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Selective Chemical Genetic Inhibition of Protein Kinase C Epsilon Reduces Ethanol Consumption in Mice

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Abstract

Reducing expression or inhibiting translocation of protein kinase C epsilon (PKCε) prolongs ethanol intoxication and decreases ethanol consumption in mice. However, we do not know if this phenotype is due to reduced PKCε kinase activity or to impairment of kinase-independent functions. In this study, we used a chemical-genetic strategy to determine whether a potent and highly selective inhibitor of PKCε catalytic activity reduces ethanol consumption. We generated ATP analog-specific PKCε (*AS*-PKCε) knock-in mice harboring a point mutation in the ATP binding site of PKCε that renders the mutant kinase highly sensitive to inhibition by 1-tert-butyl-3-naphthalen-1-ylpyrazolo[3,4-d]pyrimidin-4-amine (1-NA-PP1). Systemically administered 1-NA-PP1 readily crossed the blood brain barrier and inhibited PKCε-mediated phosphorylation. 1-NA-PP1 reversibly reduced ethanol consumption by *AS*-PKCε mice but not by wild type mice lacking the *AS*-PKCε mutation. These results support the development of inhibitors of PKCε catalytic activity as a strategy to reduce ethanol consumption, and they demonstrate that the AS-PKCε mouse is a useful tool to study the role of PKCε in behavior.

Keywords

protein kinase C; alcohol; ethanol; 1-NA-PP1; *AS*-kinase

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Chemical compounds

1-tert-butyl-3-naphthalen-1-ylpyrazolo[3,4-d]pyrimidin-4-amine

1. Introduction

Alcohol use disorder (AUD) is highly prevalent and incurs great cost to society (Rehm et al., 2009). Despite this fact, there are currently only three drugs approved in the U.S to treat AUD: Disulfuram, naltrexone, and acamprosate (Johnson, 2008). Although all are effective, disulfuram is only useful for short-term treatment of highly motivated patients in supervised settings, while naltrexone and acamprosate suffer from compliance issues and small effect sizes (Johnson, 2008). Hence, there is considerable need to develop novel therapies to combat alcoholism.

Studies from our laboratory suggest that protein kinase C epsilon (PKCε) is a target for development of drugs to reduce ethanol consumption. Previous studies found that *Prkce*$^{-/-}$ mice drink substantially less ethanol than wild type mice (Hodge et al., 1999) and show heightened aversion to ethanol (Newton and Messing, 2007), possibly because of impaired acute functional tolerance to its ataxic and hypnotic effects (Wallace et al., 2007). These behaviors do not result from developmental changes since inducible transgenic expression of PKCε in the amygdala and striatum restores normal sensitivity to intoxication and increases drinking in *Prkce*$^{-/-}$ mice to levels observed in wild type mice (Choi et al., 2002). Moreover, knockdown of PKCε in the amygdala by RNA interference (Lesscher et al., 2009) or inhibition by a peptide designed to block translocation of activated PKCε (Cozzoli et al., 2015) reduces ethanol consumption in adult wild type mice. PKCε may modulate ethanol intoxication and consumption through phosphorylation of at least 2 substrates: $GABA_A$ γ2 subunits at Ser-327 (Qi et al., 2007) and the N-ethylmaleimide sensitive factor at Ser-460 and Thr-461 (Chou et al., 2010). However, all of the evidence implicating PKCε in behavioral responses to ethanol is derived entirely from genetic or shRNA-mediated reductions in PKCε expression or use of a peptide translocation inhibitor. The effect of selective, pharmacological inhibition of PKCε kinase activity with a small molecule inhibitor has not been tested. Such studies are needed to determine if PKCε is a viable drug candidate for the treatment of alcohol use disorder.

Unfortunately, there are no compounds currently available to selectively inhibit the catalytic activity of PKCε. To circumvent this problem, we have used a chemical-genetic approach to study kinase inhibition by selective, cell-permeable, small molecule inhibitors. The strategy targets the ATP-binding pocket conserved in all kinases, replacing a bulky gatekeeper residue with an alanine or glycine to generate mutant alleles that can utilize ATP analogs in addition to ATP, and that are uniquely sensitive to novel kinase inhibitors, such as analogs of PP1 (Bishop et al., 2001). We have generated such an ATP analog-sensitive PKCε (*AS*-PKCε) carrying the mutation M486A and have used it successfully to probe PKCε function in cell lines (Durgan et al., 2008, Qi et al., 2007). Here, we report the generation of an *AS*-PKCε knock-in mouse to examine the effects of PKCε on behavior. Using the *AS*-kinase inhibitor 1-Naphthyl-PP1 (1-NA-PP1) and *AS*-PKCε mice, we found that selective

inhibition of *AS*-PKCε prolongs the ataxic and hypnotic effects of ethanol and reduces ethanol consumption. These results are consistent with our previous findings in *Prkce*$^{-/-}$ mice (Choi et al., 2002, Hodge et al., 1999) and validate PKCε as a candidate for drug development, while demonstrating the utility of the *AS*-PKCε mouse as a useful tool for investigating the role of PKCε in behavior.

2. Materials and methods

2.1. Generation of AS-PKCε mice

Knock-in mice were generated by Caliper Discovery Alliances and Services (Hanover, MD). The Ensembl database was used to identify the BAC clone RP23-75J18 containing the genomic sequence of mouse chromosome 17 from nt # 86451480 to 86613962. This sequence includes the exon encoding *Prkce* M486. 5' arm (~1.9 kb) and 3' homology arms (~6.0 kb) were generated by PCR and cloned into the targeting vector pLoxNwCD, which contains a floxed neo expression cassette for positive selection and a DTA expression cassette for negative selection. The M486A mutation was introduced into the 5' arm by site-directed mutagenesis. The final vector was confirmed by restriction digestion and end sequencing analysis, and then linearized and electroporated into C57BL/6 ES cells. Approximately 192 ES clones that survived selection were screened using a 5' external probe and 4 clones were expanded. Southern analysis of the ES cell DNA using 5' external, 3' external and neo cassette probes identified three correctly aligned clones with a single neo insertion. Two were transfected with Cre recombinase and one was confirmed to be neo deleted by PCR. Presence of the mutation in that clone was confirmed by PCR and sequencing. These ES cells were injected into tyrosinase deficient blastocysts and transplanted into pseudo-pregnant mice. Germ line transmission of the mutation from chimeras was confirmed by PCR using the following primers; CAGCACGGAGTGATCTACAGGTATTCTC (forward primer) and CGGACACAAACAGCAGGTCAAATCT (reverse primer). Heterozygous mutant progeny were then intercrossed to generate homozygous *AS*-PKCε mice, which were subsequently intercrossed and maintained as an inbred line on a C57BL/6NTac background. Mice used for experiments were housed under a reverse light dark cycle (lights off at 10AM; lights on at 10PM). Only male mice were tested so that we could compare results with prior studies that used male *Prkce*$^{-/-}$ mice (Choi et al., 2002, Hodge et al., 1999). All procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 2011) and were approved by the Institutional Animal Care and Use Committees of the Ernest Gallo Clinic and Research Center and the University of Texas at Austin. All efforts were made to minimize animal suffering and to reduce the number of animals used in experiments.

2.2. Administration of 1-NA-PP1

1-NA-PP1 was obtained from Dr. Kevin Shokat (UCSF) or from Tocris Biosciences (Bristol, UK). For ethanol, saccharin, and quinine consumption studies, we dissolved 1-NA-PP1 in 100% DMSO at 20 or 30mg/ml and then diluted it 20-fold in deionized water containing 10% Tween-80 with sonication. For studies using oral administration, we prepared 1-NA-PP1 as a 100mM stock solution in 100% DMSO by gentle heating and sonication. This

stock was diluted to 500μM in water containing 1% cremophor-RH40 (Sigma-Aldrich, St. Louis, MO) and 2g/L sucralose (Sigma-Aldrich) to increase palatability. Control animals received an equivalent amount of DMSO vehicle in cremophor-sucralose-water. 1-NA-PP1 food pellets (1g/kg) were obtained from Research Diets (New Brunswick, NJ). Control food pellets contained an equivalent amount of vehicle (DMSO). To determine the effects of 1-NA-PP1 on protein phosphorylation, we dissolved 1-NA-PP1 in vehicle containing 5% DMSO and 20% Cremophor EL (Sigma-Aldrich).

2.3. Western blot analysis

Animals were sacrificed with CO_2 asphyxiation or cervical dislocation and the amygdala and striatum were rapidly dissected on ice. Brain regions were homogenized with a glass Dounce homogenizer using 20 strokes in 0.1-0.5ml of ice-cold extraction buffer (25mM HEPES –pH7.8, 300mM NaCl, 1.5mM $MgCl_2$, 1% Triton X-100, 0.1mM DTT) containing Phosphatase Inhibitor 1 and 3 (Sigma-Aldrich) and Protease inhibitor Complete™ (Roche Diagnostics USA, Indianapolis, IN.). Lysates were clarified by centrifugation at 10,621×g for 15 minutes at 4°C and then resolved by SDS-PAGE using 4–12% gradient gels (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes which were blocked with Tris buffered saline (TBS: 50mM Tris, pH 7.6, 150mM NaCl) containing 0.1%Tween-20 (TBS-T) and 5% BSA. The blots were incubated with anti-phospho-$GABA_A$ γ2-S(P)327 antibody (Qi et al., 2007) at 1:1000 dilution in 5% BSA overnight at 4°C. Blots were washed 3 times in TBS-T, 8 minutes per wash, incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA; 1:1000 in 5% nonfat dry milk) for 1h at room temperature, washed again, and visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher, Waltham, MA). Immunoreactive bands were quantified by densitometric scanning using Image J (Schneider et al., 2012). Blots were stripped and re-probed with total $GABA_A$ γ2 antibody (Alamone labs, Jerusalem, Israel; 1:1000 dilution) or GAPDH (Cell Signaling, Danvers, MA; 1:10,000 dilution).

2.4. Behavioral screen

We examined mice for morphological abnormalities, startle response, righting reflex, and body weight (Crawley, 2008). Strength was measured using the hanging wire test. Motor learning and coordination were assessed as in prior work (Lee et al., 2013) using a rotarod treadmill (AccuRotor Rota-Rod; Omnitech Electronics, Columbus, OH) that accelerated from 0 to 40 rpm in 5 min. Locomotor activity was recorded as the distance traveled in an open field chamber over 60 min (Hodge et al., 1999). Anxiety-like behavior was measured using an elevated plus maze as in previous work (Hodge et al., 2002). Thermal sensation was tested using a tail-flick apparatus (Columbus Instruments, Columbus, OH).

2.5. Ethanol, saccharin, and quinine consumption

Continuous access two-bottle choice drinking was performed as described previously (Lim et al., 2012). Once a stable level of drinking of 10% ethanol was achieved, mice were habituated to 3 intraperitoneal injections of vehicle. The effects of 1-NA-PP1 on drinking were studied using a within-subjects design, under which each animal received vehicle, 20, or 30mg/kg 1-NA-PP1 on different days. 1-NA-PP1 was administered 5-10 min before the

onset of the dark cycle. The amount of ethanol and water consumed was monitored for 48 hours after injection. Ethanol preference was calculated by dividing the amount of ethanol-containing solution consumed by total fluid intake. Mice were allowed to recover for 1-2 days between doses of 1-NA-PP1.

Following the ethanol consumption procedure mice were tested for two-bottle choice saccharin (0.03% then 0.06%) and quinine (0.015, 0.03 and 0.06mM) consumption. Each concentration of saccharin was presented for 4 or 5 days. After 2 days of exposure to 0.06% saccharin, mice were habituated to 2 intraperitoneal injections of vehicle. The effect of 1-NA-PP1 on saccharin consumption was tested using a within-subjects design as outlined for the ethanol consumption study. Mice were then allowed to consume water containing increasing concentrations of quinine. After exposure to 0.06mM quinine for 2 days, mice were habituated to 2 vehicle injections and then administered 1-NA-PP1 in a within-subjects design.

2.6. Responses to acute ethanol administration

Ethanol-induced ataxia was evaluated as described (Wallace et al., 2007) with the rotarod treadmill set to a fixed speed of 6 rpm. The ethanol-induced loss of the righting reflex was examined as described in prior work (Choi et al., 2002, Hodge et al., 1999, Lee et al., 2014, Wallace et al., 2007).

2.7. Ethanol clearance

Mice were administered 4g/kg of ethanol intraperitoneally and the 20μl of blood were obtained via tail puncture at 30, 60, 90, 120, and 180 min post-injection. Blood samples were stored at −80°C, until BECs were determined using an NAD-ADH enzymatic assay (Carnicella et al., 2009).

2.8. Statistical Analysis

Data were analyzed using Prism 6.0e (GraphPad Software, La Jolla, CA). Data were expressed as mean ± SEM values and analyzed by two-tailed *t*-test or repeated measures ANOVA with *post-hoc* Dunnet's multiple comparisons test as appropriate.

3. Results

3.1. Generation of AS-PKCε mice

AS-PKCε mice were generated by altering the sequence of exon 11 in the *Prkce* gene to encode the M486A mutation (Fig. 1A and B). Homozygous *AS*-PKCε and wild type C57BL/6N mice showed similar abundance and pattern of PKCε immunoreactivity in the brain (Figs. 1C and D). *AS*-PKCε mice were like wild type littermates in appearance and home cage behavior, and showed a similar startle response and righting reflex. They were also like wild type animals in other behaviors including open field exploration, anxiety like-behavior on the elevated plus maze, hot plate tail-flick latency, ability to remain on an accelerating rotarod, and ability to hang suspended from a wire (Table 1). These results indicate that the PKCε M486A knock-in mutation did not disrupt development or baseline behavior.

3.2. Pharmacokinetics of 1-NA-PP1

To determine the abundance and half-life of 1-NA-PP1 in plasma and brain, a pharmacokinetic study was performed following intraperitoneal administration of 30mg/kg 1-NA-PP1 in 5% DMSO and 10% Tween-80 to wild type C57BL/6J mice (Fig. 2A). Plasma levels of 1NA-PP1 reached 7.3 ± 0.43μM thirty minutes after injection and declined biphasically (R^2= 0.94) with half-lives of 0.47 and 11.62 hours (Fig. 2A). Brain levels reached 2167 ± 85 ng/g (~6.8 ± 0.27μM) one hour after injection and declined in a single-phase (R^2 = 0.93) with a half-life of 0.57 hours (Fig. 2B). These results indicate that 1-NA-PP1 enters the brain rapidly and efficiently after intraperitoneal administration and achieves concentrations predicted to inhibit *AS*-PKCε (*K*i = 18.7nM) based on *in vitro* studies (Qi et al., 2007).

Plasma and brain concentrations of 1-NA-PP1 were also determined following repeated oral administration. Wild type C57BL/6N mice were provided food pellets containing 1g/kg 1-NA-PP1 and water containing 500μM 1-NA-PP1 in 1% Cremophor-RH40 and 0.2% sucralose. Control animals were fed food and water containing the corresponding vehicles. Mice were sacrificed after 3 days and the concentration of 1-NA-PP1 was determined by LC-MS/MS. Oral administration of 1-NA-PP1 yielded a plasma concentration of 117 ± 23nM (n=5) and brain concentration of 140 ± 54ng/g protein (~ 441 ± 172nM; n=5). These results indicate that repeated administration of 1-NA-PP1 in food and water leads to levels of 1-NA-PP1 in the brain and plasma predicted to inhibit *AS*-PKCε (Qi et al., 2007).

To determine whether systemic administration of 1-NA-PP1 inhibits *AS*-PKCε-mediated phosphorylation in the brain, we examined phosphorylation of the $GABA_A$ receptor γ2 subunit since we previously found that PKCε phosphorylates this subunit at S327 (Qi et al., 2007). We administered 1-NA-PP1 by intraperitoneal injection rather than orally in this experiment to better control the dosage relative to the timing of tissue collection. *AS*-PKCε mice were administered 25mg/kg 1-NA-PP1 or vehicle and sacrificed 1 hour later. Although we used a different vehicle (5%DMSO/20% Cremophor-EL) to dissolve 1-NA-PP1 for this experiment, pharmacokinetic analyses after intraperitoneal injection of 30mg/kg 1-NA-PP1 in this vehicle revealed plasma (6.47 ± 0.25μM; n = 2) and brain concentrations (2055 ± 455ng/g; ~4.43 ± 2.03μM; n = 2) similar to those observed for 1-NA-PP1 dissolved in 5%DMSO/10% Tween-80. Compared with vehicle-injected mice, there was a 33% reduction in γ2-S(P)327 phosphoimmunoreactivity in the striatum of 1-NA-PP1-treated mice (Fig. 3).

3.3. 1-NA-PP1 reduces ethanol consumption by AS-PKCε mice

To determine whether 1-NA-PP1 alters ethanol consumption, we subjected *AS*-PKCε mice to a continuous access, two-bottle choice-drinking procedure whereby the ethanol concentration was escalated from 3% to 6%, and finally to 10% over 8 days. After mice were habituated to vehicle injections and had attained a stable level of drinking 10% ethanol for three consecutive drinking sessions [$F(2, 34) = 1.474$, $P = 0.2433$; Fig. 4A], they were administered 1-NA-PP1 using a within-subjects design in which all animals received vehicle or 1-NA-PP1 on different days. 1-NA-PP1 at 20 or 30mg/kg reduced ethanol consumption during the first 24 h [$F(2, 34) = 10.69$; $P = 0.0003$; Fig. 4B]. This effect was reversible since ethanol consumption was similar 48 h after treatment with vehicle or 1-NA-PP1 [$F(2, 34) =$

3.058; $P = 0.0601$; Fig. 4C]. 1-NA-PP1 did not significantly alter ethanol preference [F(2, 34) = 0.9508; $P = 0.3965$; Fig. 4D]. Although there was a trend towards reduced water consumption at 30mg/kg, this effect was not statistically significant [F(2, 34) = 1.722; $P = 0.1940$; Fig. 4E].

To determine whether 1-NA-PP1 alters taste perception, we examined its effect on consumption of saccharin- and quinine-containing solutions. 1-NA-PP1 at 30 mg/kg significantly reduced saccharin consumption [F(2, 26) = 11.22; $P = 0.0003$; Fig. 4F], but did not alter the amount of quinine consumed [F(2, 26) = 0.099; $P = 0.906$; Fig. 4G]. These results suggest that at 30mg/kg, 1-NA-PP1 affects perception of sweet, but not bitter taste.

To examine the possibility that 1-NA-PP1 reduced ethanol intake by altering ethanol metabolism, we measured clearance of ethanol administered to *AS*-PKCε mice 4 hours after intraperitoneal injection of 30mg/kg 1-NA-PP1. Blood ethanol concentrations were measured every 30 minutes for 3 hours (Fig. 4H). 1-NA-PP1 did not significantly alter the time course of ethanol clearance [F_{Time} (4, 40) = 6.423; $P = 0.0004$; F_{Drug} (1, 10) = 0.867; $P = 0.3738$; $F_{Drug \times Time}$ (4, 40) = 1.034; $P = 0.4015$].

Finally, to determine whether the effects of 1-NA-PP1 on ethanol consumption were specific for *AS*-PKCε, we also investigated whether 1-NA-PP1 had an effect in wild type C57BL/6NTac mice that were habituated to vehicle injections and had achieved a stable baseline level of ethanol consumption for three consecutive drinking sessions [F(2,14) = 1.263, $P = 0.3132$; Fig. 5A]. 1-NA-PP1 did not reduce ethanol intake [F (2, 12) = 0.64; $P = 0.54$] by C57BL/6NTac mice (Fig. 5B). However, these mice consumed much less ethanol than *AS*-PKCε mice, causing concern that our inability to observe an effect of 1-NA-PP1 could be do to a floor effect. Hence, we also examined ethanol consumption by C57BL/6J mice, which consume large amounts of ethanol in the continuous access paradigm (Hwa et al., 2011). Mice were habituated to vehicle injections and allowed to achieve a stable baseline level of ethanol consumption for three consecutive drinking sessions prior to administration of 1-NA-PP1 [F(2,18) = 2.162, $P = 0.1441$; Fig. 5C]. 1-NA-PP1 caused a small increase in ethanol intake by C57BL/6J mice at the 20mg/kg dose only [F (2, 18) = 6.41; $P = 0.0079$; Fig 5D]. These results demonstrate that 1-NA-PP1 does not reduce ethanol intake in two strains of wild type mice that lack the *AS*-PKCε mutation.

3.4. 1-NA-PP1 prolongs ethanol intoxication in AS-PKCε mice

We previously found that *Prkce*$^{-/-}$ *mice* show prolonged signs of ethanol intoxication due to impaired acute functional tolerance to ethanol (Hodge et al., 1999, Wallace et al., 2007). Therefore, to determine if inhibiting PKCε alters ethanol intoxication, and to test whether oral administration of 1-NA-PP1 was effective in producing a phenotype, we fed *AS*-PKCε mice 1-NA-PP1 or control food and water for 11 days. On average, mice in the 1-NA-PP1 group consumed 3.00 ± 0.14g of 1-NA-PP1 food pellets/day, which was less than the amount consumed by the control group (3.65 ± 0.16g/day; $P = 0.02$). Mice in the 1-NA-PP1 group also consumed less water (2.00 ± 0.01ml) than mice in the control group (3.5 ± 0.25ml of control liquid /day; $P < 0.0001$). Nevertheless, despite these differences in food and water intake, body weights were similar in 1-NA-PP1-fed (25.5 ± 0.18g) and control-fed (25.8 ± 0.23g) animals.

Three days after the start of the feeding protocol, mice were tested for ethanol-induced ataxia (Fig. 6A). 1-NA-PP1 impaired recovery from ataxia induced by 1.5g/kg ethanol [$F_{Time}(6, 132) = 84.60$, $P < 0.0001$; $F_{Drug}(1, 22) = 5.572$, $P = 0.0275$; $F_{Time\times Drug}(6, 132) = 1.618$, $P = 0.147$]. The same cohort was tested 2 days later for ethanol-induced loss of the righting reflex (LORR) after receiving 5 days of 1-NA-PP1 in food and water. A second cohort of mice underwent the same feeding protocol for 5 days but also received an *i.p.* injection of 25mg/kg 1-NA-PP1 thirty min before the test. We observed a similar effect of 1-NA-PP1 in both cohorts, and therefore combined data from both. 1-NA-PP1 significantly increased the duration of the LORR induced by 3.6g/kg ethanol ($P = 0.0014$, $t_{49}=3.392$; Fig. 6B).

To determine whether the effects of 1-NA-PP1 were specific for *AS*-PKCε, we tested the effects of 1-NA-PP1 in wild type C57BL/6NTac mice. Mice that were provided 1-NA-PP1 consumed similar amounts of food (4.15g ± 0.34g/day) and fluid (2.76 ± 0.3ml/day) as mice fed a control diet (4.14 ± 0.28g/day and 2.4 ± 0.21 ml/day). 1-NA-PP1 did not affect the duration of ataxia induced by 1.5g/kg ethanol [$F_{Time}(4,52) = 69.93$, $P < 0.0001$; $F_{Drug}(1, 13) = 0.2997$; $P = 0.593$; $F_{Time\times Drug}(4,52) = 0.2612$; $P = 0.902$; Fig. 6C]. Likewise, the durations of the LORR induced by 3.6g/kg ethanol were similar in mice administered the 1-NA-PP1 diet and the control diet (Fig. 6D). These results indicate that 1-NA-PP1 has no effect on ethanol-induced ataxia or LORR in wild type mice.

4. Discussion

In this study, we used a chemical-genetic strategy to determine whether a potent and highly selective inhibitor of PKCε could mimic phenotypes we have observed in PKCε knockout mice, namely reduced ethanol consumption and prolonged ethanol intoxication (Hodge et al., 1999). We generated a novel *AS*-PKCε mouse line harboring a point mutation in the ATP binding site rendering it highly sensitive to inhibition by nanomolar concentrations of the PP1 analog 1-NA-PP1. Systemically administered 1-NA-PP1 crossed the blood-brain barrier and reached high enough concentrations in the brain to inhibit *AS*-PKCε. 1-NA-PP1 prolonged the ataxic and hypnotic effects of ethanol and reduced ethanol consumption by *AS*-PKCε mice. These effects of 1-NA-PP1 were not observed in wild type mice lacking the *AS*-PKCε mutation. These results suggest that compounds that inhibit the catalytic activity of PKCε could be useful in reducing ethanol consumption.

Pharmacokinetic analyses indicated that 1-NA-PP1 is rapidly and readily detected in the plasma and brain after parenteral administration. We also detected significant amounts of 1-NA-PP1 in the brain after chronic oral administration. 1-NA-PP1 inhibited phosphorylation of the $GABA_A$ γ2 subunit at S327 in mouse striatum, indicating that 1-NA-PP1 is able to inhibit PKCε-mediated phosphorylation *in vivo*. We had previously found that $GABA_A$ γ2-S(P)327 immunoreactivity is reduced by 60 ±6% in the frontal cortex of *Prkce*$^{-/-}$ mice (Qi et al., 2007), and phosphatase treatment did not further reduce this residual immunoreactivity, indicating that the antibody also detects dephosphorylated protein. Therefore, a 60% reduction in $GABA_A$ γ2-S(P)327 immunoreactivity represents 100% reduction in phosphorylation at this site. Based on these results, we conclude that

intraperitoneal administration of 25mg/kg 1-NA-PP1 reduced PKCε-mediated phosphorylation of $GABA_A$ γ2 in *AS*-PKCε mice by approximately 50%.

1-NA-PP1 reduced ethanol consumption in a reversible manner, without significantly reducing alcohol preference at either of the doses tested. Although water intake was not significantly altered, there was some variability in water intake that may have masked a significant reduction in ethanol preference. At the 30mg/kg dose, there was a trend towards reduced water consumption that was not statistically significant. Saccharin, but not quinine consumption, was significantly reduced at the 30mg/kg dose of 1-NA-PP1. This result is different from what was observed in *Prkce*$^{-/-}$ mice (Hodge et al., 1999), which showed no deficit in saccharin consumption. It is possible that at the 30mg/kg dose, 1-NA-PP1 reduced ethanol consumption by altering the perception of taste for sweet substances, or by effects on brain reward mechanisms or fluid intake. Of note, a reduction in saccharin and sucrose intake has been observed for naltrexone, which is FDA approved to treat alcohol use disorder (Czachowski and Delory, 2009, Ripley et al., 2015).

Baseline ethanol consumption by wild type C57BL/6NTac mice was much lower than by *AS*-PKCε mice even though both are on a C57BL/6NTac background. This difference in ethanol consumption could be due to differences in rearing environments and to genetic drift in our *AS*-PKCε colony from inbreeding. Hence, in addition to the C57BL/6NTac strain, we decided to examine the effects of 1-NA-PP1 on ethanol consumption in C57BL/6J mice, which display high intake and preference for alcohol. Importantly, 1-NA-PP1 did not reduce ethanol drinking in either strain of wild type mice, which both lack the *AS*-PKCε mutation, indicating that the effects of 1-NA-PP1 on ethanol consumption are specific for *AS*-PKCε.

Our previous molecular studies suggested that PKCε mediates its effects on ethanol-related behaviors by reducing inhibitory GABA neurotransmission through actions at $GABA_A$ receptors. We have identified two substrates of PKCε that could contribute to decreased $GABA_A$ receptor function: the $GABA_A$ γ2 subunit, which when phosphorylated at S327 shows a reduced response to the positive allosteric effects of benzodiazepines and ethanol (Qi et al., 2007), and the N-ethylmaleimide sensitive factor, which when phosphorylated at S460 and T461 reduces the number of cell surface $GABA_A$ receptors (Chou et al., 2010). It is likely that additional PKCε substrates play a role in regulating $GABA_A$ receptor function and behavioral responses to ethanol. The M486A mutation allows *AS*-PKCε to use bulky ATP analogs such as N^6-benzyl-ATP as phosphate donors, while native kinases cannot use such ATP analogs (Bishop et al., 2001, Zhang et al., 2013). ATP analogs with a thiophosphate at the γ-phosphate position can generate a kinase-transferable tag, allowing use of a covalent capture-and-release method to purify tagged peptides from digests of protein mixtures (Hertz et al., 2010, Ultanir et al., 2012). Mass spectrometric analysis of these peptides reveals the identity of the corresponding proteins and the location of the phosphorylation sites. Use of this methodology with tissues from *AS*-PKCε mice could identify novel substrates of PKCε in the brain that regulate $GABA_A$ receptor function and behavioral responses to ethanol in an unbiased manner.

5. Conclusions

In summary, our results demonstrate that specific inhibition of PKCε reduces ethanol consumption and prolongs ethanol intoxication, confirming phenotypes we have observed previously using strategies that reduce PKCε expression in the brain. Our results strengthen the rationale for developing small molecule inhibitors of PKCε catalytic activity as therapeutics to decrease ethanol consumption. In addition, our findings demonstrate the utility of the *AS*-PKCε mouse as a tool for studying the role of PKCε in behavior and for identifying direct substrates of PKCε.

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Highlights

- Novel knock-in *AS*-PKCε mice were generated with an ATP analog-specific gatekeeper mutation in the purine-binding site of PKCε.
- Administration of the selective *AS*-kinase inhibitor 1-NA-PP1 to *AS*-PKCε mice reduced their ethanol consumption.
- Administration of 1-NA-PP1 to *AS*-PKCε mice prolonged ethanol intoxication.
- Selective inhibitors of PKCε catalytic activity may prove useful for decreasing ethanol consumption.

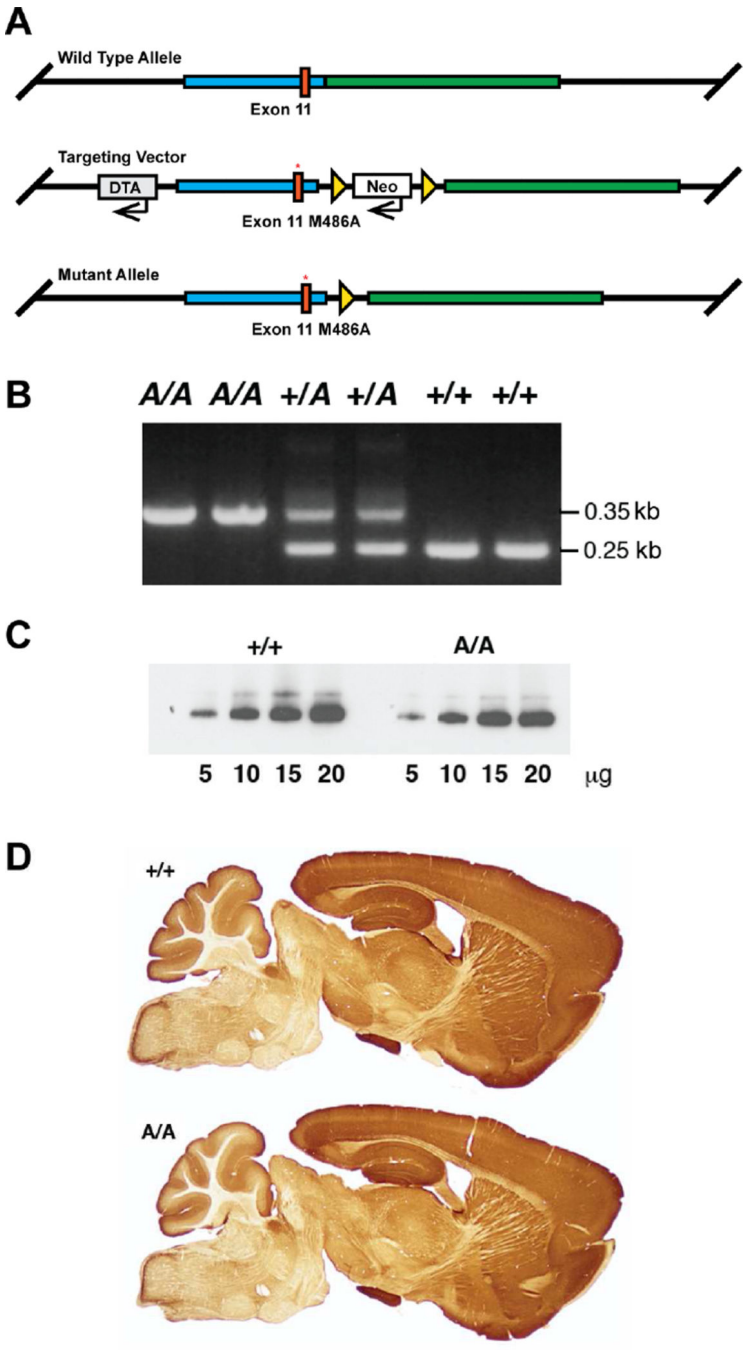

Fig. 1.

Generation of *AS*-PKCε knock-in mice. (A) Schematic showing targeting strategy for generating the M486A mutation (red asterisk) in exon 11 of the mouse *Prkce* gene. DTA = diphtheria toxin A expression cassette for negative selection; Neo = neomycin expression cassette for positive selection. Triangles represent loxP sites for Cre-recombinase mediated excision of the Neo cassette in embryonic stem cell clones. (B) PCR of tail DNA demonstrated presence of mutant (A) and wild type (+) alleles. (C) Western blot analysis showed similar levels of PKCε immunoreactivity in *AS*-PKCε (A/A) and wild type (+/+)

hippocampus. (D) The distribution of brain PKCε immunoreactivity was similar in *AS*-PKCε (A/A) and wild type (+/+) mice.

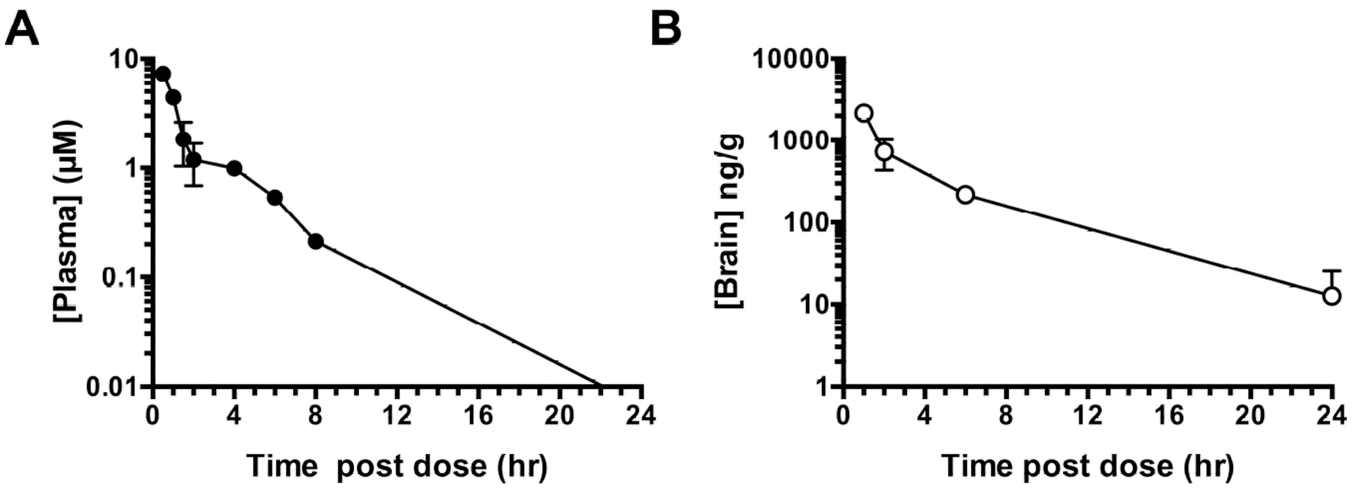

Fig. 2.

1-NA-PP1 pharmacokinetics after intraperitoneal injection of 30mg/kg 1-NA-PP1. Data shown are (A) plasma and (B) brain concentrations of 1-NA-PP1, with $n = 3$ for each data point.

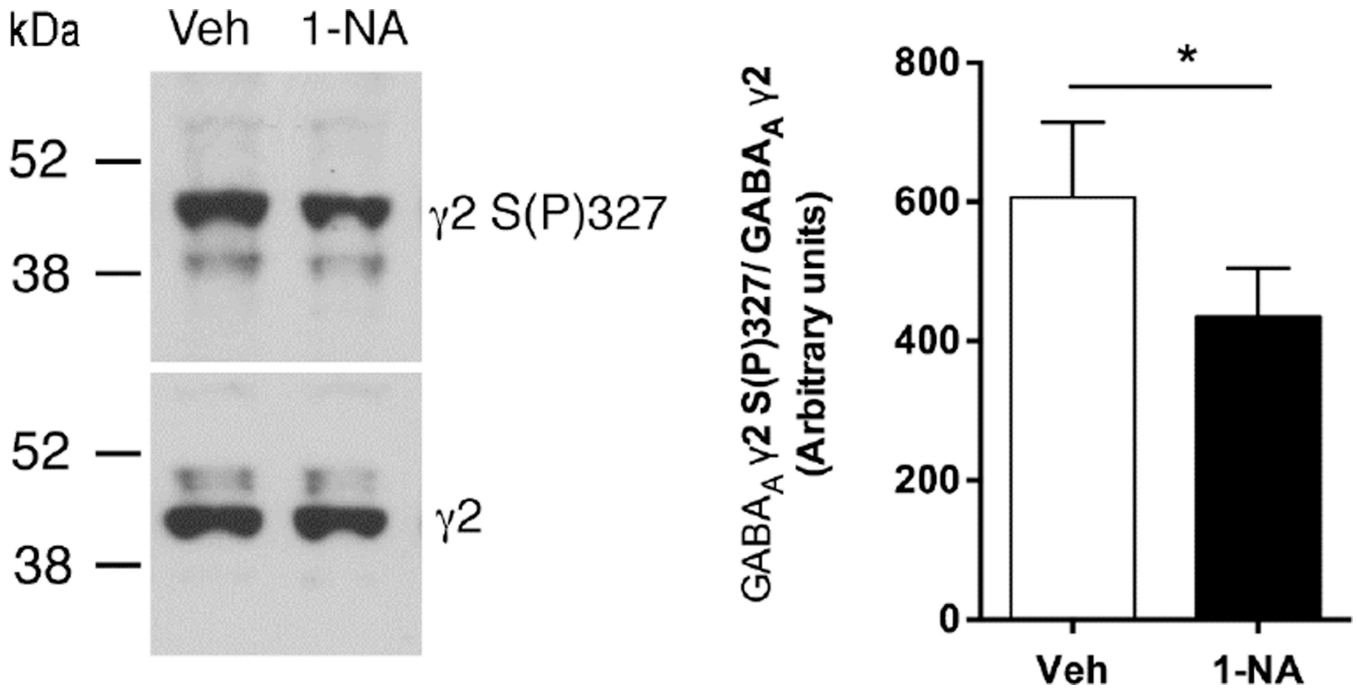

Fig. 3.

GABA$_A$ γ2 receptor subunit phosphorylation. Intraperitoneal injection of 25mg/kg 1-NA-PP1 decreased GABA$_A$ γ2-S(P)327 immunoreactivity compared with vehicle. Left panel shows representative western blots for anti GABA$_A$ γ2S(P)327 immunoreactivity (top) and total GABA$_A$ γ2 immunoreactivity (bottom) from the same vehicle (Veh)- and 1-NA-PP1 (1-NA)-treated samples. Right panel shows mean ± S.E.M. results from all animals. *P = 0.0175, t(8) = 2.98, two-tailed, unpaired t-test; n = 5 per group.

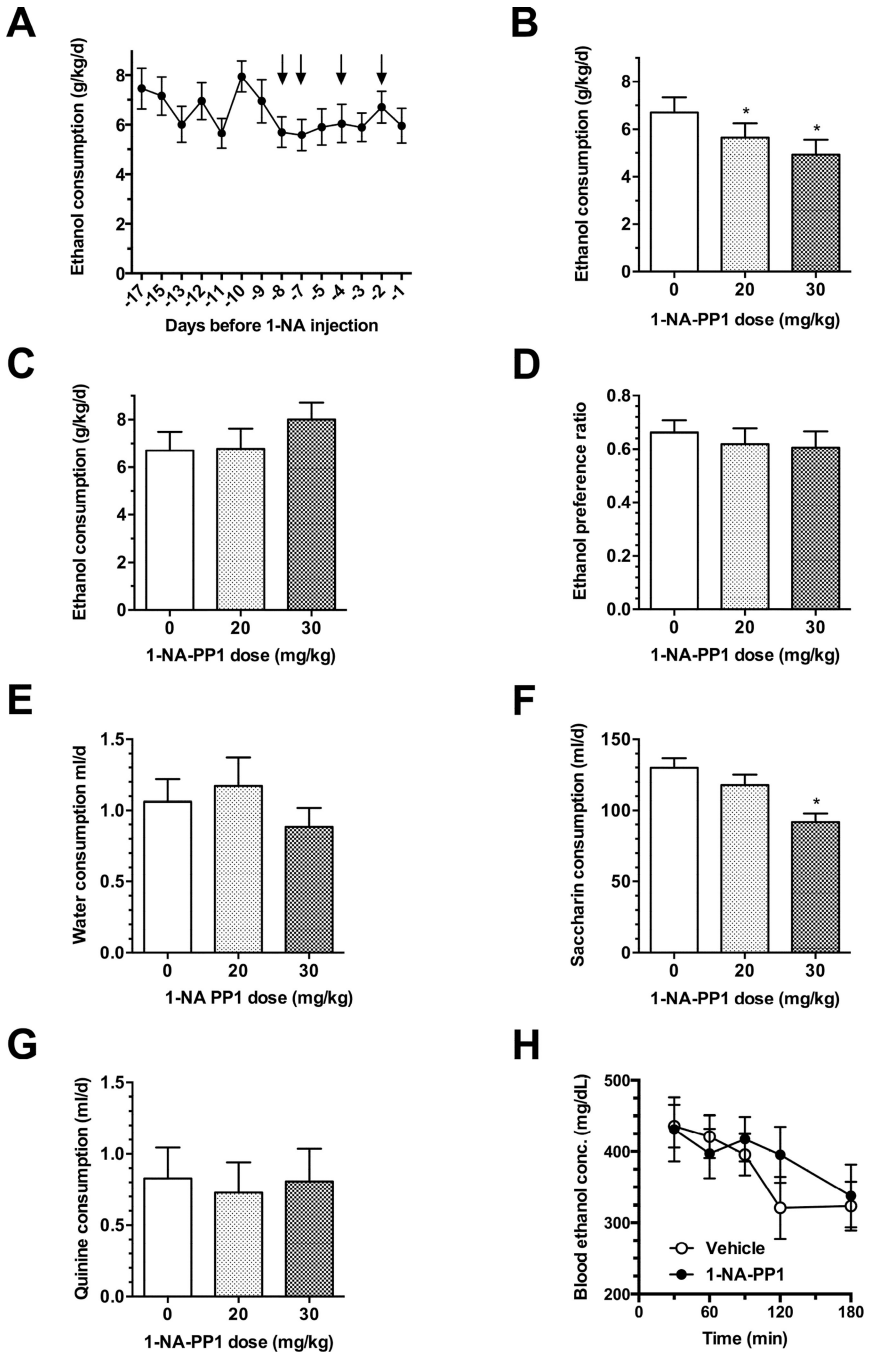

Fig. 4.

Ethanol consumption by *AS*-PKCε mice. (A) *AS*-PKCε mice were habituated to vehicle injections and allowed to achieve a stable baseline level of drinking. Arrows point to days when animals received vehicle injections. 1-NA-PP1 reduced ethanol consumption (B) and this effect was reversible since it was no longer present 48 hours after administration of 1-NA-PP1 (C). (D) 1-NA-PP1 did not alter preference for ethanol over water or water intake (E) 1-NA-PP1 (30 mg/kg) reduced saccharin intake (F), but not quinine (G) intake.(H) 1-

NA-PP1 (30 mg/kg) did not alter ethanol clearance. *$P < 0.05$, Dunnett's test; $n = 18$ per group (A-E), $n = 14$ per group (F and G), $n = 7$ per group (H).

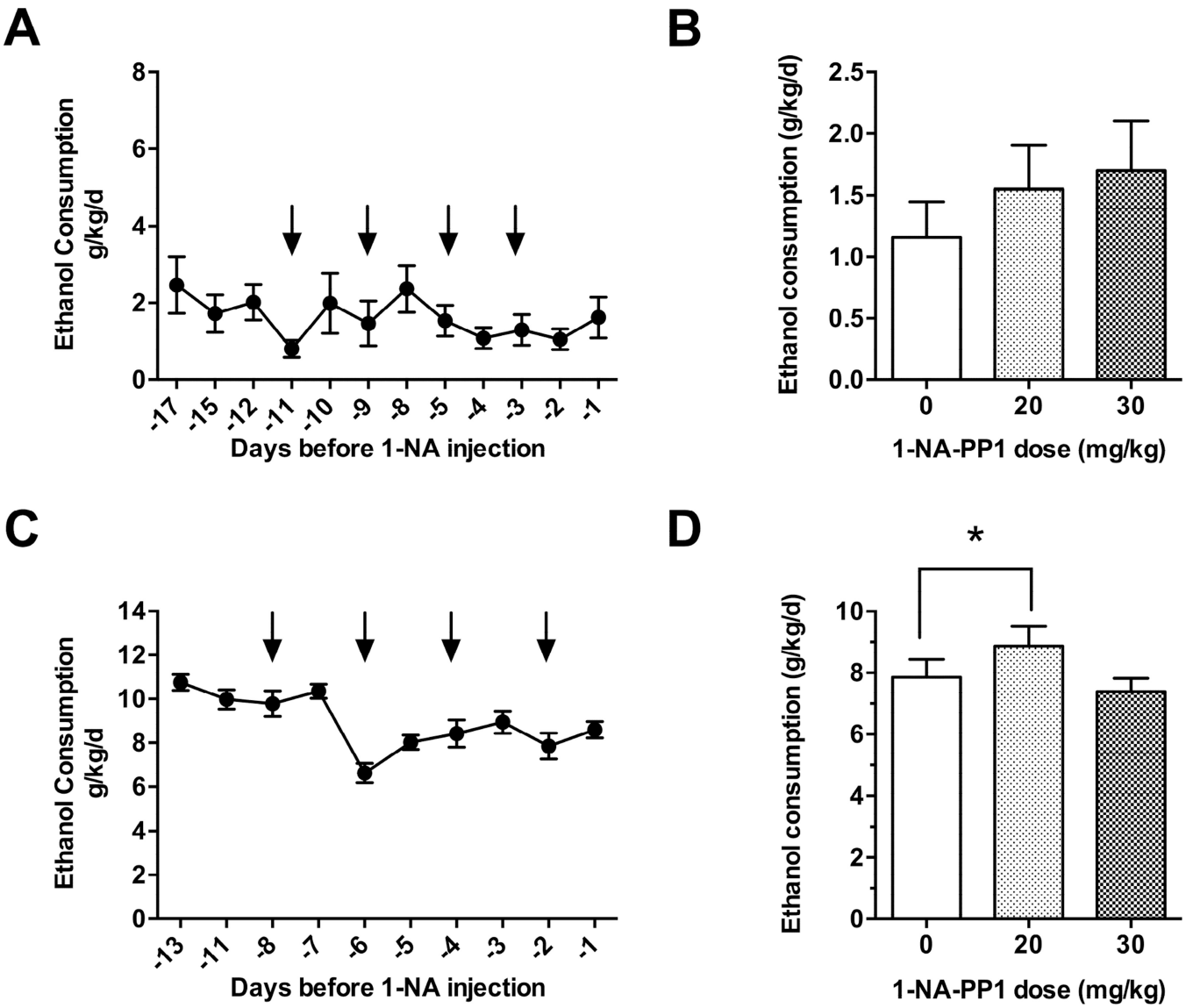

Fig. 5.

Ethanol consumption by wild type mice. (A) C57BL/6NTac mice were habituated to vehicle injections and stable baseline drinking was attained prior to administration of 1-NA-PP1. Arrows point to days when animal received vehicle injections. (B) Administration of 1-NA-PP1 did not significantly alter drinking by C57BL/6NTac mice ($n = 7$ per group). (A) C57BL/6J mice were habituated to vehicle injections and stable baseline drinking was attained prior to administration of 1-NA-PP1. Arrows point to sessions when animals received vehicle injections. (B) 1-NA-PP1 produced a small but significant increase in ethanol consumption in C57BL/6J mice at 20mg/kg, but not at 30mg/kg ($n = 10$ per group). *$P < 0.05$, Dunnett's test.

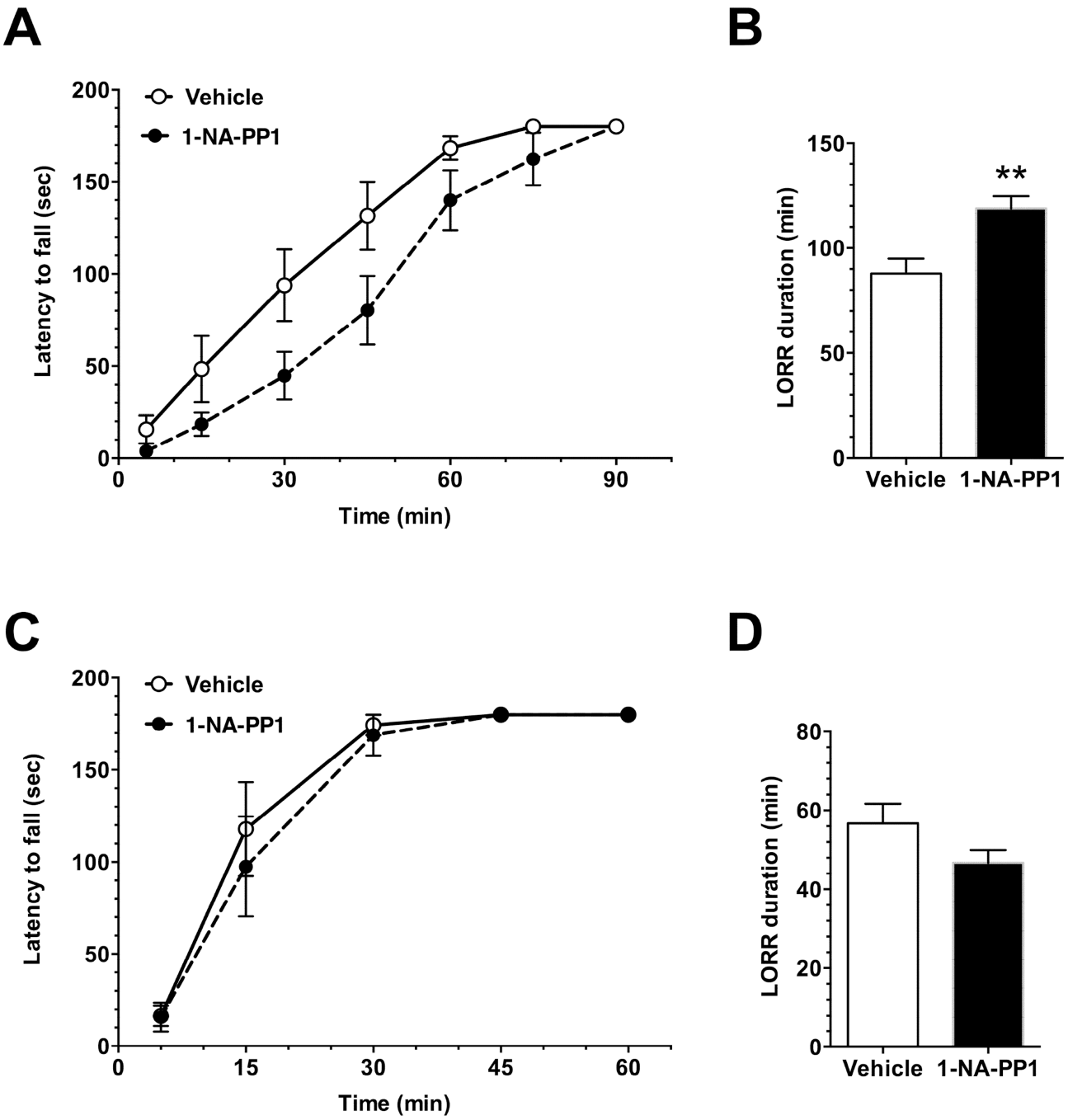

Fig. 6.

Ethanol-induced ataxia and LORR. In *AS*-PKCε mice, 30mg/kg 1-NA-PP1 prolonged recovery from ataxia induced by 1.5g/kg ethanol; $n = 11$ (vehicle), $n = 13$ (1-NA-PP1). (B) In *AS*-PKCε mice, 1-NA-PP1 also increased the duration of LORR induced by 3.6g/kg ethanol; $n = 25$ (vehicle), $n = 26$ (1-NA-PP1). (C) In wild type mice, 1-NA-PP1 did not alter recovery from ataxia induced by 1.5g/kg ethanol; $n = 8$ (vehicle), $n = 7$ (1-NA-PP1). (D) In

wild type mice, 1-NA-PP1 also did not alter the duration of the LORR induced by 3.6g/kg ethanol ($n = 8$ per group). * $P = 0.0014$, $t_{49} = 3.392$, two-tailed, unpaired t-test.

Table 1

Baseline behaviors are similar in wild type and *AS*-PKCε mice

Test (males, age P75-P165)	**Wild type (*n*)**	***AS*-PKCε (*n*)**	***P* value**
Weight (g) at age P90-P120	27.76 ± 0.92 (10)	26.43 ± 0.56 (7)	0.289
Wire suspension fall latency (sec)	111.7 ± 30.7 (10)	74.3 ± 37.6 (7)	0.450
Accelerating rotarod fall latency (sec)	56.1 ± 4.8 (19)	64.1 ± 4.2 (7)	0.350
Open field total distance (cm)	6292 ± 462.9 (8)	5532 ± 733.5 (7)	0.319
Open field % time in center	22.4 ± 3.9 (8)	21.8 ± 4.6 (7)	0.929
Elevated plus maze (5 min)			
% Open time	41.0 ± 9.4 (10)	38.3 ± 10.9 (7)	0.858
% Open entries	43.9 ± 5.5 (10)	43.4 ± 7.6 (7)	0.959
# Closed entries	11.9 ± 1.1 (10)	15.0 ± 2.0 (7)	0.158
Hot plate latency at 56°C (sec)	5.80 ± 0.27 (8)	6.01 ± 0.34 (7)	0.640