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# Severity of Alcohol-Induced Painful Peripheral Neuropathy in Female Rats: Role of Estrogen and Protein Kinase (A and Cε)

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## Abstract

Small-fiber painful peripheral neuropathy, a complication of chronic ethanol ingestion, is more severe in women. In the present study, we have replicated this clinical finding in the rat and evaluated for a role of estrogen and second messenger signaling pathways. The alcohol diet (6.5% ethanol v:v in Lieber-DeCarli formula) induced hyperalgesia with more rapid onset and severity in females. Following ovariectomy, alcohol failed to induce hyperalgesia in female rats, well past its time to onset in gonad intact males and females. Estrogen replacement reinstated alcohol neuropathy in the female rat. The protein kinase A (PKA) inhibitor (WIPTIDE) only attenuated alcohol-induced hyperalgesia in female rats. Inhibitors of protein kinase Cε (PKCε-I) and ERK1/2 (PD98059 and U0126) attenuated hyperalgesia in males and females, however the **degree of attenuation produced by** PKCε-I was much greater in females. In conclusion, estrogen plays an important role in the expression of pain associated with alcohol neuropathy in the female rat. In contrast to inflammatory hyperalgesia, in which only the contribution of PKCε signaling is sexually dimorphic, in alcohol neuropathy PKA as well as PKCε signaling is highly sexually dimorphic.

### Keywords

Ethanol; Hyperalgesia; Estrogen; Second Messenger Signaling

## Introduction

The neurological effects of ethanol consumption are complex, encompassing both the central (Pentney and Quackenbush, 1990, Zou et al., 1993) and peripheral (Victor, 1975) nervous system. Although centrally alcohol acts as an analgesic (James et al., 1978, Woodrow and Eltherington, 1988), in the periphery it produces a small-fiber dying back painful neuropathy (Bosch et al., 1979, Dina et al., 2000). Over time, pain far outweighs analgesia, producing a

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neuropathic pain syndrome with symptoms that have been described as “like tearing flesh off the bones” (Brain and Walton, 1969).

In recent years, ethanol consumption in Western industrialized countries has increased among women with an associated increase in the rates of alcohol-related health problems **(Fillmore, 1987, Gomberg, 1993)**. Thus, understanding the basis of the more severe clinical expression of ethanol-induced neuropathy in females (Ammendola et al., 2000) is an issue of growing importance and, adequate treatment of this symptom may require therapeutic strategies tailored to gender-specific targets. We have evaluated, in a rat model, mechanisms that may contribute to the increased severity of symptoms in females with alcohol neuropathy.

## Experimental Procedures

### Animals

Experiments were performed on male and female Sprague-Dawley rats (Charles River, Hollister, CA, USA). Three-week (21-day) old female rats were ovariectomized and used in experiments 4 weeks later, when they were adults. In all other cases, same aged adult male and female (250–400g) gonad-intact rats were used. **At the commencement of ethanol diet, male and female animals were weight-matched. They** were housed individually in a controlled environment in the Animal Care Facility of the University of California, San Francisco, under a 12 h light/dark cycle. Care, **health, general condition** and use of **experimental** animals conformed to NIH guidelines. Experimental protocols were approved by the UCSF Committee on Animal Research. All efforts were made to minimize **both animal suffering and number used**.

### Chronic alcohol consumption

The protocol for feeding the alcohol-containing diet to rats has been previously described (Lieber and DeCarli, 1982, Lieber and DeCarli, 1989, Lieber et al., 1989, Dina et al., 2000). Briefly, all experimental rats were fed an ethanol-containing Lieber-DeCarli liquid diet (ED, 6.5% ethanol; Dyets Inc., Bethlehem, PA, USA) daily over a period of 12 weeks. The control (CD) rats were pair-fed (i.e. calorically-matched to the ethanol-fed rats) by giving them a diet in which equal calories of maltose-dextrin was consumed in place of ethanol (Lieber, 1995). The ethanol used in this study (190-proof ethyl alcohol) was obtained from Gold Shield Distributors, Hayward, CA, USA. Alcohol-containing diet or corresponding control diet was fed only to adult rats. Three-week old ovariectomized (Ovx) or estrogen-implanted ovariectomized (OvxE) female rats were fed standard laboratory chow until they were adults (i.e., 4 weeks after Ovx or OvxE). **Animals in all the experimental groups were healthy, and displayed normal reflexes and alertness. Ethanol diet-fed rats showed signs of normal grooming behavior, albeit, were more alert than naïve or control diet fed rats. Rats on alcohol diet were hyperactive - a response which was enhanced by sound stimuli - without apparent sexual dimorphism. No similar findings of hyperactivity were observed in control diet fed animals of either sex. Because the rats were pair-fed, rats fed Lieber-DeCarli liquid diet with ethanol (6.5%, v:v ethanol; ED) did not gain weight faster than those consuming control Lieber-DeCarli diet (CD) (data not shown, (Dina et al., 2000)).**

### Ovariectomy

Ovariectomy (Ovx) was performed on 21-day old rats (i.e., before sexual maturity) and the animals used for experiments 4 weeks later (i.e., as adults). The ovariectomy procedure was performed using bilateral, cutaneous, upper flank incisions to access the abdominal cavity (Wayneforth and Flecknell, 1992, Green et al., 1999). The ovaries were located, their vascular bundles tied off with 4-0 silk suture, and they were excised. The fascia was closed with 5-0 chromic gut and the cutaneous incisions closed with 7.5 mm metal wound clips. These

procedures were carried out under inhalational anesthesia ((2.5% isoflurane (Matrix, Orchard Park, NY, USA)) in 97.5% $O_2$.

## Administration of estrogen

Chronic administration of estrogen by subcutaneous implants, to a subset of Ovx female rats (OvxE) was performed as described previously (Smith et al., 1977, Green et al., 1999, Dina et al., 2001). Briefly, 10-mm-long segments of silastic® tubing (1.67 mm inner diameter x 3.18 mm outer diameter; Fisher Scientific, Santa Clara, CA, USA) were filled with 17β-estradiol (Sigma, St. Louis, MO, USA) and the ends of the implants capped with silastic plugs (Goodfellow, Berwyn, PA, USA). Implants were washed in absolute ethanol and equilibrated in four changes of warm phosphate-buffered saline over a 24-h period before placement in the rat. Implants were placed subcutaneously on the back at the time of ovariectomy to produce sustained levels of estrogen over an extended period of time (Smith et al., 1977). Implants were replaced **6 weeks after the first implants were inserted** and remained in place through the remainder of the experiment.

## Mechanical nociceptive threshold

The mechanical nociceptive flexion reflex was quantified using the Randall–Selitto paw pressure test (Randall and Selitto, 1957), which produces a force that increases linearly over time (Analgesymeter®, Stoelting, Chicago, IL), applied to the dorsum of the rat's hind paw, a protocol that has been used in alcohol-naive **(Taiwo et al., 1989, Dina et al., 2001, Dina et al., 2004, Hucho et al., 2006)**, alcohol-fed (Dina et al., 2000) and alcohol-withdrawing (Dina et al., 2006) rats. Animals were lightly restrained using cylindrical Perspex® restrainers designed to provide adequate comfort and ventilation, minimize restraint stress and accommodate size differences between individual rats. All experimental animals used in this study were acclimated to the testing procedure such that restraint and test techniques were parallel across groups. Briefly, animals were placed individually in the restrainers for 1 hour prior to the commencement of each study and for 30 minutes prior to testing on each test day (Dina et al., 2006). The nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three readings before alcohol-containing (or control) liquid diet was administered. Each paw was treated as an independent measure and each experiment was performed on a separate group of rats. **Rats in each experimental group were tested weekly in order to determine the change in nociceptive threshold in response to ethanol (ED) or control (CD) diet.** All behavioral testing was done between 10 am and 4 pm.

## Pharmacological interventions

Since sex-specific differences in second messenger signaling have been described in inflammatory hyperalgesia (Dina et al., 2001, Khasar et al., 2005, Hucho et al., 2006) and protein kinase C epsilon (PKCε), protein kinase A (PKA) and extracellular-signal related kinase (ERK) signaling pathways are known to mediate mechanical hyperalgesia (Dina et al., 2001), we determined the sexual dimorphism for the effects of a selective PKCε inhibitor peptide (PKCε-I; EMD Biosciences, La Jolla, CA, USA; (Johnson et al., 1996)), the PKA inhibitor Walsh inhibitor peptide (WIPTIDE; Bachem Biosciences Inc., San Carlos, CA, USA; (Dragland et al., 1985, Glass et al., 1989)) and ERK pathway inhibitors, PD98059 (2′-amino-3′-methoxyflavone; a selective inhibitor of mitogen and ERK kinase (MEK) (Kultz et al., 1998)) and U0126 (1,4-Diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene; a specific inhibitor of ERK 1/2 (DeSilva et al., 1998)) in alcohol-induced hyperalgesia. ERK pathway inhibitors were obtained from EMD Biosciences.

### Drug preparation and administration

WIPTIDE and PKCε-I were dissolved in distilled water; PD98059 and U0126 were dissolved in 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) and diluted in distilled water before use (final concentration of DMSO <1%). Stock solutions (1 μg/μl) of inhibitors were stored at −20°C. With the exception of estrogen, which was administered by subcutaneous implants, all injections were made by the intradermal route in the hind paw as previously described **(Taiwo et al., 1989, Dina et al., 2003). In the subsets of animals used in this study,** all inhibitors were injected at a concentration of 1 μg/2.5 μl **after alcohol-induced mechanical hyperalgesia had been established**. These **inhibitors** have been shown previously to produce a significant attenuation of mechanical hyperalgesia induced by inflammatory agents in male **(Khasar et al., 1999b, Dina et al., 2005)** and female (Dina et al., 2001, Khasar et al., 2005) rats. Because they are less membrane permeable, injections of the protein kinase inhibitors were always preceded by administration of 2.5 μl of distilled water in the same syringe to produce hypo-osmotic shock, thereby transiently enhancing cell membrane permeability **(Tsapis and Kepes, 1977, Widdicombe et al., 1996).** Protein kinase inhibitors were separated from the distilled water by drawing up a small air bubble (<1μl) into the syringe after drawing up the protein kinase inhibitor but before drawing up the distilled water; thus the distilled water was injected first, before the kinase inhibitor.

### Statistical analysis

Repeated measures ANOVA with between-subjects factors, as appropriate, was employed to compare the effects of interventions in experimental groups ($\alpha$=0.05). For each ANOVA the Mauchly criterion was used to determine if the assumption of sphericity for the within-subjects effects was met; if the Mauchly criterion was not satisfied, Greenhouse-Geisser adjusted *p* values were calculated.

## Results

### Onset of alcohol-induced hyperalgesia

To induce neuropathy, the Lieber-DeCarli liquid diet with ethanol (ED) or control Lieber-DeCarli diet (CD) was consumed by gonad-intact male and female rats, over a period of 12 weeks, a protocol that has been previously shown to produce mechanical hyperalgesia in male rats (Dina et al., 2000). Before the initiation of ED or CD, the mean baseline paw-withdrawal threshold of male rats (104.9±1.1 g; n=36) was greater than that of females (92.4±0.9 g; n=12) ($p < 0.05$; Fig. 1a). **Consumption of ED resulted in a reduction in mechanical nociceptive thresholds in both male and female rats (both $p<0.05$**, Fig. 1a and 1b). Repeated measures ANOVA with one within-subjects factor (time with 6 levels, including the beginning of the first week) and two between-subjects factors (diet with two levels, ethanol (ED) and control (CD), and sex with two levels, male and female) demonstrated a significant three-way (time × diet × sex) interaction ($F(5,400)=2.897$, **$p<0.003$). To determine the basis of this significant difference, 2 two-way repeated measure ANOVAs were performed, one for males and females receiving the ethanol diet, the other for males and females receiving the control diet. Rats receiving the ethanol diet demonstrated a significant time × sex interaction ($F(5,230)=3.932$, $P<0.006$) as well as a significant main effect of sex ($F(1,46)=8.624$, $p<0.005$), indicating that the hyperalgesic effect of the ethanol diet was significantly greater in females.** In both sexes, a significant decrease in mechanical nociceptive threshold was produced after 4 weeks on ED and was maximal at 12 weeks, when nociceptive threshold was 84.4±1.1 g (n=36) and 63.9±1.5 g (n=12) in males and females, respectively. However, beginning at 4 weeks, there was a significantly greater decrease in nociceptive threshold in female rats consuming ED, a difference that was sustained until week 12, the final week of the study (Fig. 1a). There was no significant change ($p>0.05$) from baseline in the paw-withdrawal threshold, in rats of either sex, consuming CD, in the same 12-week study period.

## Role of estrogen

Having established a significantly more rapid onset and greater magnitude of hyperalgesia in female rats consuming alcohol, we next examined the role of estrogen in determining the severity of mechanical hyperalgesia in females **(**Fig. 1b). In separate groups of female rats the effect of ovariectomy and estrogen replacement were studied. Prior to the consumption of ED, the baseline mechanical nociceptive threshold of ovariectomized female rats (114.7±1.8 g, n=12) was significantly higher **(*p*<0.05)** than that of gonad-intact (92.4±0.9 g, n=12) or estrogen-replaced ovariectomized (97.9±1.7 g, n=12) rats. Unexpectedly, even after 12 weeks on ED or CD there was *no* decrease ($p>0.05$) in the mechanical threshold of ovariectomized female rats (baseline:114.7±1.8 g vs. ED: 111.4±1.8 g, or CD: 112.2±2.2 g, all n=12). When estrogen was administered to ovariectomized female rats, the female phenotype was reconstituted and the paw-withdrawal threshold of the ovariectomized, estrogen-replaced females (97.9±1.7 g, n=12) closely approximated that in gonad-intact females (92.4±0.9 g, n=12). Compared to gonad-intact females, estrogen-replaced ovariectomized rats on ED did not differ significantly with respect to mechanical nociceptive threshold ($p>0.05$). **Two-way repeated measures ANOVA, with one within subjects factor (time with 6 levels) and one between subjects factor (estrogen status with three levels, intact, ovariectomized, and ovariectomized with estrogen supplement) demonstrated a significant time × estrogen status interaction ($F(10,165)=20.529$, $p<0.001$) and a significant main effect of estrogen status ($F(2,33)=41.520$, $p<0.001$).**

## Role of PKCε and PKA

In gonad-intact female rats we found that both PKCε–1 μg/2.5 μl) as well as WIPTIDE (1 μg/2.5 μl), injected intradermally at the site of nociceptive testing **after establishing alcohol-induced hyperalgesia** significantly inhibited alcohol-induced hyperalgesia ($p<0.05$, Fig. 2a and 2b). **The testing was performed by the 9$^{th}$ week of the study, on rats that were hyperalgesic by the 4$^{th}$ week.** The somewhat greater effect of inhibitors in estrogen-treated rats, may be due to slightly supraphysiological levels of estrogen. WIPTIDE also attenuated alcohol-induced hyperalgesia in estrogen-replaced female rats (Fig. 2a and 2b). However, as reported previously in the male rat (Dina et al., 2000), the PKCε inhibitor, but not WIPTIDE, attenuated alcohol-induced hyperalgesia (Fig. 2a and 2b, **respectively**). In addition, the magnitude of analgesia induced by PKCε inhibitor was greater in female rats. **In** Figure 2a, two-way ANOVA demonstrated a significant two-way interaction ($F(2,27)=21.379$, ***p*<0.001). Post-hoc one-way repeated measures ANOVAs showed a significant difference between pre- and post-PKCε-inhibitor administration responses for gonad intact females (IF; $F(1,11)=193.633$, $p<0.001$; n=12) and ovariectomized estrogen-implanted females (OvxE; $F(1,5)=101.104$, $p<0.001$; n=6), and for gonad intact males (IM; $F(1,11)= 7.106$, $p<0.03$; n=12). To determine if there was a significant difference in the effect of PKCε inhibitor between intact females and males, post-treatment scores were subtracted from the pre-treatment scores and a one-way between subjects ANOVA (difference with two levels, males and females) was performed. This analysis revealed that the effect of PKCε inhibitor was significantly greater for females than for males ($F(1,22) = 22.642$; $p<0.001$). In** Figure 2b, two-way ANOVA also demonstrated a significant two-way interaction ($F(2,27) =106.237$, ***p*<0.001) for the effect of the PKA-inhibitor, WIPTIDE. Post-hoc one-way repeated measures ANOVAs showed significant difference between pre-and post-WIPTIDE administration responses for intact females ($F(1,11)=298.820$, $p<0.001$; n=12) and ovariectomized estrogen-implanted females ($F(1,5)=161.252$, $p<0.001$; n=6), but not for males (n=12).**

### Role of MEK/ERK

Since previous reports have described a role for MEK/ERK signaling in inflammatory pain in male (Aley et al., 2001, Dina et al., 2003, Dina et al., 2005, Zhuang et al., 2005) and female (Dina et al., 2001) rats, we also evaluated the contribution of MEK/ERK to alcohol-induced peripheral neuropathy. We found that intradermal injection of PD98059 (1 μg/μl) and U0126 (1 μg/μl) **after establishing a state of hyperalgesia in alcohol-fed rats of either sex**, attenuated ED-induced hyperalgesia similarly in male and female rats (Fig. 2c and 2d), consistent with a comparable role for MEK/ERK signaling in chronic alcohol-induced hyperalgesia in rats of both sexes. **In** Figure 2c, two-way ANOVA demonstrated a significant two-way interaction ($F(2,21)=7.514$, $p<0.005$). **Post-hoc one-way repeated measures ANOVAs showed a significant difference between pre-and post-PD98059 administration responses for intact females ($F(1,5)= 65.649$, $p<0.001$; n=6) and ovariectomized estrogen-implanted females ($F(1,11)= 97.107$, $p<0.001$; n=6), and for males ($F(1,5)=110.100$, $p<0.001$; n=12). In** Figure 2d, two-way ANOVA also demonstrated a significant two-way interaction for the effect of U0126 ($F(2,15)= 4.382$, $p<0.005$) **while post-hoc one-way repeated measures ANOVAs showed significant difference between pre- and post-U0126 administration responses for intact females ($F(1,5)= 143.146$, $p<0.001$; n=6) and ovariectomized estrogen-implanted females ($F(1,5)=55.592$, $p<0.001$; n=6), and for males ($F(1,5)= 185.327$, $p<0.001$; n=6).**

## Discussion

Using a model of **painful peripheral** neuropathy induced by a**n ethanol-containing** diet (Dina et al., 2000), we have replicated in rats the human clinical finding that females develop a more severe painful alcoholic polyneuropathy than males (Ammendola et al., 2000). **The pain phenotype of alcohol neuropathy (Dina et al., 2000) is, at least in part, consequent to alcohol-induced primary afferent hypersensitivity in which there is a lowered mechanical threshold for C-fibers in ethanol diet-fed rats (Dina et al., 2000).** Ethanol fed female rats developed mechanical hyperalgesia more rapidly and the mechanical hyperalgesia was more severe. **This delayed onset and difference in magnitude of hyperalgesia, albeit statistically significant if not large, was sufficient to inform the need to analyze the intracellular mechanisms underlying hyperalgesia in male and female rats, which are markedly different.** To evaluate the role of sex hormones in determining the severity of symptoms in females, we evaluated the effect of ovariectomy with and without estrogen replacement. Quite unexpectedly, we observed a complete loss of the ability of the ethanol diet to induce hyperalgesia even up to a point in time well beyond that required to induce hyperalgesia in gonad-intact and male and female rats (Dina et al., 2000). That this dramatic phenotypic switch was estrogen-dependent was confirmed using estrogen-replaced ovariectomized female rats. Interactions between estrogen and alcohol-induced neurotoxicity have been reported. Thus, while estrogen tends to be neuroprotective in the central nervous system (Jung et al., 2005, Rewal et al., 2005), it can enhance toxic effects of alcohol in peripheral tissues (Bershtein et al., 2002, Enomoto et al., 2004). **Although it is conceivable that both estrogen and alcohol are major determining factors for consideration in interpreting the present data, it is known that ethanol exerts a direct neurotoxic action on the peripheral nervous system, resulting in a neuropathy that mostly involves small-diameter fibers (Diamond, 1994, Monforte, 1995, Kielhorn, 1996, Ortiz-Plata et al., 1998, Tredici et al., 1999). While, on the other hand, estrogen receptors have been described on DRG neurons (Sohrabji et al., 1994, Taleghany et al., 1999, Papka and Storey-Workley, 2002, Purves-Tyson and Keast, 2004), whether the effect of this sex hormone is direct or indirect has not been addressed by the present experiments, even with the demonstrated changes in second messenger signaling.** While the explanation for the *complete* loss of ED-induced hyperalgesia will have to await additional experiments, our findings do suggest that the incidence and severity of

alcohol-induced painful peripheral neuropathy may be less in post-menopausal women, which could lead to an underestimation of the severity of alcohol neuropathy in younger women. **While evaluation of fiber loss is beyond the scope of the present study, it is an interesting point to consider. In fact, pain is frequently an early manifestation of peripheral neuropathy, when anatomical changes are less likely to have occurred. Thus, elucidating the mechanism of the pain associated with peripheral neuropathy may provide insight into the mechanisms that produce the late manifestations such as nerve fiber loss.**

To evaluate the contribution of specific signaling pathways to alcohol-induced mechanical hyperalgesia we used selective inhibitors of different protein kinases. We confirmed our previous observation that PKCε mediates alcohol-induced hyperalgesia (Dina et al., 2000) in males. This resembles the important role PKCε also plays in mediating inflammatory hyperalgesia in male rats and mice (Khasar et al., 1999a). However, in female rats the role of PKCε is quite different. Here we observed a major contribution of PKCε to alcohol-induced hyperalgesia in females, whereas previously we found no role for PKCε in inflammatory hyperalgesia in female rats (Dina et al., 2001); only after ovariectomy did we observe an inhibitory effect of PKCε-I which was attenuated by estrogen replacement (Dina et al., 2001). **That the contribution of PKCε shown in the present study was significantly greater for females than for males not only contrasts with our findings for inflammatory-mediated hyperalgesia induced by $\beta_2$-adrenergic receptor agonists (Dina et al., 2001) but also by hyperalgesic priming (Joseph and Levine, 2003b) - a model of chronic inflammatory pain. In a study of diabetic neuropathy, the contribution of PKCε was similar in male and female rats (Joseph and Levine, 2003a). However, in a model of chemotherapy neuropathy, induced by vincristine, PKCε did not contribute to hyperalgesia in female rats (Joseph et al., 2003). Thus, sexual dimorphism in the contribution of PKCε may be dependent on the pain syndrome being studied.** Whether estrogen activates PKCε in females or recruits a PKCε-dependent pathway in female nociceptors remains to be determined. However we predict either is likely since estrogen stimulates translocation of PKCε in cultured DRG neurons and produces a PKCε-dependent hyperalgesia in male rats (Hucho et al., 2006).

We also observed a gender-specific effect of PKA on alcohol-induced hyperalgesia. In males the PKA inhibitor had no effect, whereas in intact females and in OvxE females it had a dramatic effect, reversing alcohol-induced in intact females and elevating the mechanical threshold in OvxE females. Of note, estrogen regulates PKA in other types of cells, and PKA in turn mediates several actions of alcohol (Martinez et al., 2003, Belcher et al., 2005, Sedej et al., 2005), including actions in the nervous system (Mize and Alper, 2002, Shingo and Kito, 2002, Shingo and Kito, 2005). The mechanism underlying the sexually dimorphic contribution of PKA **and** PKCε to pain associated with alcohol-induced neuropathy remains to be determined. **While we and others have described a significant role for ERK signaling in rodent models of inflammatory (Aley et al., 2001, Obata and Noguchi, 2004, Dina et al., 2005, Karim et al., 2006, Seino et al., 2006) or neuropathic (Ciruela et al., 2003, Ji, 2004, Ma and Quirion, 2005) pain, our present studies have determined for the first time a major role, albeit not sexually dimorphic, for ERK signaling in alcohol-induced neuropathy, the significance of which will await further investigations.**

In conclusion, the present experiments establish a model to study the mechanism underlying the greater severity of alcohol-induced neuropathy in females compared to males. Our results also raise several questions for future research to identify mechanisms that underlie: (1) sexual dimorphism in inflammatory versus alcohol-induced hyperalgesia, (2) the differential contribution of PKA to alcohol-induced neuropathy in males and females and, perhaps of most interest, and (3) the profound resistance of ovariectomized females to alcohol-induced peripheral neuropathy.

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## List of abbreviations

**ED**
: ethanol (6.5 %, v:v) diet

**CD**
: control diet

**PKA**
: protein kinase A

**PKCε**
: protein kinase C (epsilon, ε isoform)

**MEK**
: mitogen and ERK (extracellular-signal related kinase) kinase

**IM**
: gonad-intact male

**IF**
: gonad-intact female

**Ovx**
: ovariectomized female rat

**OvxE**
: ovariectomized and estrogen replaced female rat

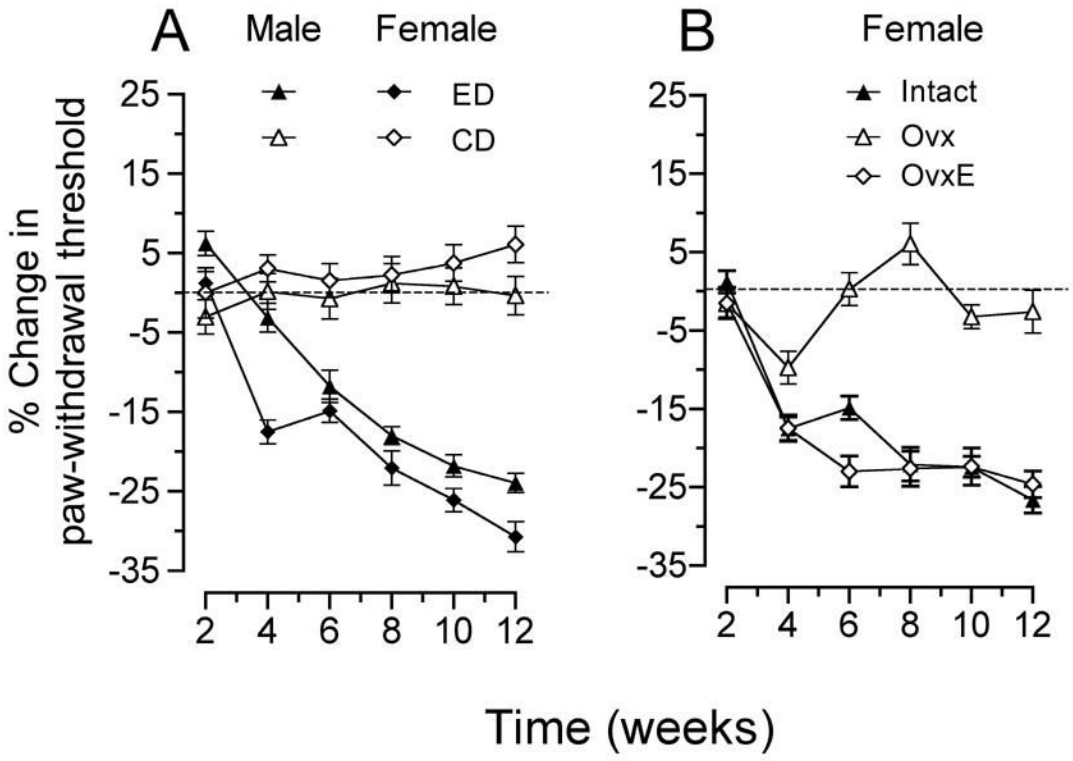


**Figure 1. Nociceptive effects of ethanol diet**

**(A)** Ethanol diet **(ED)** reduces the mechanical nociceptive threshold in male and female rats. Males and females receiving the control diet **(CD)** did not differ significantly. In this and subsequent figures data are plotted as mean ± standard error of the mean (s.e.m.). **(B) Ovariectomy (Ovx)** prevents ethanol diet-induced hyperalgesia in females. Scheffé post hoc analysis indicated that the ovariectomized group was significantly different from **the intact females and the ovariectomized group that received estrogen supplement (OvxE)** ($p<0.001$**,** in both cases), but the intact group was not significantly different from the **OvxE group**.

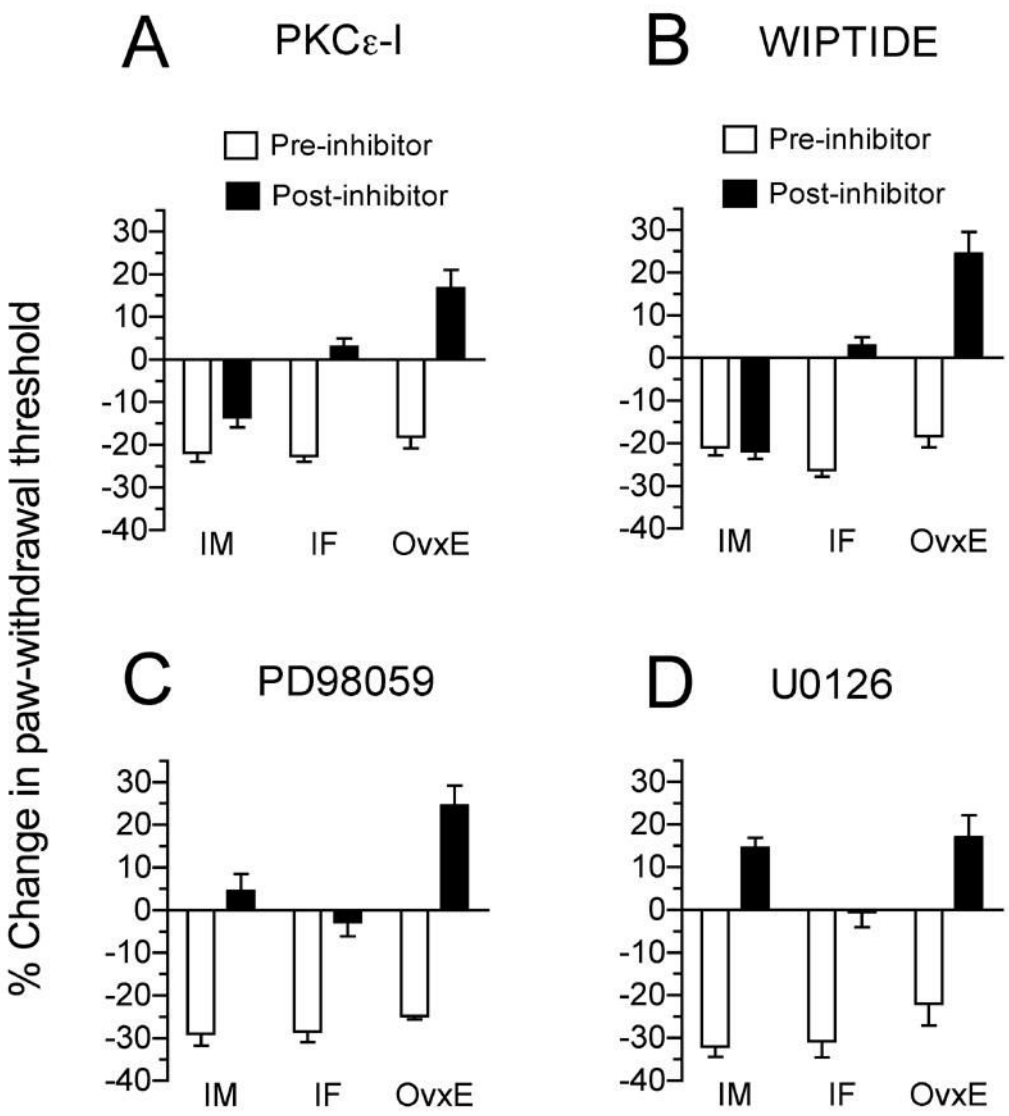


**Figure 2. Protein kinase inhibitors differentially modulate alcohol-induced hyperalgesia**

Baseline responses were measured **in intact males (IM), intact females (IF) and ovariectomized females with estrogen implants (OvxE)** prior to commencement of the ethanol diet; pre-inhibitor and post-inhibitor responses are plotted as percentage change from the baseline scores. Data were analyzed by two-way repeated measures ANOVA with one within-subjects factor (treatment with two levels, pre-inhibitor and post-inhibitor) and sex with three levels (males, females, and ovariectomized females plus estrogen). If there was a significant treatment × sex interaction, separate one-way repeated measures were performed for each sex to determine the basis of the significant differences. **Effect of the inhibitors, namely; PKCε, WIPTIDE, PD98059 and U0126 are shown in** Figures 2a, 2b, 2c and 2d, respectively. Statistical analysis and data are described in the results, as appropriate.