The κ-opioid receptor antagonist JDTic decreases ethanol intake in alcohol-preferring AA rats

Johanna Uhari-Väänänen[1,2], MSc (Pharm); Atso Raasmaja[1], PhD; Pia Bäckström[2], PhD; Ville Oinio[1,2], MSc (Pharm); F. Ivy Carroll[4], PhD; Mikko Airavaara[3], PhD; Kalervo Kiianmaa[2], PhD; Petteri Piepponen[1], PhD

[1]Department of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, P.O. Box 56, 00014 University of Helsinki, Helsinki, Finland

[2]Department of Health, National Institute for Health and Welfare, P.O. Box 30, 00271 Helsinki, Finland

[3]Institute of Biotechnology, P.O. Box 56, 00014 University of Helsinki, Helsinki, Finland

[4]RTI International, P.O. Box 12194 Research Triangle Park, NC, USA Research Triangle Park

Corresponding author: Johanna Uhari-Väänänen, Department of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, P.O. Box 56, 00014 University of Helsinki, Helsinki, Finland

Tel.: +358-(0)294 1911

Fax: +358-(0)2941 59138

E-mail: johanna.uhari@helsinki.fi

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ABSTRACT

Rationale. Studies suggest that the κ-opioidergic system becomes overactivated as ethanol use disorders develop. Nalmefene, a currently approved treatment for ethanol use disorders, may also elicit some of its main effects via the κ-opioidergic system. However, the exact role of κ-opioid receptors on regulating ethanol intake and contribution to the development of ethanol addiction remains to be elucidated.

Objectives. The aim of the present study was to clarify the role of accumbal κ-opioid receptors in controlling ethanol intake in alcohol-preferring Alko Alcohol (AA) rats.

Methods. Microinfusions of the long-acting and selective κ-opioid receptor antagonist JDTic (1 - 15 µg/site) were administered bilaterally into the nucleus accumbens shell of AA rats voluntarily consuming 10% ethanol solution in the intermittent, time-restricted two-bottle choice access paradigm. JDTic (10 mg/kg) was also administered subcutaneously. Both the acute and long-term effects of the treatment on ethanol intake were examined. As a reference, nor-BNI (3 µg/site) was administered intra-accumbally.

Results. Systemically administered JDTic decreased ethanol intake significantly two days and showed a similar trend four days after administration. Furthermore, intra-accumbally administered JDTic showed a weak decreasing effect on ethanol intake long-term but had no acute effects. Intra-accumbal administration of nor-BNI tended to decrease ethanol intake.

Conclusions. The results provide further evidence that κ-opioid receptors play a role in controlling ethanol intake and that accumbal κ-opioid receptors participate in the modulation of the reinforcing effects of ethanol. Furthermore, the results suggest that κ-opioid receptor antagonists may be a valuable adjunct in the pharmacotherapy of ethanol use disorders.

Keywords

κ-opioid receptor, intermittent ethanol intake, JDTic, nor-BNI, nucleus accumbens shell, Alko Alcohol rat

INTRODUCTION

Ethanol is known to not only cause positive reinforcing effects, but ethanol also has negative reinforcing effects (Sirohi et al. 2012). It is widely acknowledged that the μ-opioidergic system participates in mediating hedonic states (Amalric et al. 1987). Conversely, the κ-opioidergic system is considered important in mediating anhedonic states (Mucha and Herz 1985). As opioidergic systems participate in mediating the reinforcing effects of ethanol, the opposing effects of these two opioidergic systems may be the cause of ethanol's bidirectional functions, further possibly reflecting on the consumed amounts of ethanol (Sirohi et al. 2012). The μ-opioidergic system may be more important than the κ-opioidergic system in controlling acute and on-going ethanol intake (Sirohi et al. 2012; Walker et al. 2012). However, following chronic ethanol exposure, neuroadaptations occur in the opioidergic systems, resulting in upregulation of the κ-opioidergic system possibly with simultaneous downregulation of the μ-opioidergic system (Lindholm et al. 2000; Przewlocka et al. 1997; Rose et al. 2016; Sirohi et al. 2012; Walker et al. 2012). These neuroadaptations may be significant for alterations in consumed amounts of ethanol as addiction to ethanol develops. In a recent study conducted in deceased alcoholics, though, no alterations have been detected in the number of κ-opioid receptor binding sites in the ventral striatum (Hermann et al. 2017).

Dopaminergic neurons from the ventral tegmental area project to the nucleus accumbens, from which GABAergic medium spiny neurons innervate the ventral pallidum, the endpoint of the reward tract (Wise 2002). The opioidergic systems interact with this reward pathway (Koob 1992; Wise 2002). Following ethanol administration, endogenous opioid peptides, β-endorphins, enkephalins and dynorphins, are released in these central brain areas of the reward tract, the ventral tegmental area, nucleus accumbens and ventral pallidum (Jarjour et al. 2009; Marinelli et al. 2003, 2005, 2006). The released opioid peptides interact with local inhibitory acting μ-, δ- and κ-opioid receptors, which are all found in these brain areas (Mansour et al. 1987, 1988, 1995; Mitrovic and Napier 1995). Opioid peptides bind with differing affinities to the local opioid receptors, with dynorphins having the most affinity towards κ-opioid receptors (Svingos et al. 1996, 1999a, b). Different opioid receptors are located at various sites on neuronal terminals and somas, thus they may also have differing roles in controlling ethanol intake. As accumbal κ-opioid receptors are located mainly on presynaptic sites, they can modulate the release of several different neurotransmitters such as dopamine, glutamate and GABA, thus also modulating ethanol intake (Hjelmstad and Fields 2001, 2003; Svingos et al. 1999b). Furthermore, κ-opioid receptor agonists oppose the effects of μ-opioid receptor agonists on dopamine release in the nucleus accumbens shell (Di Chiara and Imperato 1988).

Clinically the opioidergic systems are an important target in treating patients with ethanol use disorders (Mason et al. 1994; Volpicelli et al. 1992). Two clinically used drugs aimed at the opioidergic systems are naltrexone and nalmefene. Naltrexone is used based on its ability to reduce craving for and consumption of ethanol (Volpicelli et al. 1992). Nalmefene is used for reducing drinking in heavy drinkers and is currently approved for use as an 'as needed' drug (Mann et al. 2013; Soyka 2016). Both naltrexone and nalmefene are non-selective opioid receptor antagonists with similar affinity for the μ-opioid receptor (Michel et al. 1985). However, nalmefene, also a κ-opioid receptor partial agonist, has two-times more affinity towards the κ-opioid receptor than naltrexone (Michel et al. 1985), which is why it is postulated to be more effective in addressing the negative reinforcing effects of ethanol (Nealey et al. 2011; Walker and Koob 2008; Walker et al. 2011). Due to encouraging results from both rodent and clinical studies on drugs acting on the κ-opioidergic system (Deehan et al. 2012; Mason et al. 1994; Nealey et al. 2011; Rorick-Kehn et al. 2014; Schank et al. 2012; Volpicelli et al. 1992; Walker and Koob 2008; Walker et al. 2011), the possibility of addressing the treatment of ethanol use disorders from a κ-opioidergic perspective has gained interest and opened up a new possible treatment strategy.

Despite vast research on the role of the κ-opioidergic system on ethanol intake, the results in rodent studies using selective κ-opioidergic drugs have been somewhat inconsistent, especially regarding results from κ-opioid receptor agonist studies. Systemically administered κ-opioid receptor agonists have been shown to inhibit ethanol induced conditioned place preference (Logrip et al. 2009) and decrease ethanol intake (Henderson-Redmond and Czachowski 2014; Hölter et al. 2000; Lindholm et al. 2001), which was blocked by pretreatment with a κ-opioid receptor antagonist (Lindholm et al. 2001). In a relapse model, chronic administration of a κ-opioid receptor agonist, however, potentiated ethanol intake during the alcohol deprivation effect (Hölter et al. 2000). Ethanol intake was also increased by a κ-opioid receptor agonist treatment in ethanol-vapor exposed mice (Rose et al. 2016). Furthermore, in our recent studies, intra-accumbal or intra-pallidal κ-opioid receptor agonist administration did not affect ethanol intake (Kemppainen et al. 2012; Uhari-Väänänen et al. 2016).

More consistent results are reported among studies using κ-opioid receptor antagonists, which have shown them to either not modify ethanol intake (Hölter et al. 2000; Kemppainen et al. 2012) or to decrease it (Deehan et al. 2012; Nealey et al. 2011; Rorick-Kehn et al. 2014; Rose et al. 2016; Schank et al. 2012; Walker and Koob 2008; Walker et al. 2011), depending possibly on the used experimental paradigm.

JDTic is a relatively new and selective κ-opioid receptor antagonist not derived from naltrexone, on the contrary to the prototypical selective κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) (Portoghese et al. 1988; Thomas et al.

2001). JDTic also has more potency and selectivity towards the κ-opioid receptor over the μ- and δ-opioid receptors than nor-BNI (Thomas et al. 2001, 2003). Both share a slow and delayed onset of κ-antagonism, taking up to 6 h for JDTic and over 4 h for nor-BNI, in addition to a long duration of κ-antagonistic activity, lasting up to 28 days for JDTic (Carroll et al. 2004; Endoh et al. 1992).

JDTic has shown efficacy in several rodent models of ethanol abuse, and overall the κ-opioidergic system appears to have significant impact on regulating ethanol intake (Deehan et al. 2012; Schank et al. 2012; Sirohi et al. 2012). Therefore, in a set of three experiments, our aim was to use JDTic to clarify the role of accumbal shell κ-opioid receptors in controlling ethanol intake. The nucleus accumbens shell was targeted because of its central role in mediating reward due to its anatomical location, in addition to the ability of local κ-opioid receptors to modulate the release of neurotransmitters, including dopamine, and oppose the effects of μ-opioid receptor agonists (Di Chiara and Imperato, 1988; Koob, 1992; Sirohi et al. 2012; Svingos et al. 1999b). JDTic was microinfused into the nucleus accumbens shell using different temporal paradigms, and the effects of the treatments on ethanol intake were examined using the intermittent, time-restricted (90 min) two-bottle choice ethanol access paradigm. To elucidate the overall effects of JDTic induced κ-opioid receptor antagonism on ethanol intake, JDTic was also administered systemically. As nor-BNI is often considered a classical κ-opioid receptor antagonist, it was included as a reference drug when assessing intra-accumbally administered JDTic's long-term effects on ethanol intake. The experiments were conducted using the alcohol-preferring Alko Alcohol (AA) rats that consume voluntarily high amounts of ethanol. The AA and alcohol non-preferring Alko Non-Alcohol (ANA) rat lines are among the earliest rodent lines produced by bi-directional, selective outbreeding for differential ethanol intake (Eriksson 1968; Sommer et al. 2006).

METHODS

Animals

Altogether 94 male alcohol-preferring AA rats (University of Helsinki, Helsinki, Finland) from generations F_{108} and F_{110} were used in the study. The rats were 3 months old and weighed at least 250 g at the onset of the experiments and they were single housed in wire mesh cages (21 x 38 x 19 cm). Water and standard rat chow (SDS RM1 [E] SQC; Witham, Essex, UK) were available *ad libitum.* Ambient temperature was maintained at 22 ± 1°C and humidity was recorded. The rats were housed in a reversed 12⁄12 hour light/dark cycle (lights off at 9 AM). Animal experiments were conducted according to the 3R principles of the EU directive 2010/63/EU governing the care and use of experimental animals, and following local laws and regulations (Finnish Act on the Protection of Animals Used for

Scientific or Educational Purposes (497/2013), Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)). The protocols were authorized by the national Animal Experiment Board of Finland.

Drugs

The κ-opioid receptor antagonist nor-binaltorphimine dihydrochloride (nor-BNI) was purchased from Tocris (Bristol, UK). The κ-opioid receptor antagonist [(3R)-7-hydroxy-N-((1S)-1-[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide] (JDTic) was a gift from RTI International (Research Triangle Park, NC, USA). All drugs were diluted in sterile saline and administered at a volume of 0.3 µl for intra-accumbal infusions and 1 ml/kg for subcutaneous (s.c.) injections. Ethanol (Etax A, 96% v/v; Altia, Rajamäki, Finland) was diluted in tap water.

For the intra-accumbal infusions, the doses of JDTic were 1, 5 and 15 µg/site and the dose of nor-BNI was 3 µg/site. For the s.c. injections, the dose of JDTic was 10 mg/kg. The doses for intra-accumbally administered JDTic were selected on the basis of our preliminary experiments and an intracranial infusion study (Knoll et al. 2011). The doses for intra-accumbal nor-BNI and systemic JDTic were based on earlier reports (Deehan et al. 2012; Kemppainen et al. 2012; Nealey et al. 2011; Rorick-Kehn et al. 2014; Schank et al. 2012; Varaschin and Morato 2009; Walker and Koob 2008).

Experimental design

In experiment 1, JDTic (15 µg/site), nor-BNI (3 µg/site) or vehicle were administered intra-accumbally 24 h prior access to ethanol for 90 min. Long-term effects of the treatments on ethanol intake were monitored for three 90 min ethanol intake sessions 1, 3 and 5 days after administration. Ethanol intake was recorded at the 30, 60 and 90 min time points.

In experiment 2, the acute effects of intra-accumbally administered JDTic on ethanol intake were tested. JDTic (1, 5 and 15 µg/site) or vehicle were administered 5 min before ethanol was presented and acute effects on 90 min ethanol intake were monitored for one ethanol intake session. Ethanol intake was recorded at 10 to 20 min intervals for 90 min.

In experiment 3, an injection of JDTic (10 mg/kg, s.c.) or vehicle was given 10 min prior access to ethanol for 90 min. Both the acute and long-term effects of JDTic on ethanol intake were monitored for three 90 min ethanol intake sessions 0, 2 and 4 days after administration. Ethanol intake was recorded at the 30, 60 and 90 min time points.

Due to the long-term actions of the used κ-opioid receptor antagonists (Carroll et al. 2004; Deehan et al. 2012; Endoh et al. 1992), each rat received only one dose of the experimental drugs.

Ethanol intake

An intermittent, time-restricted (90 min) two-bottle choice ethanol access paradigm was used to study the effects of κ-opioid receptor antagonists on ethanol intake (modified from Simms et al. 2010; Uhari-Väänänen et al. 2016).

Briefly, the rats gained access to 10% ethanol solution (v/v) 3 times a week – on Mondays, Wednesdays and Fridays –in their home cage. Ethanol was presented in Richter tubes within 30 min after the lights went out. At the onset of experiments, the rats received ethanol for 24 h for at least 3 weeks. After a stable intake of ethanol solution had been reached, the time of access to ethanol was reduced to 90 min. This was continued for at least 3 weeks or until a stable ethanol intake level had been reached. Thereafter, the rats in experiments 1 and 2 underwent surgery. Access to ethanol was continued during the post-surgery recovery period (2 weeks). After sham and training vehicle infusions, ethanol intake was further monitored and a new ethanol intake baseline was established, according to which the rats were assigned into treatment groups. The rats were also habituated for 2 weeks to the procedure of recording ethanol intake levels every 30 min (experiments 1 and 3) or 10 to 20 min (experiment 2) before the onset of the experiments.

One water tube and food were present at all times *ad libitum*. To avoid any side-preference between the water and ethanol tubes, the right-left position of the tubes was changed for each ethanol drinking session. For all rats 24 h fluid consumption was recorded daily (in ml), while measurements for 24 h food consumption (in g) and body weight were taken three times a week. Due to the acute nature of experiment 2, we also recorded the 90 min water and food intake for this particular experiment.

Surgery and microinfusions

For implantation of guide cannulas in experiments 1 and 2, the rats were anesthetized with isoflurane (4% during induction for 5 min and then 2 - 2.5% for anesthesia maintenance) and attached to a stereotaxic frame. The guide cannulas (OD = 1.0 mm) were implanted bilaterally 2 mm above the nucleus accumbens shell and attached to the skull with the help of two stainless steel screws and dental cement. Dummy stylets were placed inside the guide cannulas to prevent occlusion. The used coordinates, AP + 1.7; ML ± 1.2; DV -5.2 from the dura, were according to the rat brain atlas by Paxinos and Watson (1998). The coordinates were selected on the basis of earlier studies by our group and others (Castro and Berridge 2014; Nealey et al. 2011; Smith and Berridge 2007; Uhari-Väänänen et al. 2016). During

surgery, the rats' body temperature was kept constant with the help of a heating pad attached to a thermostatic sensor. The rats received carprofen (Rimadyl®, 5 mg/kg s.c., Vericore, Dundee, UK) 30 min before surgery and for two days post-surgery. After surgery, the rats were returned to their home cage to recover.

The microinfusions were given as described earlier (Uhari-Väänänen et al. 2016). Briefly, after surgery and before the onset of the experiments, the rats were allowed to recover for at least two weeks. During this recovery period, the rats were habituated to the intra-cranial injection procedure by removing the dummy stylets from the guide cannulas. After at least four training sessions the rats received a sham infusion during which the injection needle (tip OD = 0.3 mm) was placed into the guide cannula but no infusion was given. The tip of the injection needle extended 2 mm beyond the guide cannula shaft. After the sham infusion, the rats received a training vehicle infusion. The sham and training vehicle infusions were given to be able to reliably discriminate pharmacological effects of the drugs from the effects of the infusion procedure itself on ethanol intake. All infusions were given bilaterally in a volume of 0.3 µl at a rate of 0.3 µl/min with a microinfusion pump (CMA, Stockholm, Sweden). Before the infusions the injection needle was left in place for 1 min. After the infusions, the injection needle was left in place for 2 min to allow spreading of the drug and to avoid leakage up the cannula track.

Histology

For experiments 1 and 2, coronal sections (100 µm) were cut from the 10% formalin fixed brains to check the positioning of the guide cannulas. The sections were compared to the rat brain atlas (Paxinos and Watson 1998). Rats were excluded from the study if the injection needle tip mark was not within the nucleus accumbens shell. Rats were also excluded if the guide cannula loosened during the study.

Statistical analysis

For data analysis the ethanol intake (ml) values were first converted to grams of 100% ethanol / body weight (kg). For evaluating the long-term effect of accumbal or systemic κ-opioid receptor antagonism by JDTic or nor-BNI on ethanol intake (experiment 1 and 3), a mixed-model two-way analysis of variance (ANOVA) was used for analysis with treatment (JDTic/nor-BNI or vehicle) as the between-groups factor and ethanol intake session (for example baseline, 1, 3 and 5 days post-administration) as the within-subjects factor. If ANOVA revealed a significant interaction, the unpaired two-sample *t*-test with the Holm-Bonferroni correction for multiple comparisons was used as the *post hoc* means. The baseline ethanol intake value was calculated as the average from the preceding two ethanol intake sessions before the treatments.

The acute effects of accumbally administered JDTic (experiment 2) were analyzed with a one-way ANOVA from the cumulative 90 min ethanol intake data.

For water and food intake data analysis, the consumed amounts (ml or g) were first converted to grams of consumed amount / body weight (kg). The acute effects of intra-accumbal JDTic administration (experiment 2) on 90 min water and food intake were analyzed using the one-way ANOVA. For long-term effects of the treatments (experiment 1 and 3) on food and water intake, a mixed-model two-way ANOVA was used for analysis with treatment as the between-groups factor and food or water intake session as the within-subjects factor. Because of the long-term effects of the used κ-antagonists, the 24 h food or water intake data from only ethanol intake days were analyzed. If ANOVA revealed a significant interaction, the unpaired two-sample *t*-test with the Holm-Bonferroni correction for multiple comparisons was used as the *post hoc* means.

In all statistical analyses the criterion for significance was set at the 0.05 level. The statistical software used was IBM SPSS Statistics V22.0 (IBM Corp., Armonk, NY, USA). The graphical software used was GraphPad Prism V6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Ethanol intake

During the initial 24 h intermittent ethanol intake, the rats consumed ethanol for the last three ethanol intake session on average: 6.11 ± 0.35 g/kg/24h (experiment 1), 5.36 ± 0.33 g/kg/24h (experiment 2) and 5.48 ± 0.35 g/kg/24h (experiment 3).

Before the rats underwent surgery for the intracranial injection studies (experiments 1 and 2), the average of ethanol intake for the last three 90 min ethanol intake sessions were 0.87 ± 0.06 g/kg/90 min and 0.83 ± 0.07 g/kg/90 min, respectively for experiments 1 and 2. After surgery, the average of the last three ethanol intake sessions during the post-surgery recovery period before the habituation infusions for experiments 1 and 2 were 0.84 ± 0.08 g/kg/90 min and 1.10 ± 0.05 g/kg/90 min, respectively. No significant changes were observed between the pre-surgery and post-surgery but pre-infusion ethanol intake levels.

The baseline 90 min ethanol intake for rats receiving systemic injections (experiment 3) was 0.94 ± 0.07 g/kg/90 min.

Experiment 1

Intra-accumbally administered JDTic showed a weak decreasing effect on ethanol intake in a long-term manner. There was a significant interaction between JDTic treatment and ethanol intake session on ethanol intake [$F(3,42) = 3.41$, $p = 0.026$], when JDTic or vehicle was infused into the nucleus accumbens shell and ethanol intake was measured at the 90 min time point (mixed-model ANOVA) (Fig. 1). The decrease in ethanol intake following JDTic administration approached significance ($p = 0.058$) at the second ethanol intake session after the infusion (3 days post infusion). When the data was analyzed from other time points (30 and 60 min), no significant interaction was found (data not shown).

Statistical analysis revealed that nor-BNI tended to decrease ethanol intake as compared to the vehicle treatment when calculated from the 90 min data (session x treatment interaction [$F(3,48) = 3.547$, $p = 0.021$]) (Fig. 2). However, the *post hoc* test did not detect significant differences between the treatments at different ethanol intake sessions. At other time points (30 and 60 min), no significant interaction was found (data not shown).

Experiment 2

The acute intra-accumbal administrations of JDTic in experiment 2 did not modify ethanol intake at the 90 min time point (Fig. 3a). This is also illustrated in the cumulative ethanol intake diagram (0 to 90 min) (Fig. 3b).

Experiment 3

Systemically injected JDTic decreased ethanol intake relative to the vehicle treatment when analyzed from the 30 min data as revealed by the mixed-model ANOVA (session x treatment interaction [$F(3,48) = 2.847$, $p = 0.047$]) (Fig. 4). *Post hoc* tests revealed that JDTic decreased ethanol intake significantly at the second ($p = 0.0073$) ethanol intake sessions after the injection (2 days post injection). There was a trend to JDTic decreasing ethanol intake at the third ($p = 0.080$) ethanol intake sessions after the injection (4 days post injection). When ethanol intake data was analyzed by ANOVA from the 60 min or 90 min time points, no statistically significant interaction was detected. No acute effect of systemic JDTic on ethanol intake was evident.

Treatment effects on water and food intake

Intra-accumbal administration of κ-opioid receptor antagonists did not have either long-term 24 h (experiment 1) or acute 90 min (experiment 2) effects on water intake (data not shown).

The mixed-model ANOVA analysis for 24 h water intake in experiment 3 (systemic JDTic administration) revealed a significant interaction on treatment and time for water intake, [$F(3,48) = 3.998$, $p = 0.03$]. *Post hoc* analysis revealed

that water intake decreased only in the vehicle treated rats on the day of vehicle or JDTic administration (day 0, $p = 0.0345$) (data not shown). The simple main-effect for treatment group, as analyzed by extra repeated measures ANOVA's run separately for both vehicle and JDTic groups, showed that indeed water intake altered after vehicle treatment [$F(3,21) = 8.065$, $p = 0.001$] but not after JDTic treatment [$F(3,27) = 1.428$, $p = 0.256$].

Food intake was not altered in any of the three experiments (data not shown).

Histology

According to histological examination of the probe placements, the microinfusions of the rats accepted for statistical analysis had been given into the nucleus accumbens shell (Fig. 5). From experiment 1 altogether 8 rats out of 34 and from experiment 2 altogether 9 rats out of 42 were excluded due to either loosening or misplacement of the guide cannulas.

DISCUSSION

The aim of the present study was to clarify the role of accumbal κ-opioid receptors in controlling ethanol intake in alcohol-preferring AA rats. This was done by using the relatively new and selective κ-opioid receptor antagonist JDTic in a set of three experiments. JDTic was microinfused into the nucleus accumbens shell before access to ethanol. In addition, to elucidate the overall effects of JDTic mediated κ-opioid receptor antagonism on ethanol intake, JDTic was also administered systemically. The acute and long-term effects of the treatments on ethanol intake were monitored using the intermittent, time-restricted (90 min) two-bottle choice ethanol access paradigm. The conventional κ-opioid receptor antagonist nor-BNI was infused intra-accumbally as a reference drug for JDTic when assessing JDTic's long-term effects on ethanol intake.

A single subcutaneous injection of JDTic decreased ethanol intake significantly 2 days after administration, with a trend to decrease ethanol intake also 4 days after administration (experiment 3). In addition, intra-accumbally administered JDTic showed a weak decreasing effect on ethanol intake 3 days after administration while nor-BNI only tended to decrease ethanol intake (experiment 1). JDTic did not have any acute effects on ethanol intake after either intra-accumbal (experiment 2) or systemic administration (experiment 3).

The used intermittent, time-restricted two-bottle choice ethanol access paradigm is a simple and well-established model used to evaluate the effects of treatments on voluntary ethanol intake (modified from Simms et al. 2010; Uhari-Väänänen et al. 2016). The results are comparable to those obtained from simple operant procedures (Hyytiä and

Sinclair 1990). Due to the restricted amount of time that ethanol is available, rats tend to consume high amounts of ethanol relatively quickly. This further results in blood ethanol concentrations that have been shown to be pharmacologically significant (Nurmi et al. 1999).

Due to the lack of uniformity in pretreatment times for JDTic in reports addressing ethanol-related behaviors (Deehan et al. 2012; Schank et al. 2012), different temporal paradimgs were used in this study. Schank et al. (2012) reported that only after pretreatment times of 2 h but not longer (24 or 48 h), were ethanol-related behaviors altered by JDTic. This is interesting, since JDTic, as well as nor-BNI have been reported to both have a slow and delayed onset of κ-antagonism (Carroll et al. 2004; Endoh et al. 1992), which is why at least 24 h pretreatment times have been used (Deehan et al. 2012; Knoll et al. 2011; Rorick-Kehn et al. 2014; Walker et al. 2011). For evaluating the long-term effects of both intra-accumbally administered JDTic and nor-BNI on ethanol intake, the pretreatment time of 24 h seemed appropriate (experiment 1). As no previous data is available on the immediate effects of intra-accumbally administered JDTic on ethanol intake, the acute effects (administration 5 min prior) of JDTic were also tested (experiment 2). To be able to examine both the acute and long-term effects of systemically administered JDTic in one experiment, JDTic was administered 10 min prior to the first ethanol intake session out of several sessions (experiment 3).

Our preliminary experiments with intra-accumbally administered JDTic at doses of 1, 5 and 15 µg/site suggested that the 15 µg/site dose was the most effective in reducing ethanol intake in a long-term manner (unpublished results of Uhari-Väänänen et al.). Thus, the sole dose of 15 µg/site was chosen for experiment 1. The only available study reporting the use of intracranial JDTic was used to select the range of JDTic doses (Knoll et al. 2011). The dose of 3 µg/site of nor-BNI was chosen on the basis of earlier reports (Kemppainen et al. 2012; Nealey et al. 2011; Varaschin and Morato 2009; Walker and Koob 2008). In experiment 2, the doses for assessing the acute effects of JDTic on ethanol intake were selected on the basis of the preliminary experiments. The 10 mg/kg dose of JDTic for experiment 3 was selected due to several reports showing it to be an effective dose in modifying different ethanol-related behaviors (Deehan et al. 2012; Schank et al. 2012).

Our results on systemic JDTic on decreasing ethanol intake and on the weak decreasing effect of intra-accumbal JDTic on ethanol intake both in a long-term manner are in line with an earlier report showing that JDTic has long-term actions on ethanol-related behaviors (Deehan et al. 2012). In operant conditions in alcohol-preferring P rats, a single injection of JDTic reduced ethanol seeking behavior when given 14 days prior and relapse to ethanol when given 25 days prior. However, JDTic did not affect maintenance ethanol self-administration. This is seemingly in line with our results, as intermittent ethanol intake paradigms have been shown to increase ethanol intake more than in paradigms where ethanol

is constantly available (Simms et al. 2008). Additionally, different variations of intermittent ethanol exposure paradigms have been shown to activate the κ-opioidergic system (Rose et al. 2016).

In the present study, intra-accumbally administered nor-BNI tended to decrease ethanol intake. In line with this finding, nor-BNI has been shown not to alter operant responding to ethanol under maintenance conditions in Long Evans rats (Doyon et al. 2006). In Wistar rats subjected to intermittent ethanol vapor conditions, intra-accumbal, intra-ventricular and systemic administration of nor-BNI, however, decreased operant ethanol responding (Nealey et al. 2011; Walker and Koob 2008; Walker et al. 2011). The authors report that the effect was specific to rats considered physically ethanol dependent as ethanol intake of non-dependent rats was not affected by nor-BNI treatment. In the same studies, the non-selective κ-opioid receptor antagonists naltrexone and nalmefene were reported to reduce responding to ethanol also in rats considered non-dependent on ethanol (Nealey et al. 2011; Walker and Koob 2008). The animals used in the present study showed high voluntary ethanol intake, but no findings are suggesting that they were physically dependent on ethanol. Blood ethanol concentrations were not measured in the present study. However, it has previously been shown that AA rats reach pharmacologically significant blood ethanol concentrations in a similar limited access paradigm as we used here (Nurmi et al. 1999).

We also tested the acute effects of JDTic on ethanol intake. In experiment 2 we administered JDTic directly into the nucleus accumbens shell immediately before the ethanol intake session, thereby bypassing the absorption phase. Acutely administered JDTic did not affect 90 min ethanol intake at any of the tested doses. Neither did systemically administered JDTic given 10 min prior the first 90 min ethanol intake session affect ethanol intake. In contrast to our findings, Schank et al. (2012) reported that JDTic decreased ethanol self-administration and cue-induced reinstatement to ethanol only when JDTic was administered systemically 2 h prior to testing. In line with the present results, it has been reported that JDTic does not antagonize κ-opioid receptor mediated analgesia at short, under 6 h pretreatment times (Carroll et al. 2004).

Since nor-BNI has been shown to have μ-opioid receptor directed effects at least 30 min after administration (Endoh et al. 1992), there would also be a concern that JDTic would have similar effects. However, JDTic did not affect morphine-induced behavior in the locomotor paradigm 2 h after administration (Schank et al. 2012), neither did JDTic antagonize the analgesic effects of a selective μ-opioid receptor agonist in the mouse tail-flick test when administered 20 min to 24 h prior testing (Carroll et al. 2004). Therefore, it is unlikely that results of the acute administration experiment were confounded with JDTic's actions on the μ-opioidergic system.

The κ-opioidergic system is seen as an attractive target for the treatment of ethanol use disorders due to its interactions with the brain's reward tract (Sirohi et al. 2012; Walker et al. 2012). In the nucleus accumbens, κ-opioid receptors are mainly located presynaptically on dopaminergic, glutamatergic and GABAergic nerve terminals casting an inhibitory tone on the release of these neurotransmitters (Hjelmstad and Fields 2001, 2003; Svingos et al. 1999b). κ-Opioid receptors are also able to control dopamine uptake via interactions with the dopamine transporter protein (Svingos et al. 2001; Thompson et al. 2000). Therefore, κ-opioid receptors can inhibit the release and regulate the uptake of neurotransmitters presynaptically, thereby affecting the activity of the postsynaptic accumbal GABAergic medium spiny neurons, the main type of accumbal neurons (Wise 2002). Especially accumbal dopamine levels are affected by the κ-opioidergic system, as genetic deletions of κ-opioid receptors, as well as κ-opioid receptor antagonists, increase accumbal dopamine levels (Chefer et al. 2005; Spanagel et al. 1992).

As described above, κ-opioid receptors can interact with the brain's reward tract by modifying the release of various neurotransmitters in the nucleus accumbens, especially that of dopamine (Hjelmstad and Fields 2001, 2003; Karkhanis et al. 2016; Rose et al. 2016; Sirohi et al. 2012; Svingos et al. 1999b, 2001; Walker et al. 2012). While acute ethanol administration has been shown to release dopamine in the nucleus accumbens (Kiianmaa et al. 1995; Koob 1992), chronic intermittent ethanol exposure has been shown to reduce accumbal dopamine release and increase dopamine uptake rates (Karkhanis et al. 2016; Rose et al. 2016). The upregulation of nucleus accumbens shell prodynorphin mRNA levels and dynorphin B concentrations reported under withdrawal circumstances from ethanol may contribute to the decrease in accumbal dopamine levels (Lindholm et al. 2000; Przewlocka et al. 1997). Thus, it has been suggested that chronic ethanol exposure gradually overactivates the κ-opioidergic system, thereby increasing the κ-opioidergic system's role in controlling ethanol intake (Sirohi et al. 2012; Walker et al. 2012).

The exact neurochemical mechanisms that underlies the suppression of ethanol intake by κ-opioid receptor antagonists is, however, very much a matter of speculation, since the experimental data on the subject obtained from different ethanol administration paradigms have not been consistent (Deehan et al. 2012; Hölter et al. 2000; Kemppainen et al. 2012; Nealey et al. 2011; Rorick-Kehn et al. 2014; Rose et al. 2016; Schank et al. 2012; Walker and Koob 2008; Walker et al. 2011). It has been hypothesized that due to the overactivation of the κ-opioidergic system following chronic exposure to ethanol, a hypodopaminergic state may develop in the nucleus accumbens, which might be opposed by κ-opioid receptor antagonists (Karkhanis et al. 2016; Rose et al. 2016). Interestingly, the number of κ-opioid receptor binding sites in the ventral striatum has been shown to be unchanged in deceased alcoholics (Hermann et al. 2017). Additionally, recent evidence also suggests a possible hyperdopaminergic state to be prevalent in the brain following

protracted abstinence (Hirth et al. 2016). Thus, it seems that multiple neuroadaptations are involved in the regulation of ethanol intake.

Intra-accumbal infusions of JDTic or nor-BNI did not have acute or long-term effects on water intake. Surprisingly, water intake decreased in the vehicle treatment group 24 h after systemic vehicle administration. However, since water intake remained stable within the systemically treated JDTic group and ethanol intake was only first affected two days after the injections, we conclude that the decrease in water intake in the vehicle treated group is not a confounding factor for the interpretation of the ethanol intake results. Also earlier reports show that κ-opioid receptor antagonists do not affect water intake (Deehan et al. 2012; Nealey et al. 2011; Walker and Koob 2008; Walker et al. 2011). None of the treatments altered food intake. Previously we have shown that repeated acute intracranial injections of vehicle do not *per se* have an effect on ethanol intake (Uhari-Väänänen et al. 2016). In addition, in the current study ethanol intake levels were not altered as compared to baseline levels after the intra-accumbal vehicle infusion or the systemic vehicle injection. Therefore, the present results on the effects of κ-opioid receptor antagonists on ethanol intake are most probably not confounded by the used experimental procedures within the used paradigm themselves, but are specific to the used experimental drugs.

In conclusion, the present study suggests that κ-opioid receptors play a role in controlling ethanol intake and that accumbal shell κ-opioid receptors participate in the modulation of the reinforcing effects of ethanol. Systemic JDTic, a selective κ-opioid receptor antagonist, was able to decrease ethanol intake in alcohol-preferring AA rats. Intra-accumbal JDTic also showed a weak decreasing effect on ethanol intake in a long-term manner. As the activational state of the κ-opioidergic system may critically affect the results, the used ethanol intake paradigm and the genetic background of used animals are to be considered. The results provide further support for the view that κ-opioid receptor antagonists may be a valuable adjunct in the pharmacotherapy of ethanol use disorders.

COMPLIANCE WITH ETHICAL STANDARDS

This study was supported by the Finnish Foundation for Alcohol Studies. JDTic was provided as a gift from RTI International (Research Triangle Park, NC, USA). The authors declare that they have no conflicts of interest.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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FIGURE LEGENDS

Fig. 1 Effects of bilateral microinfusions of the κ-opioid receptor antagonist JDTic 15 µg/site (n = 8) or vehicle (n = 8) (Veh) given 24 h prior into the nucleus accumbens shell on ethanol intake of alcohol-preferring AA rats (experiment 1). Doses are given in µg/site in 0.3 µl. Ethanol intake is given in g/kg (mean ± SEM) at 90 min. Baseline (BL) ethanol intake is the average of the last two ethanol intake sessions preceding the treatments. JDTic showed a weak decreasing effect on ethanol intake as compared to vehicle ($p = 0.058$) three days after the microinfusions (unpaired *t*-test with the Holm-Bonferroni correction after a significant mixed-model two-way ANOVA interaction, $F(3,42) = 3.41$, $p = 0.026$)

Fig. 2 Effects of bilateral microinfusions of the κ-opioid receptor antagonist nor-BNI 3 µg/site (n = 10) or vehicle (n = 8) (Veh) given 24 h prior into the nucleus accumbens shell on ethanol intake of alcohol-preferring AA rats (experiment 1). Doses are given in µg/site in 0.3 µl. Ethanol intake is given in g/kg (mean ± SEM) at 90 min. Baseline (BL) ethanol intake is the average of the last two ethanol intake sessions preceding the treatments. Nor-BNI tended to decrease ethanol intake. Significant mixed-model two-way ANOVA interaction, $F(3,48) = 3.547$, $p = 0.021$

Fig. 3 Cumulative ethanol intake **(a)** at 90 min and **(b)** at 0-90 min after acute (administration 5 min prior ethanol intake session) bilateral microinfusions of the κ-opioid receptor antagonist JDTic into the nucleus accumbens shell of alcohol-preferring AA rats (experiment 2). Doses are given in µg/site in 0.3 µl. Ethanol intake is given in g/kg (mean ± SEM). Vehicle (n = 9) (Veh), JDTic 1 µg/site (n = 9), JDTic 5 µg/site (n = 7), JDTic 15 µg/site (n = 8)

Fig. 4 Effects of subcutaneous injections of the κ-opioid receptor antagonist JDTic 10 mg/kg (n = 10) or vehicle (n = 8) (Veh) on ethanol intake of alcohol-preferring AA rats (experiment 3). The treatments were given 10 min prior the first ethanol intake session. Ethanol intake is given in g/kg (mean ± SEM) at 30 min. Baseline (BL) ethanol intake is the average of the last two ethanol intake sessions preceding the treatments. Asterisks denote a significant difference (**$p < 0.01$) from the vehicle treatment at 2 days post injection and a trend at 4 days post injection ($p = 0.08$) (unpaired *t*-test with the Holm-Bonferroni correction after a significant mixed-model two-way ANOVA interaction, $F(3,48) = 2.847$, $p = 0.047$)

Fig. 5 Schematic diagram of representative infusion sites in the nucleus accumbens shell for long-term (black circles) and acute (gray circles) experiments (experiments 1 and 2, respectively). The infusion sites are fixed to the coronal section closest to the actual anterior-posterior site of the infusions relative to the bregma. One circle represents two individual rats. The coronal sections were adapted from the rat brain atlas of Paxinos and Watson (1998)

Figure 1:

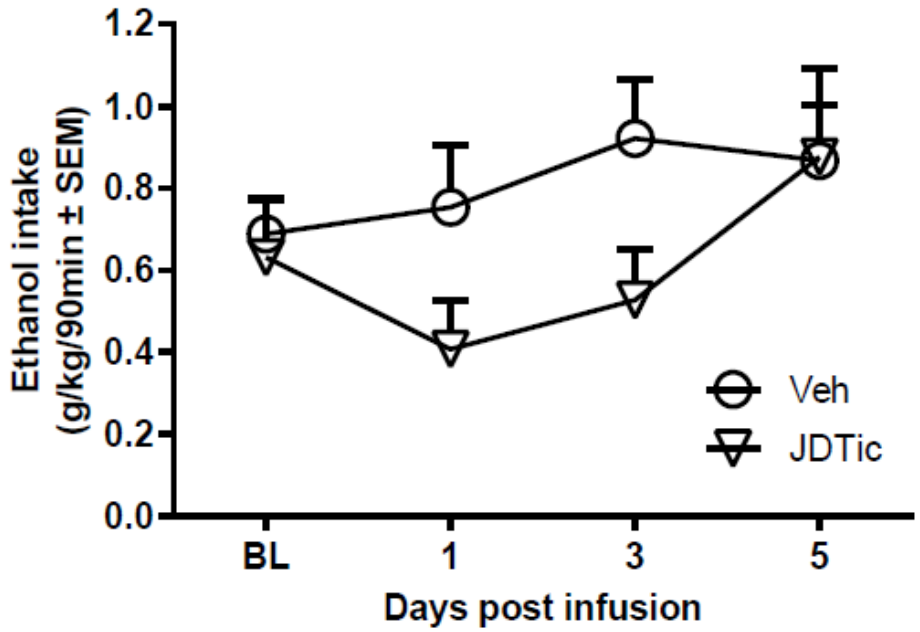
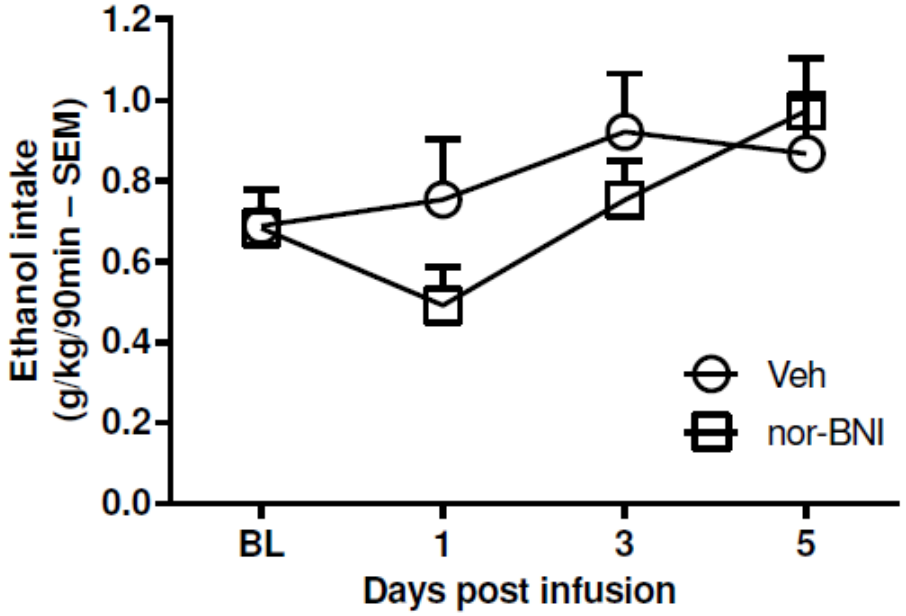


Figure 2:



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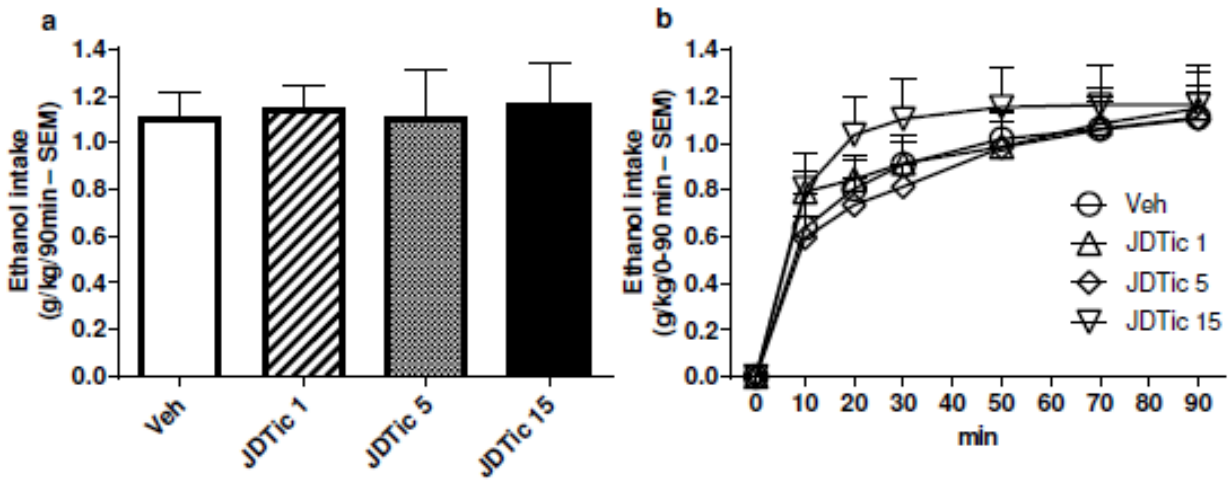

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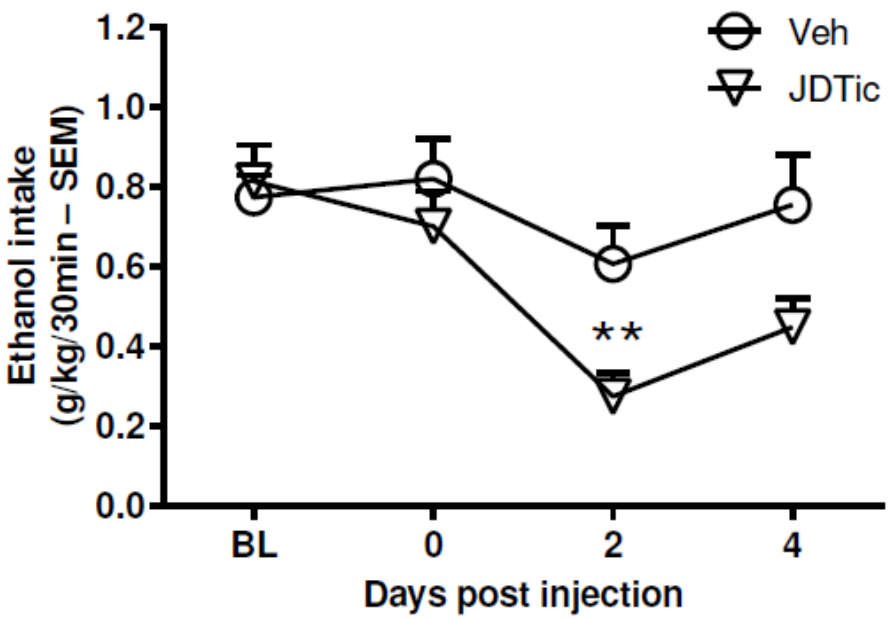

Figure 5: