases serotonin metabolite in urine and blood, indicating increase 5-HT release in brain (Lovinger, 1999). Other studies also confirmed that acute ethanol exposure elevate 5-HT level within the brain (Storvik et al., 2006). There are very less evidences suggesting that serotonin (5-HT) involved in both alcohol abuse and anxiety. Therefore, the present investigation explored the role of central serotonergic transmission in the ethanol induced anxiolysis, tolerance to antianxiety effect of ethanol and withdrawal induce anxiety.

Prolonged ethanol consumption led to the development of tolerance to acute ethanol (2 g/kg, i.p., 8% w/v) anxiolysis as compared to pair-fed liquid diet group.

In summary, we report that administration of 5-HT_{1A} analogue 8-OH-DPAT and WAY 100635 attenuated the development of tolerance to ethanol anxiolysis. 5-HT_{1A} receptors activation presynaptically might reduce increase in 5-HT transmission during ethanol tolerance and withdrawal implicating this receptor agonist in the treatment of alcoholism.

In conclusion, finally it can be suggested that during chronic ethanol increase in 5-HT transmission might be attributed to increase in tolerance to ethanol antianxiety effect and withdrawal induced anxiety. Moreover, 5-HT_{1A} receptors activation presynaptically might reduce increase in 5-HT transmission during ethanol tolerance and withdrawal implicating this receptor agonist in the treatment of alcoholism.

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