The selective κ-opioid receptor antagonist JDTic attenuates the alcohol deprivation effect in rats

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ABSTRACT

The mechanisms behind relapse to ethanol intake in recovering alcoholics are still unclear. The negative reinforcing effects contributing to ethanol addiction, including relapse, are considered to be partly driven by the κ-opioidergic system. As the κ-opioidergic system interacts with the mesolimbic reward pathway, the aim of the study was to clarify the role of nucleus accumbens shell κ-opioidergic mechanisms in relapse to ethanol intake by using the alcohol deprivation effect (ADE) paradigm. The ADE is defined as a transient increase in voluntary ethanol intake after a forced period of abstinence. Male Long-Evans rats were trained to voluntarily consume 10% (v/v) ethanol solution. Ethanol access and deprivation cycles were initiated after stable ethanol intake baselines had been reached and bilateral guide cannulas had been implanted above the nucleus accumbens shell. One cycle consisted of 10 days of 90 min access to ethanol followed by 6 days of ethanol deprivation. The ADE was measured in the beginning of a new cycle. Rats received JDTic, a selective κ-antagonist, either subcutaneously (10 mg/kg) or intra-accumbally (15 μg/site) or, as a reference substance, systemic naltrexone (0.3 mg/kg) before ethanol re-access, and the effects on the ADE were evaluated. Systemic and intra-accumbal JDTic significantly attenuated the ADE on the first day of ethanol re-access, as did systemic naltrexone. Additionally, naltrexone decreased ethanol intake levels. These results suggest that nucleus accumbens shell κ-opioidergic mechanisms may have a role in mediating relapse to ethanol intake. Additionally, κ-antagonism could be a valuable adjunct in ethanol relapse prevention.

KEYWORDS

ethanol, relapse, kappa opioid receptor, nucleus accumbens

1. Introduction

One of the major challenges in ethanol addiction research is to understand the mechanisms leading to relapse, as only 24% of patients remain abstinent one year after treatment (Miller et al., 2001). Opioidergic systems have been the focus of ethanol research due to their role in controlling ethanol intake, with shown clinical relevancy as demonstrated by the currently approved drugs naltrexone and nalmefene, both non-selective opioid receptor antagonists (Mann et al., 2013; Soyka, 2016; Volpicelli et al., 1992).

Ethanol releases endogenous opioid peptides, β-endorphins, enkephalins and dynorphins, in central areas of the reward tract, the ventral tegmental area, nucleus accumbens and ventral pallidum (Jarjour et al., 2009; Marinelli et al., 2003, 2005, 2006), after which they bind with differing affinity to local, inhibitory acting μ-, δ- or κ-opioid receptors, with dynorphins having the highest affinity for κ-opioid receptors (Mansour et al., 1987, 1988, 1995; Mitrovic and Napier, 1995; Svingos et al., 1996, 1999a, 1999b). In the reward pathway, the nucleus accumbens is situated in a manner that a large portion of reward-related signals pass through it, with dopaminergic ventral tegmental area afferents and GABAergic efferents projecting to the ventral pallidum (Wise, 2002). In the nucleus accumbens, κ-opioid receptors are mainly located on presynaptic neurons, and due to this location they are able to control the release of several different neurotransmitters, such as dopamine, glutamate and GABA, and thus modulate forward signaling (Hjelmstad and Fields, 2001, 2003; Svingos et al., 1999b).

The κ-opioidergic system is suggested to be a central player to ethanol's negative reinforcing effects (Sirohi et al., 2012; Walker et al., 2012). It is hypothesized that as ethanol intake gradually progresses, the role of the anhedonic depicted κ-opioidergic system increases while the role of the hedonic portrayed μ-opioidergic system decreases (Amalric et al., 1987; Lindholm et al., 2000; Mucha and Herz, 1985; Przewlocka et al., 1997; Rose et al., 2016; Sirohi et al., 2012; Walker et al., 2012). In rats made physically ethanol dependent, ethanol self-administration was decreased more after nalmefene than naltrexone treatment, which was postulated to be due to the dependent animals' overactivated κ-opioidergic system and the twice higher affinity of nalmefene to κ-opioid receptors than naltrexone (Michel et al., 1985; Walker and Koob, 2008). Overactivation of the accumbal κ-opioidergic system under both acute and prolonged withdrawal

circumstances has also been shown in rodents (Lindholm et al., 2000; Przewlocka et al., 1997), which could possibly contribute to ethanol relapse. Post-mortem studies in deceased alcoholics show alterations in the accumbal prodynorphin and κ-opioid receptor gene co-expression pattern (Bazov et al., 2018). However, no alterations of κ-opioid receptor binding sites in the ventral striatum were shown (Hermann et al., 2017).

The encouraging results on the role of the κ-opioidergic system in ethanol use disorders have drawn attention to it as a plausible new target for drug treatment (Deehan et al., 2012; Domi et al., 2018; Nealey et al., 2011; Rorick-Kehn et al., 2014; Schank et al., 2012; Sirohi et al., 2012; Uhari-Väänänen et al., 2018; Walker et al., 2011; Walker and Koob, 2008). New treatment strategies are urgently needed because the currently available drugs, including naltrexone and nalmefene, have been shown to have relatively limited clinical efficacy in recovering alcoholics (Jonas et al., 2014).

Surprisingly little data are available on the role of the κ-opioidergic system on ethanol intake under relapse conditions. The ability of the non-selective opioid receptor antagonist naltrexone to suppress relapse to ethanol intake in rodents is well documented (Heyser et al., 2003; Hölter and Spanagel, 1999; Orrico et al., 2014), but reports on selective κ-opioid receptor antagonists are scarce and the results are controversial (Deehan et al., 2012; Hölter et al., 2000). The prototypical selective κ-opioid receptor antagonist norbinaltorphimine (nor-BNI) was reported not to have an effect on relapse-like ethanol intake in rats (Hölter et al., 2000). On the contrary, systemic JDTic, a relatively new selective κ-opioid receptor antagonist not derived from the opioid class of compounds (Thomas et al., 2001), reduced operant responding for ethanol in rats under relapse conditions (Deehan et al., 2012). JDTic is a long-acting κ-antagonist with effects persisting up to 4 weeks (Carroll et al., 2004).

As the role of the central κ-opioidergic system in relapse to ethanol intake is unclear (Deehan et al., 2012; Hölter et al., 2000), the aim of the current study was to clarify the matter by using JDTic in the alcohol deprivation effect (ADE) paradigm. The ADE is characterized by a transient increase in voluntary ethanol intake following a period of deprivation (Sinclair and Senter, 1967), and it has been shown in several species, including rats (Sinclair and Senter, 1967), monkeys (Sinclair, 1971) and humans (Burish et al., 1981). The ADE paradigm is considered to have high face validity to relapse behavior seen in recovering

alcoholics (Samson and Chappell, 2001; Sinclair and Senter, 1967; Vengeliene et al., 2014). In this study, rats with a history of voluntary ethanol intake were predisposed to several cycles of ethanol access and deprivation and the effects of treatments on the ADE were evaluated.

JDTic was administered into the nucleus accumbens shell, on which special focus was set because of its enhanced activity in response to ethanol and other drugs of abuse (Koob, 1992; Marinelli et al., 2003, 2006; Sirohi et al., 2012; Wise, 2002), the ability of local κ-opioid receptors to modulate the release of neurotransmitters attributed to mediating ethanol-related behaviors (Hjelmstad and Fields, 2001, 2003; Svingos et al., 1999b), and because chronic ethanol intake and withdrawal have been shown to activate the local κ-opioidergic system (Lindholm et al., 2000; Nealey et al., 2011; Przewlocka et al., 1997). The nucleus accumbens shell is also a critical component of the extended amygdala (Alheid and Heimer, 1988), a neurocircuitry associated with motivation and emotion (Walker et al., 2012). Systemic JDTic was tested to be able to elucidate the overall role of this brain area on ethanol relapse. Systemic naltrexone was used as a reference substance since the effects of naltrexone in this kind of a model are well known (Heyser et al., 2003; Hölter and Spanagel, 1999; Orrico et al., 2014).

2. Experimental procedures

2.1. Animals

Altogether 51 male Long-Evans rats (HsdBlu:LE, Envigo, Indianapolis, IN, USA) were used in the study. The rats were 6-7 weeks old upon arrival and they were let to settle for 2 weeks before the onset of the study. The rats weighed 270-300 g at the beginning of the experiments. The rats were housed in standard individually ventilated cages in groups of 2-3 rats/cage. Water and standard rat chow (SDSRM1[E]SQC, Witham, Essex, UK) were available at all times *ad libitum*. The rats were housed and the experiments were performed in reversed light/dark cycle (lights off at 0800 hours). Ambient temperature was maintained at 22±1°C. For monitoring of ethanol intake, the rats were transferred to individual wire mesh cages (38x21x19 cm). 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 (license ESAVI/5705/04.10.07/2013).

2.2. Drugs

The selective 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). Naltrexone hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were diluted into sterile saline. The used volume for subcutaneous injections was 1 ml/kg and for intra-accumbal microinfusions 0.3 µl/site. Ethanol (EtaxA, 96% v/v; Altia, Rajamäki, Finland) was diluted into tap water.

For the subcutaneous injections, the used dose of JDTic was 10 mg/kg and that of naltrexone was 0.3 mg/kg. For intra-accumbal JDTic microinfusions, the used dose was 15 µg/site. The single acute systemic JDTic dose was chosen due to several reports by us and others showing it to be able to affect ethanol-related behaviors (Deehan et al., 2012; Schank et al., 2012; Uhari-Väänänen et al., 2018). The dose of naltrexone was chosen based on previous work showing low doses to be selective for ethanol and effective in preventing the ADE in rats (Heyser et al., 2003; Hölter and Spanagel, 1999). The single acute dose of intra-accumbal JDTic was based on previous intracranial microinfusion and ethanol intake experiments by us and others (Knoll et al., 2011; Uhari-Väänänen et al., 2018).

The pretreatment time for JDTic was 24 h before ethanol re-access to ensure maximal κ-antagonistic activity due to its slow and delayed onset (Carroll et al., 2004). This is a pretreatment time used previously for JDTic (Knoll et al., 2011; Uhari-Väänänen et al., 2018). Following systemic JDTic, κ-antagonism starts first at 6 h and the plasma and brain half-lives of JDTic are roughly 28 and 52 h, respectively (Carroll et al., 2004; Owens et al., 2016). As naltrexone is a rapidly acting opioid receptor antagonist (Meyer et al., 1984), the pretreatment time of 20 min before ethanol re-access was used.

2.3. Ethanol access and deprivation cycles

Initially, the rats were trained to drink 10% ethanol solution using the intermittent, time restricted two-bottle choice ethanol access paradigm (modified from Simms et al., 2010; Uhari-Väänänen et al., 2016). To initiate ethanol intake, a standard saccharin fade paradigm was used, starting with 0.2% (w/v) saccharin in 10% ethanol solution (modified from Samson 1986). Ethanol was available for 24 h three times a week – on Mondays, Wednesdays and Fridays – in the individual wire mesh cages to which the rats were moved to for the ethanol intake sessions within 1 h after the lights went out. This was continued for at least 3 weeks or until stable 24 h ethanol intake levels had been attained, after which ethanol availability was reduced to 90 min. The left-right positions of the water or ethanol containing Richter tubes were altered each session to prevent side preference.

After stable 90 min intermittent ethanol intake levels, the ethanol access and deprivation cycles were initiated. By this time the rats had had access to ethanol for 8-10 weeks. Initial ethanol experience of 6-8 weeks is regarded as the minimum length of time needed for a reliable ADE to develop following the deprivation period (Vengeliene et al., 2014; Wolffgramm and Heyne, 1995). One cycle consisted of 10 days of access to ethanol for 90 min/day in the individual wire mesh cages, followed by a 6 day deprivation period in home cages, after which a new cycle was initiated. In the beginning of each new cycle (first 2 days), the ADE was evaluated. Repeated cycles were mainly used to enable habituation to the used procedures. Additionally, repeated cycles have been shown to produce a more robust and reliable ADE than after a single deprivation period (Spanagel et al., 1996).

The ADE was determined from the cumulative 90 min ethanol intake data. The average baseline ethanol intake of 3 previous days before the deprivation period was compared to ethanol intake on days 1 and 2 after deprivation. Ethanol access periods ranging from 0.5-24 h/day have previously shown to result in a successful ADE (Heyser et al., 1997, 2003; Hölter et al., 2000; Hölter and Spanagel, 1999; McKinzie et al., 1998; Sinclair and Senter, 1967; Vengeliene et al., 2014). Water intake was measured at the 90 min time point. The level of ethanol intake during the ADE had to be at least 20% higher than during baseline, and the baseline ethanol intake level had to be at least 0.2 g/kg/90 min for the rat to be included in the experiments. If these criterions were not met, the rat was excluded before statistical analysis.

2.4. Outline of experimental design

The study consisted of four experiments outlined in Figure 1. In experiment 1, the manifestation of the ADE in the current paradigm was examined in non-treated rats. In experiment 2, systemic JDTic (10 mg/kg) was utilized to evaluate the overall role of κ-antagonism on the ADE. The animals in experiment 2 were re-used in experiment 3, where systemic naltrexone (0.3 mg/kg) was used to assess non-selective opioid receptor antagonism on the ADE. In experiment 4, intra-accumbens shell microinfusions of JDTic (15 µg/site) were used to assess the role of local κ-opioid receptors on the ADE.

Experiment 1 was conducted before the onset of experiments 2-4 with 20/51 rats, which consumed stable, over 0.2 g/kg/90 min amounts of ethanol. All but 5 rats used in experiment 1 were randomly re-assigned to the rest of the experiments. Altogether 20/51 rats (including 9 from experiment 1) were assigned to experiment 2, out of which 12 rats (5 vehicle and 7 JDTic rats) were re-used in experiment 3. Altogether 26/51 rats (including 6 from experiment 1) were assigned to experiment 4.

For both subcutaneous and intra-accumbens shell JDTic experiments (experiments 2 and 4), a between-subjects design was used due to the long-term κ-antagonistic effects of JDTic (Carroll et al., 2004; Deehan et al., 2012). In experiment 3, due to the relatively short-term effects of naltrexone (Meyer et al., 1984), a within-subjects design was used in two consecutive ADE cycles.

2.5. Experiment 1 - Expression of the ADE

The ethanol intakes of two groups of non-treated rats were compared to one another after the first deprivation period (Fig.1). While one group was deprived of ethanol for 6 days, the other group continued to have 90 min/day ethanol access. After ethanol was re-accessible to the deprived rats, the magnitude and duration of the ADE, as well as its relevant manifestation when compared to continuous ethanol access, was determined by comparing ethanol intakes of the two groups of rats to one another.

2.6. Experiments 2 and 3 – The effects of systemic JDTic and naltrexone on the ADE

To assess the effects of systemic JDTic on the ADE (experiment 2), the rats first underwent two ethanol intake and deprivation cycles, during which habituation vehicle injections were given 24 h before ethanol re-

access (Fig.1). The rats were assigned to treatment groups according to baseline ethanol intake levels and magnitude of the ADE (expressed as percentage from baseline) of the last cycle. JDTic or vehicle was administered during the third cycle, 24 h before the end of the deprivation period and ethanol re-access.

The same rats that first received JDTic or vehicle were subsequently used to test the effects of systemic naltrexone on the ADE (experiment 3) (Fig.1). To ensure that the previous treatment would not be a confounding factor for result interpretation, there was one ethanol access and deprivation cycle in between the administrations (4.5 weeks). Naltrexone (0.3 mg/kg) or vehicle was administered subcutaneously 20 min before ethanol re-access in 2 consecutive ethanol access and deprivation cycles in random order.

2.7. Experiment 4 - The effects of intra-accumbens shell JDTic on the ADE

The rats for intra-cranial microinfusions underwent surgery after stable 90 min intermittent ethanol intake baselines had been established (Fig.1). Isoflurane was used for anesthesia (4% during induction and 2-2.5% for anesthesia maintenance). The rats were attached to a stereotaxic frame, the skull was exposed and two guide cannulas cut from stainless steel hypodermic tubes (23 G, Component Supply Company, Sparta, TN, USA) were implanted bilaterally 2 mm above the nucleus accumbens shell according to the stereotaxic coordinates by Paxinos and Watson (1998). The used coordinates (AP+1.7; ML±1.2; DV-5.2 from the dura) were chosen based on earlier work by us and others (Castro and Berridge, 2014; Nealey et al., 2011; Uhari-Väänänen et al., 2016, 2018). The guide cannulas were attached to the skull with three stainless steel screws and dental cement. Dummy stylets cut out of stainless steel wire (Component Supply Company, Sparta, TN, USA) were used to prevent occlusion. During surgery, the rat's body temperature was kept constant with a heating pad attached to a thermostatic sensor. Sterile saline was given intraperitoneally to prevent dehydration. After surgery, the rats were returned to their home cage to recover. Carprofen (Rimadyl, 5 mg/kg, subcutaneously; Vericore, Dundee, UK) was given for analgesia 30 min before surgery and for two consecutive days. The rats were let to recover for at least three days before they were presented with ethanol solution and the repeated cycles of ethanol access and deprivation were initiated.

The rats were habituated to the microinfusion procedure during the course of three ethanol access and deprivation cycles after the surgeries and before JDTic or vehicle were administered (Fig.1). Habituation was

done by removing and replacing the wire dummy stylets to the guide cannulas. During the first cycle, the rats received sham microinfusions, during which the infusion needles, constructed from stainless steel hypodermic tubes (30 G, Component Supply Company, Sparta, TN, USA) and extending 2 mm beyond the ventral tip of the guide, were placed into the guide cannulas but no microinfusions were given. During the two consequent cycles, training vehicle microinfusions were given 24 h before ethanol re-access during the deprivation period. The aim of these three training cycles was to ensure that neither the surgery nor the microinfusion procedure itself would affect result interpretation. Based on the baseline ethanol intake level and magnitude of the ADE (expressed as percentage from baseline) during the last ethanol access and deprivation cycle, the rats were assigned to treatment groups. JDTic (15 µg/site) or vehicle were administered during the fourth cycle (9-10 weeks after surgery) 24 h before the end of the deprivation period and before ethanol re-access. The microinfusions 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 microinfusion, the injection needle was left in place for 1 min. After the microinfusion, the needle was left in place for 2 min to avoid leakage up the cannula track and to allow spreading of the drug.

2.8. Histology

For experiment 4, the placement of the guide cannulas and success of intra-cranial injections was verified from 100 µm coronal brain slices made from 10% formalin fixed brains. Only rats with marks of the injection needle tip within the nucleus accumbens shell were accepted. The brain slices were compared to the rat brain atlas by Paxinos and Watson (1998). Rats were also excluded if the guide cannulas loosened during the experiments.

2.9. Statistical analysis

For statistical analysis, the cumulative 90 min ethanol intake data (ml) was first converted to grams of 100% ethanol/body weight (kg). The effects of all treatments on the ADE were analyzed from percentual changes in ethanol intake. The ethanol intake data from days 1 and 2 after deprivation were first converted to percentage of baseline ethanol intake values. Baseline intake was calculated as an average of the cumulative 90 min consumption from the last 3 ethanol intake sessions before ethanol deprivation. These percentage of

baseline values from either the different ethanol availability schemes (experiment 1) or drug and vehicle treatments (experiments 2-4) were then compared to one another either by the unpaired or paired two-sample t-test, where appropriate, with the Holm-Bonferroni correction for multiple comparisons.

Further, a mixed-model two-way analysis of variance (ANOVA) was used to analyze ethanol intake (g/kg) with treatment (experiment 1: continuous access or ethanol deprivation; experiments 2 and 4: JDTic or vehicle) as the between-groups factor and ethanol intake session (baseline, days 1 and 2 after deprivation) 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 for post hoc means.

The effects of naltrexone on the ADE (experiment 3) were analyzed from the ethanol intake data (g/kg) by a within-subjects two-way ANOVA with repeated measures on treatment (naltrexone or vehicle). Following significance, the paired two-sample t-test with the Holm-Bonferroni correction for multiple comparisons was used for post hoc means.

For water intake, the 90 min data (ml) was first converted to grams of water/body weight (kg). The effects of the different treatments on water intake were analyzed similarly as were the effects of the treatments on ethanol intake.

The accepted level of significance was set at $p<0.05$. The statistical software used was IBM SPSS Statistics V22.0 (IBM Corp., Armonk, NY, USA). The graphical software used was GraphPad PrismV6 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Ethanol intake

Before the onset of the ethanol access and deprivation cycles, average ethanol intake of rats scheduled for systemic injections was 0.46±0.10 g/kg/90 min. The average ethanol intake of rats scheduled for the intra-accumbal microinfusions was 0.43±0.08 g/kg/90 min before surgery and 0.50±0.09 g/kg/90 min after surgery but before the onset of the first deprivation period. Plots of 10-day ethanol intake before deprivation preceding drug administrations are provided in Supplement S1 (for experiments 1, 2 and 4).

Altogether 6 rats (2 from experiment 2; 4 from experiment 4) had to be excluded from the study because of low, below 0.2 g/kg/90 min baseline ethanol intake levels despite showing an ADE and 1 rat had to be excluded from experiment 4 because it did not show an ADE.

3.2. Experiment 1 – Expression of the ADE

Ethanol intake increased significantly on the first day after deprivation in the deprived rats as compared to the non-deprived rats ($t(18)=3.63$, $p=0.0019$) (Fig.2A), when the ethanol intake data were expressed as percentage of baseline. Also the g/kg ethanol intake data showed that ethanol deprivation led to a significant increase in 90 min ethanol intake in the deprived rats (mixed-model ANOVA, ethanol intake session x treatment interaction, $F(2,36)=3.982$, $p=0.027$). The post hoc test (Holm-Bonferroni) confirmed that the increase in ethanol intake was significant on the first day of ethanol re-access ($t(18)=2.304$, $p=0.0334$) (Fig.3A).

The increase in ethanol intake was 67±12% in the deprived rats on the first day after deprivation as compared to baseline levels while the non-deprived rats' ethanol intake levels remained unchanged. An additional paired t-test (baseline vs. day 1 in g/kg) confirmed that the deprived rats consumed more ethanol on the first day of ethanol re-access than during baseline ($t(9)=9.698$, $p<0.0005$).

3.3. Experiment 2 - The effects of systemic JDTic on the ADE

A significant ADE was verified in the vehicle treated rats for the first day of ethanol re-access with a paired t-test (baseline vs. day 1 in g/kg) ($t(8)=4.811$, $p=0.001$).

Systemically administered JDTic attenuated the ADE on the first day of ethanol re-access after the deprivation period as compared to controls ($t(16)=2.460$, $p=0.0257$), when analyzed from the percentage of baseline ethanol intake data (Fig.2B). The g/kg ethanol intake data showed no significant ethanol intake session x treatment interaction (Fig.3B).

3.4. Experiment 3 - The effects of systemic naltrexone on the ADE

A significant ADE was evident after vehicle treatment during the first day of ethanol re-access as shown with a paired t-test (baseline vs. day 1 in g/kg) ($t(11)=5.118$, $p<0.0005$).

Naltrexone significantly inhibited the ADE. The percentage of baseline ethanol intake data showed that naltrexone inhibited ethanol relapse on the first day after deprivation ($t(11)=4.002$, $p=0.0021$) (Fig.2C). Also a significant ethanol intake session x treatment interaction was shown when the data were expressed as g/kg ethanol intake, $F(2,22)=76.14$, $p<0.0005$ (within-subjects ANOVA). The post hoc test (Holm-Bonferroni) showed that the difference in ethanol intake between the treatments was significant on the first day after ethanol deprivation ($t(11)=8.802$, $p<0.0005$) (Fig.3C). No rebound ADE was evident on re-access days 2-4 (data not shown for days 3-4). Naltrexone treatment decreased ethanol intake below baseline levels on the first day of ethanol re-access, with a decrease of ethanol intake of roughly 64±5.5%.

3.5. Experiment 4 - The effects of intra-accumbens shell JDTic on the ADE

A paired t-test (baseline vs. day 1 in g/kg) verified that the ADE was significant on the first day of ethanol re-access in vehicle treated rats ($t(6)=3.187$, $p=0.019$).

Intra-accumbally administered JDTic attenuated the ADE on the first day of ethanol re-access as compared to controls ($t(14)=3.527$, $p=0.0034$), when analyzed from the percentage of baseline ethanol intake data (Fig.2D). When the g/kg ethanol intake data were analyzed, a significant ethanol intake session x treatment interaction was found, $F(2,28)=4.179$, $p=0.026$ (mixed-model ANOVA). The post hoc test (Holm-Bonferroni) showed that JDTic tended to attenuate the ADE on the first day after the deprivation period as compared to controls ($t(14)=1.906$, $p=0.077$) (Fig.3D).

3.6. Water intake

Neither systemic JDTic or naltrexone nor intra-accumbal JDTic modified 90 min water intake (experiments 2-4; data not shown). Neither was water intake affected in experiment 1 in non-treated rats (data not shown).

3.7. Histology

No rats in the intra-accumbens shell microinfusion experiment 4 needed to be excluded due to misplacement of guide cannulas (Fig.4). However, 3 rats had to be excluded due to loosening of the guide cannulas and 2 rats died of complications related to intracranial infusions.

4. Discussion

The aim of the present study was to examine the role of κ-opioid receptors especially in the nucleus accumbens shell in relapse to ethanol intake using the ADE paradigm in Long-Evans rats. Our results showed that both systemically and intra-accumbally administered JDTic attenuated the ADE. Systemic naltrexone inhibited the ADE as expected and decreased the amount of consumed ethanol. Water intake was not affected by JDTic, naltrexone or the ADE paradigm itself.

The ADE paradigm has been used as an ethanol relapse model as it directly measures the amount of ethanol consumed during a relapse situation (Heyser et al., 1997, 2003; Hölter et al., 2000; Hölter and Spanagel, 1999; McKinzie et al., 1998; Sinclair and Senter, 1967; Vengeliene et al., 2014). There is, however, not always a correlation between baseline ethanol intake levels and the occurrence, robustness or duration of the ADE in individual animals (Vengeliene et al., 2014). The magnitude of the ADE also varies among different rodent strains.

Using the current ADE paradigm, the increase in ethanol intake after ethanol re-access in ethanol deprived rats was 67±12% over baseline levels, which were significantly higher than in non-deprived rats. The magnitude of the ADE is in line with previous studies in outbred rats which show a roughly 40-50% increase in ethanol lever responding as compared to baseline (Heyser et al., 1997, 2003; Rodd et al., 2004). Baseline ethanol intake levels were in the same range as those reported earlier for outbred rats (Heyser et al., 1997; Hölter et al., 2000). Blood ethanol levels were not measured in the current study. However, it has previously been shown that ethanol intake levels of 0.7-0.8 g/kg produce pharmacologically meaningful blood ethanol concentrations (Heyser et al., 1997; Nurmi et al., 1999), amounts which vehicle treated and non-treated rats consumed during the ADE. In the current ADE paradigm, the ADE was relevant during the first day of ethanol re-access and this was consistently shown in each experiment. This is in line with previous experiments showing that the transient increase in ethanol intake lasts 1-4 days (Heyser et al., 1997; Hölter et al., 2000; McKinzie et al., 1998; Rodd et al., 2004).

There are only a few previous studies examining the effects of selective κ-opioid receptor antagonists on relapse to ethanol intake. In the current study, both systemically and intra-accumbally administered JDTic attenuated the ADE. Our results are in line with an earlier study showing that JDTic was able to reduce

ethanol relapse responding in operant conditions after a single systemic dose of JDTic (1-10 mg/kg) given during abstinence 25 days before relapse testing (Deehan et al., 2012). To increase the robustness of their and our results, it would be interesting to re-test the effects of systemic and intra-accumbens shell JDTic on ethanol relapse in operant conditions after similar pretreatment times as used here. On the contrary to these results, the prototypical κ-opioid receptor antagonist nor-BNI did not affect the ADE in long-term ethanol-experienced rats (Hölter et al., 2000). However, the authors reported a tendency towards a decrease in ethanol intake and lever pressing during the first hour of ethanol re-access. Since JDTic has greater potency and selectivity for the κ-opioid receptor over the μ- and δ-opioid receptors than nor-BNI and the drugs are derived from dissimilar compounds (Portoghese et al., 1988; Thomas et al., 2001, 2003), the discrepancy in the results may at least partly be attributed to the pharmacological differences between the two drugs.

In line with the current results, chronic administration of a κ-opioid receptor agonist increased the ADE in long-term (16-18 months) ethanol experienced rats (Hölter et al., 2000). Interestingly, an acute dose of the κ-opioid receptor agonist Mesyl Salvinor B decreased the ADE in mice with a shorter (3 weeks) ethanol experience history (Zhou et al., 2018). The discrepancy in results may partly be explained by the different ethanol pre-exposure times, which could also differently affect the activational state of the κ-opioidergic system.

Even though the results obtained with JDTic are not fully in line with nor-BNI on relapse to ethanol intake (current results; Deehan et al., 2012; Hölter et al., 2000), compelling evidence suggests that selective κ-opioid receptor antagonism is effective in decreasing ethanol relapse-related behaviors (Deehan et al., 2012; Domi et al., 2018; Harshberger et al., 2016; Le et al., 2018; Rorick-Kehn et al., 2014; Schank et al., 2012). In ethanol seeking models where ethanol is not available for consumption, systemic JDTic decreased cue-induced, but not stress-induced reinstatement of ethanol seeking, and operant responding for ethanol in the Pavlovian Spontaneous Recovery paradigm (Deehan et al., 2012; Schank et al., 2012). Accordingly, both systemic and bed nucleus of the stria terminalis injections of nor-BNI attenuated κ-agonist-induced reinstatement of ethanol seeking in rats (Harshberger et al., 2016; Le et al., 2018). CERC-501 (previously LY2456302), a relatively short-acting and selective κ-antagonist, blocked stress but not cue-induced reinstatement of ethanol seeking, in addition to reducing responding for ethanol under operant conditions

using a progressive ratio schedule of reinforcement, a model considered to measure the motivation to obtain ethanol (Domi et al., 2018; Rorick-Kehn et al., 2014).

Relapse to ethanol intake may be attributed to the negative reinforcing effects of ethanol, which are suggested to be partially driven by the κ-opioidergic system (Sirohi et al., 2012; Walker et al., 2012). The κ-opioidergic system is suggested to become overactivated following chronic ethanol intake and withdrawal (Karkhanis et al., 2016; Lindholm et al., 2000; Nealey et al., 2011; Przewlocka et al., 1997; Rose et al., 2016; Sirohi et al., 2012; Walker et al., 2011; Walker and Koob, 2008). This is supported by studies showing that nucleus accumbens shell prodynorphin mRNA levels and dynorphin B concentrations are upregulated after chronic ethanol exposure and during ethanol withdrawal (Lindholm et al., 2000; Przewlocka et al., 1997). Additionally, systemic and intra-accumbens shell administrations of nor-BNI decreased ethanol self-administration in rats made physically ethanol dependent but not in non-dependent animals (Nealey et al., 2011; Walker et al., 2011; Walker and Koob, 2008).

As κ-opioid receptors are located presynaptically in the nucleus accumbens shell, they are able to inhibit the release of several neurotransmitters (Hjelmstad and Fields, 2001, 2003; Svingos et al., 1999b). Additionally, κ-opioid receptors interact with dopamine transporter proteins (DAT) and can thus modulate dopamine uptake (Svingos et al., 2001; Thompson et al., 2000). Consequently, overactivation of the κ-opioidergic system could have various effects on local neurotransmitter levels. Chronic intermittent ethanol exposure has been shown to reduce accumbal core dopamine release and increase dopamine uptake rates (Karkhanis et al., 2016; Rose et al., 2016). Additionally, in rats exposed to repeated ethanol access and deprivation cycles, the level of accumbens shell dopamine was lower during periods of deprivation than 24 h after ethanol re-access (Hadar et al., 2017). Sensitized κ-opioid receptors have been suggested to be one reason behind these changes as κ-agonism reduced accumbal dopamine levels more in chronic intermittent ethanol exposed animals than in controls (Karkhanis et al., 2016; Rose et al., 2016). Nor-BNI was also able to normalize, in this case increase, accumbal dopamine levels after an acute ethanol challenge in chronic intermittent ethanol exposed animals (Karkhanis et al., 2016).

As a consequence of the previous results, it has been speculated that overactivation of the κ-opioidergic system could cause a hypodopaminergic state to prevail in the nucleus accumbens (Karkhanis et al., 2016; Rose et al., 2016), which could also provoke relapse to ethanol intake. Thus, κ-opioid receptor antagonism could theoretically oppose these effects and attenuate relapse to ethanol intake. On the contrary, a hyperdopaminergic state has also been proposed following protracted abstinence based on reductions in dopamine D1 receptor and DAT binding and increased extracellular dopamine levels (Hirth et al., 2016), highlighting the complexity of the neuroadaptations that follow chronic ethanol intake and periods of deprivation.

Systemic naltrexone was used as a reference substance to test the current ADE model. As expected, the 0.3 mg/kg dose inhibited the ADE and decreased ethanol intake on the first day of ethanol re-access, which is in line with previous reports on the effects of naltrexone on the ADE (Heyser et al., 2003; Hölter and Spanagel, 1999; Orrico et al., 2014). In addition, no rebound ADE was evident on days 2-4 of ethanol re-access, during which ethanol intake levels returned to baseline. These results mirror those observed after chronically administered low doses of naltrexone (0.125-0.250 mg/kg) (Heyser et al., 2003), according to which naltrexone attenuated the ADE and decreased operant responding rates for ethanol specifically on the first day of ethanol re-access, without a rebound ADE on subsequent days. On the contrary, higher doses of chronically administered naltrexone (5 mg twice a day) showed a delayed ADE (Orrico et al., 2014).

In the current study it is noteworthy that naltrexone was more effective than JDTic in preventing the ADE. A similar effect was seen in a previous reinstatement of ethanol seeking study using both naltrexone and nor-BNI, in which naltrexone was more effective than nor-BNI in reducing ethanol-seeking behavior induced by a κ-opioid receptor agonist (Harshberger et al., 2016). Naltrexone has the most affinity towards the μ-opioid receptor with lower affinity to δ- and κ-opioid receptors (Michel et al. 1985). Thus, it seems that all three opioidergic systems may participate in controlling relapse behavior. Naltrexone's effects on ethanol relapse might mainly be mediated by the reward-attributed μ-opioidergic system, though it is possible that as ethanol dependence progresses its role may decrease (Sirohi et al., 2012; Walker et al., 2012). This could explain why not all recovering alcoholics benefit from naltrexone treatment (Jonas et al. 2014), which is why additional or alternative κ-antagonism could be beneficial.

In conclusion, the results suggest that κ-opioidergic mechanisms participate in mediating the ADE and that nucleus accumbens shell κ-opioid receptors may play a role in relapse to ethanol intake. These results provide evidence that κ-opioid receptor antagonists should be investigated further for their potential in preventing relapse to ethanol intake.

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Contributors

JUV, TE, PB, AR, KK and PP designed the study. JUV and TE conducted the experiments and undertook the statistical analysis. JUV, TE, PB, AR, KK and PP were involved in interpretation of the data. JUV drafted the manuscript. All authors have contributed to revising the manuscript critically for its intellectual content and have approved the final version.

Conflict of Interest

The authors declare that they do not have any conflicts of interest.

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Figure legends

Fig.1. Outline of experimental design for experiments (Exp.) 1-4. The vertical solid arrows indicate the administration of training vehicle and experimental drug systemic injections or intra-accumbens shell microinfusions before ethanol re-access after a deprivation period. ADE cycle=ethanol access and deprivation cycle, EtOH=ethanol, Veh=vehicle

Fig.2. Effects of treatments on the alcohol deprivation effect of Long-Evans rats, as expressed as percentage of baseline (BL) ethanol intake on Days 1 and 2 after deprivation. **A)** A 6 day deprivation period (Depr, n=10) or continuous ethanol access (Cont, n=10), **B)** systemic JDTic (10 mg/kg, sc, n=9), a κ-opioid receptor antagonist, or vehicle (n=9) administered 24 h before, **C)** systemic naltrexone (NTX, 0.3 mg/kg, sc), a non-selective opioid receptor antagonist, or vehicle (n=12, within-design) administered 20 min before and **D)** bilateral intra-accumbens shell microinfusions of JDTic (15 µg/0.3 µl/site, n=9) or vehicle (0.3 µl/site, VEH, n=7) administered 24 h before ethanol re-access. Baseline ethanol intake=average of the last three 90 min ethanol intake sessions (days) before ethanol deprivation. Asterisks indicate a significant difference from vehicle treatment (*p<0.05, **p<0.01, Holm-Bonferroni's paired or unpaired t-test).

Fig.3. Effects of treatments on the alcohol deprivation effect of Long-Evans rats. Ethanol intake is expressed as g/kg (mean±SEM). **A)** A 6 day deprivation period (Depr, n=10) or continuous ethanol access (Cont, n=10), **B)** systemic JDTic (10 mg/kg, sc, n=9), a κ-opioid receptor antagonist, or vehicle (n=9) administered 24 h before, **C)** systemic naltrexone (NTX, 0.3 mg/kg, sc), a non-selective opioid receptor antagonist, or vehicle (n=12, within-design) administered 20 min before and **D)** bilateral intra-accumbens shell microinfusions of JDTic (15 µg/0.3 µl/site, n=9) or vehicle (0.3 µl/site, VEH, n=7) administered 24 h before ethanol re-access. Baseline (BL) ethanol intake=average of the last three 90 min ethanol intake sessions (days) before ethanol deprivation. Asterisks indicate a significant difference from vehicle treatment (*p<0.05, ***p<0.0005) after a significant mixed-model two-way ANOVA treatment x ethanol intake session (day) interaction (A) or a within-subjects repeated two-way ANOVA treatment x day interaction (C). Intra-accumbal JDTic tended to decrease ethanol intake (p=0.077) on Day 1 of ethanol re-access after a significant mixed-model two-way ANOVA (D)

Fig.4. Schematic diagram of representative microinfusion sites (black circles) in the nucleus accumbens shell. The microinfusion sites were fixed to the coronal section closest to the actual anterior-posterior site of the microinfusions relative to the bregma. The coronal sections were adapted from the rat brain atlas by Paxinos and Watson (1998). A representative brain section is shown in the inset.

Fig. 1

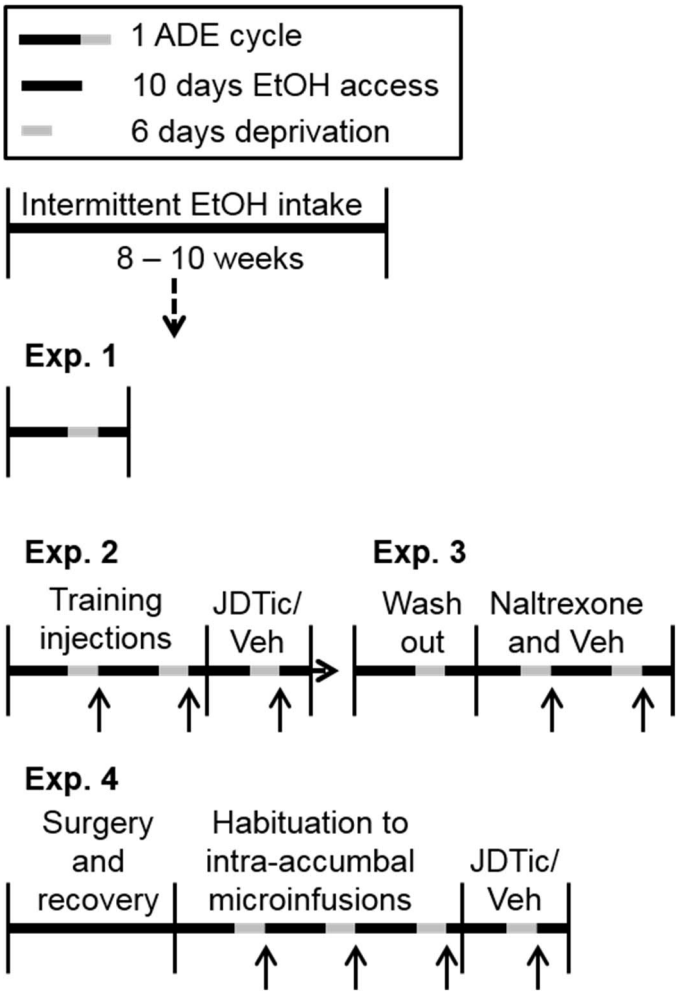

1 ADE cycle
10 days EtOH access
6 days deprivation
Intermittent EtOH intake
8 – 10 weeks
Exp. 1
Exp. 2
Training injections
JDTic/ Veh
Exp. 3
Wash out
Naltrexone and Veh
Exp. 4
Surgery and recovery
Habituation to intra-accumbal microinfusions
JDTic/ Veh

Fig.2.

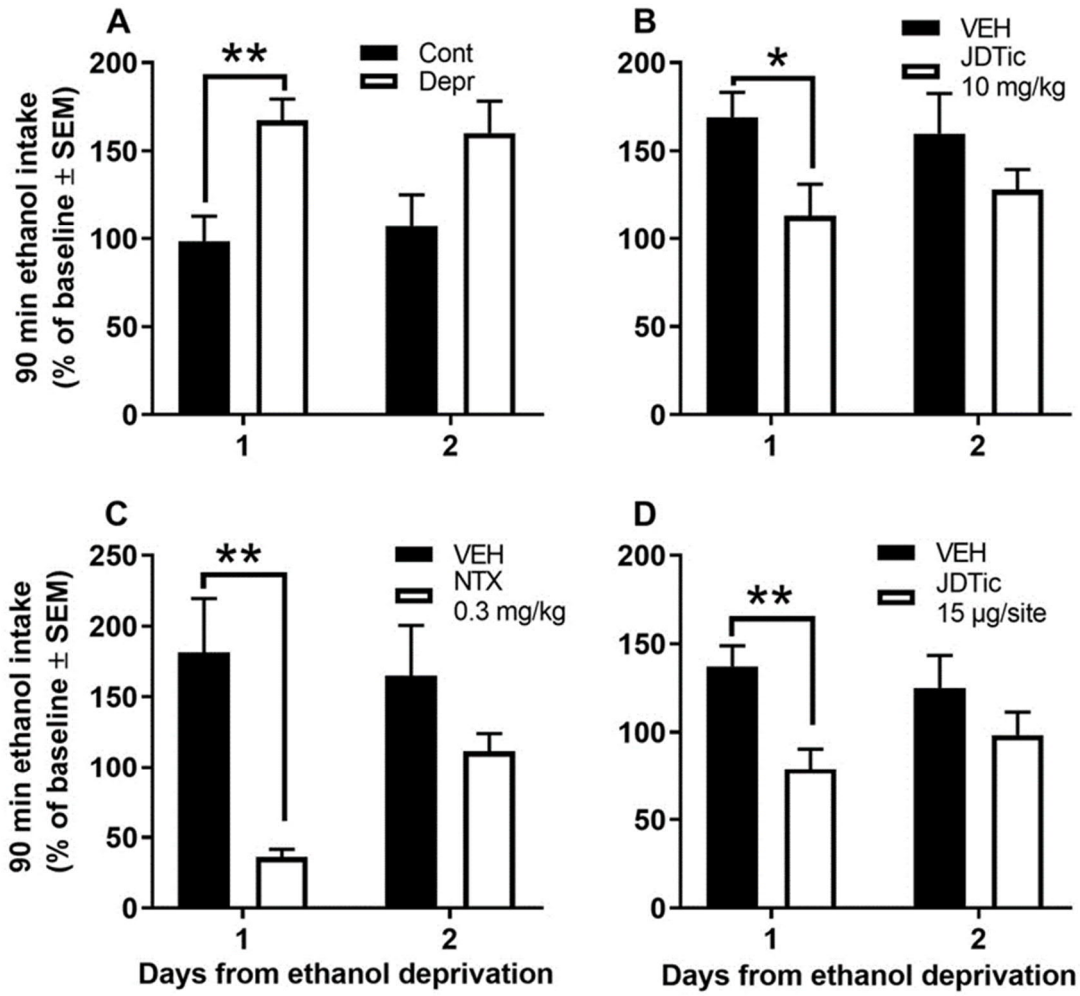

A
B
C
D
90 min ethanol intake
(% of baseline ± SEM)
Cont
Depr
VEH
JDTic
10 mg/kg
NTX
0.3 mg/kg
JDTic
15 µg/site
**
*
Days from ethanol deprivation

Fig.3.

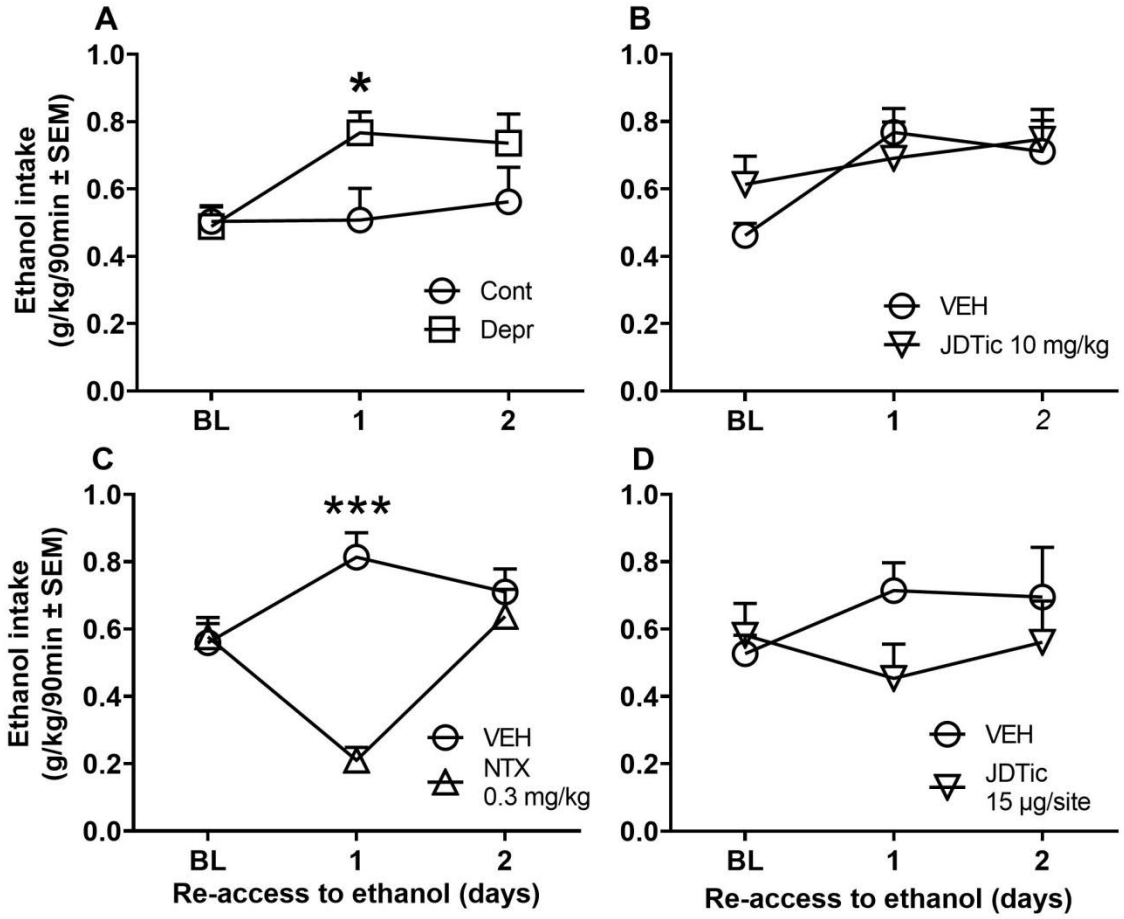
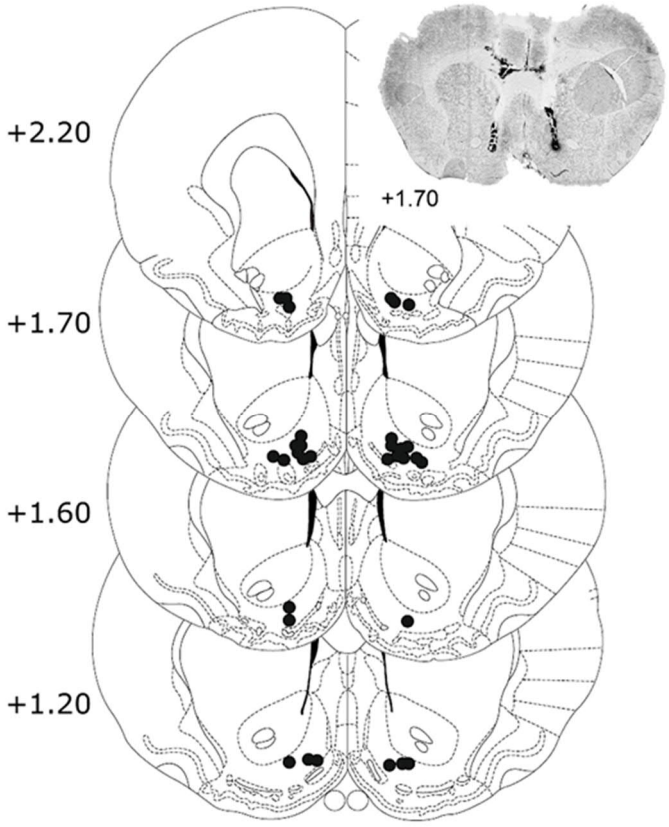

A
B
C
D
Ethanol intake
(g/kg/90min ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
***
BL
1
2
Cont
Depr
VEH
JDTic 10 mg/kg
NTX
0.3 mg/kg
JDTic
15 µg/site
Re-access to ethanol (days)

Fig.4.



SUPPLEMENTARY MATERIAL

The selective κ-opioid receptor antagonist JDTic attenuates the alcohol deprivation effect in rats

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Supplement S1Figure legend

Plots of 10 day ethanol intake preceding ethanol deprivation and re-access to ethanol of Long-Evans rats. The 6 day deprivation period is marked with a vertical line, after which the alcohol deprivation effect (ADE) was evaluated on ethanol re-access days 1 and 2 (ADE 1 and ADE 2, respectively). Ethanol intake is expressed as g/kg (mean±SEM) from 90 min ethanol intake sessions per day. The different treatments were A) a 6 day deprivation period (Depr, n=10) or continuous ethanol access (Cont, n=10) prior to ethanol re-access, B) systemic JDTic (10 mg/kg, sc, n=9), a κ-opioid receptor antagonist, or vehicle (n=9) administered 24 h prior to ethanol re-access and C) bilateral intra-accumbens shell microinfusions of JDTic (15 µg/0.3 µl/site, n=9) or vehicle (0.3 µl/site, VEH, n=7) administered 24 h prior to ethanol re-access.