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RESEARCH ARTICLE

Glucagon-like peptide-1 (GLP-1) receptor agonist prevents development of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats

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Abstract Despite major advances in the understanding about ethanol actions, the precise underlying neurobiological mechanisms for ethanol dependence remain largely elusive. We recently reported that inhibition of dipeptidyl-peptidase IV (DPP-IV), an enzyme responsible for metabolism of endogenous glucagon-like peptide-1 (GLP-1), delays tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats. Intrigued with this report, present study examined the role of glucagon-like peptide-1 (GLP-1) receptor agonist, liraglutide in (1) acute anti-anxiety effect of ethanol; (2) tolerance to ethanol's anti-anxiety-effect and (3) ethanol withdrawal-induced anxiety using elevated plus maze (EPM) test in rats. Ethanol (2 g/kg, i.p.; 8 % *w/v*) and liraglutide (50 μg/kg, i.p.) treatments exhibited anti-anxiety effect in EPM test. Doses of ethanol (1.0 or 1.5 g/kg, i.p.) that were not effective per se elicited anti-anxiety when combined with sub-effective dose of liraglutide (25 μg/kg, i.p.). Rats consuming ethanol-diet (6 % *v/v*) exhibited tolerance to anti-anxiety effect of ethanol from day-7 of ethanol consumption. Peak ethanol withdrawal-induced anxiety was observed at 8–10 h upon abstinence from ethanol-diet after 15-days consumption. Rats on simultaneous once-daily liraglutide treatment (50 μg/kg, i.p.) neither had any signs of tolerance to anti-anxiety effect of ethanol nor did they exhibit withdrawal-induced anxiety. In conclusion: (1) GLP-1 agonist, liraglutide exhibited anti-anxiety effect per se; (2) potentiated anti-anxiety effect of ethanol; (3) prevented development tolerance to anti-anxiety effect of ethanol and (4) prevented withdrawal-induced anxiety. Further studies examining intracellular cascade of events contributing to these effects may help to improve understanding about role of GLP-1 receptors in ethanol mediated behaviors.

Keywords Ethanol dependence · Tolerance · Anxiety · GLP-1 receptor · Liraglutide

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Introduction

Anxiety is one of the major contributing factors for heavy ethanol drinking and relapse (Kushner et al. 2001; Kushner et al. 2000). While acute ethanol consumption helps to relieve from anxiety, its chronic consumption results in tolerance to ethanol's anti-anxiety effect. Animal studies examining motivational effects of ethanol have shown that its ability to relieve anxiety as a major determinant to ethanol craving (Cunningham et al. 2000). However, with repeated consumption, significantly higher amount of ethanol is required to elicit similar anti-anxiety effect, indicating development of tolerance to ethanol actions (Sharma et al. 2007). Moreover, abstinence from heavy ethanol consumption results in withdrawal-induced anxiety symptoms (Sharma et al.

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2007), and serves as a driving force for relapse to ethanol drinking (Samson & Harris 1992; Roelofs 1985). The current treatment strategies for the management of alcoholism and pertaining anxiety employ medications with diverse biological targets such as aldehyde dehydrogenase (Sinclair & Lindros 1981), glutamatergic system (Mann et al. 2008), gamma-amino butyric acid system (Nutt et al. 1989) and opioidergic system (Jarosz et al. 2013). However, due to their limited benefits accompanied with cluster of adverse effects such as sedation, ataxia, amnesia, tolerance, dependence, cross-tolerance and cross-dependence (Lader 1999) persuading researchers to examine for alternatives. Several alternative targets such as neurosteroides (Sharma et al. 2007; Hirani et al. 2005), neuropeptides (Bhisikar et al. 2009) etc. were examined in preclinical settings. Still, there is no definitive understanding in regard to the biological basis for the deleterious effects on ethanol on the brain and behavior. Interestingly, endocrine system molecules affecting metabolism and glucose homeostasis such as insulin (de la Monte et al. 2008), leptin (Rojdmark et al. 2001) and ghrelin (Leggio et al. 2014) etc. are currently being scrutinized for their role in ethanol actions. Among such molecules affecting glucose homeostasis, glucagon-like peptide-1 (GLP-1) receptor agonists are relatively new candidates and are approved by the US FDA for the management of type II diabetes (Gough 2012). Apart from role of these hormones in the management of glucose homeostasis, food intake and body weight gain (Jelsing et al. 2012; Shirazi et al. 2013), recent findings points to their ability to cross blood brain barrier, induce neurogenesis (Hunter & Holscher 2012) and modulate a wide range of centrally mediated effects (Chen et al. 2012; Dixit et al. 2013; Egecioglu et al. 2013a; Egecioglu et al. 2013b). There is significant expression of GLP-1 receptors in several important brain regions (Gu et al. 2013). Moreover, preclinical studies support to their role in neuroprotection (Hunter & Holscher 2012). Additionally, a recent clinical study reported that GLP-1 receptor agonist, liraglutide helps to overcome anxiety and depression scores (Grant et al. 2011). Recently, Egecioglu and co-workers reported that GLP-1 receptor agonist exendine-4 helps to suppress ethanol induced motor stimulation, conditioned place preference, ethanol seeking behavior and dopamine release in the nucleus accumbens in mice (Egecioglu et al. 2013b). Additionally, exendine-4 was shown to attenuate similar behaviors induced by other drugs of abuse such as nicotine, cocaine and amphetamine in mice (Egecioglu et al. 2013a). Further, GLP-1 agonists were shown to be devoid of any adverse hypoglycemic effects in normoglycemic non-diabetic subjects (Vella et al. 2002). We recently reported that inhibitor for endogenous GLP-1 metabolizing enzyme dipeptidyl-peptidase IV (DPP-IV), sitagliptin delays appearance of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats (Sharma et al. 2014a). Intrigued with these reports, present study aimed to examine for the role of GLP-1 receptor agonist, liraglutide in i) anti-anxiety effect of ethanol, ii) tolerance to anti-anxiety effect of ethanol and iii) withdrawal induced anxiety in rats.

Materials and methods

Animals

Subjects were Male Wistar albino rats, born and reared in the animal house of Sinhgad Technical Education Society's (S.T.E.S.s) Smt. Kashibai Navale College of Pharmacy (SKNCOP), Kondhwa (Bk), Pune, India. Young healthy male rats (220–250 g body weight and 80–90 days old) were maintained at 25±2 °C under 12:12 h light/dark cycle (lights on 0700–1900 h) with rodent chow (Nutrivet Life Sciences, India) and water ad libitum. Animals were handled once daily for 1 week before experiments began and acclimatized to laboratory conditions at least 12 h before conducting experiments. All procedure and ethical principles of laboratory animal care were carried out in accordance with the guidelines approved by the Institutional Experimental Animal Ethical Committee.

Drugs

The drugs used were the GLP-1 agonist liraglutide (Novo Nordisk, India) and absolute alcohol (MSSIDC, Mumbai, India). Liraglutide and absolute ethanol were diluted with 0.9 % saline solution. Liraglutide was given by intraperitonial route (i.p.). Ethanol was diluted with 0.9 % saline to a concentration of 8 % *w/v* for intraperitoneal injection and to 6 % *v/v* in a liquid diet for 15-days *per oral* consumption.

Elevated plus maze test (EPM)

The EPM test is an experimental tool to evaluate anxiogenic behavior and/or anti-anxiety effect in rodents. This test takes advantage of conflict behavior in rodents because of conditions such as elevation from the ground and open spaces. The wooden EPM apparatus was consisted of opposite facing two open arms (no sidewalls; 50×10 cm) and two closed arms (with 40 cm high sidewalls; 50×10×40 cm) connected by a central platform (10×10 cm). The whole maze was raised 50 cm above the ground level. Rats were tested on the plus maze in a room with low, indirect incandescent light and very low noise levels. On EPM test day, individual rats were placed on the central platform of the EPM apparatus facing towards

one of the two open arms and allowed to explore it for 5 min. The number of entries into and time spent in each arm was recorded by an observer blind to the treatments. If rat placed all four paws on one of the four arms, an entry to the arms was registered. After 5 min test session for each rat, the maze was wiped clean with 70 % ethanol by damp cotton. The time spent and number of entries to each arm by rats was recorded to evaluate for ethanol and liraglutide induced changes in rat behaviour on EPM test. An increase in the percent time spent and number of entries to the open arms compared to vehicle treated rats was used as an index of anti-anxiety effect. In contrast to this, a decrease in the time spent and number of entries to the open arms compared to vehicle treated rats was used as an index of anxiogenic effects. Additionally, the number of entries to the closed arms was recorded to rule the possibility of changes in open arms behaviour was due to changes in rat motor behavior. Separate groups of animals were used for each treatment. All rats were tested between 0900 and 1400 h to minimize the influence of circadian rhythm on EPM behavior.

Acute study

Effect of acute ethanol treatment on rat behavior in EPM test

Different groups of rats (n=6–7 rats/group) were injected with either vehicle (0.9 % saline) or different doses of ethanol (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg, i.p.; 8 % *w/v* in saline) and 30 min thereafter subjected to EPM test. Rats were observed for the time spent in open arms and number of entries in open as well as closed arms. The outcomes of this experiment were used to determine the optimal anti-anxiety dose of ethanol for further studies.

Time-course of effect of ethanol on EPM test in rats

To determine the optimal time for anti-anxiety effect of ethanol using EPM test, separate groups of rats were injected with ethanol (2.0 g/kg, i.p.). Selection of ethanol dose for this study dose was based on *experiment 2.4.1.* results. Rats were subjected to EPM test at 10, 20, 30, 40, 50, or 60 min post-ethanol treatment. The optimal time for acute ethanol-induced anti-anxiety effect was determined based on the outcomes of this experiment.

Effect of GLP-1 receptor agonist treatment on EPM behavior in rats

To study effect of GLP-1 receptor agonist on rat plus maze behavior, they were injected with either vehicle or different doses of liraglutide (25, 50, 75, 100 μg/kg, i.p.) and 4 h thereafter were subjected to EPM test. Selection of liraglutide dose and lag-time for EPM test post-liraglutide injections was based on our previous report (Shukla et al. 2012c) and pharmacokinetics of liraglutide.

Evaluation of the effects of combination of sub-effective doses of ethanol and GLP-1 receptor agonist on EPM behavior in rats

Experiment *2.4.1.* and *2.4.3.* results were used to select sub-effective doses of ethanol and GLP-1 receptor agonist, liraglutide. Rats were injected with liraglutide (25 μg/kg, i.p.) at 4 h and ethanol (0.5, 1.0 and 1.5 g/kg, i.p.) at 40 min before studying their behavior in EPM test. As discussed above, rats were scored for time spent and number of entries in open arms. Rats were also monitored for their closed arms entries.

Chronic study

Effect of prolonged ethanol consumption on rat plus maze behavior

Rats were randomly assigned to various groups (n=6–7 rats/group) and housed individually in polypropylene cages. To persuade rats to consume nutritionally complete 'liquid diet' (Bournvita®, Cadbury, India), they were deprived of food and water for 12 h prior to introducing bottles pre-filled with 'liquid diet'. Following three days of habituation to 'liquid diet', some groups of rats were introduced to liquid diet mixed with 6 % *v/v* ethanol (called as 'ethanol diet'). From fourth day onward, every morning rats received a bottle of freshly prepared aliquot (100 ml/rat) of either 'liquid diet' or 'ethanol diet' for 15 days. During this period, rats were having free access to food pellets. During chronic ethanol consumption period, rats were injected with acute anti-anxiety dose of ethanol (i.e. 2 g/kg, i.p. based on *experiment 2.4.1.* results) and subjected to EPM test on day 1, 3, 5, 7, 9, 11, 13, and 15. Rats fed with 'liquid diet' (without ethanol) in identical manner served as pair-fed controls for tolerance to >anti-anxiety effect of ethanol study. We previously reported that this experimental design helps in the development of tolerance to anti-anxiety action of ethanol [66]. Separate groups of rats were used for each time-point before subjecting them to EPM test (i.e. day 1, 3, 5, 7, 9, 11, 13 and 15). Acute anti-anxiety dose of ethanol (2 g/kg, i.p.) was used to evaluate for the development of tolerance to anti-anxiety effect of ethanol in rats that were consuming 'ethanol diet'.

Effect of GLP-1 receptor agonist, liraglutide on tolerance to anti-anxiety effect of ethanol in rats

To examine the effect of GLP-1 receptor agonist, liraglutide on development of tolerance to anti-anxiety effect of ethanol, separate groups of rats consuming 'ethanol diet' were

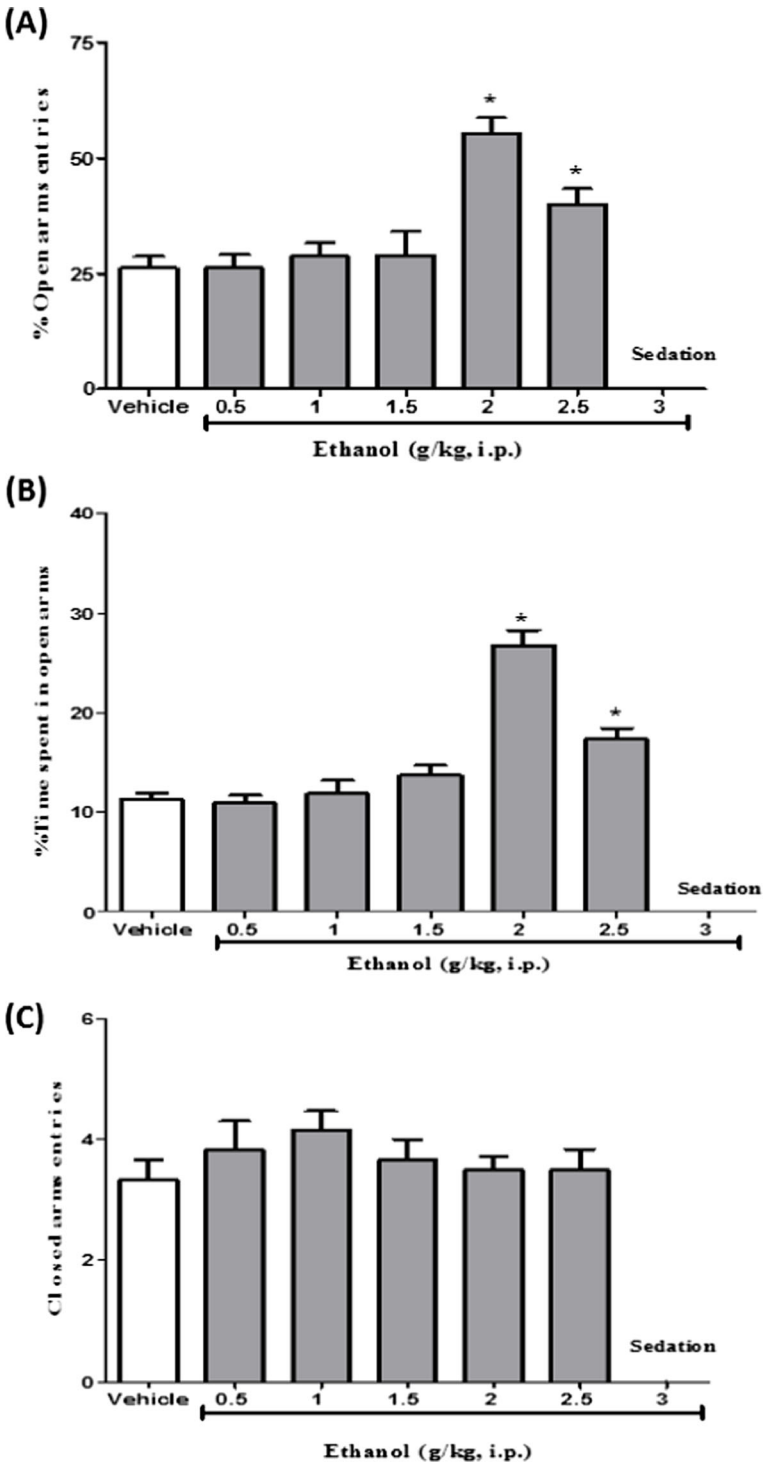

Fig. 1 Dose dependent anti-anxiety effect of ethanol in rat elevated plus maze test. **a** % time spent in open arms **b** % entries into open arms and (C) number of closed arms entries in elevated plus maze test. Separate groups of rats were injected with vehicle (n=6) or different doses of ethanol (0.5 or 1.0 or 1.5 or 2.0 or 2.5 or 3 g/kg, i.p.; n=7/group for 0.5–2.0 g/kg groups and n=6/group for 2.5 and 3.0 g/kg groups) and 30 min thereafter were subjected to 5-min elevated plus maze test. Each bar represents mean ± SEM of data from 6 to 7 rats per group. *p<0.05 versus vehicle treated rats (one-way ANOVA followed by *post-hoc* Bonferroni test)

subjected to daily liraglutide (50 μg/kg, i.p.) or vehicle (1 ml/kg) treatment. On test day, rats were injected with liraglutide (50 μg/kg, i.p.) 4-h prior to and ethanol (2 g/kg, i.p., 8 % *w/v*) 40-min prior to evaluating them for their behavior in EPM test.

Effect of discontinuation of chronic ethanol consumption on rat behavior in EPM test

As described above (chronic study; experiment 2.4.1), separate groups of rats were forced to consume 'liquid

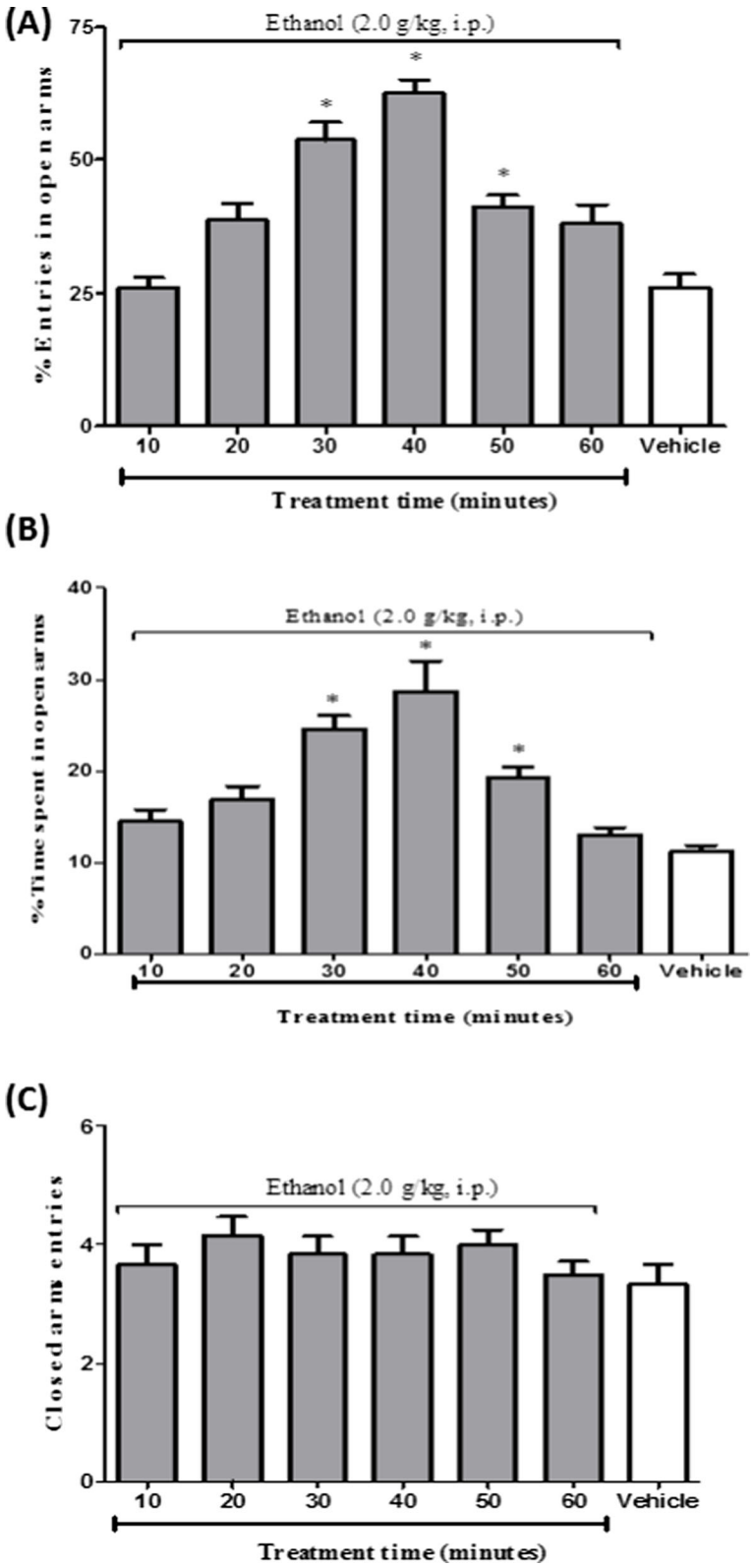

Fig. 2 Time course of anti-anxiety effect of ethanol in rats. **a** % entries into open arms, **b** % time spent in open arms and **c** number of closed arms entries in elevated plus maze test. Separate groups of rats were injected with ethanol (2 g/kg, i.p.) and subjected to 5-min elevated plus maze test at 10 or 20 or 30 or 40 or 50 or 60 min post-ethanol injection (n=7/group for 10–40 min groups and n=6/group for 50 and 60 min groups). A separate group of rats was injected with vehicle (n=6) and 30-min post-vehicle injection, they were subjected to elevated plus maze test. Each bar represents mean ± SEM of data from 6 to 7 rats per group. *p<0.05 vs. vehicle treated rats (one-way ANOVA followed by *post-hoc* Bonferroni test)

diet' or 'ethanol diet' for 15-days. On day 16 mornings (9:00 am), rats that were on 'liquid diet' were continued on the same diet, whereas 'ethanol diet' groups were introduced with ethanol free 'liquid diet'. All rats were

given free access to food pellets and water. Following ethanol withdrawal, rats were subjected to EPM test at 1, 4, 8, 10 or 24 h to evaluate for the impact of discontinuation from chronic ethanol consumption on rat EPM behavior.

Effect of GLP-1 receptor agonist on ethanol withdrawal induced changes in rat EPM behavior

Separate groups of rats were forced to consume 'liquid diet' or 'ethanol diet' for 15-days and simultaneously

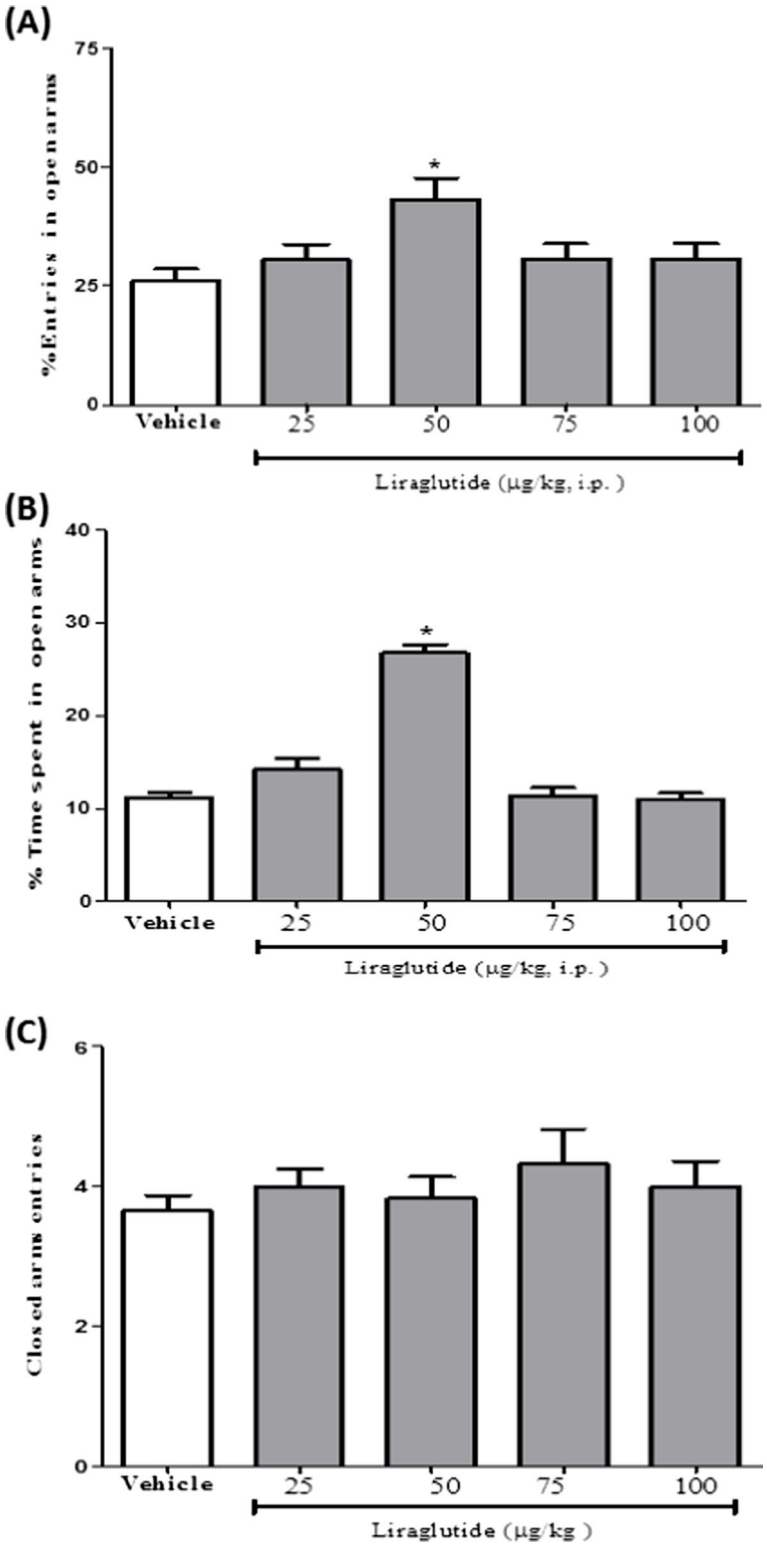

Fig. 3 Anti-anxiety effect of GLP-1 receptor agonist, liraglutide in EPM test in rats. **a** % entries into open arms, **b** % time spent in open arms and **c** number of closed arms entries in elevated plus maze test. Separate groups of rats were injected with liraglutide (25 or 50 or 75 or 100 µg/kg, i.p.; n=7/group) or vehicle (1 mL/kg, i.p.; n=6), and 4 h thereafter, individual rat was subjected to elevated plus maze for 5 min test session. Each bar represents mean ± SEM of data from 6 to 7 rats per group. *p<0.05 vs. vehicle treated rats (one-way ANOVA followed by *post-hoc* Bonferroni test)

subjected to daily GLP-1 receptor agonist, liraglutide (50 μg/kg, i.p.) or vehicle (1 ml/kg) treatment. As described above (experiment 2.5.3.), on day 16, chronic ethanol consumption was discontinued and rats were examined for impact of daily liraglutide treatment on ethanol-withdrawal induced changes in rat EPM behavior at 1, 4, 8, 10 or 24 h post-withdrawal.

Data analysis

The results are expressed as means ± S.E.M. and analyzed by GraphPad Prism 5 software. Data obtained from acute study were analyzed by One-way analysis of variance (ANOVA) followed by *post-hoc* Bonferroni test. Chronic study data was analyzed by Two-way ANOVA followed by *post-hoc* Bonferroni test. Differences were considered significant at $p<0.05$.

Results

Acute study

Dose-dependent anti-anxiety effect of ethanol in rats

Acute ethanol injection to rats at doses 2.0 and 2.5 g/kg resulted in significant increase in the time spent in- and number of entries to the open arms suggesting its anti-anxiety effect at these doses ($p<0.05$; Fig. 1). However, this effect of ethanol was devoid of changes in motor behavior as there were no significant changes in closed arm entries

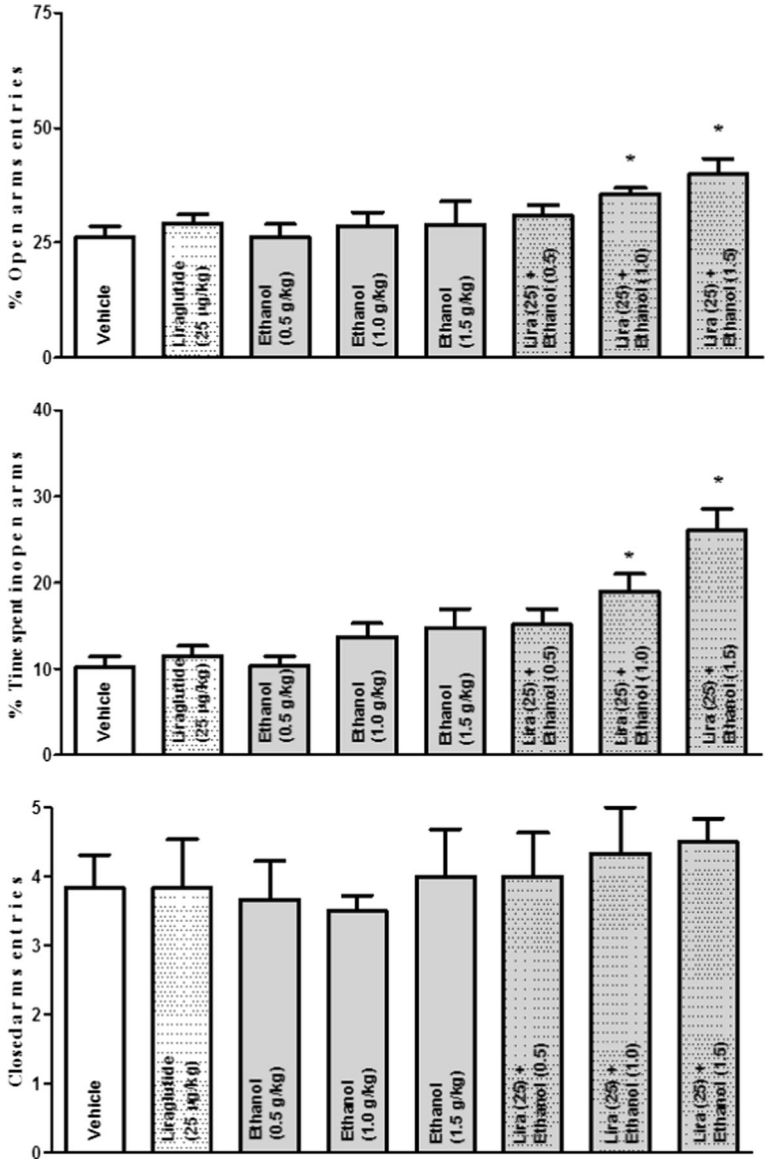

Fig. 4 Potentiation of anti-anxiety effect of ethanol by GLP-1 receptor agonist in rat EPM test. **a** % entries into open arms, **b** % time spent in open arms and **c** number of closed arms entries in elevated plus maze test. Separate groups of rats were injected with vehicle (1 mL/kg, i.p.; $n=6$) or liraglutide (25 μg/kg, i.p.; $n=6$) or ethanol (0.5 ($n=6$) or 1.0 ($n=7$) or 1.5 ($n=7$) g/kg, i.p.) or liraglutide (25 μg/kg, i.p.) + ethanol (0.5 or 1.0 or 1.5 g/kg, i.p.; $n=7$/group) and subjected to elevated plus maze test for 5 min test session. Each bar represents mean ± SEM of data from 6 to 7 rats per group. *$p<0.05$ vs. vehicle treated rats (one-way ANOVA followed by *post-hoc* Bonferroni test)

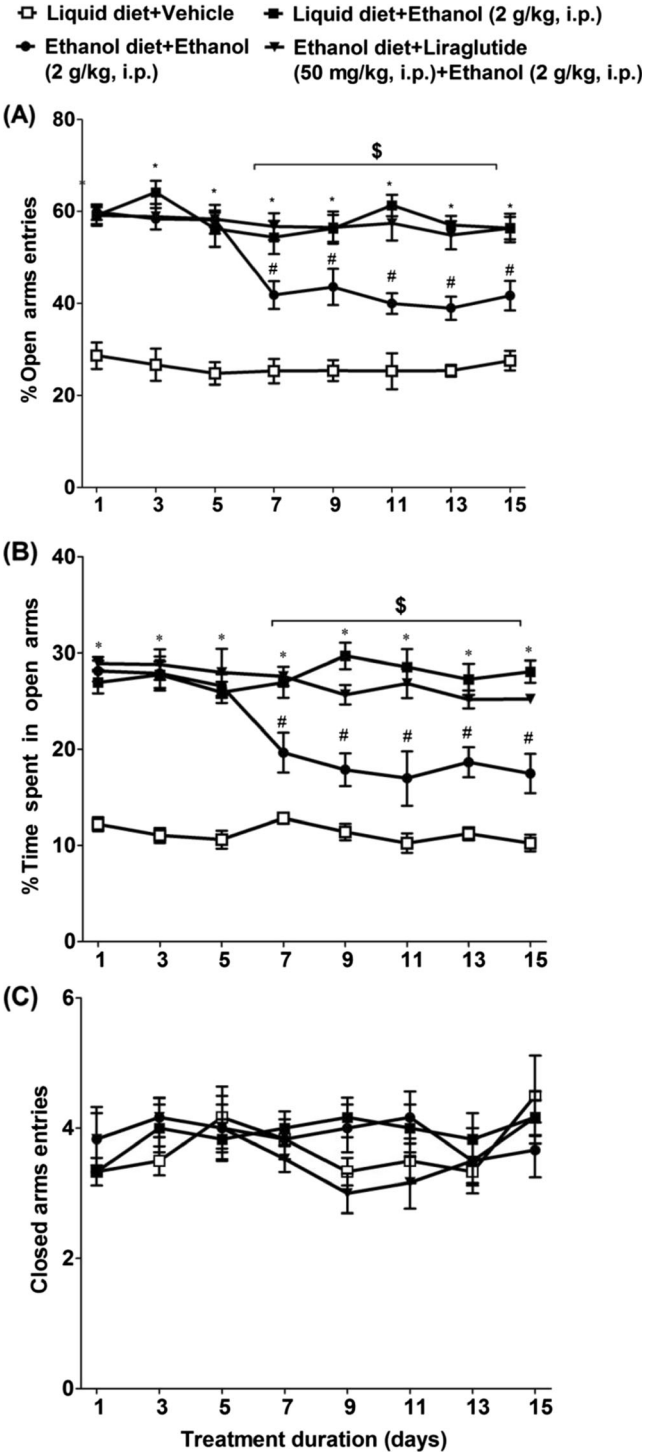

Fig. 5 Effect of daily liraglutide administration on tolerance to anti-anxiety effect of ethanol in rats: **a** % entries into open arms, **b** % time spent in open arms and **c** number of closed arms entries in elevated plus maze test. Different groups of rats consuming 'liquid diet' or 'ethanol diet' (upto 15 days) were injected on the EPM test day with either vehicle (5 mL/kg, i. p.; 'Liquid diet + Vehicle' groups: total 8 groups with $n=6$/group) or ethanol (2.0 g/kg, i. p.; 'Liquid diet + Ethanol' groups: total 8 groups with $n=6$/group and 'Ethanol diet + Ethanol' groups: total 8 groups with $n=6$/group). 40 min thereafter, individual rats were evaluated during 5 min test session for their plus maze behavior. Separate group of rats on 'ethanol diet' were administered daily with liraglutide (50 μg/kg, i.p.) and on the test day were injected with ethanol (2 g/kg, i.p.; i.e. 'Ethanol diet + Liraglutide + Ethanol' groups: total 8 groups with $n=7$/group) and 40 min thereafter, were evaluated for impact of liraglutide treatment on anxiety scores using EPM test. Each data point represents means ± S.E.M. of data from 6 to 7 rats per group. *$p<0.05$ versus 'liquid diet + vehicle' treated rats; #$p<0.05$ versus 'liquid diet + ethanol (2 g/kg, i.p.)' treated rats (indicated '*tolerance to anti-anxiety effect of ethanol*'); $$p<0.05$ versus 'Ethanol diet' + Liraglutide (50 mg/kg, i.p.) + Ethanol (2 g/kg, i.p.) treated rats (indicated '*prevention of tolerance to anti-anxiety effect of ethanol*') [two-way ANOVA followed by *post-hoc* Bonferroni test; factors: 'treatment' X 'time (days)']

($p>0.05$). At doses lower than 2 g/kg, ethanol did not significantly alter rat EPM behavior. At higher doses (>2.5 g/kg), ethanol injections led to ataxia and sedation in rats.

Time course of anti-anxiety effect of ethanol in rats

As shown in Fig. 2, onset of anti-anxiety effect was observed at 20 min post-ethanol (2 g/kg, i.p.) injection. Peak anti-anxiety effect was observed at 40-min post-ethanol (2 g/kg, i.p.) injection as reflected by significant increase in the time spent and number of open arms entries in EPM test ($p<0.05$). However, there were no significant changes in closed arm entries at this time-point ($p>0.05$). At later time-points (>40 min), anti-anxiety effect of ethanol was gradually receded.

Anti-anxiety effect of GLP-1 receptor agonist, liraglutide in EPM test in rats

Evaluation of rat EPM behavior following 4-h post liraglutide treatment resulted in significant increase in time spent and number of entries to open arms at dose (50 μg/kg, i.p.; $p<0.05$; Fig. 3) indicating its per se anti-anxiety effect . The anti-anxiety dose of liraglutide did not affect closed arms entries in EPM test by rats (p>0.05). Rest of tested doses of liraglutide was devoid of anti-anxiety effect as there were no significant changes in rat EPM behavior with their treatments ($p>0.05$).

GLP-1 receptor agonist, liraglutide potentiated anti-anxiety effect of ethanol in rats

Treatment of rats with ethanol (1.0 and 1.5 g/kg, i.p.) and liraglutide (25 μg/kg, i.p.) in combination at doses that were not effective per se (Figs. 1 and 3) resulted in significant increase in time spent and number of entries to open arms in EPM test ($p<0.05$; Fig. 4). Co-treatment with ethanol and liraglutide did not affect rat closed arms entries in EPM test ($p>0.05$). In contrast, combined treatment with ethanol (0.5 g/kg, i.p.) and liraglutide (25 μg/kg, i.p.) produced only marginal increase in open arms behavior that was not statistically significant ($p>0.05$).

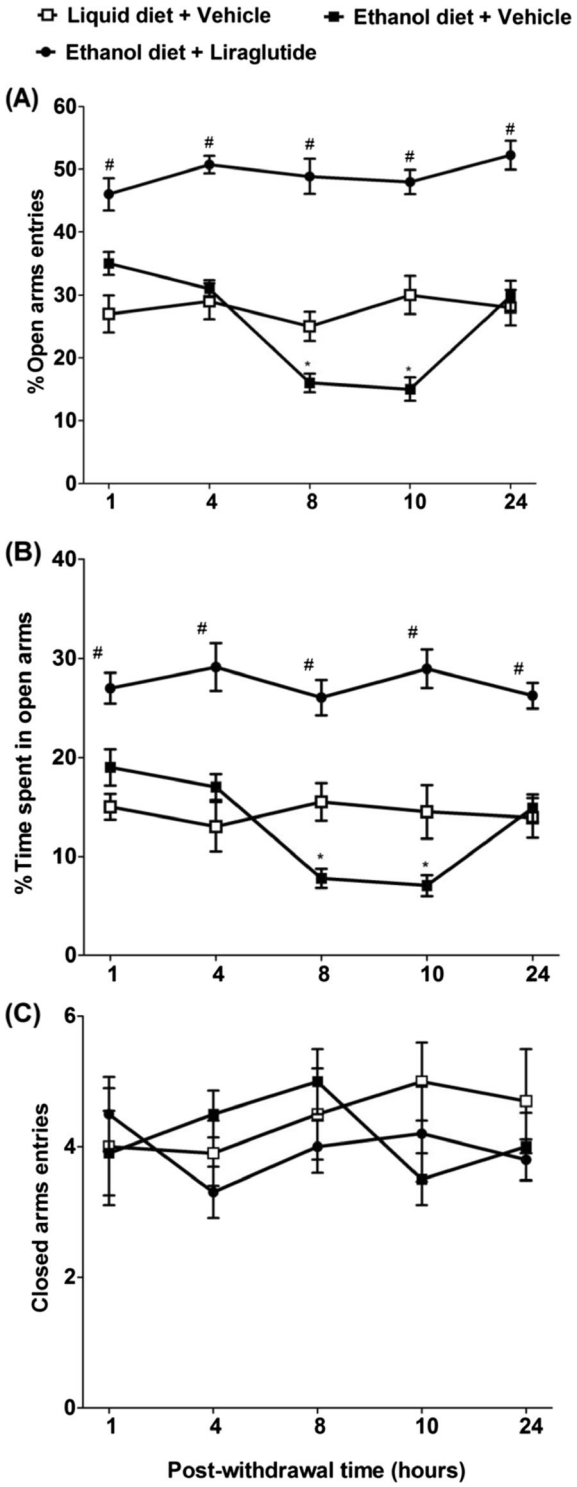

Fig. 6 GLP-1 receptor agonist, liraglutide prevents appearance of ethanol withdrawal-induced anxiety in rats: **a** % entries into open arms, **b** % time spent in open arms and **c** number of closed arms entries in elevated plus maze test. Different groups of rats were forced to consume 'liquid diet' or 'ethanol diet' for 15 days. Rats consuming 'liquid diet' were injected daily with vehicle (1 mL/kg, i.p.; 'Liquid diet + Vehicle' groups: total 5 groups with n=6/group). Rats on 'ethanol diet' were injected daily with either GLP-1 receptor agonist, liraglutide (50 μg/kg, i.p.; 'Ethanol diet + Liraglutide' groups: total 5 groups with n=7/group) or vehicle (1 mL/kg, i.p.; 'Ethanol diet + Vehicle' groups: total 5 groups with n=7/group). On day 16, 'ethanol diet' bottles were removed from rat cages and replaced with 'liquid diet' bottles. Rats were subjected to elevated plus maze test for 5 min at 1, 4, 8, 10 or 24 h post-withdrawal. Each data point represents means ± S.E.M. of data from 6 to 7 rats per group. * P<0.05 versus 'Liquid diet' + Vehicle treated rats; # P<0.05 versus 'Ethanol diet' + Vehicle treated rats [two-way ANOVA followed by *post-hoc* Bonferroni test; factors: 'treatment' X 'withdrawal-duration (hours)']

Chronic study

Chronic liraglutide treatment prevented development of tolerance to anti-anxiety effect of ethanol in rats

Ethanol injection (2 g/kg, i.p.) to rats consuming 'ethanol diet' resulted in significant reduction in time spent and number of entries to open arms from day 7 and onwards of consumption (p<0.05; Fig. 5). These results indicated development of tolerance to anti-anxiety effect of ethanol. However, there were no significant changes in closed arms entries suggesting changes observed were not because of changes in motor behavior of rats (p>0.05). In contrast to this, co-administration of GLP-1 receptor agonist, liraglutide prevented development of tolerance to anti-anxiety effect of ethanol as there was no such reduction in time spent and number of entries to open arms (p>0.05; Fig. 4).

Reversal of ethanol withdrawal anxiety in liraglutide treated rats

As shown in Fig. 6, significant reduction in time spent and number of entries to open arms was observed in rats after 8 and 10 h post-ethanol withdrawal compared to pair-fed 'liquid diet' rats (p<0.05). However, concomitant treatment with liraglutide (50 μg/kg, i.p. once daily for 15 days) prevented development of ethanol withdrawal-induced anxiogenic symptoms in rats (p<0.05 compared to 'ethanol diet' rats). Further, there were no significant changes in closed arms entries during these treatments (p>0.05).

Discussion

Alcoholism is among the major healthcare challenges and there is an immediate need to search for innovative medicines for it. Despite considerable progress in understanding about the neurobiology of alcoholism, the available therapeutic options failed to meet healthcare sector expectations. The data presented here in this article provides first experimental evidence for the favorable role of GLP-1 receptor agonist, liraglutide against alcoholism related anxiety behavior in rats. We recently reported that pharmacological inhibition of enzyme metabolizing endogenous GLP-1 peptide delays

development of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats. Since endogenous GLP-1 is vulnerable to metabolic degradation by enzyme DPP-IV, present study was designed to examine the effect of direct stimulation of receptors for GLP-1, employing agonist for these receptors, on tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats.

New lines of evidence suggest beneficial role of pharmacological agents affecting metabolism and glucose homeostasis on the central nervous system (Sharma et al. 2012). Mounting evidences in literature points toward the role of common molecular pathways in neurological disorders and endocrine metabolic disorders (Sharma et al. 2014b; Ernst et al. 2013; Sharma et al. 2010). Recent advances in the field led to growing interest in the molecules modulating metabolism and energy homeostasis for their role in ethanol's effect on the brain functions (de la Monte et al. 2008; Rojdmark et al. 2001; Leggio et al. 2014). GLP-1 receptor agonists are recently approved pharmacological agents for the management of type II diabetes (Gough 2012). In view of increasing clinical trials cost with high attrition rate, repositioning of approved drugs for new indications is gaining popularity among researchers and drug developers. Here, we provide a comprehensive assessment of the role of GLP-1 receptor agonist in acute anti-anxiety effect of ethanol and chronic ethanol consumption associated tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety using EPM test in rats. We followed a simple approach to first conduct dose–response (Fig. 1) and time-course (Fig. 2) studies for optimal dose and time for anti-anxiety effect of ethanol using EPM test in rats. Based on the outcomes of these two experiments and previous reports (Sharma et al. 2007; Hirani et al. 2005), we selected ethanol (2 g/kg, i.p., 8 %*w/v*) as an acute challenge dose for chronic studies. Moreover, we selected 40 min as a time-point post-ethanol injection to examine rats for their EPM behavior. We also conducted a dose–response study with GLP-1 receptor agonist, liraglutide to examine its influence on rat EPM behavior (Fig. 3). At selected doses, liraglutide produced an inverted U-shaped response-curve with anti-anxiety effect at dose (50 μg/kg, i.p.) as indicated by significantly increased time spent and entries to open arms in rat EPM test. Liraglutide doses, that were lesser than or greater than (50 μg/kg, i.p.), failed to elicit anti-anxiety effect in rats.

The central hypothesis of this study was 'activation of GLP-1 receptors modulates acute anti-anxiety effect of ethanol, tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety'. To test this hypothesis, we first examined the impact of treating rats with sub-effective ethanol in combination with sub-effective dose of GLP-1 receptor agonist, liraglutide on rat EPM behavior (Fig. 4). Liraglutide potentiated anti-anxiety effect of ethanol as there was significant increase in the time spent and number of entries to open arms when both (i.e. liraglutide and ethanol) were administered at their sub-effective doses. There are many novel peptides (Bhisikar et al. 2009) and steroids (Sharma et al. 2007; Hirani et al. 2005) proposed till date as targets for ethanol actions on the central nervous system (CNS). However, it is important to note that there is no agreement among researchers on the mechanisms for ethanol action. And the search for novel biological mechanisms contributing to ethanol actions on the CNS is a continuing process which still in its early stages.

Rats consuming 'ethanol diet' when challenged with acute ethanol injection (2 g/kg, i.p.), from day 7 and onwards,

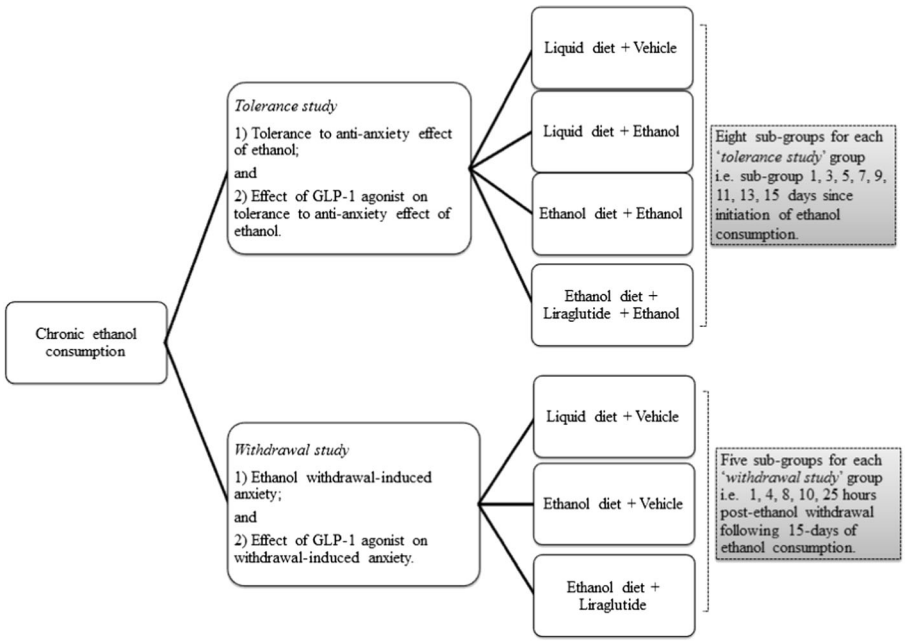

Fig. 7 Schematic representation of timeline of chonic study experiments

exhibited significant reduction in the time spent and number of entries to open arms (Fig. 5) compared to pair-fed (liquid diet) rats. This suggests that, with prolonged ethanol consumption, there was a development of tolerance to anti-anxiety effect of ethanol in rats. This was in agreement to our previous report (Sharma et al. 2007). Interestingly, rats on 'ethanol diet' when subjected to daily liraglutide treatment did not exhibit such reduction in the time spent and number of entries to open arms. Thus, stimulation of GLP-1 receptors employing its agonist liraglutide helped to attenuate development of tolerance to ethanol's anti-anxiety effect in rats. Thus, it is plausible that chronic ethanol consumption could lead to suppression of GLP-1 receptor mediated intracellular events and may contribute to development of ethanol tolerance. Since development of tolerance to pharmacologically effective dose of ethanol could trigger heavy ethanol consumption, it is tempting to speculate that GLP-1 receptors could be an important target to examine neurobiological mechanisms for ethanol dependence.

Rats discontinued from chronic (i.e. 15 days) ethanol consumption exhibited withdrawal-induced anxiety at 8 and 10 h post-withdrawal as indicated by significant reduction in the time spent and number of entries to the open arms compared to rats consuming pair-fed 'liquid diet' (Fig. 6). This was in agreement with our previous report (Sharma et al. 2007). However, daily co-administration liraglutide to rats consuming 'ethanol diet' for 15-days, prevented development of ethanol withdrawal-induced anxiety in rats (Fig. 6). Appearance of withdrawal symptoms upon abstinence from ethanol consumption is one of the important reasons for ethanol craving and relapse to heavy ethanol drinking. Given the ability of GLP-1 receptor agonist to prevent occurrence of withdrawal symptoms, modulation of GLP-1 receptor system could potentially serve as a novel neurobiological target for the management of ethanol withdrawal symptoms (Fig. 7).

In conclusion, using rat model for anxiety we report that: (1) GLP-1 agonist, liraglutide exhibit anti-anxiety effect per se; (2) liraglutide potentiates anti-anxiety effect of ethanol; (3) its co-administration helps to prevent development tolerance to anti-anxiety effect of ethanol; and (4) withdrawal-induced anxiety. Studies examining intracellular cascade of events contributing to these effects are needed to improve understanding about role of GLP-1 receptors in ethanol mediated behaviors. In addition to incretin mimetics, recent clinical study points to the role of hormones such as melatonin in alcoholism related behavioral alterations (Grosshans et al. 2014). Melatonin is a hormone with vital physiological functions (Cajochen et al. 2003; Shukla et al. 2014; Vonnahme et al. 2013; Tunstall et al. 2011). Melatonin was shown to modulate GLP-1 mediated endocrine functions (Kemp et al. 2002). Melatonin is known to act via NO/cyclic GMP (Shukla et al. 2012a; Shukla et al. 2012b; Shukla & O'Rourke 2009; Shukla & O'Rourke 2011) and potassium channel signaling (Shukla et al. 2012c; Shukla et al. 2011; Shukla et al. 2012d). Studies examining pharmacological interactions of GLP-1 and melatonin may help unravel complex interlinked molecular pathways involved in GLP-1 agonist mediated prevention of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety. Evaluation of influence of incretin mimetics on anti-anxiety effect of non-benzodiazepine anxiolytics (Ugale et al. 2007) represents another novel strategy for the effective management of anxiety disorders. Additionally, studies on endogenous GLP-1 levels and on the expression pattern of its receptors in the brain following chronic ethanol consumption may help to strengthen the role of GLP-1 receptors in ethanol dependence. Moreover, studies on how GLP-1 receptor agonist co-treatment to rodents on chronic ethanol consumption affects classic neurotransmitter systems (such as dopamine, glutamate, gamma-aminobutyric acid etc.) and several neuromodulator levels (viz. neuropeptides, neurosteroids, neurotrophic factors etc.) may shed more light on the role of GLP-1 receptors in ethanol dependence.

Conflict of interest Authors declare no conflict of interest.

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