

# Dissociation of $\mu$ -opioid receptor and CRF-R1 antagonist effects on escalated ethanol consumption and mPFC serotonin in C57BL/6J mice

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## ABSTRACT

Both the opioid antagonist naltrexone and corticotropin-releasing factor type-1 receptor (CRF-R1) antagonists have been investigated for the treatment of alcoholism. The current study examines the combination of naltrexone and CP154526 to reduce intermittent access ethanol drinking [intermittent access to alcohol (IAA)] in C57BL/6J male mice, and if these compounds reduce drinking via serotonergic mechanisms in the dorsal raphe nucleus (DRN). Systemic injections and chronic intracerebroventricular infusions of naltrexone, CP154526 or CP376395 transiently decreased IAA drinking. Immunohistochemistry revealed CRF-R1 or  $\mu$ -opioid receptor immunoreactivity was co-localized in tryptophan hydroxylase (TPH)-immunoreactive neurons as well as non-TPH neurons in the DRN. Mice with a history of IAA or continuous access to alcohol were microinjected with artificial cerebral spinal fluid, naltrexone, CP154526 or the combination into the DRN or the median raphe nucleus (MRN). Either intra-DRN naltrexone or CP154526 reduced IAA in the initial 2 hours of fluid access, but the combination did not additively suppress IAA, suggesting a common mechanism via which these two compounds affect intermittent drinking. These alcohol-reducing effects were localized to the DRN of IAA drinkers, as intra-MRN injections only significantly suppressed water drinking, and continuous access drinkers were not affected by CRF-R1 antagonism. Extracellular serotonin was measured in the medial prefrontal cortex (mPFC) using *in vivo* microdialysis after intra-DRN microinjections in another group of mice. Intra-DRN CP154526 increased serotonin impulse flow to the mPFC while naltrexone did not. This suggests the mPFC may not be an essential location to intermittent drinking, as evidenced by different effects on serotonin signaling to the forebrain yet similar behavioral findings.

**Keywords** CRF-R1, dorsal raphe nucleus, intermittent alcohol, naltrexone.

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## INTRODUCTION

The neural adaptations during the transition to alcohol dependence remain to be fully characterized, since they may reveal targets for therapeutic intervention. One model of heavy drinking in animals is based on intermittent access to alcohol (IAA), which engenders high-level binge drinking, as observed in alcohol use disorders. IAA generates high levels of voluntary and preferential ethanol drinking up to 9 g/kg/day in rats and greater than 20 g/kg/day in C57BL/6J mice

which eventually results in convulsive withdrawal symptoms (Wise 1973; Simms *et al.* 2008; Hwa *et al.* 2011).

Several pharmacotherapies have been explored to reduce IAA drinking. One United States Food and Drug Administration (FDA)-approved medication for alcoholism, the opioid antagonist naltrexone, decreased 20 percent ethanol drinking both during intermittent and continuous access schedules (Simms *et al.* 2008; Sabino *et al.* 2013). Naltrexone can be contrasted with other compounds that target the negatively reinforcing effects

of alcohol withdrawal and stress-induced relapse-like corticotropin-releasing factor type-1 receptor (CRF-R1) antagonists (Heilig & Koob 2007). Systemic injections of CRF-R1 antagonists like antalarmin or R121919 were not effective in reducing IAA in rats (Cippitelli *et al.* 2012; Sabino *et al.* 2013) but other compounds like CP376395 decreased IAA (Simms *et al.* 2013). Microinjection of CRF-R1 antagonist CP154526 into the dorsal raphe nucleus (DRN) selectively reduced high but not low ethanol drinking (Hwa, DeBold & Miczek 2013), which suggests some site specificity for CRF-R1 in heavy drinkers.

The DRN contains most serotonin (5-HT) neurons that project to the forebrain (Imai, Steindler & Kitai 1986) and is a target for neuropeptides such as opioid peptides and CRF. Opioids, like morphine, have excitatory effects on DRN 5-HT neurotransmission indirectly through GABAergic inhibition on 5-HT neurons (Staub *et al.* 2012). At low to moderate doses, CRF inhibits 5-HT neuronal activity in the DRN and neuronal release via CRF-R1 on GABA afferents while higher doses stimulate DRN 5-HT neuronal activity (Kirby, Rice & Valentino 2000; Lowry *et al.* 2000). These studies imply that opioid and CRF receptors may differentially modulate 5-HT signaling in the DRN. 5-HT has been long implicated in regulating ethanol intake, abuse and dependence (Rolf, Matz & Brune 1978), so the anti-alcohol effects of neuropeptide antagonists may be caused by modulating 5-HT neurons in the DRN that project to the forebrain. Alcoholics have substantially reduced 5-HT neurotransmission in the prefrontal cortex (PFC; Mantere *et al.* 2002).

Altogether, this behavioral and neurochemical evidence suggests the hypothesis that both opioid receptors and CRF-R1 may play a role in excessive drinking, possibly through 5-HTergic mechanisms in the DRN. The current studies investigated whether naltrexone and CRF-R1 antagonist CP154526 act dependently or independently in the DRN to reduce IAA drinking in C57BL/6J mice. Co-infusion of naltrexone and CP154526 into the DRN may or may not produce an additive suppression of IAA drinking, revealing common or distinct neural pathways. We also use *in vivo* microdialysis in a terminal region for the 5-HT DRN system, the PFC, to measure extracellular 5-HT. It is hypothesized that CP154526 may more potently disinhibit 5-HT neurons than naltrexone, increasing 5-HT in the PFC, as its efficacy may be more selective to high-level drinkers rather than moderate-level drinkers. In this, we test if behavioral reductions in alcohol drinking are akin to the effects on 5-HT impulse flow, confirming or refuting another hypothesis that 5-HT transmission to the medial prefrontal cortex (mPFC) is crucial for IAA drinking.

## METHODS

### Animals

Eight-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed in groups of six or more upon arrival. Mice acclimated to a 12-hour reversed light/dark cycle with constant temperature ( $21 \pm 2^\circ\text{C}$ ) and humidity (25 percent). After 5 days of group housing, mice were singly housed in polycarbonate cages ( $28 \times 17 \times 12$  cm) with stainless steel wire mesh lids and pine shavings as bedding. Water and standard food (LabDiet 5001 Rodent Diet, St. Louis, MO, USA) were available *ad libitum*. The Tufts University Institutional Animal Care and Use Committee approved all procedures, which followed the guidelines set by the NIH Guide for Care and Use of Laboratory Animals (2011).

### Ethanol drinking procedures

Mice were given intermittent access to 20 percent ethanol and water, which has been previously described in detail (Hwa *et al.* 2011). Briefly, 20 percent ethanol solution (w/v) was prepared from 95 percent ethanol (Pharmaco-AAPER, Brookfield, CT, USA) in one bottle accompanied by a bottle of water on Mondays, Wednesdays and Fridays for 24 hours. Two bottles of water were presented to mice on the remaining days of the week. In experiment 4, a group of mice was given continuous access to 20 percent ethanol and water for 12 consecutive days, the equivalent number of drinking days to 4 weeks in the IAA schedule. A total of 2, 4 and 24 hours of fluid intakes were measured by assessing bottle weights before and after drug manipulations. In order to quantify water and ethanol lost from bottles not due to drinking, full bottles were weighed, then placed on a cage without a mouse, and weighed again at the end of 24 hours. This was done daily, and the average volume lost to 'drip' was subtracted from individual values.

Blood ethanol concentrations (BECs) were measured in a subset of animals given intermittent access ( $n = 11$ ) and continuous access ( $n = 12$ ) to alcohol for 2 hours. Blood was collected from the submandibular vein and centrifuged for 10 minutes at 1000 G. Plasma was extracted and BECs were analyzed with the AM1 Analox Analyzer (Analox Instruments Inc., Lunenburg, MA, USA).

### Experiment 1: systemic injections of naltrexone and CRF-R1 antagonists

After 4 weeks of IAA, mice were assessed for ethanol drinking behavior after systemic drug manipulations. Repeated doses of 0.9 percent saline and 1 and 10 mg/kg naltrexone were administered *i.p.* 20 minutes before ethanol and water bottles were given to mice ( $n = 12$ ). In another group of mice ( $n = 12$ ), repeated doses of 0.6

percent methylcellulose and 10, 17 and 30 mg/kg CP154526 were administered i.p. 20 minutes before two-bottle choice. In order to test another highly potent, more water-soluble CRF-R1 antagonist, a third group of mice ( $n = 12$ ) was given 0.9 percent saline and 10, 17 and 30 mg/kg CP376395 20 minutes before IAA. Injection volumes for systemic drugs were 10 ml/kg. Drug doses were chosen to preserve logarithmically scaled dosing and counterbalanced across four two-bottle choice test sessions. Ethanol intake in grams per kilogram of bodyweight (g/kg) ethanol intake in milliliters (ml) and water intake (ml) were measured at 2, 4 and 24 hours after drug injections.

### Experiment 2: chronic intracerebroventricular (icv) infusion of naltrexone and CRF-R1 antagonists

Separate groups of mice ( $n = 6-8$  per group) were implanted with osmotic minipumps for chronic icv drug infusion after 4 weeks of IAA. Mice were anesthetized with a 100 mg/kg ketamine and 10 mg/kg xylazine combination i.p. before surgery and given carprofen (5 mg/kg) subcutaneously (s.c.) for analgesia. Osmotic minipumps (Model 1002, Alzet, Cupertino, CA, USA) connected through a 20-mm-long polyvinylchloride catheter to a 30-gauge cannula (Brain Infusion Kit 3, Alzet). Cannulae were stereotactically implanted targeting the lateral ventricle at coordinates AP +0.5 mm, ML -0.6 mm from bregma, DV -3.0 mm from dura. Minipumps rested s.c. over the left shoulder blade. Animals recovered for 2 days before tests for ethanol drinking were resumed on day 3.

Groups of mice were given minipumps containing artificial cerebral spinal fluid (aCSF), 3  $\mu$ g/ $\mu$ l naltrexone, 30  $\mu$ g/ $\mu$ l naltrexone or 0.3  $\mu$ g/ $\mu$ l CP376395. Other mice were given 4 percent dimethyl sulfoxide (DMSO) or 0.3  $\mu$ g/ $\mu$ l CP154526. CP154526 did not stay in aCSF for longer than 1 hour at 37°C (personal observations), so we chose to apply an additional water-soluble CRF-R1 antagonist, CP376395, for the 14-day chronic infusion. All minipumps had flow rates of 0.25  $\mu$ l/hour. Two-bottle choice drinking behaviors were assessed on minipump infusion days 3, 5, 7, 10 and 12, which corresponded to a normal schedule of IAA. A total of 2, 4 and 24 hours of ethanol (g/kg) and water intake (ml) were monitored over the 14-day duration of the minipump.

To verify that naltrexone was chronically administered across the 14-day pump duration, the animals that received naltrexone or aCSF in the minipump were tested in a morphine-sensitive tail withdrawal procedure (adapted from Miczek & Winslow, 1987). After ethanol was removed on minipump day 13, mice were gently restrained so that the distal end of the tail could be submerged in a 54°C water bath. Latency to

withdraw the tail (seconds) was measured after 0 (saline baseline), 10, 20, 30 and 40 mg/kg i.p. morphine administered through cumulative dosing every 20 minutes. If the tail withdrawal did not occur within a 20-second cut-off, the test was discontinued, and the mice were not tested with further doses of morphine.

After the drinking tests and/or the tail withdrawal procedure, mice were given an overdose of 100 mg/kg ketamine and 10 mg/kg xylazine combination and perfused with saline and 4 percent paraformaldehyde. Brains were removed and sliced using a Leica Cryostat (CM1900, Bannockburn, IL, USA). Cannula placement was verified using Nissl staining. Animals with incorrect placements ( $n = 2$ ) into the lateral ventricle were excluded from analysis.

### Experiment 3: immunohistological identification of CRF-R1 or $\mu$ -opioid receptor (MOR) on DRN 5-HT cells

C57BL/6J mice with a history of 4 weeks of intermittent alcohol drinking ( $n = 4$ ) or 4 weeks water ( $n = 4$ ) were transcardially perfused with 0.1 M phosphate buffer solution (PBS) and with 4 percent paraformaldehyde in PBS. Brains were removed and post-fixed overnight at 4°C. They were then placed in a sucrose solution with 0.1 percent sodium azide and shipped to the Children's Hospital of Philadelphia for immunohistochemistry. Coronal serial sections (14  $\mu$ m thick) were cut onto slides (Fisherbrand ProbeOn Plus, Fisher Scientific). Slides were placed in 4 percent paraformaldehyde for 1 hour followed by rinses. Sections were incubated with a cocktail of goat anti-CRF1 (C-20, 1:500, Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit anti-MOR (1:1000, Millipore, Billerica, MA, USA) and mouse anti-tryptophan hydroxylase (TPH, 1:500, Abcam, Cambridge, MA, USA) for 72 hours at 4°C. Sections were then rinsed and incubated with secondary antibodies for 90 minutes at room temperature followed by rinses in phosphate buffer. For the CRF1/TPH labeling, AlexaFluor 647-conjugated donkey anti-mouse IgG (1:200, Life Technologies, Carlsbad, CA, USA) was used to visualize TPH immunoreactivity and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch, West Grove, PA, USA) was used to visualize CRF1 immunoreactivity. For MOR/TPH labeling, FITC-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch) was used to visualize TPH immunoreactivity and rhodamine isothiocyanate (RITC)-conjugated donkey anti-rabbit (1:200, Jackson ImmunoResearch) was used to visualize MOR. Finally, sections were coverslipped and visualized using a Leica DM5000B. Images were captured using a Hamamatsu ORCA-ER digital camera (Hamamatsu, Middlesex, NJ, USA) and Leica Microsystems software.

#### Experiment 4: intra-DRN microinjections of naltrexone and CRF-R1 antagonist

Mice with a history of 4-week intermittent ethanol drinking were prepared with intra-DRN cannulae for microinjections of naltrexone and CP154526. In similar surgical methods as experiment 2, mice were implanted with 26-gauge, 6-mm cannulae (Plastics One, Roanoke, VA, USA) targeting the DRN at the coordinates AP  $-4.2$  mm, ML  $+1.5$  mm from bregma, DV  $-1.9$  mm from dura with  $26^\circ$  angle. To investigate if changes in drinking behavior were site specific to the DRN, another group of mice was implanted with cannulae targeting the median raphe nucleus (MRN) at AP  $-4.2$  mm, ML  $+1.2$  mm, DV  $-3.0$  mm from bregma with  $14^\circ$  angle. Fitted obdurators protruded  $0.5$  mm beyond the guide cannulae. Mice were allowed 3 days of recovery before resuming 2–3 days of ethanol and water drinking to return to pre-surgical drinking behavior. During this period, obdurators were handled daily to habituate animals to microinjection procedures.

Microinjection procedures have been previously described in Hwa *et al.* (2013). On test days, 33-gauge microinjectors (Plastics One) infused  $0.2\ \mu\text{l}$  drug into the DRN at a flow rate of  $0.1\ \mu\text{l}/\text{min}$ . Injectors were connected through polyethylene tubing to a glass syringe controlled by a microinjection pump (CMA/100, CMA Microdialysis, Holliston, MA, USA). One group of mice ( $n = 8$ ) was injected with intra-DRN with aCSF,  $6\ \mu\text{g}$  naltrexone,  $0.6\ \mu\text{g}$  CP154526, and the combination of  $6\ \mu\text{g}$  naltrexone and  $0.6\ \mu\text{g}$  CP154526 on four intermittent ethanol-water test days. In addition to the group of mice that received the high dose combination, we studied a second group of mice ( $n = 7$ ) that was injected intra-DRN with aCSF,  $3\ \mu\text{g}$  naltrexone,  $0.3\ \mu\text{g}$  CP154526, and a lower combination of  $3\ \mu\text{g}$  naltrexone and  $0.3\ \mu\text{g}$  CP154526. To consider if microinjections were specific to the DRN, another group of intermittent access mice ( $n = 9$ ) was microinjected intra-MRN with aCSF,  $6\ \mu\text{g}$  naltrexone,  $0.6\ \mu\text{g}$  CP154526 and the combination. Finally, to assess the drug effects upon intermittent versus continuous access drinkers, continuous access mice ( $n = 9$ ) were injected intra-DRN with aCSF,  $6\ \mu\text{g}$  naltrexone,  $0.6\ \mu\text{g}$  CP154526 and the combination.

During microinjections, mice were allowed to move about freely. Injectors were kept in place for 1 minute after microinfusion to minimize capillary backflow up the cannula. Alcohol and water bottles were given to mice 9 minutes later to measure the drug effects on drinking behavior. A total of 2, 4 and 24 hours of fluid consumption were assessed after each microinjection. Similar to experiment 2, animals were perfused after testing procedures, and brains were collected for histological verifica-

tion of the cannula placement in the DRN or MRN. Mice with missed placements ( $n = 6$ ) were eliminated from analysis.

#### Experiment 5: 5-HT measurement in the mPFC after intra-DRN naltrexone or CRF-R1 antagonist

To further investigate how opioid receptor and CRF-R1 antagonism in the DRN affect 5-HT impulse flow to the forebrain, IAA mice were dually implanted with 26-gauge, 6-mm cannula for intra-DRN microinjections and a microdialysis probe in the mPFC for 5-HT measurement. Mice that had 4 weeks of intermittent access were surgically prepared with cannulae targeting the DRN, as described in experiment 4. Additionally, they had microdialysis guide cannulae (CMA 7, Harvard Apparatus, Holliston, MA, USA) targeting the pre- and infralimbic regions of the mPFC (AP  $+2.0$  mm, ML  $-0.3$  mm from bregma, DV  $-1.0$  mm from dura). Animals were allowed 3 days of recovery before resumption of IAA drinking and daily handling of the obdurator.

The night before the test day, mice were anesthetized with isoflurane (Webster Veterinary, Devens, MA, USA) and the dummy probe was replaced with the microdialysis probe with a 1-mm active membrane (CMA 7, Harvard Apparatus). The probe was infused with aCSF at a flow rate of  $0.5\ \mu\text{l}/\text{minute}$  overnight (CMA 400 microinfusion pump).

On the test day, the flow rate was increased to  $1.5\ \mu\text{l}/\text{minute}$ . Mice were habituated to the increased flow rate for 1 hour before dialyzate sample collection. Samples were collected every 20 minutes. In similar methods as experiment 4, about  $0.2\ \mu\text{l}$  microinjections of either aCSF,  $6\ \mu\text{g}$  naltrexone or  $0.6\ \mu\text{g}$  CP154526 occurred 10 minutes before hour 3 of the dark cycle at a flow rate of  $0.1\ \mu\text{l}/\text{minute}$ , which is when access to ethanol and water is normally given. Microinjection and placement verification procedures were identical to those described in experiment 4. Mice with missed placements in either the DRN or the mPFC ( $n = 7$ ) were excluded from analysis.

5-HT was measured using electrochemical detection equipped with high performance liquid chromatography (Takahashi *et al.* 2010; Shimamoto *et al.* 2011). A stabilizing agent of 20 mM phosphate buffer with 25 mM ethylenediaminetetraacetic acid (EDTA) was added to  $30\ \mu\text{l}$  dialyzate samples. A cation-exchange column (Capcell Pak SCX,  $1.5\ \text{mm} \times 250\ \text{mm}$ ,  $5\ \mu\text{m}$  I.D., Shiseido, Tokyo, Japan) separated monoamines at a column temperature of  $30^\circ\text{C}$  and a flow rate of  $0.2\ \text{ml}/\text{minute}$ . The mobile phase consisted of 150 mM ammonium acetate, 50 mM citric acid,  $27\ \mu\text{M}$  EDTA, 10 percent methanol and 1 percent acetonitrile with pH adjusted to 4.6. 5-HT concentrations were determined by using standard curves with known amounts of 5-HT in a



range of 0.125–0.5 pg. The limit of detection was 2 fg under these conditions with a 10.5 percent recovery rate.

## Drugs

For systemic injections in experiment 1, naltrexone HCl (NIDA, Rockville, MD, USA) was dissolved into 0.9 percent sterile saline at 1 and 10 mg/kg for i.p. delivery. CP154526 is a prototypic CRF-R1 antagonist with high affinity for CRF-R1 ( $K_i < 10$ ; Schulz *et al.* 1996). CP376395 also has high selectivity for CRF-R1 ( $IC_{50} = 5$ ) with an advantage of being more water soluble than CP154526 (Chen *et al.* 2008). CP154526 (Tocris, Minneapolis, MN, USA) was freshly suspended in a vehicle of 0.6 percent methylcellulose, and CP376395 (Tocris) was dissolved in 0.9 percent sterile saline. For chronic icv infusion in experiment 2, about 3  $\mu\text{g}/\mu\text{l}$  naltrexone, 30  $\mu\text{g}/\mu\text{l}$  naltrexone and 0.3  $\mu\text{g}/\mu\text{l}$  CP376,395 were dissolved in filtered aCSF. About 0.3  $\mu\text{g}/\mu\text{l}$  CP154526 was also chronically delivered in the minipump, though it required a vehicle of 4 percent DMSO. Four percent DMSO was used as a control. Minipump doses were chosen based on a log step reduction from the microinjection doses. Morphine HCl (NIDA), used for the tail withdrawal procedure, was dissolved in sterile saline at 10 mg/kg. For microinjections in experiment 4, drug doses were chosen based on previous intracerebral studies in C57BL/6J mice with CP154526 in the laboratory (Hwa *et al.* 2013). Naltrexone and CP154526 were freshly suspended in filtered aCSF on the day of testing. The most effective intra-DRN doses for decreasing IAA, 6  $\mu\text{g}$  naltrexone and 0.6  $\mu\text{g}$  CP154526, were used to evoke changes in 5-HT in experiment 5 microdialysis.

## Statistical analyses

BECs of mice that underwent either IAA or continuous access were compared with a one-way analysis of variance (ANOVA). Ethanol intake (g/kg and ml) and water intake (ml) were measured in experiments with drug manipulations. For experiment 1, one-way repeated measures ANOVAs were run at each timepoint after naltrexone, CP154526 or CP376395 injection. For experiment 2, two-way repeated measures ANOVAs were conducted (Drug  $\times$  Day) to see if drugs infused icv via minipump affected fluid intake over 14 days. A two-way repeated measures ANOVA (drug treatment versus morphine dose) was also used to test whether morphine-induced analgesia was effective on minipump day 13 in aCSF and naltrexone-infused animals. For experiment 4, one-way repeated measures ANOVAs were run for 2, 4 and 24 hours of drinking data to assess if microinfusions into the DRN or MRN affected intermittent or continuous access drinking. For experiment 5, a two-way repeated

measures ANOVA (Drug  $\times$  Time) was conducted to test if the microinjected drugs affected mPFC 5-HT over repeated samples. In all of the statistical tests, *post hoc* analyses were Bonferroni *t*-tests when a main effect of drug was found ( $P < 0.05$ ).

## RESULTS

### Intermittent access promotes excessive alcohol drinking

At the end of 4 weeks of IAA drinking, male C57BL/6J mice ( $n = 103$ ) consumed, on average,  $22.44 \pm 0.67$  g/kg/day. BECs in a subset of IAA mice ( $n = 11$ ) were  $134 \pm 7.53$  mg/dl, ranging from 97.9 to 179.4 mg/dl, after 2 hours access to two-bottle choice. In contrast, continuous access mice ( $n = 12$ ) consumed 14.97 g/kg/day after 12 days of continuous two-bottle choice. BECs for that group were  $38.4 \pm 5.64$  mg/dl during the initial 2 hours after bottle measurements. A one-way ANOVA confirmed that BECs from the IAA group were significantly higher than the continuous access group [ $F_{(1,21)} = 106.28$ ,  $P < 0.001$ ].

### Systemic injections of naltrexone and CRF-R1 antagonists reduce intermittent ethanol drinking

The opioid antagonist naltrexone (0, 1, 10 mg/kg, i.p.) was administered to mice ( $n = 12$ ) to reduce IAA. A one-way repeated measures ANOVA showed that naltrexone decreased 2 hours of ethanol drinking (g/kg) [ $F_{(2,22)} = 12.76$ ,  $P < 0.001$ ] at both doses compared with saline vehicle (1 mg/kg,  $P < 0.01$ ; 10 mg/kg,  $P < 0.001$ ). This suppression of ethanol drinking (g/kg) lasted after 4 hours of access [ $F_{(2,22)} = 9.20$ ,  $P < 0.01$ ] at both doses (1 mg/kg,  $P < 0.01$ ; 10 mg/kg,  $P < 0.01$ ) and after 24 hours of access [ $F_{(2,22)} = 11.13$ ,  $P < 0.001$ ] also at both naltrexone doses (1 mg/kg,  $P < 0.05$ ; 10 mg/kg,  $P < 0.001$ ). In accordance with reduced ethanol drinking (g/kg), volume of ethanol intake (ml) was decreased by naltrexone at the 2, 4 and 24 hours of timepoints [2 hours:  $F_{(2,22)} = 12.17$ ,  $P < 0.001$ ; 4 hours:  $F_{(2,22)} = 8.76$ ,  $P < 0.01$ ; 24 hours:  $F_{(2,22)} = 10.52$ ,  $P < 0.001$ ; Table 1] by the same effective doses (2 hours: 1 mg/kg,  $P < 0.01$ ; 10 mg/kg,  $P < 0.001$ ; 4 hours: 1 mg/kg,  $P < 0.01$ ; 10 mg/kg,  $P < 0.01$ ; 24 hours: 1 mg/kg,  $P < 0.001$ ; 10 mg/kg,  $P < 0.001$ ). The fluid-altering effects of naltrexone were not exclusive to ethanol. Naltrexone also increased water intake (ml) at the 4 and 24 hours of timepoints [4 hours:  $F_{(2,22)} = 5.62$ ,  $P < 0.05$ ; 24 hours:  $F_{(2,22)} = 3.90$ ,  $P < 0.05$ ; Table 1] at the 10 mg/kg dose (4 hours:  $P < 0.05$ ; 24 hours:  $P < 0.05$ ).

The CRF-R1 antagonist CP154516 (0, 10, 17, 30 mg/kg, i.p.) was given to another group of IAA mice ( $n = 9$ ). A one-way repeated measures ANOVA revealed

**Table 1** Systemic injections of naltrexone and CRF-R1 antagonists on intermittent alcohol drinking.

| Drug       | Dose     | Hour | ETOH (ml)     | H2O (ml)     |
|------------|----------|------|---------------|--------------|
| Naltrexone | Saline   | 2    | 0.37 ± 0.03   | 0.45 ± 0.05  |
|            |          | 4    | 0.93 ± 0.07   | 0.78 ± 0.08  |
|            |          | 24   | 3.25 ± 0.13   | 2.08 ± 0.19  |
|            | 1 mg/kg  | 2    | 0.23 ± 0.03*  | 0.31 ± 0.06  |
|            |          | 4    | 0.65 ± 0.09*  | 0.66 ± 0.12  |
|            |          | 24   | 2.95 ± 0.13   | 2.18 ± 0.24  |
|            | 10 mg/kg | 2    | 0.18 ± 0.03** | 0.52 ± 0.09  |
|            |          | 4    | 0.65 ± 0.09*  | 1.18 ± 0.18* |
|            |          | 24   | 2.65 ± 0.15** | 2.66 ± 0.24* |
|            | CP154526 | 2    | 0.29 ± 0.06   | 0.36 ± 0.15  |
|            |          | 4    | 0.46 ± 0.07   | 0.83 ± 0.24  |
|            |          | 24   | 2.37 ± 0.42   | 3.00 ± 0.50  |
| CP376395   | 10 mg/kg | 2    | 0.15 ± 0.06   | 0.44 ± 0.08  |
|            |          | 4    | 0.37 ± 0.10   | 0.71 ± 0.09  |
|            |          | 24   | 1.95 ± 0.20   | 3.28 ± 0.28  |
|            | 17 mg/kg | 2    | 0.12 ± 0.02*  | 0.14 ± 0.07  |
|            |          | 4    | 0.47 ± 0.12   | 0.40 ± 0.13  |
|            |          | 24   | 2.39 ± 0.37   | 2.43 ± 0.38  |
|            | 30 mg/kg | 2    | 0.04 ± 0.01** | 0.23 ± 0.05  |
|            |          | 4    | 0.32 ± 0.08   | 0.34 ± 0.11  |
|            |          | 24   | 2.19 ± 0.30   | 2.00 ± 0.38  |
|            | Saline   | 2    | 0.21 ± 0.06   | 0.16 ± 0.06  |
|            |          | 4    | 0.89 ± 0.07   | 0.24 ± 0.07  |
|            |          | 24   | 3.45 ± 0.15   | 1.15 ± 0.16  |
| CP376395   | 10 mg/kg | 2    | 0.14 ± 0.04   | 0.34 ± 0.07  |
|            |          | 4    | 0.76 ± 0.12   | 0.50 ± 0.10  |
|            |          | 24   | 3.31 ± 0.25   | 0.99 ± 0.16  |
|            | 17 mg/kg | 2    | 0.06 ± 0.03*  | 0.90 ± 0.67  |
|            |          | 4    | 0.48 ± 0.11*  | 0.99 ± 0.68  |
|            |          | 24   | 3.07 ± 0.15   | 0.92 ± 0.14  |
|            | 30 mg/kg | 2    | 0.06 ± 0.04*  | 0.58 ± 0.22  |
|            |          | 4    | 0.46 ± 0.12*  | 0.67 ± 0.24  |
|            |          | 24   | 2.94 ± 0.16   | 1.15 ± 0.18  |

Opioid antagonist naltrexone (0, 1, 10 mg/kg, i.p.,  $n = 12$ ) and CRF-R1 antagonists CP154526 (0, 10, 17, 30 mg/kg, i.p.,  $n = 9$ ) and CP376395 (0, 10, 17, 30 mg/kg, i.p.,  $n = 11$ ) reduced intermittent ethanol drinking in male C57BL/6J mice. Shown are mean ethanol (ml) and water (ml) ± SEM consumed over 2, 4 and 24 hours of fluid access after drug treatment. \* $P < 0.05$  versus vehicle. \*\* $P < 0.001$  versus vehicle.

that CP154526 initially suppressed 2 hours of ethanol drinking (g/kg) [ $F_{(3,23)} = 6.03$ ,  $P < 0.01$ ] at the two higher doses compared with 0.6 percent methylcellulose vehicle (17 mg/kg,  $P < 0.05$ ; 30 mg/kg,  $P < 0.01$ ). This reduction was also observed in volume of ethanol intake (ml) [ $F_{(3,23)} = 6.40$ ,  $P < 0.01$ ; Table 1] specifically at the higher doses (17 mg/kg,  $P < 0.05$ ; 30 mg/kg,  $P < 0.001$ ). Systemic CP154526 was not effective at decreasing alcohol after 4 or 24 hours. The transient alcohol-suppressing effects were exclusive to alcohol, as water intake (ml) was not affected (Table 1).

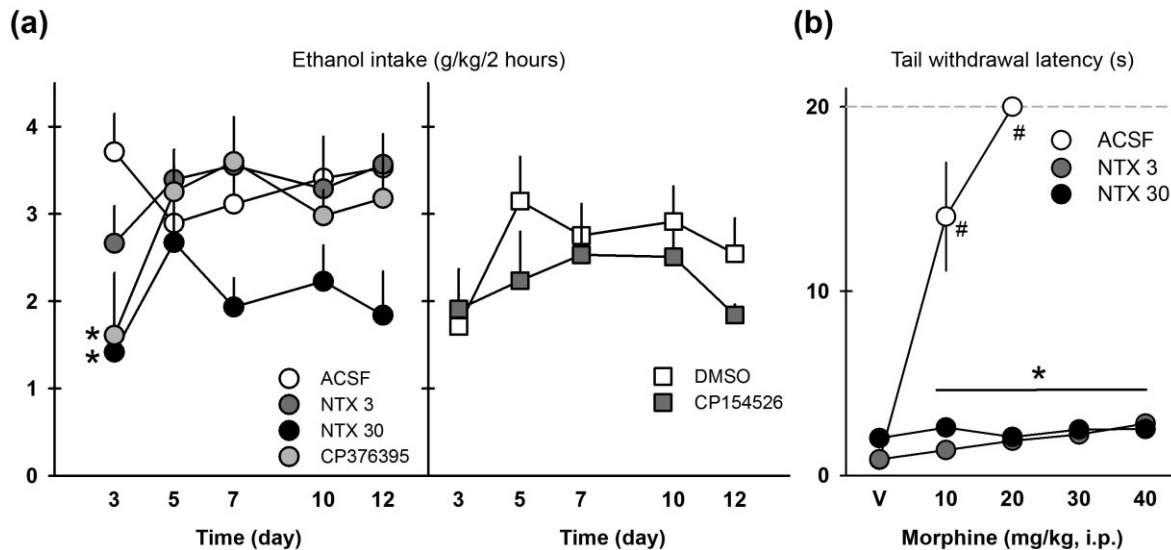
Another CRF-R1 antagonist, CP376395 (0, 10, 17, 30 mg/kg, i.p.), was used to reduce IAA drinking in another group of mice ( $n = 11$ ). One-way repeated meas-

ures ANOVAs revealed that CP376395 significantly reduced 2 and 4 hours of ethanol drinking (g/kg) [2 hours:  $F_{(3,30)} = 3.74$ ,  $P < 0.05$ ; 4 hours:  $F_{(3,30)} = 4.76$ ,  $P < 0.05$ ]. *Post hoc* tests confirm that the 17 mg/kg ( $P < 0.05$ ) and 30 mg/kg ( $P < 0.05$ ) doses were different from saline vehicle. This trend was similar for volume of ethanol intake (ml) during the initial 4 hours of IAA [2 hours:  $F_{(3,30)} = 3.54$ ,  $P < 0.05$ ; 4 hours:  $F_{(3,30)} = 4.42$ ,  $P < 0.05$ ; Table 1] also at the higher doses (2 hours: 17 mg/kg,  $P < 0.05$ ; 30 mg/kg,  $P < 0.05$ ; 4 hours: 17 mg/kg,  $P < 0.05$ ; 30 mg/kg,  $P < 0.05$ ). Water drinking was not significantly affected by CP376395 at any timepoint (Table 1).

#### Chronic icv infusion of naltrexone and CRF-R1 antagonists transiently reduce IAA

Mice with a history of 4 weeks of IAA were implanted with icv cannulae attached to minipumps that chronically infused aCSF ( $n = 8$ ), 3 µg/µl naltrexone ( $n = 7$ ), 30 µg/µl naltrexone ( $n = 8$ ) or 3 µg/µl CP376395 ( $n = 8$ ) across 14 days in separate groups of mice. These groups were compared statistically apart from the CP154526 and 4 percent DMSO groups, as aCSF was a vehicle for these groups. A two-way (Drug × Day) repeated measures ANOVA indicated that the only significant interaction for drug effect on ethanol drinking (g/kg) was seen on day 1 during the initial 2 hours of IAA time period [ $F_{(3,100)} = 5.74$ ,  $P < 0.01$ ; Fig. 1a]. Specifically, 30 µg/µl naltrexone ( $P < 0.01$ ) and 3 µg/µl CP376395 ( $P < 0.05$ ) were different from aCSF infusion within test day 1. There were no other significant interactions for drug group differences on ethanol drinking (g/kg) or water intake (ml) across the 14 days of chronic infusion at the 2, 4 or 24 hours of timepoints.

To verify that the minipumps were chronically infusing naltrexone during the 14-day pump duration, the mice given with aCSF, 3 µg/µl naltrexone and 30 µg/µl naltrexone were subjected to a test of morphine-induced analgesia on day 13 after IAA drinking tests. Mouse tails were held in 54°C water, and latency to withdraw the tail was measured after 0, 10, 20, 30 and 40 mg/kg morphine, administered i.p. with cumulative dosing every 20 minutes. There was a significant Minipump Drug × Morphine dose interaction [ $F_{(8,90)} = 32.94$ ,  $P < 0.001$ ] where during the 10, 20, 30 and 40 mg/kg morphine doses, mice given with aCSF had shorter tail withdrawal latencies than mice given with 3 and 30 µg/µl naltrexone (all  $P < 0.001$ ; Fig. 1b). Mice given with aCSF via minipump showed lengthy tail withdrawal latencies of  $14.04 \pm 2.94$  seconds with 10 mg/kg morphine, and all aCSF mice met the experimenter-controlled maximum (20 seconds) in the hot water with 20 mg/kg morphine, suggesting morphine-induced analgesia. Mice that were



**Figure 1** Drugs were chronically administered icv via osmotic minipumps to affect intermittent access drinking. The left side of panel (a) shows ethanol drinking (g/kg/2 hours) over time during naltrexone infusion (3  $\mu$ g/ $\mu$ l/hour; NTX3,  $n=7$  and 30  $\mu$ g/ $\mu$ l/hour; NTX30,  $n=8$ ), CRF-R1 antagonist CP376395 infusion (0.3  $\mu$ g/ $\mu$ l/hour;  $n=8$ ) and their vehicle aCSF ( $n=8$ ). The right side of panel (a) shows ethanol intake (g/kg/2 hours) over time during CRF-R1 antagonist CP154526 infusion (0.3  $\mu$ g/ $\mu$ l/hour;  $n=6$ ) and its vehicle 4 percent DMSO ( $n=6$ ). Data points are mean ethanol intake  $\pm$  SEM. \* $P < 0.05$  versus ACSF vehicle. Panel (b) shows the tail withdrawal latency in seconds to a hot water bath after cumulative doses of morphine (mg/kg, i.p.) in mice given with ACSF or naltrexone on day 13 of minipump infusion. Data points are mean latencies  $\pm$  SEM. \* $P < 0.05$  versus ACSF group. # $P < 0.05$  versus saline vehicle

given naltrexone via minipump exhibited short tail withdrawal latencies of  $2.81 \pm 0.20$  seconds after 40 mg/kg morphine, suggesting blockade of morphine-induced analgesia.

Other groups of IAA mice were implanted with minipumps infusing 4 percent DMSO ( $n=6$ ) and 3  $\mu$ g/ $\mu$ l CP154526 ( $n=6$ ) for 14 days that were also tested for IAA drinking behavior. These DMSO-based groups were statistically analyzed separately from the aCSF-based vehicle groups. Unlike the other minipump drugs that reduced ethanol drinking on the first day, chronic CP154526 infusion was not different from DMSO infusion on day 1 (Fig. 1a). A two-way repeated measures (Drug  $\times$  Day) ANOVA showed that there was a Drug  $\times$  Day interaction for 4 hours of ethanol drinking (g/kg) [ $F_{(4,40)} = 2.93$ ,  $P < 0.05$ ], where day 5 showed a difference between DMSO and CP154526 treatments ( $P < 0.05$ ). On day 5, DMSO-treated mice consumed  $0.73 \pm 0.08$  g/kg/4 hours while CP154526-treated mice consumed  $0.51 \pm 0.05$  g/kg/4 hours. There were no drug effects on water drinking.

#### TPH-immunoreactive DRN neurons co-localize with CRF-R1 or MOR

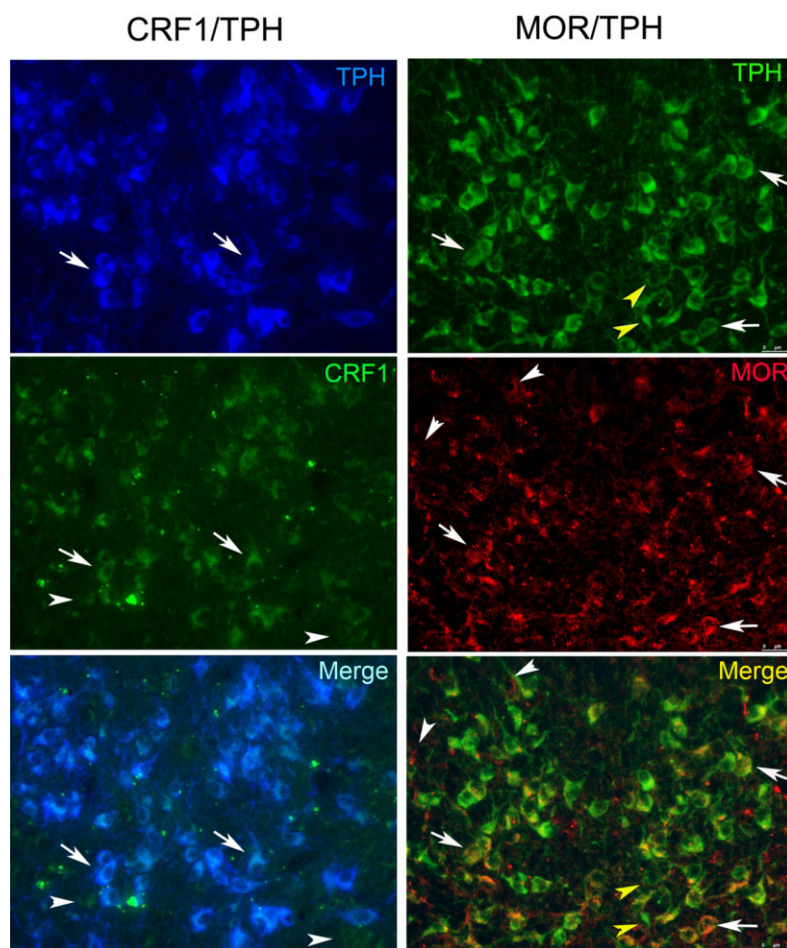
The brains of some IAA and water-drinking B6 mice ( $n=4$  per group) were taken for immunohistochemistry to visualize CRF-R1 and TPH or MOR and TPH in DRN sections. Figure 2 (left panel) shows a representative section through the DRN with dual labeling of CRF-R1

and TPH. Additionally, some cells were CRF-R1 only, which we hypothesize to be GABAergic cells. CRF-R1 immunoreactivity was present within the DRN as well as in the lateral regions. Many TPH-immunoreactive neurons in the DRN were also dual labeled for MOR, although there were occasional single-labeled TPH and single-labeled MOR neurons (Fig. 2, right panel). There were no evident differences between water-drinking and ethanol-drinking mice.

#### Distinct effects of intra-DRN naltrexone and CRF-R1 antagonist on drinking patterns

A group of mice ( $n=8$ ) with a history of IAA was outfitted with cannula targeting the DRN and tested for IAA drinking after microinjections of aCSF, 6  $\mu$ g naltrexone, 0.6  $\mu$ g CP154526, or the combination of 6  $\mu$ g naltrexone and 0.6  $\mu$ g CP154526. Cannula placements in the DRN are shown in Fig. 3e. A one-way repeated measures ANOVA revealed that drug treatment affected IAA ethanol drinking (g/kg) at the 2 and 4 hours of timepoints [2 hours:  $F_{(3,21)} = 6.24$ ,  $P < 0.01$ , Fig. 3a; 4 hours:  $F_{(3,20)} = 5.28$ ,  $P < 0.01$ ]. *Post hoc* tests showed that naltrexone, CP154526 and the combination reduced drinking compared with aCSF at both the 2 and 4 hours of timepoints (all  $P < 0.05$ ). The suppression in IAA drinking caused by the combination of naltrexone and CP154526 was not statistically different from naltrexone or CP154526 administered alone. Water intake (ml) was not affected by any microinjection.





**Figure 2** Fluorescent photomicrographs of sections through the DRN that were dual labeled to visualize either CRF1 and tryptophan hydroxylase (CRF1/TPH) or MOR and tryptophan hydroxylase (MOR/TPH). The leftmost panel shows TPH cells in blue and CRF1 in green. Dual-labeled cells in the merged panel appear whitish blue. Arrows point to examples of dual-labeled cells. Arrowheads point to single-labeled CRF1 neurons. The rightmost panel shows TPH cells in green and MOR in red. Dual-labeled cells in the merged panel appear yellow/orange. Arrows point to examples of dual-labeled cells. White arrowheads point to single-labeled MOR neurons and yellow arrowheads point to single-labeled TPH neurons

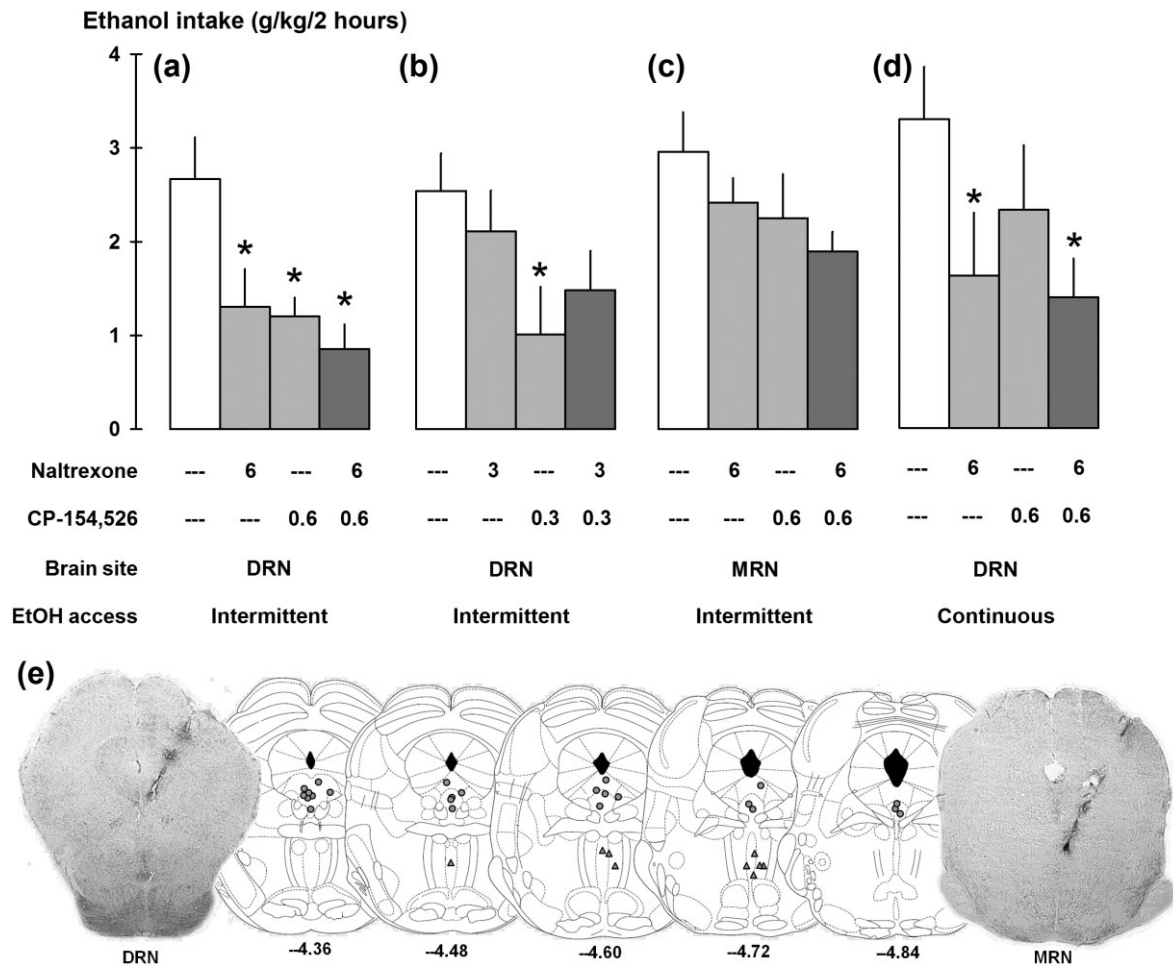
Another group of IAA mice ( $n = 7$ ) was microinjected with aCSF and lower doses of naltrexone ( $3 \mu\text{g}$ ), CP154526 ( $0.3 \mu\text{g}$ ), and the combination of  $3 \mu\text{g}$  naltrexone and  $0.3 \mu\text{g}$  CP154526 into the DRN. A one-way repeated measures ANOVA showed that 2 hours of ethanol drinking (g/kg) was affected by intra-DRN drug [g/kg:  $F_{(3,16)} = 5.45$ ,  $P < 0.01$ ; Fig. 3b]. *Post hoc* tests revealed that only  $0.3 \mu\text{g}$  CP154526 decreased ethanol drinking (g/kg) ( $P < 0.01$ ) compared with aCSF. Lower dose drug treatment did not affect consumption further than 2 hours. Water drinking (ml) was not changed due to drug infusion.

A group of IAA mice ( $n = 9$ ) was microinjected with aCSF,  $6 \mu\text{g}$  naltrexone,  $0.6 \mu\text{g}$  CP154526 and the combination into the MRN to contrast drug effects in the DRN. Intra-MRN cannula placements are shown in Fig. 3e. A one-way repeated measures ANOVA indicated that water intake (ml) was initially affected by drug treatment in the MRN at 2 hours [ $F_{(3,24)} = 5.67$ ,  $P < 0.01$ ]. Naltrexone ( $P < 0.01$ ), CP154526 ( $P < 0.05$ ) and the combination ( $P < 0.01$ ) reduced 2 hours of water intake compared with aCSF. The reduction of water intake (ml) continued until 4 hours of timepoint [ $F_{(3,24)} = 4.17$ ,  $P < 0.05$ ] caused by all drug doses, again (all  $P < 0.05$ ). Ethanol

drinking (g/kg) was only affected at the 4 hours of timepoint [g/kg:  $F_{(3,24)} = 4.00$ ,  $P < 0.05$ , Fig. 3c] but not at the 2 hours of timepoint. *Post hoc* tests indicated that CP154526 and the combination of naltrexone and CP154526 were different from aCSF for both ethanol drinking (g/kg) at 4 hours (all  $P < 0.05$ ; not shown). CP154526, naltrexone and the combination reduced baseline ethanol drinking from  $6.64 \pm 0.87$  after aCSF to  $4.29 \pm 0.73$ ,  $5.43 \pm 0.25$  and  $4.30 \pm 0.48$  g/kg/4 hours, respectively. The drugs suppressed baseline water drinking from  $0.92 \pm 0.14$  after aCSF to  $0.48 \pm 0.10$  after CP154526,  $0.51 \pm 0.14$  after naltrexone and  $0.42 \pm 0.13$  ml/4 hours after the combination.

A final group of mice ( $n = 9$ ) with a history of continuous access to alcohol was tested for drinking behavior after intra-DRN microinjections of aCSF,  $6 \mu\text{g}$  naltrexone,  $0.6 \mu\text{g}$  CP154526, and the combination of naltrexone and CP154526 to reveal if drug effects were exclusive to excessive drinkers compared with moderate-level drinkers. A one-way repeated measures ANOVA indicated that the initial 2 hours of ethanol drinking (g/kg) was affected by drug treatments [g/kg:  $F_{(3,24)} = 4.70$ ,  $P < 0.05$ , Fig. 3d]. Continuous access to alcohol (g/kg) was specifically decreased by intra-DRN naltrexone





**Figure 3** Separate groups of mice were microinjected with aCSF, naltrexone, CP154526, and the combination of naltrexone and CP154526 to reduce ethanol drinking. Panel (a) shows intra-DRN microinjections of higher doses in intermittent access drinkers ( $n=8$ ). Panel (b) shows intra-DRN microinjections of lower doses in intermittent drinkers ( $n=7$ ). Panel (c) shows intra-MRN microinjections in intermittent drinkers ( $n=9$ ). Panel (d) shows intra-DRN microinjections in continuous access drinkers ( $n=9$ ). Bars represent mean ethanol intake (g/kg/2 hours)  $\pm$  SEM. \* $P < 0.05$  versus aCSF. Panel (e) shows representative schematics of correct placements of microinjection cannulae in the DRN (gray circles,  $n=24$ ) or MRN (gray triangles,  $n=9$ ). Numbers below coronal slices are the distances (mm) from bregma. Photos show correct placement in the brain areas

( $P < 0.05$ ) and the combination of naltrexone and CP154526 ( $P < 0.01$ ), but not by CP154526 alone. The naltrexone effects did not last longer than 2 hours. No drug treatments affected water drinking in the continuous access mice.

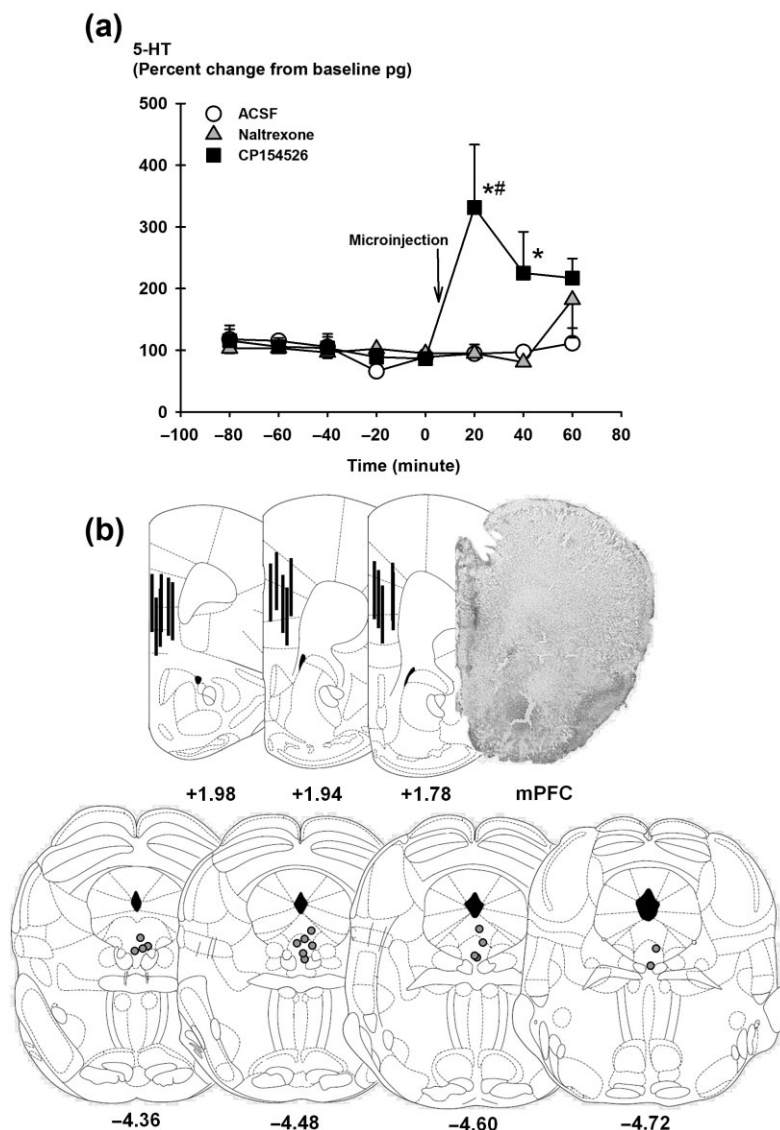
#### Intra-DRN CRF-R1 antagonist, but not naltrexone, increased prefrontal cortical 5-HT impulse flow

IAA mice were concurrently implanted with cannulae in the DRN and microdialysis probes in the mPFC for 5-HT measurements after drug microinjections. Correct placements of DRN cannulae and mPFC probes are depicted in Figs 3e and 4b. Eight samples were collected every 20 minutes, and aCSF ( $n=6$ ), 6  $\mu$ g naltrexone ( $n=7$ ) and 0.6  $\mu$ g CP154526 ( $n=7$ ) were infused in different groups of mice 10 minutes before the sixth sample was collected. A two-way repeated measures ANOVA showed

that there was a significant main effect of intra-DRN drug microinjection on 5-HT concentrations [ $F_{(2,86)} = 4.13$ ,  $P < 0.05$ ; Fig. 4a], where CP154526, but not naltrexone, was different from aCSF ( $P < 0.05$ ). There was also a Drug  $\times$  Time interaction [ $F_{(14,86)} = 1.83$ ,  $P < 0.05$ ]. *Post hoc* analysis revealed that 5-HT concentrations 10 and 30 minutes after the microinjection, the sixth and seventh samples, respectively, were significantly increased when CP154526 was injected compared with those when after aCSF ( $P < 0.001$ ) or naltrexone ( $P < 0.001$ ) was injected.

## DISCUSSION

The current studies demonstrate divergent roles of CRF-R1 and opioid receptors in the DRN in volitional excessive ethanol drinking. Persistently high levels of



**Figure 4** Panel (a) shows extracellular concentrations of serotonin (5-HT; percent change from baseline pg) were collected from the mPFC after intra-DRN micro-injections of aCSF (white circles,  $n=6$ ), 6  $\mu\text{g}$  naltrexone (gray triangles,  $n=7$ ) or 0.6  $\mu\text{g}$  CP154526 (black squares,  $n=7$ ) in mice with a history of intermittent access to alcohol. Data points are mean percent change  $\pm$  SEM. \* $P < 0.05$  versus aCSF, # $P < 0.05$  versus baseline timepoints. Panel (b) shows representative schematics of correct placements of microinjection canulae in the DRN and microdialysis probes in the mPFC ( $n=16$ ). Numbers below coronal sections are the distances (mm) from bregma. The photo shows correct placement in the mPFC

ethanol drinking and BECs were generated using an intermittent schedule of access to two-bottle choice, and systemic and intra-DRN administration of either FDA-approved naltrexone or CP154526 decreased this type of drinking. Intra-DRN naltrexone also reduced moderate-level ethanol drinking, engendered by a continuous schedule of access, but CP154526 did not. These specific anti-ethanol drug effects were limited to the DRN of IAA drinkers, as identical infusions into the MRN had a greater effect on water drinking. Because there is no evidence for an additive effect by CP154526 and naltrexone, this suggests that these compounds accomplished their ethanol-suppressing effects via a common downstream target. In contrast, in our microdialysis experiments, intra-DRN CP154526 increased extracellular concentrations of 5-HT, presumably reflecting release in the mPFC, while naltrexone did not. These differential patterns of impulse flow to the mPFC caused by CP154526 and

naltrexone also suggest that the mPFC may not be as relevant for the suppression of IAA drinking.

At the level of the DRN, we co-localized CRF-R1 on 5-HT and non-5-HT cells. This is the first study to confirm CRF-R1 and TPH immunoreactivity in the DRN in the mouse brain, as previous studies have examined co-localization of CRF and TPH in the rat brain (Kirby *et al.* 2000; Price *et al.* 2002) and CRF-R1 within the DRN in the rat brain (Waselus *et al.* 2009). The nearby non-TPH-expressing cells with CRF-R1 immunoreactivity most likely represent GABAergic neurons (Kirby *et al.* 2008). Our behavioral findings did not reveal any additive effects of co-infusion of both antagonists (e.g. Liu & Weiss 2002), suggesting that the opioid and CRF-R1 systems may be acting on a common mechanism in the DRN to suppress escalated drinking in the current preparation. From a pharmacological perspective, this pattern suggests that they may work through the same mechanism because

when one mechanism is saturated, the other drug cannot exert a greater effect to reduce drinking further. We speculate that this putative, common mechanism is located downstream and requires further investigation. At the intracellular level, both naltrexone and CP154526 inhibit adenylate cyclase activity and cyclic adenosine monophosphate (Childers 1991; Schulz *et al.* 1996). At the receptor level, both morphine and CRF increase GABAergic tone and act on GABAergic and glutamatergic afferents to indirectly influence 5-HT neurons in the DRN (Kirby *et al.* 2008). Therefore, it is possible that naltrexone and CRF-R1 antagonists similarly influence GABAergic tone in the DRN to decrease excessive ethanol drinking. Alternatively, CP154526 may act through additional interneurons to increase 5-HT flow, as seen in the current study. Future studies measuring GABAergic tone in efferent sites like the PFC would be required to confirm this hypothesis.

In the current preparation, intra-DRN CP154526 significantly increased extracellular 5-HT concentrations in the mPFC in IAA mice, but naltrexone did not. Because we speculate the treatments work via one common neural mechanism to reduce IAA drinking, but they have differential effects on mPFC 5-HT, it suggests that the mPFC may not be a critical site for the suppression of IAA drinking. One limitation of our microdialysis study was that the probes spanned both the infralimbic and prelimbic subregions of the mPFC. We cannot ignore the fact that these regions have discrete projections and are functionally distinct for aspects of drug-seeking behaviors (reviewed by George & Koob 2010). For example, previous morphological studies have identified high 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor localization and expression throughout both the infralimbic and prelimbic subregions (Amargós-Bosch *et al.* 2004), which can be interpreted as a collective 5-HT-dense region for extracellular 5-HT measurement. The mPFC involved in higher order cognitive and motivational functions is a site that is dysfunctional in alcoholics (Goldstein & Volkow 2002). Alcoholics can have up to 35 percent loss of 5-HT transporter density in the PFC compared with non-alcoholics, suggesting an association between excessive drinking and dysfunctional 5-HT neurotransmission in the mPFC (Mantere *et al.* 2002). Connectivity to the PFC becomes disrupted in rats with a history of IAA, which has been shown to be CRF dependent (George *et al.* 2012). 5-HT in the mPFC appears important for heavy drinking, but our divergent effects of the two compounds on mPFC 5-HT suggest that downstream effects in other brain sites are more important for naltrexone and CP154526.

Neurochemically, CRF at low to moderate doses inhibits 5-HT DRN activity and local release via CRF-R1 receptors on GABA afferents (Kirby *et al.* 2000; Kirby *et al.* 2008). Furthermore, stressful events mediated by endog-

enous CRF release acting on DRN neurons lead to inhibition of 5-HT release in the lateral septum (Price *et al.* 2002). One interpretation of the increased 5-HT is that IAA may contain stressful elements, possibly causing a CRF-R1 antagonist to disinhibit impulse flow from the DRN to the mPFC. Another hypothesis for the increased mPFC 5-HT is that blockade of CRF-R1 may cause relative activation of CRF-R2, stimulating 5-HT flow. A further limitation of the present microdialysis study was that 5-HT was measured only in IAA-experienced mice. It would be interesting to also investigate the CRF-R1 influence of mPFC 5-HT in mice with continuous access to ethanol to confirm if there is a dose-dependent influence of CP154526-induced increase in 5-HT. Although there was not an ethanol-naïve group in the current study, Tanahashi *et al.* (2012) importantly showed that neither perfusion of CRF-R1 nor CRF-R2 antagonists intra-DRN affect DRN/mPFC 5-HT in ethanol-naïve animals. Paired with the fact that there were no significant differences in CRF-R1 and TPH immunoreactivity between the IAA and water-drinking mice in our study, these results suggest that there may not have been a change in 5-HT caused by CP154526 in water-drinking mice. However, our experiments with CRF-R1 antagonism in mice with a history of chronic, excessive drinking experience do reveal neuroadaptations in the CRF-R1 system that affect 5-HT.

Systemic morphine increases 5-HT release in the mPFC, which is mediated by the DRN and not MRN (Tao & Auerbach 1995). Like the excitatory effects of acute morphine, MOR agonist DAMGO disinhibits 5-HT neurons in DRN (Tao & Auerbach 2005). Dimatellis *et al.* (2012) found that naltrexone inhibits striatal 5-HT levels, which is in accordance with MOR activation and disinhibition of 5-HT. Our microdialysis findings did not reveal any changes from baseline 5-HT concentrations caused by intra-DRN naltrexone, suggesting no tonic action of opioids. However, naltrexone may influence DRN function under phasic conditions where endogenous opioid release is stimulated. Future experiments with morphine microinjections can possibly answer this question. This is the first report to test how naltrexone affects DRN 5-HT impulse flow to the forebrain in excessively drinking mice, so perturbations of 5-HT may be different in more modestly drinking animals, or more heavily influenced by other circuits, like CRF-R1.

We also tested the CRF-R1 antagonist in the MRN where both CRF-R1 and CRF-R2 are moderately expressed (Van Pett *et al.* 2000). This suggests CRF-R1 antagonists intra-MRN might also alter ethanol-related behavior. For example, non-specific CRF receptor antagonist, d-Phe-CRF, into the MRN blocked footshock-induced ethanol reinstatement (Lê *et al.* 2002). The current results showed intra-MRN CP154526 and the combination of CP154526 and naltrexone reducing ethanol intake after

4 hours, with a trend at the 2 hours of timepoint, confirming its latent involvement in decreasing IAA. However, under the present conditions, it was an issue that intra-MRN CP154526 and naltrexone also decreased water intake in addition to alcohol. 5-HT<sub>1A</sub> receptors in the MRN are implicated by evidence showing that 8-OH-DPAT microinjections increased locomotor activity, feeding and ethanol drinking (Currie, Fletcher & Coscina 1994; Tomkins, Sellers & Fletcher 1994). As the DRN and MRN have distinct 5-HTergic systems (reviewed by Lechin, van der Dijs & Hernández-Adrián 2006), it is possible that microinjections of a CRF-R1 antagonist suppressed both ethanol and water drinking by indirectly influencing 5-HT regulation in the MRN.

We found that systemic administration of naltrexone decreased intermittent ethanol drinking in B6 mice, confirming our previous reports (Hwa *et al.* 2014), and intra-DRN naltrexone infusions reduced intermittent and continuous ethanol drinking. Because naltrexone and CP154526 decreased IAA, and naltrexone but not CP154526 reduced continuous access drinking, the data suggest that the mechanism underlying continuous drinking is different from IAA, and it may be opioid related. These results point to reductions in behavior that are not exclusive to the excessive drinkers. Naltrexone treatment in alcoholics is useful for controlled consumption, but there is a high degree of non-compliance and negative side effects (Croop, Faulkner & Labriola 1997). Based on these problems, some clinicians do not support the use of naltrexone for the treatment of men with chronic, severe alcohol dependence (Krystal *et al.* 2001). In preclinical studies, naltrexone has been known to decrease voluntary drinking since the 1980s (Altshuler, Phillips & Feinhandler 1980), but in both dependent and non-dependent animals (Sabino *et al.* 2006; Gilpin, Richardson & Koob 2008). Under two-bottle choice conditions, systemic naltrexone suppressed 20 percent ethanol intermittent intake, 20 percent ethanol continuous intake, and 10 percent ethanol continuous intake in Long-Evans and sP rats (Simms *et al.* 2008; Sabino *et al.* 2013). Given these limitations, other medications need to be developed to target excessive drinking.

It has been hypothesized that *crhr1* does not have a role in basal alcohol intake or relapse-like drinking situations with a low stress load (Molander *et al.* 2012). Our studies found that the CRF-R1 antagonist CP154526 intra-DRN decreased IAA, but did not affect continuous access intake. Although IAA and continuous access ethanol drinking (g/kg) values were similar at 2-hour baseline, there were large significant differences in BECs (threefold higher in the IAA group), confirming different patterns of overconsumption and the role of CRF-R1 in each group. CRF-R1 antagonists' effects are most convincing in animals that drink in excess or are dependent on ethanol

(see Lowery & Thiele 2010 for review). For example, CRF-R1 antagonists MPZP and LWH-63 reduced responding for ethanol reinforcement in dependent P and sP rats but not in the non-dependent rats (Sabino *et al.* 2006; Gilpin *et al.* 2008). In studies using volitional two-bottle choice, MPZP did not affect binge-like ethanol drinking and R121919 did not affect intermittent intake in rats (Sabino *et al.* 2013). However, these studies were conducted in non-dependent rats. Comparing treatment efficacies between rats and mice may not be straightforward due to innate differences in ethanol preference and drug metabolism between these species (Martignoni, Groothuis & de Kanter 2006). Nevertheless, our current studies using B6 mice demonstrate that CRF-R1 antagonism in the DRN is effective in reducing >20 g/kg daily intake, as this may be more excessive drinking to dependence, replicating previous reports (Hwa *et al.* 2013).

Despite the efficacy of CRF-R1 antagonists in decreasing alcohol drinking, we only observed transient effects with CP154526 and CP376395 chronic infusion, so future studies of chronic administration need to overcome the short-lasting behavioral effects. To date, this is the first set of experiments to investigate chronic icv infusions of CRF-R1 antagonists to affect alcohol drinking. Others have seen in voles that had lost their partners, chronic icv infusions of CP154526 reduced immobility time in the forced swim test (Bosch *et al.* 2008), but this was a single test, unlike our repeated testing of IAA. We also speculate that the lack of effect of CP154526 was caused by non-specific reductions in drinking due to the vehicle, DMSO. It is also possible that the minipumps did not deliver CP154526 consistently over the 14 days, but results with the water-soluble CP376395 confirm the short-lasting behavioral findings. With repeated testing, it is necessary to avoid compensatory changes with chronic naltrexone. Kaminski, Duke & Weerts (2012) found that naltrexone only reduces alcohol self-administration in the initial drinking bout, but when administered chronically, naltrexone did not decrease progressive ratio break points. To exclude the possibility that the minipumps had failed working, we tested long-lasting opioid receptor antagonism with a morphine-sensitive tail withdrawal test. Mice receiving chronic naltrexone withdrew their tails from the hot water even after 40 mg/kg morphine, suggesting MORs were still potently antagonized on day 13. We speculate that chronic naltrexone induced specific behavioral neuroadaptations such as the development of tolerance to the ethanol drinking effects of MOR antagonism, but not to other behaviors such as the analgesic effect of opiates. It will be useful to confirm the nature of the neuroadaptations that accompany chronic naltrexone and CRF-R1 antagonist treatment.

In addition to continued exploration of CRF-R1 treatments for excessive drinking, there are apparent future



directions with other endogenous opioid receptor systems, like targeting dynorphin/kappa-opioid receptor (KOR) systems to treat alcohol abuse and dependence (Walker *et al.* 2012). Stress and CRF each cause dynorphin-dependent KOR activation in DRN, suggesting KOR antagonists may be therapeutic for stress-related psychiatric disorders like alcoholism (Land *et al.* 2008). Similarly, naltrexone blocks both MOR and KOR, so it is possible that naltrexone exerts secondary effects on KOR to suppress the escalated component of IAA, in addition to the primary reinforcing effects of ethanol by inhibiting MOR. Altogether, there is an intricate balance between reward-related neuropeptides and stress-related neuropeptides. Perhaps these receptor systems are time sensitive in the transition to alcohol dependence, first opioid systems when naltrexone may be more helpful, then CRF-R1 systems when CRF-R1 antagonists may be more efficacious.

### Acknowledgements

The authors would like to acknowledge the research assistance of Jillian Tayeh, Allison Wilens, Danna NiSai and Johana Alvarez. We appreciate Elizabeth Holly for her technical aid with the HPLC and Dr. Lucas Albrechet-Souza for his assistance with histology. This research was supported by NIH funding R01 AA013983 (K.A.M.), F31 AA021622 (L.S.H.) and R01 MH058250 (R.J.V.).

### Disclosure/Conflict of Interest

The authors declare no competing financial interests.

### Authors Contribution

LSH, JFD and KAM were responsible for the experimental design. LSH, TK and KJN acquired the behavioral data while LSH and AS collected the neurochemical data. RJV performed the immunohistochemistry. LSH drafted the manuscript, and RJV, JFD and KAM provided critical revision and contributed to the interpretation of findings. All authors reviewed the content and approved the final version for publication.

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