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Research article

Alcohol amplifies cingulate cortex signaling and facilitates immobilization-induced hyperalgesia in female rats

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ABSTRACT

Complex Regional Pain Syndrome (CRPS) is a musculoskeletal pain condition that often develops after limb injury and/or immobilization. Although the exact mechanisms underlying CRPS are unknown, the syndrome is associated with central and autonomic nervous system dysregulation and peripheral hyperalgesia symptoms. These symptoms also manifest in alcoholic neuropathy, suggesting that the two conditions may be pathophysiologically accretive. Interestingly, people assigned female at birth (AFAB) appear to be more sensitive to both CRPS and alcoholic neuropathy. To better understand the biobehavioral mechanisms underlying these conditions, we investigated a model of combined CRPS and alcoholic neuropathy in female rats. Animals were pair-fed either a Lieber-DeCarli alcohol liquid diet or a control diet for ten weeks. CRPS was modeled via unilateral hind limb cast immobilization for seven days, allowing for the other limb to serve as a within-subject control for hyperalgesia measures. To investigate the role of circulating ovarian hormones on pain-related behaviors, half of the animals underwent ovariectomy (OVX). Using the von Frey procedure to record mechanical paw withdrawal thresholds, we found that cast immobilization and chronic alcohol drinking separately and additively produced mechanical hyperalgesia observed 3 days after cast removal. We then examined neuroadaptations in AMPA GluR1 and NMDA NR1 glutamate channel subunits, extracellular signal-regulated kinase (ERK), and cAMP response element-binding protein (CREB) in bilateral motor and cingulate cortex across all groups. Consistent with increased pain-related behavior, chronic alcohol drinking increased GluR1, NR1, ERK, and CREB phosphorylation in the cingulate cortex. OVX did not alter any of the observed effects. Our results suggest accretive relationships between CRPS and alcoholic neuropathy symptoms and point to novel therapeutic targets for these conditions.

1. Introduction

Chronic pain impacts over 20% of the population [1] and exacts a heavy toll on both physical and mental health [2]. This relationship appears to be particularly true for people assigned female sex at birth (AFAB; [3]). Complex Regional Pain Syndrome (CRPS) describes a post-traumatic neuropathic pain condition of the limb [4,5], and according to the McGill Pain Index is one of the most subjectively painful conditions known. CRPS disproportionally affects AFABs and often develops following limb injuries that require casting or limb immobilization. Extending beyond a simple somatic pain disorder, CRPS patients often suffer from a range of affective disorders, suggesting a dysregulation of higher emotional brain centers [6]. A recent examination found that 60% of CRPS patients suffered from depression while 18% misused alcohol or other substances [7]. Moreover, pain catastrophizing and other elements of pain-related negative affect contribute significantly to CRPS-related disability [8,9].

Many chronic pain patients report higher levels of alcohol drinking and suffer from alcohol use disorder (AUD) at rates higher than the general population [10,11]. Chronic pain and AUD are characterized by

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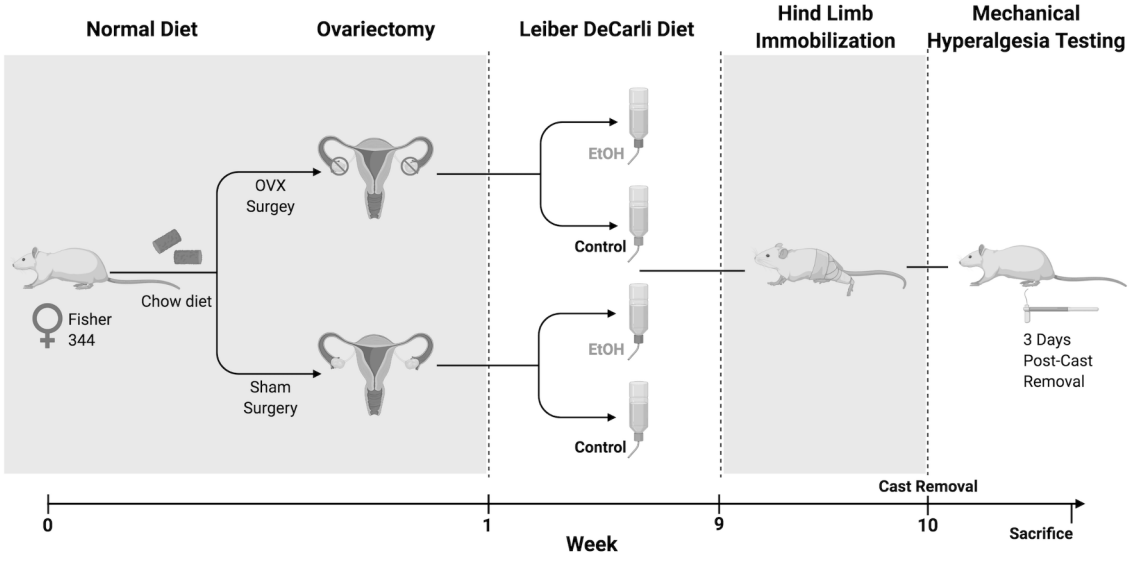
similar behavioral attributes, including cognitive dysfunction and enhanced negative affect [12,13]. The profound negative emotional state generated by chronic pain is proposed to increase the risk of development AUD [14]. Heavy alcohol drinking itself often exacerbates nociceptive hypersensitivity in both humans and animal models [15,16]. Self-reports of alcohol use with intention of pain management are common [17,18]. Moreover, problem drinkers not only report more severe pain symptoms compared to non-drinkers, but also report a higher incidence of using alcohol to manage their pain [19]. Such data also suggest that frequent drinking in the context of AUD may be motivated in part by a desire to alleviate enhanced nociceptive sensitivity, or hyperalgesia [20].

Strong evidence demonstrates that the neurobiological substrates associated with alcohol reward overlap considerably with the supraspinal substrates of the emotional aspects of pain processing [21]. Specifically, the affective component of pain appears to be strongly mediated by the cingulate cortex [22]. A recent role for the cingulate in the social transfer of pain has also been elucidated [23]. Various types of chronic pain are associated with specific neuronal plasticity in the cingulate cortex that closely associates with the affective or emotional dimension of pain, including increases in glutamatergic signaling [24], extracellular signal-regulated kinase (ERK) activity [25,26], and closely associated phosphorylation and transcriptional activation of nuclear cAMP response-element binding protein (CREB; [27]). Much less is known about how chronic alcohol drinking dysregulates the cingulate cortex, although increases in glutamatergic activity and ERK phosphorylation are known to manifest throughout the brain in animal models of AUD [28,29], indicating that this pathway may serve as a useful biomarker to interrogate how pain and alcohol interact to dysregulate nociceptive brain regions such as the cingulate.

To better understand the biobehavioral outcomes resulting from chronic alcohol drinking and limb immobilization, the objectives of the current study were to first examine mechanical nociceptive thresholds in a cast immobilization animal model of CRPS [30] in female rats exposed to an alcohol-containing diet. Half of the animals received ovariectomies (OVX) to determine the contributions of circulating ovarian hormones to



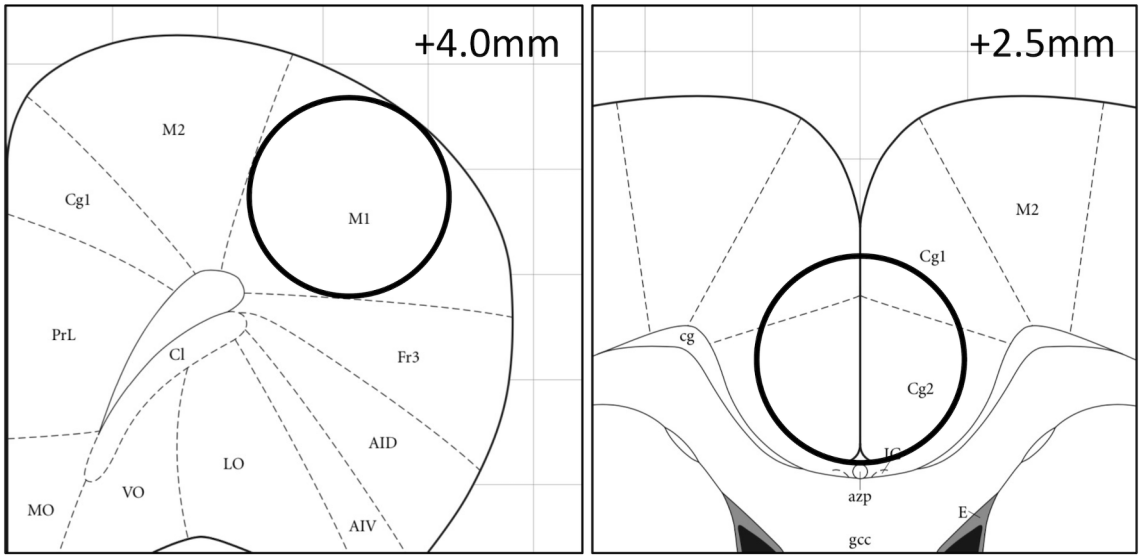


Fig. 1. Experimental timeline and regional brain dissections for Western analyses.

development of CRPS. We also investigated resultant neuroadaptations in glutamatergic subunit (GluR1 and NR1) and intracellular signaling protein (ERK and CREB) phosphorylation levels within the motor cortex (ipsilateral and contralateral to the casted limb) and the cingulate cortex, representing potential points of intersection between peripheral injury, central brain nociceptive processing, and alcohol-mediated plasticity.

2. Material and methods

2.1. Animals

Adult female Fischer 344 rats (n = 4–5/group), 3 months old upon arrival, were purchased from Charles River and single-housed in a temperature and humidity controlled vivarium. Rats were maintained on a 12-hour light/dark cycle throughout the duration of the study. Rats were given one week to acclimate to the colony room prior to the start of experimental procedures. All animal care, use, and procedures in this study were approved by Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center (LSUSHC) in New Orleans, LA, and were in accordance with the National Institute of Health guidelines. For experimental timeline, see Fig. 1. We utilized rats of the age typically employed in similar studies (e.g., [31]). It should also be pointed out that CRPS can affect individuals of any age, with age of onset ranging from as low as 18 to as high as 90 years of age [32].

2.2. Ovariectomy

At the start of the experiment, animals were randomly assigned to receive an ovariectomy (OVX) or sham surgery, as previously described [33]. Animals were anesthetized using ketamine/xylazine and were given a dose of slow-release buprenorphine prior to surgery. To perform the OVX, a bilateral skin and muscle incision were made and ovaries located in the fat pad were drawn out using blunt forceps. Ovarian blood vessels were ligated with 4–0 silk, the ovaries were excised, and skin and muscle wall incisions were sutured separately. Animals receiving sham surgery underwent a similar procedure but did not receive clamping or excision of the ovaries. All animals were allowed to recover for three days. Ovarian hormone loss was confirmed via uterine weight at the time of sacrifice.

2.3. Lieber-DeCarli liquid diet

One week following surgery, animals were randomly assigned to receive either an alcohol-containing (ethanol diet F1258SP; Bio-Serve, Frenchtown, NJ, USA) or a pair-fed control (control diet F1259SP; Bio-Serve) Lieber-DeCarli liquid diet for the duration of the experiment. Animals were transitioned onto the Lieber-DeCarli liquid diet over a five-day period of decreasing solid food and increasing the liquid diet. In a recent cohort of animals run in parallel with the current study (Levitt et al., 2020), administration of a Lieber-DeCarli diet produced average blood alcohol levels of 103.9 mg/dL (n = 7, SEM = 15.5), consistent with other studies utilizing this model to examine alcohol-related neuropathy [34].

2.4. Hind limb immobilization

Previous work has demonstrated that hind limb immobilization produces mechanical hypersensitivity, even in the absence of bone fracturing [30], similar to that reported in clinical studies [35]. All animals underwent unilateral hind limb immobilization 9 weeks following OVX surgery. Animals were anesthetized using ketamine/xylazine and hind limbs were casted using plaster of Paris as previously described [33]. The casted leg was randomized across animals such that there was an equal number of right and left casted legs. Animals were casted with slight hip flexion and knee extension, with dorsal aspects of the ankle and the feet left un-casted to allow for movement and unobstructed blood flow. The animals were immobilized for seven days and allowed to recover for three days prior to mechanical hyperalgesia testing.

2.5. Von Frey filament mechanical hyperalgesia testing

One day prior to sacrifice, mechanical sensitivity was determined by obtaining hind paw withdrawal thresholds, similar to what is described in [15]. Animals were first acclimated for 10 min to individual Plexiglas compartments set on top of a mesh stand. A series of nylon von Frey filaments were applied perpendicularly to the plantar surface of the hind paw until they buckled for 2 s, and a sharp withdrawal of the stimulated hind paw before or within the 2 s indicated a positive response. Testing was initiated with the filament corresponding to 15 g of force and continued in accordance with the up-and-down method [36]. The 50% paw withdrawal threshold was determined by the formula $Xf + k\delta$, where Xf = last von Frey filament used, k = Dixon value corresponding to response pattern, and δ = mean difference between stimuli.

2.6. Western blot analysis

Western analyses of brain tissue were performed as previously described [37]. Briefly, all animals were sacrificed by decapitation under light isofluorane anesthesia procedures that permit detection of brain protein phosphorylation levels. Brains were rapidly dissected, snap-frozen in −30 °C isopentane, and stored at −80 °C until microdissection, during which time three 0.5 mm thick regional punches were taken from coronal brain sections using a 13 gauge needle (motor cortex dissections ranged from 4.0 mm to 2.5 mm from Bregma and cingulate cortex dissections ranged from 2.5 to 1.0 mm from Bregma, Fig. 1). A homogenization buffer consisting of 320 mm sucrose, 5 mm HEPES, 1 mm EGTA, 1 mm EDTA, 1% SDS, protease inhibitor cocktail (diluted 1:100), and phosphatase inhibitor cocktails II and III (diluted 1:100) (Sigma, St. Louis, MO, USA) was added to brain punches prior to sonication to homogenize the tissue. Following sonication, tissue homogenates were heated to 90 °C for 5 min, and total protein concentration was measured using a detergent-compatible Lowry protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of tissue homogenates were loaded into 10% acrylamide gels and samples of protein were separated by SDS-polyacrylamide gel electrophoresis using a Tris/Glycine/SDS buffer system (Bio-Rad). Gels were electrophorectically transferred onto polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA), and following transfer, membranes were blocked for 1 h at room temperature in 5% non-fat milk. Primary antibody incubation for the following antibodies occurred overnight at 4 °C in 2.5% non-fat milk: phospho-ERK (1:5,000–1:10,000; Cell Signaling, Cat #9106), total ERK (1:5000–1:10,000; Cell Signaling, Cat #9102), phospho-CREB (1:20,000; Millipore, Cat #06-519), total CREB (1:20,000; Millipore, Cat #06-863), phospho-NR1 (Ser897) (1:5000; Millipore, Cat #ABN99), total NR1 (1:5000; Cell Signaling, Cat #5704), phospho-GluR1 (Ser845) (1:2000–1:5000; Cell Signaling, Cat #8084S), total GluR1 (1:2000–1:5000; Cell Signaling, Cat #13185S), and Tubulin (1:1,000,000; Santa Cruz Biotechnology, Inc., Cat #sc-53140). Membranes were then washed, incubated with species-specific peroxidase-conjugated secondary antibody (1:10,000; Bio-Rad) for 1 h at room temperature, washed again, incubated in Immobilon® Crescendo Western HRP substrate chemiluminescent reagent (MilliporeSigma, Cat #WBLUR0500) and exposed to film. After films were developed, membranes were stripped for 20 min at room temperature (Restore; Thermo Scientific) and re-probed for either total protein levels or Tubulin. Band immunoreactivity was detected using densitometry (Image J 1.45S, Bethesda, MD, USA). Densitized phosphoprotein values were normalized to either total protein densitometry values to generate phosphorylated:total protein ratio values or to loading control Tubulin if significant changes were observed in total protein between experimental groups. Densitized values are expressed as a percentage of the mean of

control (control diet-sham surgery) values for each gel to normalize data across blots.

2.7. Statistical analysis

All data were analyzed using Prism 8 (GraphPad Software, Inc.; La Jolla, CA, USA). Behavioral data was analyzed using a three-way ANOVA (alcohol × OVX × limb). Two-way ANOVAs (alcohol × OVX) were used to measure the effects of each treatment and their interaction on phosphoprotein and total protein levels. Pearson's *r* correlations and linear regressions were used to analyze the relationships between individual phosphoprotein levels and paw withdrawal thresholds. All data are presented as mean ± SEM. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Hind limb cast immobilization and chronic alcohol separately and additively produce mechanical hyperalgesia.

Both hind limb cast immobilization and chronic alcohol induced mechanical hyperalgesia as measured by the von Frey test, indicating that we have successfully modeled both CRPS and alcoholic neuropathy (Fig. 2). Three-way ANOVA indicated a significant main effect of alcohol ($F_{1,30} = 5.721$, $p = 0.0232$) and a significant main effect of casting ($F_{1,30} = 8.318$, $p = 0.0072$) to decrease mean paw withdrawal threshold. However, there was no effect of OVX ($F_{1,30} = 0.1548$, $p = 0.6968$) or alcohol × casting ($F_{1,30} = 0.9746$, $p = 0.3314$), alcohol × OVX ($F_{1,30} = 0.1836$, $p = 0.5595$), or alcohol × OVX × casting ($F_{1,30} = 0.2457$, $p = 0.6237$) interaction on mechanical hyperalgesia.

3.2. Hind limb cast immobilization-induced mechanical hyperalgesia correlates with ERK phosphorylation in the contralateral motor cortex

We have previously examined within-subjects correlations between protein phosphorylation status and pain behavior as a marker of neurobiological signaling mechanisms underlying pain states [38]. Utilizing this approach, we discovered a significant positive correlation ($r = 0.6211$, $p = 0.0045$) between ERK phosphorylation in the motor cortex contralateral to the casted limb and mean paw withdrawal thresholds of the casted limb (Fig. 3A). Conversely, there was no correlation ($r = -0.1020$, $p = 0.6777$) between ERK phosphorylation in the motor cortex contralateral to the non-casted limb and mean paw withdrawal thresholds of the non-casted limb (Fig. 3B). Because this correlation is only observed in the motor cortex corresponding to the immobilized limb, these data suggest reduced capacity for plasticity in association with immobilization-induced hyperalgesia.

3.3. Chronic alcohol increases total and PKA-phosphorylated levels of glutamate receptor channel subunits in the cingulate cortex

To determine if chronic alcohol in the context of CRPS altered excitatory signaling within the cingulate cortex, we first examined the phosphorylation status of glutamate receptors AMPA GluR1 and NMDAR1 at the serine 845 and 897 phosphorylation sites, respectively. Phosphorylation at these residues is specific to protein kinase A (PKA) and corresponds to increased AMPA and NMDA receptor currents and membrane trafficking. We observed a significant effect of alcohol to increase both phosphorylated and total levels of GluR1 ($F_{1,16} = 13.96$, $p = 0.0018$; $F_{1,16} = 5.157$, $p = 0.0395$) and NMDAR1 ($F_{1,16} = 7.502$, $p = 0.0146$; $F_{1,16} = 5.277$, $p = 0.0354$; Fig. 4). The observed increase in total GluR1 (Fig. 4B) and NMDAR1 (Fig. 4D) levels suggests that chronic alcohol produces an increase in GluR1- and NR1- containing AMPA and NMDA receptors in the cingulate cortex. However, when normalized to tubulin, we still observed an increase in receptor phosphorylation (Fig. 4A,C), suggesting an increase in receptor activity and glutamatergic signaling. There was no effect of OVX ($F_{1,16} = 0.06364$, $p = 0.8041$; $F_{1,16} = 0.3746$, $p = 0.5491$) or alcohol × OVX interaction ($F_{1,16} = 0.9300$, $p = 0.3492$; $F_{1,16} = 0.009589$, $p = 0.9232$) on phosphorylated or total levels of GluR1 or NMDAR1. Increases in phosphorylation of both AMPA GluR1 and NMDAR1 suggest an increase in excitatory glutamatergic signaling in the cingulate cortex following chronic alcohol use. Representative images are shown in Fig. 6A.

3.4. Chronic alcohol increases intracellular activity markers ERK and CREB in the cingulate cortex

We next investigated the phosphorylation status of intracellular and nuclear activity markers in the cingulate cortex. We first examined ERK phosphorylation as a general marker of activity and observed a significant effect of alcohol ($F_{1,16} = 5.649$, $p = 0.0303$) to increase ERK phosphorylation in the cingulate cortex (Fig. 5 A). We observed a similar alcohol effect ($F_{1,16} = 5.602$, $p = 0.0309$) on phosphorylation of the transcription factor CREB at the serine 133 site, which initiates target gene transcription (Fig. 5C). Alcohol also significantly increased total levels of ERK ($F_{1,16} = 5.694$, $p = 0.0297$, Fig. 5B), but not CREB ($F_{1,16} = 2.899$, $p = 0.1080$, Fig. 5D). Neither ERK phosphorylation or CREB phosphorylation were affected by OVX ($F_{1,16} = 0.00136$, $p = 0.9710$; $F_{1,16} = 0.5084$, $p = 0.4861$), and no alcohol × OVX interactions were observed ($F_{1,16} = 0.03742$, $p = 0.8491$; $F_{1,16} = 8.011e\text{-}005$, $p = 0.9930$). These findings indicate that chronic alcohol in the context of CRPS increases intracellular and nuclear activity within the cingulate cortex, further suggesting a widespread increase in cingulate cortex activity following chronic alcohol drinking. Representative western blot images are shown in Fig. 6.

4. Discussion

Heavy alcohol use and chronic pain are highly comorbid [39,40]. Moreover, chronic alcohol use is associated with increased risk of injury [41], and regular use of alcohol is frequently reported as an analgesic in both chronic pain and alcohol use disorder (AUD) patients [17,42]. The current study sought to develop and validate an animal model of combined CRPS and alcoholic neuropathy and identify associated

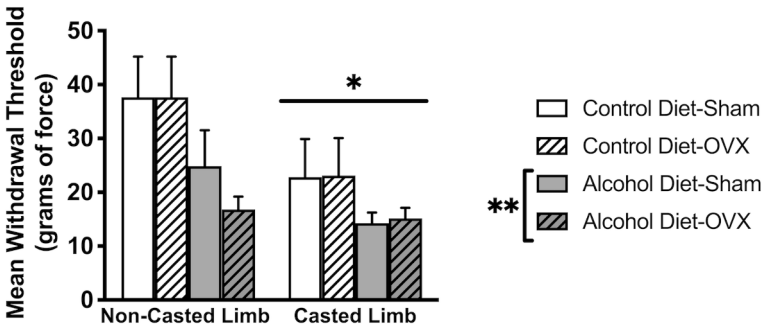


Fig. 2. Hind limb cast immobilization and chronic alcohol separately and additively produce mechanical hypersensitivity. There was a significant main effect of alcohol (p = 0.0072) and a significant main effect of casting (p = 0.0232) to decrease paw withdrawal thresholds. There was no significant main effect of ovariectomy (OVX) or significant OVX interactions. Data were analyzed using 3-way ANOVA. Data are represented as mean ± SEM. Control diet + sham surgery, solid white (n = 5); control diet + OVX, hatched white (n = 5); alcohol diet + sham surgery, solid gray (n = 5); alcohol diet + OVX, hatched gray (n = 4).

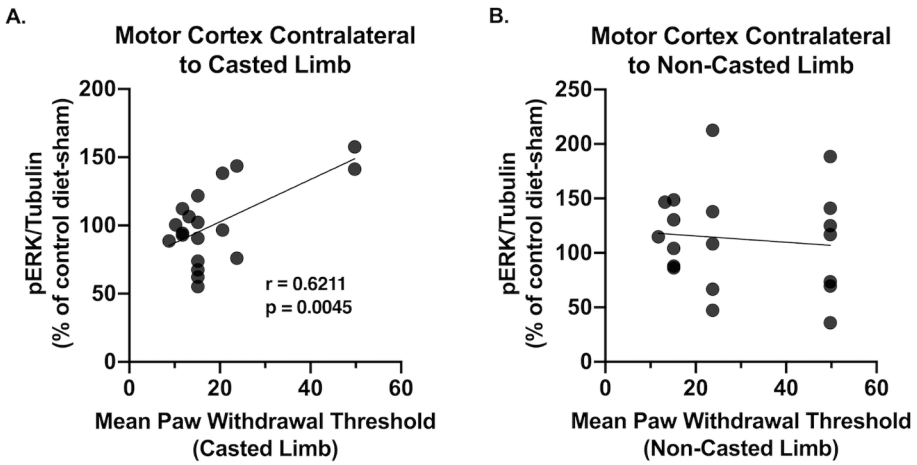


Fig. 3. (A) CRPS-induced mechanical hyperalgesia correlates with ERK phosphorylation in the contralateral motor cortex (r = 0.6221; p = 0.0045). (B) There is no correlation between ERK phosphorylation in the motor cortex contralateral to the non-casted limb and paw withdrawal thresholds in the non-casted limb. Data were analyzed using Pearson's linear regression. n = 4–5/group.

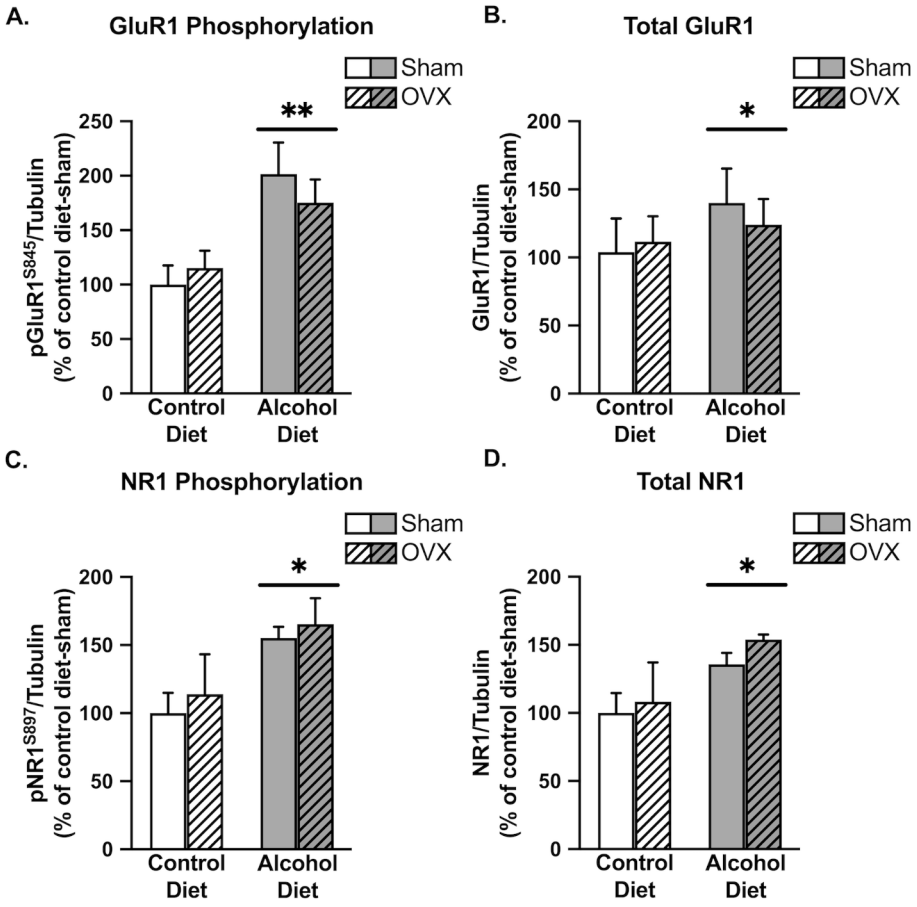


Fig. 4. Chronic alcohol increases both phosphorylated and total levels of glutamate receptor channel subunits in the cingulate cortex. There was a significant main effect of alcohol to increase GluR1 phosphorylation (A; p = 0.0018), total levels of GluR1 (B; p = 0.0395), NR1 phosphorylation (C; p = 0.0146), and total levels of NR1 (D; p = 0.0354). Control diet + sham surgery, solid white (n = 5); control diet + OVX, hatched white (n = 5); alcohol diet + sham surgery, solid gray (n = 5); alcohol diet + OVX, hatched gray (n = 4).

neuroadaptations in a key central pain-related brain area (the cingulate cortex). Of particular interest is the role of the cingulate cortex in organizing behavioral goals (such as avoiding pain) in the context of injury-associated nociception [43]. Evidence for this intersection in frontocortical regions, including the cingulate cortex, has been demonstrated in preclinical pain models [25,27]. Thus, we hypothesized that significant neurobiological interactions would exist between chronic alcohol drinking and pain within the cingulate as reflected by altered neuronal activity (indexed via both synaptic and intracellular protein phosphorylation measures). Dysregulated cingulate activity has also been described in relation to pain processing by CRPS patients [44–46]. Based on its regulation of sympathetic outflow [47–49], over-activation of the cingulate cortex may also underlie the characteristic autonomic symptoms of CRPS [50]. Indeed, increased cingulate cortex activity has been observed in several animal models of neuropathic pain [51,52]. Further, glutamate-mediated long-term potentiation (LTP) in the cingulate cortex and glutamate receptor neuroadaptations have also been observed in animal models of neuropathic pain and following

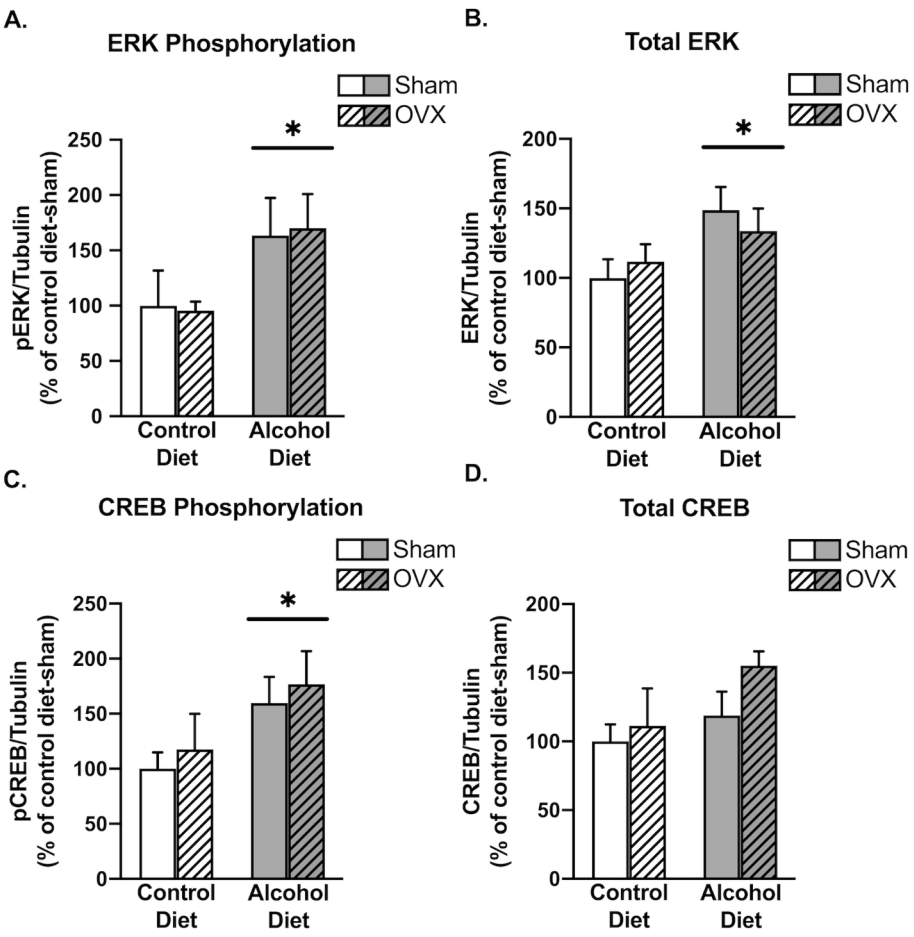


Fig. 5. Chronic alcohol increases intracellular activity markers in the cingulate cortex. There was a significant main effect of alcohol to increase ERK phosphorylation (A; $p = 0.0303$), total levels of ERK (B; $p = 0.0297$), and CREB phosphorylation (C; $p = 0.0048$). Alcohol did not increase total levels of CREB (D; $p = 0.1080$). Control diet + sham surgery, solid white ($n = 5$); control diet + OVX, hatched whte ($n = 5$); alcohol diet + sham surgery, solid gray ($n = 5$); alcohol diet + OVX, hatched gray ($n = 4$).

injury [53–55]. Interestingly, increased activity in the cingulate cortex also appears to serve as a risk factor for alcohol dependence [56]. Additional studies using this model are recommended to parse out neuroadaptations in excitatory (pro-nociceptive) versus inhibitory (anti-nociceptive) neurons within the cingulate cortex (e.g., by utilizing a c-Fos mapping strategy).

In addition to the observed change in the cingulate cortex, our results show a significant correlation between paw withdrawal thresholds and ERK phosphorylation levels in the motor cortex contralateral (but not ipsilateral) to the immobilized limb across all animals, indicating that hyperalgesia symptoms are linked to compromised ERK activity in the motor cortex. Although this may appear to conflict with clinical data suggesting decreased inhibition [57,58] and increased activation in the CRPS-corresponding motor cortex [59], a limitation of the current level of analysis (Western blot) is the inability of discriminating ERK phosphorylation changes in excitatory pyramidal neurons vs. inhibitory interneurons (or other cell types) within the motor cortex. Our previous study confirmed a reduction in corresponding quadriceps muscle mass with hind limb immobilization [33]. The current findings extend these functional deficits into associated motor centers in the brain. Interestingly, CRPS patients exhibit altered functional connectivity between motor and cingulate cortices that associate with neuropathic pain intensity [60].

CRPS and alcoholic neuropathy present with similar symptoms, including neurogenic inflammation, central and autonomic nervous system dysregulation, and peripheral hyperalgesia. While the bidirectional relationship between alcohol use and pain has been an intense area of recent research [18,61], alcohol use in the context of CRPS remains under-investigated. Our current findings are consistent with other studies describing the development of mechanical and thermal hyperalgesia in animal models of AUD [15,34,62]. Importantly, we observed hyperalgesia symptoms in animals continuously exposed to the Liber-DeCarli diet (even in the absence of alcohol withdrawal), consistent with previous studies [34]. Future studies should examine additional analgesic substances that may be frequently used by CRPS patients, including opioids. Interestingly, combined morphine and NMDA glutamate receptor antagonist treatment attenuates cingulate activation during movement of the affected limb in CRPS patients [63]. However, opioid therapy should be approached with caution as, similar to alcohol, chronic opioid use also gradually produces paradoxical hyperalgesia symptoms [15,38]. Collectively, our findings suggest that alcohol drinking worsens CRPS-induced pain, in line with clinician recommendations for CRPS patients to avoid alcohol consumption.

Future studies should also examine how alcohol-drinking levels are altered in the casting model of CRPS. A recent study of C57BL/6J male and female mice that were in a state of chronic inflammatory pain and given continuous access to alcohol reported that male (but not female) mice consumed significantly more alcohol in the context of pain [64]. Another study in male rats suggested that relationships between alcohol drinking and hyperalgesia symptoms are altered over the course of time in an inflammatory pain state [65]. In humans, a confluence of studies appear to indicate that pain may be more likely to increase drinking and relapse in those who have begun the transition to AUD [19,66–68]. Another transitory relationship exists between the analgesic efficacy of alcohol in humans relative to AUD status. While regular alcohol consumption is associated with reduced pain symptoms in most chronic pain sufferers [66,69], alcohol appears to increase pain and pain-related disability in problem drinkers and those with an AUD diagnosis [19,68,70]. Our findings warrant additional longitudinal studies examining how CRPS-related pain affects alcohol physiology in subjects

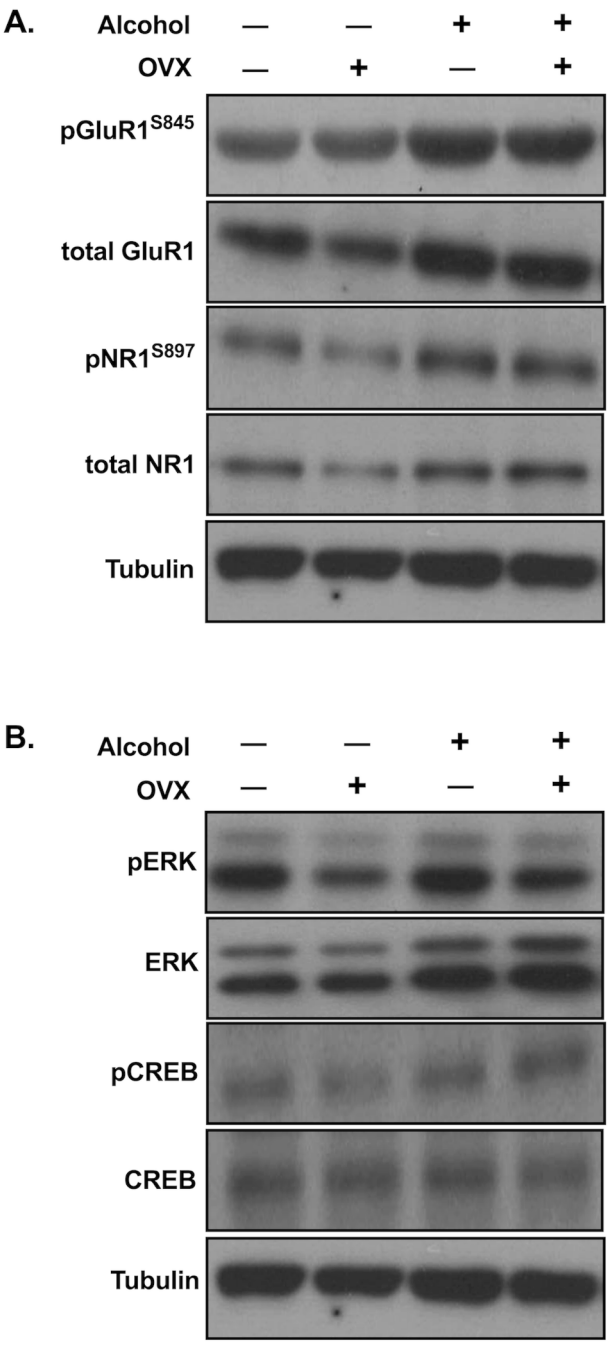


Fig. 6. Representative Western blots corresponding to Fig. 4 (A) and Fig. 5 (B).

transitioning from non-dependent to alcohol-dependent states to better understand how these relationships change over time.

A recent meta-analysis of alcohol-mediated analgesia in human subjects discovered a linear relationship between alcohol dose and analgesia, with blood alcohol levels that corresponded to binge-like alcohol exposure (0.08 g/dL) producing a clinically relevant reduction in pain intensity [71]. Administration of a Liber-DiCarli alcohol diet approximates this level of drinking over an extended period, producing an average blood alcohol concentration of 0.10 g/dL [33]. While alcohol produces reliable analgesia in rodent models [72], additional studies are necessary to determine the efficacy of alcohol to manage CRPS-related pain in either animal models or humans. However, binge alcohol exposure is also considered a primary risk factor for the eventual development of AUD based on its engagement of brain reward areas, including the cingulate cortex [73].

In addition to shared overlapping symptomology, CRPS and alcoholic neuropathy also disproportionately affect people assigned female at birth (AFAB). CRPS is two to four times more prevalent in AFAB people [74], and AFAB individuals display both higher rates and more severe symptoms of alcoholic neuropathy [75]. Further, AFAB individuals report higher incidence of chronic pain, greater sensitivity to painful stimuli, and more frequent pain compared to their male counterparts [76,77]. Additionally, AFAB individuals display more rapid progression from onset of drinking to dependence [78,79], greater rates of alcohol-related health consequences [80], and are the fastest-growing population of alcohol users in the United States [81]. Based on these sex differences, the present study investigated only female animals and utilized ovariectomy (OVX) to determine the effects of circulating ovarian hormones on mechanical pain sensitivity and underlying neurobiology. We originally hypothesized that OVX would facilitate hyperalgesia symptoms, since AFAB individuals suffering from CRPS exhibit reduced estradiol levels [82]. It is possible that hyperalgesia produced by limb immobilization and chronic alcohol drinking precluded the expected OVX effects on pain sensitivity. The absence of OVX-related factors in the current study may also suggest that non-sex hormonal factors may disproportionately predispose individuals to CRPS, including psychosocial determinants of pain sensitivity, brain organizational factors, and/or sex differences in inflammation and oxidative stress status following immobilization-related injury [83]. Indeed, various genetic and psychosocial factors have been shown to influence the development of pain in AFAB individuals [84–87]. The first and only study to date investigating the role gender identity on chronic pain found that transgender and cisgender women report similar pain summation and chronic pain severity, both of which were greater than their cisgender male counterparts [88]. This supports anecdotal evidence that gender identity plays a more significant role in pain than genetic sex. While the present study focused on an animal model of CRPS, these findings may also contribute to our understanding of other under-investigated pain syndromes that disproportionally affect AFAB individuals, as well as sex-dependent medication strategies for treating alcohol-induced hyperalgesia symptoms [89].

Some limitations of the current study should be pointed out. First, our measure of hyperalgesia was limited to von Frey analysis of mechanical hypersensitivity, and no morphological or electrophysiological measures were conducted to verify a neuropathic state. Future studies should examine additional pain modalities (e.g., thermal) as well as measures of unprovoked or spontaneous pain, such as pain-avoidance assays [90]. While the current design incorporated a within-subject control for the casted leg, future studies including a non-casted control could shed additional light on how alcohol drinking impacts CRPS-related symptoms. We did not track estrous cycle in the current study due to potentially confounding influences on the measured behavior. However, in relation to this, a case-control study of AFAB CRPS patients found no association between current or cumulative endogenous estrogen exposure and CRPS [91]. Finally, our study employed a relatively low number of animals. While we were able to detect clear main effects of some of the factors under investigation, future studies incorporating additional variables may require considerably more subjects to uncover valuable interactions.

5. Conclusions

To our knowledge, this is the first study investigating pain behavior and neuroadaptations in a combined model of CRPS and alcoholic neuropathy. We found that 1) CRPS and alcohol drinking separately and additively produced mechanical hyperalgesia, 2) immobilization-induced hyperalgesia is associated with a potentially altered capacity for motor cortex plasticity, 3) chronic alcohol drinking in the context of CRPS appears to facilitate cingulate cortex hyperexcitability, and 4) circulating ovarian hormones do not affect mechanical hyperalgesia or associated neuroadaptations in our model of combined CRPS and

alcoholic neuropathy. These findings suggest that the cingulate cortex may serve as a novel target for understanding the pathophysiology and treatment of these disorders.

CRediT authorship contribution statement

Jessica A. Cucinello-Ragland: Investigation, Formal analysis, Visualization, Writing - original draft. **Roshaun Mitchell-Cleveland:** Investigation, Formal analysis. **W. Bradley Trimble:** Investigation. **Amy Urbina Lopez:** Investigation. **Alice Y. Yeh:** Investigation. **Kimberly N. Edwards:** Investigation. **Patricia E. Molina:** Conceptualization, Investigation, Supervision, Resources. **Liz Simon Peter:** Conceptualization, Funding acquisition, Resources, Methodology. **Scott Edwards:** Conceptualization, Investigation, Supervision, Writing - original draft, Writing - review & editing, Resources.

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