**Increased Alcohol Consumption in Urocortin 3 Knockout Mice Is Unaffected by Chronic Inflammatory Pain**

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**Abstract — Aims:** Stress neurocircuity may modulate the relationship between alcohol drinking and chronic pain. The corticotropin-releasing factor (CRF) system is crucial for regulation of stress responses. The current study aimed to elucidate the role of the endogenous CRF ligand Urocortin 3 (Ucn3) in the relationship between alcohol drinking behavior and chronic pain using a genetic approach.

**Methods:** Ucn3 (KO) and wildtype (WT) littersmates were subjected to a 24-h access drinking procedure prior to and following induction of chronic inflammatory pain. **Results:** Ucn3 KO mice displayed significantly increased ethanol intake and preference compared with WT across the time course. There were no long-term effects of chronic pain on alcohol drinking behavior, regardless of genotype, nor any evidence for alcohol-induced analgesia. **Conclusion:** The increased drinking in Ucn3 KO supports a role for this peptide in alcohol-related behavior. These data suggest the necessity for more research exploring the relationship between alcohol drinking, chronic pain and the CRF system in rodent models.

**INTRODUCTION**

Alcohol Use Disorders (AUDs) affect 140 million people worldwide (World Health Organization) and are often comorbid with other pathologies, such as chronic pain. Approximately one in five people suffer from unrelenting pain for which there is no other pathologies, such as chronic pain. Approximately one in wide (World Health Organization) and are often comorbid with AUDs affect 140 million people worldwide (World Health Organization). Conversely, chronic pain disorders can develop following alcohol dependence (Katon et al., 1985). Furthermore, it has been demonstrated that there is a bidirectional relationship between family history of AUDs and chronic pain, such that the occurrence of one of these disorders can predict family occurrence of the other (Katon et al., 1985; Stewart et al., 1995; Goldberg et al., 1999). Despite compelling evidence for a relationship between pain and alcohol disorders, this connection has not been thoroughly investigated in preclinical models.

A wide body of research suggests that alcohol-induced neuroadaptations within stress regulatory systems contribute to the transition into alcoholism (Heilig and Koob, 2007; Breese et al., 2011; Ryabinin et al., 2012; Koob, 2013). Similarly, other pathological states including chronic pain are characterized by functionally altered stress neurocircuitry (Gianoulakis et al., 2003; Ji et al., 2007; Edwards and Koob, 2010; Rouwette et al., 2012). Therefore it is possible that aberrant stress neurocircuitry may modulate the relationship between alcohol drinking and chronic pain behaviors (see Egli et al., 2012). This modulation may be traced to the corticotropin-releasing factor (CRF) system, which plays an important role in the regulation of stress, AUDs and pain disorders. Within the CRF family, there exist multiple endogenous ligands (CRF and the Urocortins: Ucn1, Ucn2 and Ucn3) as well as multiple receptors (CRFR1, CRFR2) and the CRF binding protein (CRFβP). Ucn1 and CRF can bind to both CRFR1 and CRFR2, while Ucn2 and Ucn3 are primarily selective for CRFR2 (Lewis et al., 2001; Reyes et al., 2001). The ability of the multiple components within this system to act in concert to regulate behavior is a process that is not fully understood (Gravanis and Margioris, 2005; Giardino and Ryabinin, 2012; Janssen and Kozicz, 2013; Zorrilla et al., 2014). Nevertheless, previous research has demonstrated that central injection of Ucn3 decreases alcohol drinking in mice in limited access procedures (Sharpe and Phillips, 2009; Lowery et al., 2010). Additionally, drinking is decreased when Ucn3 is administered into the central nucleus of the amygdala of ethanol (EtOH)-dependent rats (Funk and Koob, 2007). These studies demonstrate that exogenously administered Ucn3, a peptide targeting CRFR2, is capable of regulating alcohol consumption. However, interpretation of the effects of exogenously administered Ucn3 is difficult, as these effects may be endogenously mediated by Ucn1, Ucn2, Ucn3 or even CRF acting on these receptors. This caveat necessitates investigation of the role of Ucn3 in alcohol consumption using non-pharmacological tools, for example, using transgenic or knockout technologies.

Another important function of the CRF system relates to pain and analgesia (McNally and Akil, 2002; Pu and Neugebauer, 2008; Ji and Neugebauer, 2008; Edwards et al., 2012). It has even been theorized that Ucn3 may be responsible for CRFR2 mediated pain inhibition within the amygdala, due to the lack of evidence for other endogenous ligands within this area (see Rouwette et al., 2012). Neuroanatomical evidence also points to potential involvement of Ucn3 in pain behaviors. Specifically, Ucn3 neurons and fibers are located in the rostral perifornical area of the hypothalamus, the periaqueductal gray and the posterior bed nucleus of the stria terminalis (Lewis et al., 2001; Li et al., 2002; Wittmann et al., 2009). All of these areas are involved in aspects of pain transmission and processing or innervate such areas (e.g.: amygdala). These converging lines of evidence highlight the potential involvement of Ucn3 in both alcohol and pain related processing. Thus, we aimed to elucidate the role of endogenous Ucn3 in the relationship between alcohol drinking behavior and chronic pain. To accomplish this aim, we examined chronic alcohol drinking during concomitant chronic inflammatory pain in Ucn3 KO mice with the hypothesis that Ucn3 KO would increase drinking and this effect may be modulated by the presence of chronic pain.
MATERIALS AND METHODS

Animals

Adult male and female Ucn3 knockout (KO) mice and wild type (WT) littermates were used in all experiments. These single gene mutant mice were generated by targeted deletion, as described in detail elsewhere (Li et al., 2007). Breeding pairs were generously provided by Drs. Wylie Vale and Mark Huising from the Salk Institute (La Jolla, California). These mice were generated on C57BL/6J × 129s background and backcrossed onto the C57BL/6J (B6) background for four generations. KO and WT mice were generated by crossing of heterozygous animals. Mice were genotyped and weaned at 28–32 days and isosexually housed 2–5 per cage. Between 30 and 33 weeks of age, mice were individually housed and transferred to the experimental room with a 12/12 reverse light/dark cycle for a 7-day acclimation period prior to the initiation of the experiments. All housing rooms were temperature and humidity controlled and food and water were available ad libitum. All protocols were approved by the Oregon Health & Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals, as well as the Guidelines for the Care and use of Mammals and Behavioral Research.

24 h access 2-bottle choice

EtOH solutions (v/v) were prepared in tap water from 95% ethyl alcohol. During the acclimation period, the mice were allowed 24-h access to two 25 ml glass cylinder bottles with metal sipper tubes (containing water) on either side of the cage, with food evenly spread across the cage top. Mice underwent 35 days of EtOH drinking, during which they received 24 h access to two bottles: one containing tap water and one containing increasing concentrations of EtOH (6–20%) dissolved in tap water. Six percent EtOH was available for 3 days, followed by access to 10% for 18 days and 20% for 14 days. All bottles were introduced and fluid levels were recorded on a daily basis between 1000 and 1100 h (2 h into the dark cycle). The locations of the bottles on the cages (left vs. right) were alternated daily to avoid the potential confound of an inherent side preference. All mice were sacrificed for BEC 2 h into the dark cycle on the final day of 20% drinking (immediately following the final von Frey test).

Mechanical testing and chronic inflammatory pain

Following acclimation to the experimental room, mice were habituated to the testing area for 40 min on 2 separate days. In addition, on each testing day, mice were allowed to acclimate to the testing rack for 20 min. The rack was located on a large table adjacent to home cages, within the experimental room. All testing and manipulation occurred 2 h into the dark cycle under a dimly lit red light. Following habituation, but prior to EtOH access, mice were tested for basal responses to punctate mechanical stimuli according to the up-down method (Chaplan et al., 1994). Briefly, the plantar surface of the animals left hindpaw was stimulated with a series of eight von Frey filaments (bending force ranging from 0.01 to 2 g). A response was defined as lifting or shaking of the stimulated paw. Beginning with the 0.4 g filament, a lack of response led to testing with a heavier filament (up), and in the presence of a response, a lighter filament (down) was attempted. This was repeated for a maximum of 4 filaments following the first response. In order to determine whether EtOH drinking altered mechanical thresholds, mice were tested again after 2 days of 10% EtOH access. To induce chronic inflammatory pain, on the fourth day of 10% EtOH, mice were removed from their cages, lightly anesthetized with 1–2% isoflurane and injected with 10 µl Complete Freunds Adjuvant (CFA; 1 mg Mycobacterium tuberculosis (H37Ra, ATCC 25177)/ml of emulsion in 85% paraffin oil and 15% mannnide monooleate – Sigma) in the intraplantar surface of the left hindpaw, which is known to reliably induce long lasting pain (Ren and Dubner, 1999). Induction of anesthesia and injection of CFA took 1–2 min, and recovery of mobility occurred within a minute post injection. Mice were then returned to their cages with access to EtOH and water. Following induction of pain, mice were tested weekly for mechanical von Frey thresholds for a total of 28 days, or four tests post-CFA.

Statistical analysis

EtOH consumption in ml was converted to grams (based on concentration) and divided by the animal’s body weight to give daily intake scores expressed in grams per kilogram (g/kg). EtOH preference was calculated by dividing EtOH consumption (ml) by total fluid consumption ml (EtOH ml + water ml). Total intake is expressed in milliliters per kilogram (ml/kg). Alcohol intake, preference and total intake were analyzed by repeated measures ANOVA examining sex (male, female) and genotype (WT, KO) as the between subject factors, and day or concentration as within subject factors. Blood ethanol content (BEC) and all basal mechanical thresholds were compared via one-way ANOVA. Weekly mechanical testing was also compared via repeated measures ANOVA with sex and genotype as the between subject factors and test session as within subject factor. For all analyses, significance threshold was set at P < 0.05. Data are expressed as mean ± standard error of the mean (SEM). Unless mentioned, all other effects and interactions were not significant (P > 0.05).

RESULTS

Body weights

Male mice weighed significantly more than female mice, and all bodyweights increased across time according to repeated measures ANOVA. Thus, there was a significant effect of sex (F(1,31) = 19.69, P < 0.0001) and day (F(34,1054) = 10.26, P = 0.0032). There were no other significant effects or interactions (Table 1).

EtOH intake and preference

As expected, EtOH intake was higher in females, and intake increased for both sexes as concentrations increased over time. Interestingly, both male and female KO mice drank slightly more than their respective WT controls (Fig. 1A and B). The effect of genotype was not confirmed when examined using day as the within subject factor, but was significant when examined using concentration as the within subject factor. Thus, a repeated measures ANOVA analyzing all days of the experiment only revealed an effect of sex (F(1,31) = 24.429, P < 0.0001), and day (F(34,1054) = 43.709, P < 0.0001), as well as an interaction between sex and day (F(34,1054) = 4.114,
Yet a repeated measures ANOVA examining intake across the three concentrations of EtOH indicated an effect of sex ($F_{(1,31)} = 27.455, P < 0.001$), concentration ($F_{(2,62)} = 126.631, P < 0.0001$) and genotype ($F_{(1,31)} = 5.523, P = 0.041$), and an interaction between sex and concentration ($F_{(2,62)} = 11.9, P < 0.0001$).

As with intake, EtOH preference was significantly increased in females compared with males and Ucn3 KO compared with WT. Additionally, preference increased in all groups across day and concentrations (Fig. 2A and B). When examining all days of the study, a repeated measures ANOVA revealed significant effects of sex ($F_{(1,31)} = 8.155, P = 0.0076$), genotype ($F_{(1,31)} = 19.803, P = 0.0001$) and day ($F_{(34,1054)} = 8.45, P < 0.0001$). Additionally, a when examining these variables across the three concentrations, a repeated measures ANOVA revealed a main effect of sex ($F_{(1,31)} = 19.54, P < 0.0001$), genotype ($F_{(1,31)} = 9.13, P = 0.005$) and concentration ($F_{(2,62)} = 9.85, P < 0.0002$) on ethanol preference, but no interactions between these variables.

All groups demonstrated a notable decrease in alcohol intake and preference for the 24 h following CFA injection (Figs 1 and 2), which likely contributed to the significant decrease in body weight in mice following CFA injection (Table 1).

### Table 1. Bodyweights of experimental animals

<table>
<thead>
<tr>
<th>GT</th>
<th>Sex</th>
<th>n</th>
<th>Pre-CFA Mean Body Weight</th>
<th>Post-CFA Mean Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Male</td>
<td>7</td>
<td>26.76 ± 0.96</td>
<td>26.10 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>21.57 ± 0.75</td>
<td>23.59 ± 0.91</td>
</tr>
<tr>
<td>KO</td>
<td>Male</td>
<td>7</td>
<td>25.29 ± 0.53</td>
<td>27.7 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11</td>
<td>22.00 ± 0.63</td>
<td>24.13 ± 0.41</td>
</tr>
</tbody>
</table>

Mice were weighed prior to acclimation to the room and following the completion of the final von Frey test on Week 5. Overall, females weighed less than males. There were no significant effects with genotype or interactions with genotype.
effect of day on this measure. However, this effect disappeared by 48 h, suggesting that chronic inflammatory pain does not alter alcohol intake or preference for alcohol.

**Total fluid intake**
There was a slight decrease in fluid intake over the course of the study, resulting in a main effect of day ($F(34,1054) = 9.964, P < 0.0001$). There were no other significant main effects or interactions when comparing the total intake levels of the groups (Fig. 3).

**Blood ethanol content**
It appeared that male KO had higher BECs than WT on the final test day, but this difference was not significant according to a one-way ANOVA including genotype and sex ($F(3,34) = 1.75, P = 0.177$; Fig. 4).

**Mechanical sensitivity**
Although male KO appeared to have higher mechanical thresholds than all other groups at baseline, this difference did not reach significance according to an ANOVA comparing genotype and sex ($F(3,34) = 2.5, P = 0.078$) (Fig. 5A). This tendency disappeared following access to EtOH, such that a one-way ANOVA demonstrated no differences in mechanical sensitivity according to genotype or sex ($F(3,34) = 0.95, P = 0.44$) (Fig. 5B).

CFA induced a similar level of pain in all groups and though pain dissipated over time, it was not altered by access to alcohol, suggesting a lack of EtOH-induced analgesia in all groups. In fact, a repeated measures ANOVA comparing the weekly test sessions revealed significant main effect of week ($F(5,155) = 35.03, P < 0.0001$), but there were no other main effects or interactions (Fig. 6).

**DISCUSSION**
In this study we provide the first evidence for the role of endogenous Ucn3 in long-term, moderate alcohol consumption. In general, Ucn3 KO mice had a significantly higher preference for alcohol and drank more than WT controls throughout the experiment. However, the pattern of this effect seemed to differ between the sexes. Although there was no statistical interaction between sex and concentration, it appears that males had a higher preference for alcohol during Weeks 2–4, or the last week of 10%, and first week of 20% access, whereas females preferred alcohol most strongly for the entire period of 20% access.

These results present another line of evidence supporting the role of Ucn3 in drinking behavior. Previous research has shown that injection of Ucn3 can decrease drinking behavior in non-dependent mice (Sharpe and Phillips, 2009; Lowery et al., 2010) and in alcohol-dependent rats (Funk et al., 2006). These previous studies used pharmacological techniques that
cannot delineate which endogenous CRF-related peptide is responsible for effects on alcohol drinking, whereas the current study investigated the role of endogenous Ucn3 in alcohol drinking using the genetic deletion approach. Although studies utilizing genetic mutations must be carefully considered, the direction of effects within our study and previous pharmacological studies are in agreement, which strongly suggests the observed effects are not due to developmental compensations.

The current findings also need to be considered in relation to the effect of this mutation on other phenotypes. Although no gross changes in these animals have been reported, it has been found that these mice can display increased resistance to glucose intolerance, hyperinsulinemia and hepatic steatosis following prolonged exposure to high fat diets (Li et al., 2007). However, since our mice were not exposed to a high fat diet, it is unlikely that these effects explain the observed drinking phenotype. Furthermore, an independently generated line of Ucn3 KO mice did not display any alterations except for an enhanced retention of social discrimination memory (Deussing et al., 2010). The latter caveat does not apply to the current studies, due to the fact that our mice were drinking in isolation.

Further considerations are that the current findings in the Ucn3 KO mice could be explained by differences in body weight, initial sensitivity to alcohol or alcohol metabolism in KO and WT mice. However, there were no significant differences in body weight when comparing genotypes at the
beginning or end of the study (Table 1). Additionally, the gradual development of this phenotype over time argues against any sensitivity differences. Finally, there were no differences between genotypes in BECs on the final day of EtOH access (Fig. 4), despite similar levels of drinking (Fig. 1A and B). This suggests that differences in alcohol metabolism do not explain the current findings. Interestingly, although females drank more than males, there were no significant differences in their blood ethanol levels at the time of sacrifice. This could be explained by the fact that blood was taken 2 h into the dark cycle, before peak drinking levels typically occur. Thus, if we had allowed the animals to drink for at least 4 h into the dark cycle, the pattern of drinking may have led to significantly higher BECs in females when compared with males. Additionally, females drank more but weighed significantly less than male mice (Table 1), therefore we cannot rule out the possibility that alcohol metabolism differs in female and male mice. In fact, previous research has demonstrated that the rate of alcohol disposition in male mice decreases with age compared with females, and this may account for differences in alcohol preference (Collins et al., 1975).

As predicted, CFA induced significant and long lasting mechanical pain. However, our studies did not support the role of Ucn3 in chronic inflammatory pain, as there were no differences in the pain behavior of KO and WT mice. This is in contrast to recent findings that Ucn3 may be modulated in certain inflammatory processes (Novembri et al., 2011; Pérez-García et al., 2011), and is located in brain areas relevant to pain modulation (Lewis et al., 2001; Li et al., 2002).

Interestingly, basal mechanical thresholds did not significantly change following alcohol access, suggesting a lack of alcohol-induced changes in mechanical sensation. Additionally, we did not find any interactions between pain and drinking. In fact, drinking was only altered following the first day following CFA treatment, and not in the expected direction. Specifically, there was a short-lived decrease in alcohol intake and preference in all mice in the first day of pain. Drinking behavior recovered within 24 h, and did not surpass pre-CFA drinking levels. Thus, the pain state of the mice did not increase alcohol intake or preference at any point in the study, suggesting a lack of alcohol-induced analgesia or alleviation of pain. This observation contrasts with extensive body of literature demonstrating analgesic effects of alcohol in humans (James et al., 1978; Saddler et al., 1985; Woodrow and Etherton, 1988) and evidence that chronic pain patients often self-medicate with alcohol (Riley and King, 2009).

Although the lack of a significant statistical interaction between CFA-induced pain and drinking suggests that presence of pain did not influence alcohol intake, we cannot completely dismiss this possibility. Nevertheless, it seems unlikely that pain is influencing alcohol intake or mediating the differences between the genotypes in the current study for a couple of reasons: (a) The primary differences in drinking between the genotypes do not occur during the peak level of CFA-induced pain, which lasts for 1–2 weeks before recovering (Millan et al., 1988; Chillingworth and Donaldson, 2003; Lu et al., 2008). In fact, in the current study, mechanical thresholds are only statistically decreased from baseline levels during the first two von Frey tests (or the first 14 days) and thus, Ucn3 KO mice appear to be drinking more during or following recovery from pain. (b) The overall level (g/kg) of drinking in the WT mice is similar to that of the typical C57Bl/6 mice in this type of paradigm (Crabbe, 1989; Belknap et al., 1993; Yoneyama et al., 2008). Although this fact, in combination with the lack of an obvious effect during peak pain suggests that pain did not influence drinking, the lack of additional control groups remains a limitation of the current study. Additionally, it is still possible that CFA treatment influenced the later drinking patterns of the KO mice specifically, and in the future it will be essential to carefully eliminate the potential effect of chronic pain on drinking behavior in Ucn3 KO mice. Finally, future replication of the increased drinking behavior in Ucn3 KO mice will be beneficial to understanding the current data set.

To date, only a limited number of animal studies demonstrate analgesic effects of alcohol (Gatch, 1999, 2006, 2009; Gatch and Lal, 1999). The lack of animal research in this area may be due to the inability to demonstrate alcohol-induced analgesia in a rodent model, as seen with the current results. One consideration is that mice may not be drinking enough to produce antinociception. In fact, previous rodent research showed that while high alcohol doses achieved by an injection or liquid diet can reverse withdrawal induced pain, this does not represent useful analgesia (Gatch, 1999, 2006; Gatch and Lal, 1999). On the contrary, in humans, alcohol can produce analgesia comparable to morphine (James et al., 1978). Another possible explanation for our findings is that alcohol-induced analgesia in rodents is highly sensitive to the timecourse and methodology used. For example, previous research utilizing ethanol-liquid diet exposure in rats found that alcohol-induced analgesia peaked at 2–4 days before dissipating (Gatch, 2009). Therefore the current study may not have captured the peak effect of this phenotype. Alternatively, there may have been developmental of tolerance to the analgesic effects of alcohol, which has been shown to develop following repeated testing in some cases (Jørgensen and Hole, 1984; Tiffany et al., 1987), but not in others (Gatch and Lal, 1999). Although the current study demonstrates a lack of alcohol analgesia following chronic drinking, we cannot rule out the possibility that alcohol induces analgesia in the rodent following acute intake. It is possible that acute and chronic effects of alcohol produce very different behavioral outcomes. In fact, it is known that abstinence from alcohol following chronic drinking can produce significant changes in pain sensitivity (Katon et al., 1985; Gatch, 2009) and that repeated administration of highly analgesic opiates often leads to hyperalgesia in animals (see Angst and Clark, 2006) and humans (see Lee et al., 2011). Thus, it is possible that the week of alcohol drinking prior to CFA treatment has already led to dysregulation within pain circuitry, and led to an alteration in the response to alcohol. The potential complexity of this phenomenon and the discrepancies in published research solidify the need for meticulous study of the time course and mechanisms of alcohol-induced analgesia. Future research will be essential to understanding the current findings regarding the lack of effect of chronic pain on alcohol drinking, as the current study was lacking certain control groups (water drinking mice and mice that did not receive CFA) necessary to clarify this.

In conclusion, we found that genetic deletion of Ucn3 leads to increased intake and preference for alcohol, as expected based on previous research and our theoretical understanding of the involvement of the Urocortin system in alcohol drinking. The direction of this effect is in contrast to that which is observed in male Ucn1 KO mice that can display decreased EtOH preference (Giardino et al., 2011) at lower EtOH
concentrations (6–10%). This fact suggests that there is a complex and orchestrated regulation of EtOH-related behavior by these two neuropeptides (capable of acting on CRFR2 receptors). Whereas Ucn3 is selective for CRFR2, Ucn1 has the capability of acting at CRFR1 and the CRFBP, and this, in combination with some anatomical variation in the expression of these peptides, may account for their unique effects on alcohol drinking. Although alcohol drinking has not been investigated in female Ucn1 KO mice, these mice do display a similar EtOH-induced conditioned place preference as male Ucn1 KO mice (Giardino et al., 2011), which implies that they may also drink less alcohol. Presently, it has not been determined whether Ucn3 KO mice have differences in Ucn1/2 peptide or CRF1/2 receptor expression, yet this could contribute to the current findings. Thus, current research suggests that the Urocortins may play differential roles in alcohol consumption based upon sex and the drinking procedures used, but this needs to be thoroughly investigated in future studies.

Finally, although induction of pain with CFA can lead to acute decreases in alcohol drinking, we did not find any long-term effects of chronic pain on alcohol drinking, regardless of genotype. Nor did we find any evidence for alcohol-induced analgesia. These findings highlight the necessity for further characterization of the relationship between alcohol and pain in rodent models in a variety of drinking procedures (e.g.: following other types of pain, or during withdrawal) in order to better understand the overlapping neural mechanisms responsible for these disorders.

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**Conflict of interest statement.** None declared.

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