RESEARCH PAPER

Neurokinin 1 receptor blockade in the medial amygdala attenuates alcohol drinking in rats with innate anxiety but not in Wistar rats

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BACKGROUND AND PURPOSE

Substance P and its preferred neurokinin receptor NK_1 have been implicated in stress and anxiety and have been proposed as possible therapeutic targets for the treatment of anxiety/depression. Attention is also being focused on the role this neuropeptide system may play in drug addiction, because stress-related mechanisms promote drug abuse.

EXPERIMENTAL APPROACH

The effects of the rat-specific NK_1 receptor antagonist, L822429, on alcohol intake and seeking behaviour was investigated in genetically selected Marchigian Sardinian alcohol preferring rats. These rats demonstrate an anxious phenotype and are highly sensitive to stress and stress-induced drinking.

KEY RESULTS

Systemic administration of L822429 significantly reduced operant alcohol self-administration in Marchigian Sardinian alcohol preferring rats, but did not reduce alcohol self-administration in stock Wistar rats. NK_1 receptor antagonism also attenuated yohimbine-induced reinstatement of alcohol seeking at all doses tested but had no effect on cue-induced reinstatement of alcohol seeking. L822429 reduced operant alcohol self-administration when injected into the lateral cerebroventricles or the medial amygdala. L822429 injected into the medial amygdala also significantly reduced anxiety-like behaviour in the elevated plus maze test. No effects on alcohol intake were observed following injection of L822429 into the dorsal or the ventral hippocampus.

Conclusions and Implications

Our results suggest that NK_1 receptor antagonists may be useful for the treatment of alcohol addiction associated with stress or comorbid anxiety disorders. The medial amygdala appears to be an important brain site of action of NK_1 receptor antagonism.

Abbreviations

AP, antero-posterior; CeA, central amygdala; CRF, corticotropin releasing factor; DH, dorsal hippocampus; DV, dorsal-ventral; EPM, elevated plus maze; ML, medial-lateral; msP rats, Marchigian Sardinian (alcohol) preferring rats; SP, substance P; TO, time out; VH, ventral hippocampus

Tables of Links

TARGETS
GPCRs
NK1 receptor
CRF1 receptor

LIGANDS
SP, substance P
CRF, corticotropin releasing factor
Yohimbine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology. org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Substance P (SP) and its preferred neurokinin NK$_1$ receptor are involved in stress and anxiety-like behaviour (Santarelli *et al.*, 2001; McLean, 2005; Ebner and Singewald, 2006; Ebner *et al.*, 2008). Neuropeptidergic systems involved in stress and anxiety have frequently attracted the interest of the researchers as targets for the pharmacotherapy of alcoholism (Ubaldi *et al.*, 2013). SP and NK$_1$ receptors are highly expressed in areas involved in the modulation of stress, anxiety and mood responses, such as the cingulate cortex, caudate putamen, nucleus accumbens, septum, hippocampus, amygdala, dorsal raphe and locus coeruleus (McLean, 2005; Ebner *et al.*, 2008). Emotional and physical stressors modulate SP tissue levels or SP immunoreactivity in brain areas that are involved in fear and anxiety (Siegel *et al.*, 1987; Nakamura *et al.*, 1990). Blockade of NK$_1$ receptors has consistently been shown to exert anxiolytic and anti-depressant effects (see Mclean, 2005).

The NK$_1$ receptor system may additionally play a role in excessive alcohol drinking and relapse (see Schank *et al.*, 2012; Schank, 2014). For instance, systemic administration of an NK$_1$ receptor antagonist attenuated stress-induced reinstatement of alcohol seeking in non-selected Wistar rats (Schank *et al.*, 2011). At the same doses, the NK$_1$ receptor antagonist did not have effects on self-administration of alcohol under baseline conditions, nor did it affect cue-induced reinstatement of alcohol seeking or novel environment-induced locomotion (Schank *et al.*, 2011). This suggests that NK$_1$ receptors affect alcohol drinking and seeking that are motivated by the need to attenuate stress and anxiety in vulnerable individuals. To investigate this hypothesis, we evaluated the effect of NK$_1$ receptor blockade on alcohol-related behaviours in genetically selected Marchigian Sardinian alcohol preferring (msP) rats.

This strain of msP rats is highly sensitive to stress and stress-induced excessive alcohol seeking (Ciccocioppo *et al.*, 2006), show an anxious phenotype (Hansson *et al.*, 2006), morphological and functional brain alterations, similar to those in depressed patients (Gozzi *et al.*, 2013), and depressive-like symptoms that recover following alcohol consumption (Ciccocioppo *et al.*, 1999). The msP rats also display a high alcohol preference and intake, with an excessive daily alcohol drinking (6–8 g·kg^{-1} body weight) in a binge-type pattern, leading to significant blood alcohol levels (Ciccocioppo, 2013). Altogether, these data suggest that rats of this strain may be useful as a model of a sub-population

of alcoholic patients in whom excessive drinking and relapse vulnerability is associated with co-morbid anxiety and is motivated by negative reinforcement, or 'tension relief' mechanisms.

It is also relevant to note that msP rats have an innate up-regulation of the corticotropin releasing factor CRF$_1$ receptor transcript and receptor density (Hansson *et al.*, 2006). This strain of rats show a reduction in alcohol drinking in response to CRF$_1$ receptor antagonists, at doses that have no effect in non-dependent, non-selected rats (Hansson *et al.*, 2006), whereas CRF$_1$ receptor gene expression is reduced following voluntary drinking in these animals (Hansson *et al.*, 2007). There could be some similarity between the actions of CRF$_1$ receptor antagonists and NK$_1$ receptor antagonists (Schank *et al.*, 2011). For instance, NK$_1$ receptor antagonists can reduce alcohol consumption in conditions of high or escalated intake similar to CRF$_1$ receptor antagonists (Cippitelli *et al.*, 2012). Furthermore, as with CRF$_1$ receptor antagonists, NK$_1$ receptor antagonists attenuated stress-induced reinstatement to alcohol seeking in non-selected Wistar lines, an effect not observed in the cue-induced model (Marinelli et al., 2007, Liu and Weiss, 2002, Schank *et al.*, 2011). It was therefore of great interest to investigate the effect of NK$_1$ receptor antagonism on alcohol drinking in the msP strain of rats.

Based on these findings, we predicted that NK$_1$ receptor blockade, contrary to what has been observed in heterogeneous, non-selected Wistar rats, should reduce drinking in msP rats and that this effect should occur concomitantly with an anxiolytic action. To explore this hypothesis, we used L822429, an NK$_1$ receptor antagonist developed to possess high affinity for the rat NK$_1$ receptor (Ebner *et al.*, 2004; Ebner *et al.*, 2008; Singewald *et al.*, 2008; Schank *et al.*, 2011), and that exhibits anxiolytic properties (Ebner *et al.*, 2004). The effect of L822429 was assessed on alcohol self-administration in msP rats and for comparison in Wistar rats. Moreover, in the msP rats, we evaluated the effect of NK$_1$ receptor blockade on stress and cue-induced reinstatement of alcohol seeking. In the attempt to determine the brain sites of action of the NK$_1$ receptor antagonist on alcohol intake, intracranial administrations of L822429 were also carried out in areas rich in SP fibres and NK1 receptors, which include the hippocampus and medial amygdala (Roberts *et al.*, 1982; Nakaya *et al.*, 1994; Borhegyi and Leranth, 1997; Ribeiro-da-Silva and Hokfelt, 2000; Ogier and Raggenbass, 2003). Finally, to link the reduction of alcohol drinking by the NK$_1$ receptor antagonist to its anxiolytic action, we explored the consequences

of injecting L822429 directly into the medial amygdala, on the results from the elevated plus maze (EPM) test, an established measure of anxiety in rodents.

Methods

Animals

All animal care and experimental procedures followed the EU directive for Care and Use of Laboratory Animals and were approved by the Ethical Committee of the University of Camerino. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2011; McGrath and Lilley, 2015). A total of 59 animals were used in the experiments described here.

Male msP and Wistar rats (Charles River, Calco, Italy) were used for the study. They were housed two per cage on a reverse 12 h light/dark cycle (lights off at 0900) at a constant temperature of $20 \pm 2\,°C$ and relative humidity of 45–55%. Food and water were available *ad libitum*. At the time of drug treatments, their body weights ranged between 450 and 550 g. Animals were handled daily for 1 week prior the start of experimentation.

Preparation of drugs used

Alcohol solution (10% v/v) was prepared by diluting 95% alcohol (F.L. Carsetti s.n.c., Italy) in tap water. The NK_1 receptor antagonist, L822429 [2-cyclopropoxy-5-(5-(trifluoromethyl) tetrazol-1-yl)ben-zyl]-(2-phenylpiperidin-3-yl)amine, was synthesized in the laboratory of Dr K. Rice at the NIDA Intramural Research Program. L822429 was dissolved in 45% w/v 2-hydroxypropyl β-cyclodextrin and the pH adjusted. The compound was injected i.p. in a volume of $1–2\,mL\cdot kg^{-1}$. Yohimbine (Sigma-Aldrich, Milano, Italy) was dissolved in distilled water and injected i.p. in a volume of $1\,mL\cdot kg^{-1}$.

Operant self-administration apparatus and training

Training and testing were conducted in standard operant conditioning chambers housed in sound-attenuating cubicles (Med Associates Inc., USA). Each operant chamber was equipped with a drinking reservoir (volume capacity: 0.3 mL) positioned 4 cm above the grid floor in the centre of the front panel of the chamber and two retractable levers positioned laterally (3 cm) to either side of the drinking receptacle. Auditory and visual stimuli were presented via a speaker located on the front panel and light located on the back panel respectively. A microcomputer controlled the delivery of the fluids, presentation of auditory and visual stimuli and recording of the behavioural data. Each response on the active lever resulted in delivery of 0.1 mL of fluid, while responses on the second lever had no consequences. For 30 min each day, rats were trained to self-administer 10% (v/v) alcohol solution on a fixed ratio 1 schedule of reinforcement with a time-out (TO) of 5 s. The TO period (during which lever responses are recorded but not reinforced) was signalled by illumination of a white house light located at the back panel for 5 s.

Experiments were commenced when a stable baseline of responding was achieved.

Stereotaxic surgery

The msP rats were anaesthetised by i.m. injection of 250–300 μL of a solution (Zoletil®) containing $58.17\,mg\cdot mL$ tiletamine chlorhydrate and $57.5\,mg\cdot ml^{-1}$ zolazepam chlorhydrate (VIRBAC, Carros, France). Atropine sulphate (80 μg in 80 μL; Fattro S.P.A., Ozzano Emilia (BO), Italy) was also administered to the rats. For intracerebroventricular (i.c.v.) injections, a stainless steel guide cannula (7 mm length) aimed at the lateral ventricle was stereotaxically implanted. Taking bregma as reference point, the following coordinates were used: AP = −1.0 mm, ML = 1.8 mm and DV = −2.0 mm. For the dorsal hippocampus (DH), ventral hippocampus (VH) and medial amygdala injections, each animal was stereotaxically implanted with a unilateral guide stainless steel cannula (12 mm length) in each hemisphere over the DH, VH or MeA. Taking bregma as reference point, the following coordinates were used: DH: AP = −4.2 mm, ML = 2.6 mm and DV = −2.9 mm; VH: AP = −4.8 mm, ML = 5.1 mm and DV = −5.0 mm; MeA: AP = −2.7 mm, ML = 3.4 mm and DV= −7.0 mm (Paxinos and Watson, 1988). Injector lengths used for the injection were 1.5 mm longer than the cannula for DH and VH and 2.0 mm longer for MeA. At the end of the surgery, each guide cannula was sealed with a stainless steel wire (0.3 mm in diameter), and rats were given a single i.m. injection (500 μL) of rubrocillin (Intervet, Schering-Plough Animal Health, Segrate (MI), Italy) as a prophylactic antibiotic. Rats were allowed to recover from surgery for 1 week during which they were monitored daily for signs of pain or distress. Correct cannula placement of the ICV guide cannulae was confirmed with angiotensin II (Gosnell *et al.*, 1990; Choi *et al.*, 2003) (Supporting Information).

At completion of the experiment, the animals were killed by CO_2 inhalation and then the brains were quickly removed and snap-frozen at −20°C in isopentane. The brains were then stored at −80°C. The correct placement of the cannulae was further verified by histological examination (Supporting Information). Only behavioural data obtained from rats that had the cannulae correctly placed in both hemispheres of the brain were analysed (N = 7 for DH, N = 9 for VH, N = 8 and N = 14 for MeA, self-administration and EPM experiments, respectively).

Effect of L822429 on alcohol and saccharin self-administration

One group of msP rats (N = 7) and another of non-selected Wistar rats (N = 10) were trained to self-administer 10% (v/v) alcohol solution as described previously. In a within-subject Latin square counterbalanced order, 1 h before the beginning of the session, rats were treated with different doses of L822429 (0, 5, 15 and $30\,mg\cdot kg^{-1}$) i.p. Drug treatment was performed every fourth day. Thus, four rounds of drug treatment were performed, with each rat receiving all four doses of L822429. The first day after drug test, rats remained in their home cage, whereas on days 2 and 3, the baseline alcohol self-administration was re-established. Under identical experimental conditions, the study was replicated in another group of msP rats (N = 7) with saccharin solution (0.2%) as the reinforcer.

Effect of L822429 on yohimbine-induced reinstatement of alcohol seeking in msP rats

A group of msP rats ($N = 9$) were trained to self-administer 10% (v/v) alcohol solution as described previously. The rats were then subjected to 30 min extinction sessions for 16 consecutive days. Sessions were identical to those of the self-administration training, with the exception that lever responses were not associated with alcohol delivery. Following the extinction phase, the rats were treated i.p. with L822429 (0, 5, 15 and 30 mg·kg^{-1}) according to a counterbalanced Latin square (within-subject) design 1 h before the reinstatement session. Yohimbine (1.25 mg·kg^{-1}) was given i.p. to all the rats 30 min after the L822429 administration. A 4-day interval, during which animals were subjected to extinction sessions, was allowed between drug tests (Cippitelli et al., 2008). Thus, four rounds of drug treatment were performed, with each rat receiving all four doses of L822429.

Effect of L822429 on cue-induced reinstatement of alcohol seeking in msP rats

These msP rats ($N = 9$) were trained to discriminate between 10% alcohol and water in 30 min daily sessions. The discriminative stimulus for alcohol was the odour of an orange extract (S$^+$), whereas water availability was signalled by an anise extract (S$^-$). In addition, each lever press resulting in alcohol delivery was paired with illumination of the chamber's house light for 5 s. The corresponding cue during water sessions was a 5 s tone (70 dB). Concurrently with the presentation of these stimuli, a 5 s TO period was in effect during which responses were recorded but not reinforced. Following the conditioning phase, rats were subjected to 30 min extinction sessions for six consecutive days. During this phase, sessions began by extension of the levers without presentation of the discriminative stimuli, associated delivery of liquids or response-contingent cues. After the extinction phase, reinstatement tests began under conditions identical to those during the conditioning phase, except that alcohol and water were not made available. The msP rats were tested under the S$^-$ condition on day 1. On day 2, the rats were treated i.p. with L822429 (0, 5, 15 and 30 mg·kg^{-1}) 1 h before the beginning of the reinstatement test under the S$^+$ condition according to a counterbalanced Latin square (within-subject) design. A 4-day interval, during which animals remained in their home cage, was allowed between drug tests (Ciccocioppo et al., 2002; Cippitelli et al., 2008). Thus, four rounds of drug treatment were performed, with each rat receiving all four doses of L822429.

Effect of L822429 administered intracranially on alcohol self-administration in msP rats

A group of msP rats ($N = 8$) was trained to self-administer 10% (v/v) alcohol solution as described previously. The rats then underwent surgery to implant a cannula into the lateral cerebroventricle and, after recovery, were subjected to alcohol self-administration again. Following acquisition of a stable baseline of alcohol self-administration, L822429 treatment began. In a within-subject Latin square counterbalanced design, rats were treated with L822429 (30 μg in·2 μL per rat) or its vehicle 10 min before the beginning of the session. Drug treatment was performed every third day. The first day

after drug test, rats remained in their home cage, whereas on day 2, the baseline alcohol self-administration was re-established. This experiment was also performed using lower doses (0, 7.5 and 15 μg·μl^{-1} per rat) of the NK$_1$ receptor antagonist (see Supporting Information for results).

In a similar experiment, three groups of msP rats ($N = 7$–9 per group) trained to self-administer 10% (v/v) alcohol were implanted bilaterally with cannulae into the DH, VH and medial amygdala respectively. The doses of L822429 evaluated here were 0, 7.5 and 15 μg per rat (0.3 μL per side). Treatment was carried out in a within-subject Latin square counterbalanced design.

Effect of injection of L822429 into medial amygdala on anxiety-like behaviour in msP rats

To measure anxiety-like responses, the elevated plus maze (EPM) test was used. The maze, elevated 50 cm above the floor, was located in a sound attenuated room illuminated by a red dim light (~30 lx). Two groups of rats ($N = 7$ per group) were injected with L822429 (15 μg per rat; 0.3 μL per side) or its vehicle into the MeA. Ten minutes later, animals were tested in the EPM arena. The 5 min test procedure began when the animals were individually placed in the centre of the maze, facing a closed arm. A rat was considered to be on the central platform when at least two of its paws were on it. An entry was defined as the presence of all four paws in the arms. The percent of time spent in the open arm, the number of open arm entries and the number of total arm entries were recorded by an automated videotracking system (Noldus Ethovision XT 7.0). Data were then analysed by an experimenter unaware of the experimental treatments.

Data analysis

Self-administration, reinstatement and intracranial experiments were evaluated by one-way ANOVA with repeated measures using drug dose as a within-subject factor. Analysis of variance was followed by the Newman–Keuls test when found to be significant. Statistical significance was set at $P < 0.05$. Data obtained from the EPM experiment were analysed using Student's t-test. Statistical significance was set at $P < 0.05$. Statistica version 8 (StatSoft, Inc. Tulsa, OK, USA) was used for statistical analysis of the data.

Results

L822429 reduces alcohol self-administration in msP but not Wistar rats

Rats rapidly acquired stable alcohol self-administration. As shown in Figure 1A, treatment with L822429 significantly reduced operant responding for alcohol in msP rats [$F_{(3,6)} = 24.5$; $P < 0.001$]. Post hoc analysis revealed a significant reduction of alcohol self-administration at the doses of 5 mg·kg^{-1} ($P < 0.01$), 15 mg·kg^{-1} ($P < 0.01$) and 30 mg·kg^{-1} ($P < 0.001$), demonstrating a dose-dependent effect. There was no significant effect of L822429 treatment on alcohol self-administration in Wistar rats (Figure 1B). ANOVA did not show any significant main effect of treatment [$F_{(3,9)}=1.34$, NS]. In msP rats, L822429
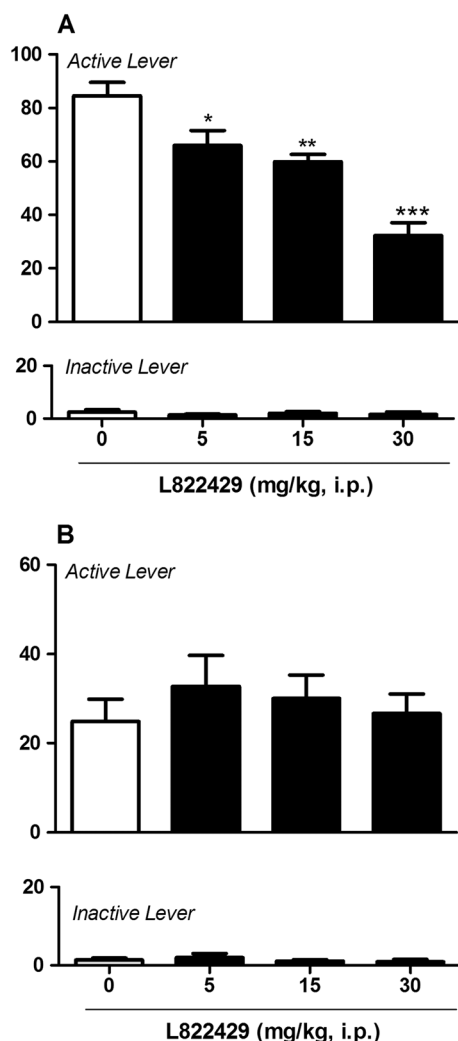
Figure 1

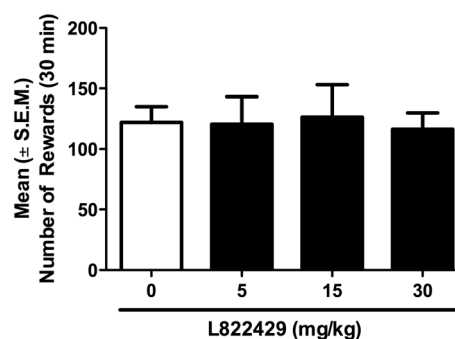Effect of L822429 (0, 5, 15 and 30 mg·kg^{-1}) on alcohol self-administration in msP (A) and Wistar (B) rats under fixed ratio 1 schedule of reinforcement. Panel A shows that alcohol self-administration is dose-dependently reduced by L822429 in msP rats ($N = 7$). Panel B shows that there is no significant effect of L822429 treatment on alcohol self-administration in Wistar rats ($N = 10$). Data are the mean (±SEM) number of rewards in 30 min. L822429 was given i.p. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, significant differences from vehicle-treated (0 mg·kg^{-1}) rats.

did not significantly modify operant responding for saccharin [[F$_{(3,6)}$=0.07, NS] (Figure 2)].

In the experiments, responses at the inactive lever were extremely low and were not affected by L822429 treatment (data not shown).

L822429 attenuates yohimbine-induced reinstatement of alcohol seeking in msP rats

After a stable baseline of responding to 10% alcohol was established during the self-administration phase (mean ± SEM of the last 3 days: 52 ± 4.6), extinction was initiated and the responses to alcohol progressively decreased. As

Figure 2

Effect of L822429 (5, 10, 15 and 30 mg·kg^{-1}) on operant saccharin self-administration in msP rats under fixed ratio 1 schedule of reinforcement. Data are the mean ±SEM of total number of rewards earned in 30 min. L822429 was given i.p. No significant differences were found between vehicle-treated (0 mg·kg^{-1}) and drug treated groups ($N = 7$).

shown in Figure 3A, administration of yohimbine (1.25 mg·kg^{-1}) significantly reinstated the operant responding for alcohol [F$_{(1,8)}$ = 9.75, $P < 0.05$]. ANOVA showed that pre-treatment with L822429 significantly reduced the effect of yohimbine [F$_{(3,8)}$ = 4.25, $P < 0.05$]. *Post hoc* analysis revealed a significant dose-related inhibition of reinstatement following administration of the NK$_1$ receptor antagonist (5, 15 and 30 mg·kg^{-1}). Statistical analysis of the inactive lever responses showed that there was no effect of yohimbine treatment, indicating its selectivity to elicit reinstatement of alcohol seeking (data not shown).

L822429 does not affect cue-induced reinstatement of alcohol seeking in msP rats

ANOVA showed a significant overall effect of conditioning [F$_{(1,16)}$= 27.82, $P < 0.001$] during the discrimination phase. Animals responded at a significantly higher level for alcohol (mean ± SEM of the last 3 days for alcohol: 53 ± 4.5) than for water (mean ± SEM of the last 3 days for water: 14 ± 2.1). During extinction, lever pressing progressively decreased. In the reinstatement test, ANOVA showed that cues had a significant overall effect on alcohol seeking [F$_{(2,8)}$ = 11.46; $P < 0.001$]. A more detailed analysis showed a robust reinstatement of responding under the S$^+$ ($P < 0.01$) but not under the S$^-$ ($P > 0.05$, NS) compared with the last day of extinction. As shown in Figure 3B, pre-treatment with L822429 did not affect conditioned reinstatement of alcohol seeking [F$_{(3,8)}$ = 16.59; NS]. Responses at the inactive lever were not influenced by the treatment.

Intracerebroventricular administration of L822429 reduces alcohol self-administration in msP rats

ANOVA revealed a significant effect of the treatment [F$_{(1,7)}$ = 24.0, $P \leq 0.01$] on alcohol self-administration. As shown in Figure 4, the i.c.v administration of L822429 at the dose of 30 μg per rat significantly reduced alcohol self-administration in the msP rats. Inactive lever responses were not influenced by the L822429
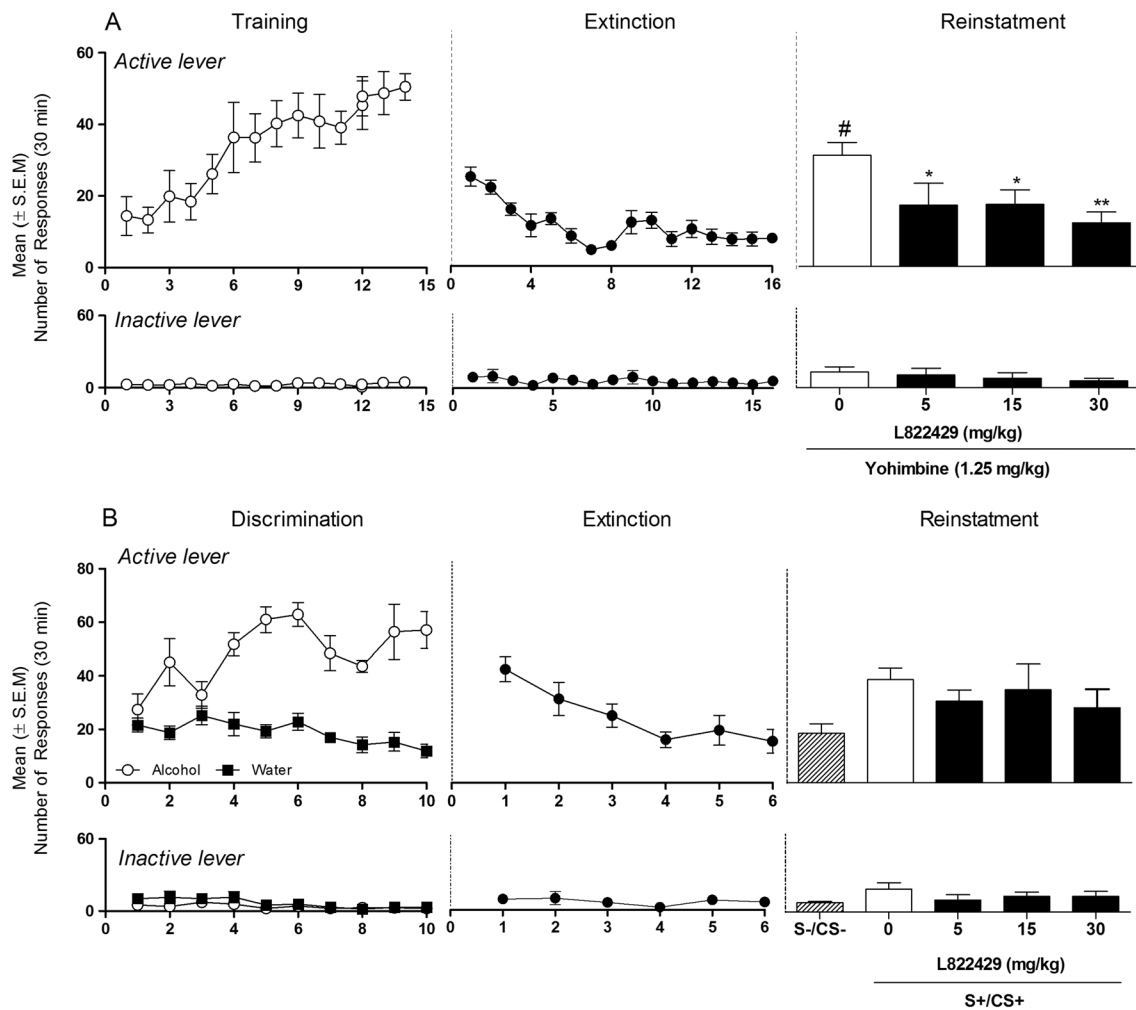
Figure 3

Effect of L822429 (0, 5, 15 and 30 mg·kg^{-1}) on (A) yohimbine-induced reinstatement of alcohol seeking and (B) cue-induced reinstatement of alcohol seeking. S−, S+ refer to the discriminative stimuli indicating water and alcohol availability respectively. Data are the mean (±SEM) number of responses on the active lever in 30 min ($N = 9$ for each experiment). L822429 was administered via the intraperitoneal route. **$P < 0.01$; *$P < 0.05$, significant differences from vehicle-treated group (0 mg·kg^{-1}). # $P < 0.01$, significant difference from extinction. Data from the last day of extinction with water-paired cues are shown as S-/CS-; the alcohol-paired cues are shown as S+/CS+.
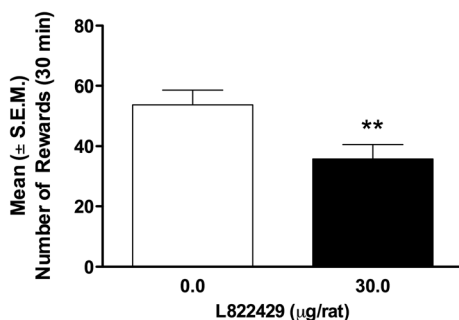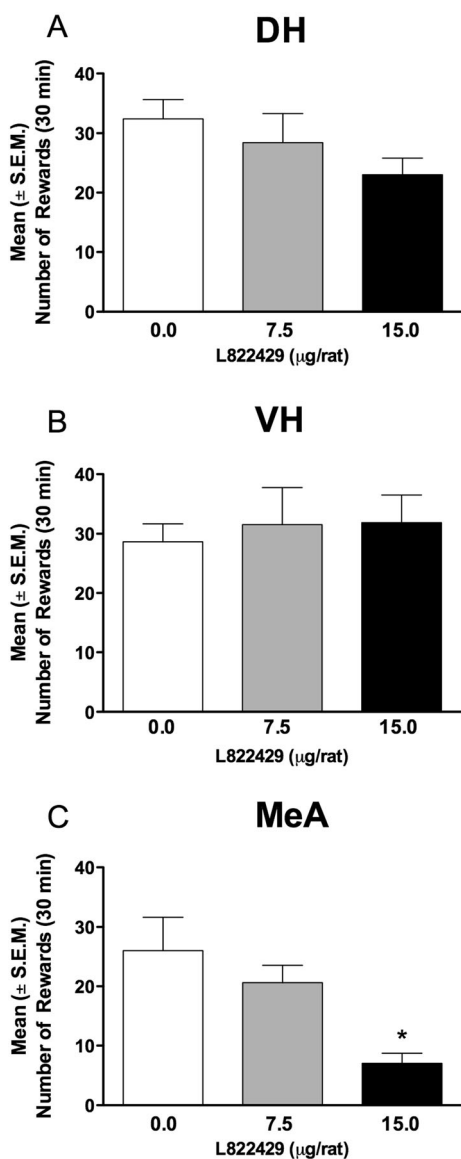

Figure 4

Effect of i.c.v. injection of L822429 (0.0, 30.0 μg per rat) on alcohol self-administration in msP rats under fixed ratio 1 schedule of reinforcement. Data are the mean ± SEM of number of rewards earned in 30 min ($N = 8$). **$P < 0.01$, significant difference from vehicle-treated group (0 μg per rat).

treatment. No appreciable effect of L822429 was found at the lower doses of 7.5 and 15 μg per rat (Figure S2).

L822429 attenuates alcohol self-administration in msP rats when administered into the medial amygdala but not the dorsal hippocampus or ventral hippocampus

ANOVA showed that the administration of L822429 into the dorsal hippocampus (Figure 5A) did not significantly modify alcohol self-administration [$F_{(2,6)} = 2.23$, NS]. As shown in Figure 5B, there was also no significant effect of L822429 administration into the ventral hippocampus [$F_{(2,8)} = 0.26$, NS]. However, there was a significant reduction of alcohol self-administration when the drug was injected into the medial amygdala [$F_{(2,7)} = 6.3$, $P < 0.05$]. As shown in Figure 5C,

A   **DH**

B   **VH**

C   **MeA**

Figure 5

Effect of L822429 injection (0, 7.5 and 15 μg per rat) into the dorsal hippocampus (A), ventral hippocampus (B) and medial amygdala (C) on alcohol self-administration in msP rats under fixed ratio 1 schedule of reinforcement. Data are the mean ± SEM of total number of rewards earned in 30 min ($N = 7–9$ per group). *$P < 0.05$, significant difference from vehicle-treated group (0 mg·kg^{-1}).

post hoc analysis revealed that alcohol self-administration was significantly reduced at the dose of 15 μg per rat.

Micro-injection of L822429 into the medial amygdala attenuates anxiety-like behaviour of msP rats in elevated plus maze test

As shown in Figure 6, L822429 treatment (15 μg per rat) significantly increased the percentage of time spent in the open arms [$t(12) = 5.69$, $P < 0.0001$] and the percentage of open arm entries [$t(12) = 4.97$, $P < 0.0001$]. Conversely, drug

A

B

C
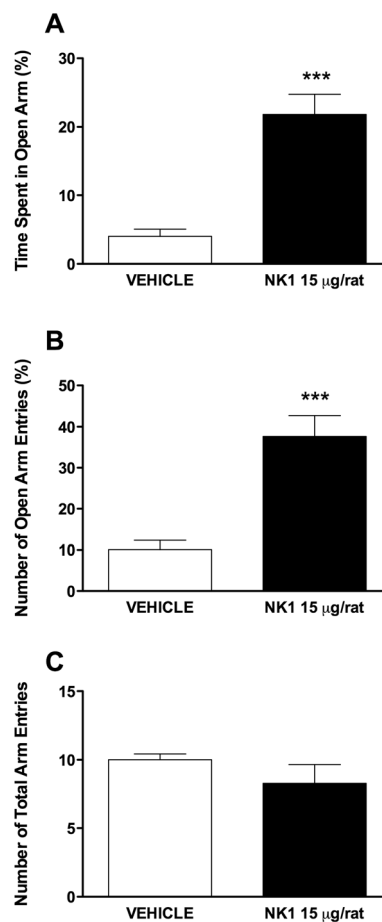
Figure 6

Effect of L822429 injection (0, 15 μg per rat) into the medial amygdala on the time spent on the open arms (A), the percentage of entries into the open arms (B) and the total number of entries (C) in the elevated plus maze test. Data are expressed as the mean ±SEM ($N = 7$ per group). ***$P < 0.001$, significant difference from vehicle-treated group.

exposure reduced the total number of closed arm entries [$t(12) = 4.97$, $P < 0.0001$]. Total arm entries were not affected by treatment [$t(12) = 1.19$, $P > 0.05$].

Discussion

In agreement with previous reports (Schank *et al.*, 2011; Schank *et al.*, 2013), we found that the NK$_1$ receptor antagonist L822429 did not modify basal alcohol self-administration in non-selected Wistar rats. In contrast, in genetically selected msP rats, L822429 did attenuate alcohol self-administration at doses that did not modify saccharin-reinforced operant responding. This indicates that the action of the NK$_1$ receptor antagonist is selective for alcohol in msP rats, consistent with previous findings with another selected line, the alcohol preferring (P) rat (Schank *et al.*, 2013). Furthermore, and also in agreement with previous work (Schank *et al.*, 2011; Schank *et al.*, 2014), NK$_1$ receptor blockade suppressed reinstatement of alcohol seeking elicited by the pharmacological stressor yohimbine, but not by cues previously

associated with alcohol availability. We also confirmed that L82242 exerted its effects via central mechanisms and that the medial amygdala is an important site of action for the NK$_1$ receptor antagonist on alcohol intake in msP rats. Finally, we demonstrated that the injection of L822429 into the medial amygdala of msP rats decreased anxiety-like behaviour in the EPM test under baseline conditions, suggesting that the reduction of alcohol intake elicited by the NK$_1$ receptor antagonist may be driven by inhibition of innate anxiety and negative emotionality in these rats.

The NK$_1$ receptor mediates anxiety and stress (McLean, 2005; Ebner and Singewald, 2006), and it is known that these negative emotional states promote the compulsivity of alcohol drinking (Koob and Le Moal, 2006). Also, NK$_1$ receptor antagonists are effective in the reduction of anxiety, depression and stress-related states (Ebner and Singewald, 2006). Our findings therefore suggest that the likely mechanism by which the NK$_1$ receptor antagonist was able to reduce alcohol drinking was by attenuating these negative emotional states that drive drinking behaviour in vulnerable individuals. This may explain why NK$_1$ receptor antagonists reduced alcohol self-administration in the msP rats, which are postulated to drink to alleviate an innate high anxiety state, but not in their Wistar counterparts. This is in agreement with the study by Schank et al. (2011) that showed no effects of the NK$_1$ receptor antagonist on alcohol drinking in non-selected Wistar rats (Schank et al., 2011). To our knowledge in fact, only one study has reported inhibition of alcohol drinking in heterogeneous rats following NK$_1$ receptor antagonism, but the effect was observed at doses that also suppressed sucrose consumption (Steensland et al., 2010). This indicates that high doses of a NK$_1$ receptor antagonist could elicit a general inhibitory effect on appetitive behaviour.

Interestingly, inhibition of the NK$_1$ receptor by pharmacological antagonism or genetic deletion reduced alcohol intake in mice of the C57/BL6 strain (George et al., 2008; Thorsell et al., 2010), which have relatively high alcohol preference (Belknap et al., 1993). This would be compatible with our observations in alcohol preferring rat strains and suggests that NK$_1$ receptor antagonists can reduce alcohol consumption in conditions of high or escalated intake similar to CRF$_1$ receptor antagonists (Cippitelli et al., 2012).

The NK$_1$ receptor antagonist attenuated stress-induced reinstatement to alcohol seeking in this study. The stressor employed was yohimbine, an α_2-adrenoceptor antagonist that increases noradrenergic cell firing (Aghajanian and VanderMaelen, 1982) and enhances noradrenaline release in terminal areas (Abercrombie et al., 1988; Pacak et al., 1992). Yohimbine induces anxiety-like responses in both humans (Holmberg and Gershon, 1961; Bremner et al., 1996b) and laboratory animals (Bremner et al., 1996a) and triggers craving in alcohol-dependent patients (Umhau et al., 2011). The ability of the NK$_1$ receptor antagonist to attenuate stress-induced reinstatement to alcohol seeking suggests the possible involvement of the SP/NK$_1$ receptor system in stress-induced craving in alcohol dependence. Indeed, a recent clinical trial using an NK$_1$ receptor antagonist found beneficial effects on baseline and stress-elicited alcohol craving (George et al., 2008). The ability of the NK$_1$ receptor antagonist to attenuate stress-induced reinstatement to alcohol seeking and not affect cue-induced reinstatement to

alcohol seeking parallels the study of Schank and colleagues, where similar effects were observed in non-selected Wistar rats (Schank et al., 2011). This further supports the notion that stress may be a necessary precondition for NK$_1$ receptor antagonists to elicit their alcohol-seeking suppressing effects. These findings also underscore another important similarity between the antagonists of SP/NK$_1$ receptor and CRF/CRF$_1$ receptor systems in the modulation of alcohol intake. CRF$_1$ receptor antagonists suppress stress-induced reinstatement to alcohol seeking in msP lines (Hansson et al., 2006) and non-selected Wistar lines (Marinelli et al., 2007) while being ineffective on cue-reinstatement models (Liu and Weiss, 2002). It is tempting to speculate that both the SP/NK$_1$ receptor and the CRF/CRF$_1$ receptor systems might share common neurobiological pathways. For instance, like CRF, SP and the NK$_1$ receptor might be involved in the neuroadaptations induced by history of alcohol dependence or escalated intake. To support this hypothesis, NK$_1$ receptor knock-out mice failed to escalate their alcohol consumption after repeated cycles of deprivation (Thorsell et al., 2010), suggesting that the SP/NK$_1$ receptor system may mediate neuroadaptive changes that contribute to the escalation of alcohol intake, a hallmark of addiction.

The ability of the NK$_1$ receptor antagonist to reduce alcohol drinking in the up-regulated CRF state of msP rats suggests that there could be an interaction between the actions of SP and CRF in the brain. In a study that investigated the differential gene expression profile induced by SP in human astronoma and rat astroglial cells using microarray techniques, SP was shown to induce the expression of CRF$_1$ receptors, an effect that was inhibited by a selective NK$_1$ receptor antagonist (Hamke et al., 2006). In addition, SP has also been shown to increase CRF$_1$ receptor mRNA and induce the expression of the CRF$_1$ receptor protein in human mast cells (Asadi et al., 2012). This indicates that SP could induce CRF$_1$ receptor expression in brain regions that mediate anxiety and stress-induced alcohol intake. Taken together, these data suggest that a cross-talk may exist between the two neuropeptide systems in the modulation of stress-induced drinking in msP rats. However, there is a possibility that NK$_1$ receptor and CRF$_1$ receptor systems use parallel pathways to mediate stress-induced behaviours, or that these systems converge on a common downstream target. Future research should focus on exploring these possibilities.

The hippocampus is a brain structure involved in many functional processes such as learning and memory as well as regulation of emotions (Eichenbaum, 2000; Engin and Treit, 2007) and has a high density of SP-containing fibres, which derive from intrinsic and extrinsic origins (Borhegyi and Leranth, 1997). In the hippocampus, SP effects are mediated by NK$_1$ receptors (Ogier and Raggenbass, 2003) even though other neurokinin receptor subtypes are present in this brain structure (Maeno et al., 1993; Quartara and Maggi, 1998). Thus, we were interested in studying the effect of NK$_1$ receptor antagonism in this brain area on alcohol intake. Our results showed that neither NK$_1$ receptor antagonism in the dorsal nor the ventral hippocampus influenced alcohol intake in msP rats. The hippocampus is known to play a role in drug reinforcement, craving and cue-induced relapse (Volkow, 2004; Martin-Fardon et al., 2008; Koob and Volkow, 2010b), whereas not much is known about its role in the

modulation of negative states associated with addiction. Based on present findings, we would speculate that the NK_1 receptor system located in this brain structure was not involved in the modulation of drinking behaviour, nor did it contribute to the regulation of negative affect associated with the innate high alcohol vulnerability of msP rats.

On the other hand, a suppression of alcohol intake was observed when the drug was injected into the MeA. Notably, the effect in the medial amygdala was obtained at a dose of L822429, which was ineffective following i.c.v. administration. This provides strong evidence for a specific role of this area in mediating the effect of NK_1 receptor antagonists on alcohol drinking. The amygdala is a critical brain structure for the processing of emotions including fear and anxiety (Aggleton, 1993; LeDoux, 2003) and has been shown to play an important role in negative reinforcement that drives compulsive drug use (Koob and Zorrilla, 2010a). SP-containing neurons as well as NK_1 receptors are highly expressed in this area (Roberts *et al.*, 1982; Nakaya *et al.*, 1994; Ribeiro-da-Silva and Hokfelt, 2000). The medial amygdala is reported to contain the highest amounts of SP within the amygdala (Roberts *et al.*, 1982) and immunohistochemical studies have demonstrated a dense plexus of SP-containing cell bodies and terminals in this area (Ribeiro-da-Silva and Hokfelt, 2000; Cippitelli *et al.*, 2012). One study showed that within the amygdala, the medial amygdala is particularly activated by emotional stress more strongly than the central amygdala, as indicated by c-fos expression (Dayas *et al.*, 1999). Another study demonstrated an immobilization stress-induced increase of SP release in the medial amygdala but not in the central amygdala (Ebner *et al.*, 2004). Thus, we considered it relevant to investigate the effect of NK_1 receptor antagonism in this brain area on anxiety in msP rats.

Results from the EPM confirmed that the administration of L822429 suppressed anxiety-like responses when microinjected into the medial amygdala. This agrees with the study of Ebner *et al* (2004), which showed that injections of the same NK_1 receptor antagonist into the medial amygdala blocked anxiogenic-like effects in previously stressed (immobilization stress) rats, as tested with the EPM (Ebner *et al.*, 2004). Additionally, Ebner and colleagues found that the NK_1 receptor antagonist did not elicit an anxiolytic effect in unstressed rats but reversed the anxiogenic effect of SP microinjected into the medial amygdala (Ebner *et al.*, 2004). Extrapolating these findings to our study, it could be that the msP rats have an innately overfunctioning SP system in the medial amygdala, which may contribute to the typical negative affective state of these animals, in turn facilitating their excessive alcohol consumption. Additionally, because it has also been shown that NK_1 receptor activation by SP increases CRF_1 receptor expression, it is tempting to speculate that up-regulation of the extrahypothalamic corticotropin system described in msP rats may result (Hansson *et al.*, 2006; Hansson *et al.*, 2007), at least in part, from over-activity of the neurokinin system. Together, these data indicate an important role for the SP/NK_1 receptor system in the medial amygdala for the modulation of anxiety and stress-induced excessive alcohol drinking and seeking. However, recent studies in P alcohol preferring rats have identified the central amygdala as the major site of NK_1 receptor antagonist activity on excessive alcohol intake in that particular strain (Schank

et al., 2013). The underlying mechanism of this divergence is not clear, but it is important to note that these two alcohol preferring lines were independently generated through genetic selection under somewhat different selection conditions.

In conclusion, the findings in this study demonstrate that the SP/NK_1 receptor system plays an important role in excessive alcohol intake mediated by stress. Like CRF_1 receptor antagonists, NK_1 receptor antagonists reduce alcohol drinking in a similar fashion and could represent a viable pharmacological target for the treatment of alcoholism associated with stress or co-morbid anxiety disorders. The medial amygdala is a probable site of action for the effect of the NK_1 receptor antagonist on alcohol intake in genetically selected msP rats.

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Author contributions

R. C., J. S. and M. H. planned and coordinated the study. L. A., S. S., M. U., A. C., C. N. and R. D. performed experimental assays. K. C. and K. R. synthesized L822429 and provided critical comments.

Conflicts of interest

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the on-line version of this article at the publisher's web-site:

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Figure S1 Effect of intracerebroventricular injection of L822429 (0.0, 7.5, 15.0 μg/rat) on alcohol self-administration in msP rats under FR-1 schedule of reinforcement. Data are the mean ± SEM of total number of rewards earned in 30 min (N = 8). L822429 did not reduce the number of rewards at these doses. No significant differences were found between the treatment groups. Vehicle-treated group: white bar; drug-treated groups: black bars.

Figure S2 Microinjections into the Medial Amygdala were performed within the highlighted area. Taking bregma as reference point, the following coordinates were used in accordance with the atlas of Paxinos and Watson, 1998: Antero-Posterior = 2.7 mm; Medio-Lateral = 3.4 mm; Dorso-Ventral = 9.0 mm.