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Protein kinase C epsilon activity in the nucleus accumbens and central nucleus of the amygdala mediates binge alcohol consumption

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Abstract

Background—Protein kinase C epsilon (PKC ϵ) is emerging as a potential target for the development of pharmacotherapies to treat alcohol use disorders, yet little is known regarding how a history of a highly prevalent form of drinking, binge alcohol intake, influences enzyme priming or the functional relevance of kinase activity for excessive alcohol intake.

Methods—Immunoblotting was employed on tissue from subregions of the nucleus accumbens (NAC) and the amygdala to examine both idiopathic and binge drinking-induced changes in constitutive PKC ϵ priming. The functional relevance of PKC ϵ translocation for binge drinking and determination of potential upstream signaling pathways involved were investigated using neuropharmacological approaches within the context of 2 distinct binge drinking procedures, Drinking in the Dark (DID) and Scheduled High Alcohol Consumption (SHAC).

Results—Binge alcohol drinking elevated p(Ser729)-PKC ϵ levels in both the NAC and the central nucleus of the amygdala (CeA). Moreover, immunoblotting studies of selectively bred and transgenic mouse lines revealed a positive correlation between the propensity to binge drink alcohol and constitutive p(Ser729)-PKC ϵ levels in the NAC and CeA. Finally,

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neuropharmacological inhibition of PKC ϵ translocation within both regions reduced binge alcohol consumption in a manner requiring intact Group1 metabotropic glutamate receptors, Homer2, phospholipase C (PLC) and/or phosphatidylinositol-3 kinase (PI3K) function.

Conclusions—Taken together, these data indicate that PKC ϵ signaling in both the NAC and CeA is a major contributor to binge alcohol drinking and to the genetic propensity to consume excessive amounts of alcohol.

Keywords

Homer; glutamate; Scheduled High Alcohol Consumption; Drinking in the Dark; alcohol use disorders; HDID-1 mice

Introduction

Globally, alcohol use is the leading cause of premature death and disability in persons aged 15–49 (1), with upwards of 50% of alcohol-drinking individuals reporting consuming 4+ alcoholic drinks per occasion (a.k.a. binge drinking) (2,3). Despite the high prevalence of binge alcohol drinking world-wide (2), a knowledge gap exists regarding the neurobiological consequences of this pattern of excessive alcohol intake relevant for understanding the etiology and treatment of this form of alcohol use disorder (AUD). In two different murine models for binge-like drinking, Drinking in the Dark (DID) and Scheduled High Alcohol Consumption (SHAC), a binge history sensitizes both pre- and post-synaptic aspects of glutamate signaling within the nucleus accumbens (NAC) and central nucleus of the amygdala (CeA) (4–7) – two mesocorticolimbic structures highly implicated in alcoholism psychopathology (8). Moreover, biochemical indices of a hyper-glutamate state in these structures are biochemical traits associated with an excessive alcohol drinking phenotype in mice (5–7,9,10). Thus, pharmacotherapeutic strategies aimed at reducing glutamate hyperactivity may well serve as a rational treatment option for curbing binge drinking behavior. In support of this assertion, neuropharmacological or transgenic manipulations that interfere with signaling through Group1 metabotropic glutamate receptors (mGluRs) in the NAC and/or CeA attenuate binge alcohol intake in murine models (6–7,11).

One emerging potential target for pharmacotherapeutic development for AUDs is protein kinase C epsilon (PKC ϵ), a serine/threonine kinase that is diacylglycerol (DAG)-dependent (12,13). PKC ϵ is activated by G $\alpha_{q/11}$ -coupled receptors, including Group1 mGluRs (14), following DAG formation from phosphatidylinositol 4,5-bisphosphate after hydrolysis by phospholipase C (PLC) (15). Alcohol interacts directly with PKC ϵ (16) and up-regulates indices of PKC ϵ phosphorylation, priming, and activity in brain (10,17–19). Activated PKC ϵ can then, in turn, phosphorylate Ser-327 on the $\gamma 2$ subunits of the GABA type A (GABA $_A$) receptor (20) to mediate acute tolerance to the intoxicating effects of alcohol. Additionally, null mutations of PKC ϵ reduce alcohol reward-related behaviors in various paradigms as determined in studies of mice (13,21–27), and PKC ϵ knock-down in the CeA blunts binge alcohol intake by mice (28). Thus, an understanding of the potential initiators of PKC ϵ signaling will provide significant insight into the molecular pathways influenced by alcohol that lead to pathological drinking.

One potential intermediary may be Group1 mGluRs. These receptors are specifically coupled to PKC ϵ in certain brain areas (14). Alcohol intake, including binge drinking, is partly mediated by mGlu5 activity (29,30) and the subsequent stimulation of PKC ϵ - and/or phosphatidylinositol-3 kinase (PI3K)-dependent transduction pathways (5–7,11,19,27), which were suggested much earlier to involve the Group1 mGluR-associated scaffolding protein Homer (19). However, no study to date has directly investigated the links between Group1 mGluRs, Homer2, PI3K, and PKC ϵ for effects on alcohol consumption. Thus, we employed a combination of immunoblotting and neuropharmacological approaches to test the hypothesis that in both the NACs and the CeA, signaling from mGlu1/5-Homer2 to PKC ϵ is critical for the manifestation of excessive alcohol drinking. Such findings further elucidate the intracellular signaling pathways influenced by alcohol that likely underpin anomalies in excitatory neurotransmission contributing to risky alcohol drinking behavior.

Materials and Methods

Overview of the Experiments

A history of binge alcohol drinking using modified DID (31) and SHAC (32) models up-regulates indices of Group1 mGluR/Homer2/PI3K signaling within the CeA and/or NACs of C57BL/6J (B6) mice (5–7,11). Therefore, we employed immunoblotting procedures (10) to determine the effects of a history of binge alcohol drinking upon PKC ϵ levels and priming in both brain regions of the B6 mice assayed for the expression of putative up-stream signaling molecules in our prior work (5–7). Across inbred mice, strain differences exist regarding PKC ϵ phosphorylation of Ser729 in the NACs suggestive of a relation between higher levels of constitutive PKC ϵ priming and alcohol intake (10). Thus, we also examined the relation between basal p(Ser729)-PKC ϵ levels in the CeA and NAC and genetic propensity to binge drink by immunoblotting tissue from alcohol-naïve S₁₅ HDID-1 [1st replicate line of mice selected for high blood alcohol concentrations (BACs) under DID procedures] vs. non-selected mice from a genetically heterogeneous HS/Npt stock (33). We also compared S₄ SHAC (selected for high BACs under SHAC procedures) vs. SLAC (selected for low BACs under SHAC procedures) mice (5) and *mGlu5*^{F1128R} transgenic (TG) mice, with a point mutation in the Homer binding site on mGlu5. TG mice exhibit low binge drinking under SHAC procedures vs. their wild-type (WT) counterparts that binge drink with this paradigm (5).

We next employed established neuropharmacological approaches (5–7,11) to examine the influence of locally inhibiting PKC ϵ translocation using a Tat- ϵ V1-2 peptide (34), either alone or in tandem with inhibition of mGlu1, mGlu5 and PLC upon binge alcohol intake by B6 mice. Finally, as Homer2 is in position to mediate the effects of mGlu1/5-PKC ϵ signaling (35,36), we examined whether or not local inhibition of PKC ϵ translocation reduced binge alcohol consumption in *Homer2* knock-out (KO) animals (37).

The details of the experimental procedures for these experiments are provided in the Supplemental Online Methods section.

Results

Drinking in the Dark up-regulates the relative expression of p(Ser729)-PKC ϵ

The average daily alcohol intake under DID procedures exhibited by the B6 mice was 4.8 ± 0.37 g/kg/2 hr (6,7,11), which is predicted to result in a mean BAC ~ 100 mg% (31) that exceeds the NIAAA criterion for binge drinking (38). In the NACs, binge drinking elevated the relative expression of p(Ser729)-PKC ϵ [PKC ϵ ratio: $t_{22} = -3.984$, $p = 0.001$], and this reflected a non-significant reduction in total PKC ϵ levels (t -test: $p > 0.05$) and a significant 50% increase in p(Ser729)-PKC ϵ (Figure 1A) [$t_{22} = -2.314$, $p = 0.03$]. These same B6 mice also exhibited an increase in the relative levels of p(Ser729)-PKC ϵ in the CeA (Figure 1B) [$t_{19} = 2.122$, $p = 0.047$]; this reflected a strong trend toward reduced total kinase levels in binge drinking mice (t -test: $p > 0.05$), as p(Ser729)-PKC ϵ levels were not different from water controls (t -test: $p > 0.05$). In contrast, no changes in total or phosphorylated PKC ϵ were apparent in the NAC core (NACc) or basolateral amygdala (BLA) (t -tests, p 's > 0.05 ; Suppl. Table S1).

Basal PKC ϵ phosphorylation as a correlate of genetic propensity to binge drink

We next determined whether or not basal p(Ser729)-PKC ϵ expression is a biochemical correlate of a high binge alcohol drinking phenotype through a comparison of tissue from selectively bred HDID-1 and genetically heterogenous HS/Npt control mice (33). We observed no genotypic differences in PKC ϵ expression within either NAC subregion (t -tests: p 's > 0.05 ; Figure 2A and Suppl. Table S2). While no genotypic differences in total PKC ϵ levels were apparent in CeA (t -test: $p > 0.05$), HDID-1 mice exhibited a robust increase in the absolute, as well as relative levels of p(Ser729)-PKC ϵ in this region (Figure 2B) [total: $t_{26} = 3.25$, $p = 0.003$; ratio: $t_{26} = 3.306$, $p = 0.003$]. However, HDID-1 mice did not differ from HS/Npt controls regarding the basal levels of Homer proteins, mGlu1/5, GluN2A/B, PI3K or AKT phosphorylation (the latter serving to index PI3K activity) in this subregion (Suppl. Table S3). Moreover, there were no genotypic differences in PKC ϵ levels in BLA (t -tests: $p > 0.05$; Table 2), nor were differences observed for any other protein examined (t -tests: p 's > 0.05 ; Suppl. Table S3).

PKC ϵ signaling within the NACs is necessary for binge drinking

We next assessed the role for PKC ϵ in maintaining binge drinking under DID procedures via intra-NAC infusions of the PKC ϵ translocation inhibitor Tat- ϵ V1-2 and employed co-infusion procedures to address whether or not the effects of PKC ϵ inhibition were shared with those produced by infusion of maximally effective doses of mGlu1, mGlu5, PLC, and PI3K inhibitors (Figure 3). Prior to any drug infusions, the B6 mice exhibited an average alcohol intake of 4.56 ± 0.39 g/kg during the 2-h drinking period, an intake correlated with BACs > 80 mg% (31). An intra-NACs infusion of Tat- ϵ V1-2 alone, as well as the co-infusion of this inhibitor with 3 g/side MTEP, 15 pg/side JNJ-16259685, 5.8 fg/side U-73122, or 0.17 ng/side LY294002 reduced alcohol intake under DID procedures (Figure 3A) [$F(5,104) = 6.54$, $p < 0.001$]. LSD *post-hoc* analyses confirmed that all intra-NACs manipulations lowered alcohol intake below that exhibited by control mice infused with the scrambled Tat peptide (all p 's < 0.05). *Post-hoc* comparisons were made also between mice infused with the PKC ϵ inhibitor alone and those co-infused with the pharmacological agents.

As illustrated in Figure 3A, co-infusion of 3 µg/side MTEP attenuated significantly the capacity of Tat-εV1-2 to inhibit binge drinking ($p=0.037$), while this effect was also significant (albeit less robust) when animals were co-infused with 15 pg/side JNJ-16259685 ($p=0.049$), 5.8 fg/side U-73122 ($p=0.047$) or 0.17 ng/side LY294002 ($p=0.047$). Representative placements of our microinjector tips in the NACs are presented in Figure 3C.

PKCε signaling in the CeA is necessary for binge drinking

An intra-CeA infusion of Tat-εV1-2 or co-infusion of the PKCε inhibitor with 3 µg MTEP, 15 pg JNJ-16259685 or 5.8 fg U-73122 inhibited binge alcohol intake under our DID procedures in B6 mice (Figure 3B) [$F(4,74)=14.612$, $p<0.001$]. B6 mice infused intra-CeA with the scrambled control peptide consumed alcohol in excess of amounts predicted to result in BACs > 80 mg% (4.73 ± 0.28 g/kg) (31) and LSD post-hoc analyses confirmed that alcohol drinking was attenuated by infusion of Tat-εV1-2 alone ($p=0.009$), as well as by co-infusion of the inhibitor with MTEP ($p<0.001$), with JNJ-16259685 ($p=0.002$) or with U-73122 ($p<0.001$) (Figure 3B). However, in contrast to the NACs (Figure 3A), the magnitude of the reduction in alcohol intake produced by an intra-CeA infusion of Tat-εV1-2 was enhanced by co-infusion with MTEP ($p=0.005$) or U-73122 ($p=0.001$). These data suggest that, at least within the CeA, mGlu5 and PLC operate to influence alcohol drinking through a pathway independent of PKCε. In contrast, the reduction in drinking produced by Tat-εV1-2 co-infusion with JNJ-16259685 was similar in magnitude to that produced by the PKCε inhibitor alone ($p>0.05$), suggesting that mGlu1 and PKCε in the CeA operate to influence binge alcohol intake via a shared pathway. Representative placements of our microinjector tips in the CeA are presented in Figure 3D.

PKCε signaling and sucrose intake

To examine potential non-specific effects of our PKCε translocation inhibitor, we infused B6 mice with 0.25 µl/side Tat-εV1-2 into the NACs or CeA and measured 5% sucrose intake under our 2-hr DID procedures. As summarized in Suppl. Table S4, PKCε inhibition did not alter sucrose intake in this model (t-tests, $p>0.05$).

Homer2 is necessary for the “anti-drinking” effect of PKCε inhibition

To test the hypothesis that Homer proteins play a role in regulating the functional interaction between mGlu5 and PKCε (19), we next examined the effects of Tat-εV1-2 infusions upon alcohol intake by *Homer2* WT and KO mice. As observed in previous reports (6,7,11), no differences were found in baseline alcohol intake between *Homer2* WT and KO mice (Figure 4). The level of alcohol intake exhibited by the mice was 3.31 ± 0.35 (NACs study) and 4.19 ± 0.27 (CeA study) g/kg/2hr. The effects of infusing Tat-εV1-2 either into the NACs or into the CeA varied as a function of genotype, but not of region [Genotype $\times$ Pretreatment: $F(1,31)=5.43$, $p=0.03$; no main effect of, or interactions with, Region, $p>0.05$]. Post-hoc comparisons confirmed that Tat-εV1-2 inhibited alcohol intake by WT mice when infused into the NACs (Figure 4A) [$t_8=3.079$, $p=0.02$] or CeA (Figure 4B) [$t_6=3.0$, $p=0.03$]. In contrast, the inhibitor had no effect in KO animals (for NACs: $p=0.97$; for CeA: $p=0.15$).

Interactions between PKC ϵ and alcohol intake under SHAC procedures

A final series of experiments were designed to determine whether or not the interactions between NAC PKC ϵ activity and alcohol drinking observed under DID procedures generalized to binge drinking under SHAC procedures. Thus, we examined relative p(Ser729)-PKC ϵ levels in tissue from the entire NAC obtained from mice with a history of repeated bouts of binge alcohol drinking under SHAC procedures (average daily intake = 1.6 ± 0.2 g/kg/30 min; resultant average BAC = 109.5 ± 37.3 mg%), which had been processed previously for binge drinking-induced changes in putative up-stream signaling molecules (5). Although binge drinking under SHAC procedures did not significantly affect either total or p(Ser729)-PKC ϵ levels in the entire NAC (*t*-tests, *p*'s > 0.05), there was an increase in the relative expression of p(Ser729)-PKC ϵ in this study (Figure 5A) [$t_{24}=2.44$, *p*=0.02]. We also immunoblotted for the relative levels of p(Ser729)-PKC ϵ in the entire NAC of mice selectively bred for high versus low alcohol intake under SHAC procedures (SHAC vs. SLAC lines, respectively), which had been processed previously for line differences in glutamate receptor/Homer/PI3K expression (5). As indicated in Figure 5B, SHAC line mice exhibited higher relative levels of p(Ser729)-PKC ϵ versus their low-drinking SLAC counterparts [$t_{11}=2.966$, *p*=0.013], and this effect reflected both an increase in p(Ser729)-PKC ϵ expression [$t_{11}=2.029$, *p*=0.06] and a modest, albeit non-significant, reduction in total kinase levels [*t*-test: *p*>0.05].

We next asked whether or not the converse might be true and assayed for genotypic differences in basal NAC PKC ϵ phosphorylation between low SHAC-intake *mGlu5^{F1128R}* transgenic (TG) mice, with a point mutation in the Homer binding site on mGlu5 versus their WT littermates (5). While total PKC ϵ levels did not differ between genotypes (*p*>0.05), TG mice exhibited 40% lower p(Ser729)-PKC ϵ levels [$t_{17}=3.747$, *p*=0.002], as well as significantly lower relative levels of phosphorylated versus non-phosphorylated kinase [PKC ϵ ratio; $t_{17}=2.513$, *p*=0.022], compared to WT animals (Figure 5C).

We also examined also the ability of the Tat- ϵ V1-2 translocation inhibitor to reduce alcohol intake under SHAC procedures when infused into the NACs of B6 mice. Indeed, Tat- ϵ V1-2 infusion significantly reduced consumption of 5% alcohol, relative to mice infused with a scrambled control peptide (Figure 5D) [$t_{10}=4.28$, *p*=0.002]. Importantly, the alcohol intake of the control mice in this study correlated with BACs > 80 mg%, while that of Tat- ϵ V1-2-infused animals was lower than that predicted to meet the criterion for binge drinking (5). Thus, PKC ϵ inhibition in the NACs is sufficient to prevent binge alcohol drinking under SHAC procedures. Finally, intra-NAC inhibition of PKC ϵ did not influence the consumption of 5% sucrose under SHAC procedures (*p*>0.05; Suppl. Table S4), providing further evidence that interfering with PKC ϵ activity selectively affects alcohol intake.

Discussion

Herein, alcohol consumption up-regulated PKC ϵ phosphorylation on Ser729 in both the NACs and the CeA and basal p(Ser729)-PKC ϵ levels in these regions were a correlate of the genetic propensity to binge drink. These data implicate either idiopathic or alcohol-induced increases in PKC ϵ priming in NACs and CeA in the etiology of excessive alcohol drinking. Furthering this hypothesis, inhibition of PKC ϵ translocation in the NACs or CeA reduced

alcohol intake under our DID and SHAC procedures, and experiments conducted in *Homer2* KO mice provided evidence that this scaffolding molecule is integrally involved. The neuropharmacological studies suggested that PKC ϵ in the NACs regulates binge alcohol drinking via signaling pathways involving mGlu1, mGlu5, PLC and PI3K, while PKC ϵ in the CeA operates in a signaling pathway involving mGlu1. These results extend current knowledge, derived from studies using constitutive *PKC ϵ* KO mice (13) and shRNA-PKC ϵ approaches (28), by demonstrating that PKC ϵ operates in both the NACs and CeA to facilitate voluntary alcohol consumption through distinct Group1 mGluR-associated signaling pathways.

***In vivo* regulation of PKC ϵ by alcohol and relation to alcoholism vulnerability**

In vitro, alcohol stimulates PKC ϵ translocation (39), and chronic alcohol exposure increases PKC ϵ immunoreactivity and PKC ϵ -mediated phosphorylation (17). The precise neuroanatomical loci of alcohol-PKC ϵ interactions are less well-described and were examined herein using phosphorylation of PKC ϵ at Ser729 as an indicator of isozyme priming (40). Binge alcohol drinking increased p(Ser729)-PKC ϵ specifically in NAC and amygdala subregions that are components of the extended amygdala circuit highly implicated in alcoholism neurobiology (41,42). Moreover, this effect was observed within the same tissue reported previously by our group to exhibit increases in Group1 mGluR, Homer2a/b levels and/or PI3K activation (5–7). Such observations indicate that elevated PKC ϵ priming in these brain regions, presumably via pathways involving Group1 mGluRs and their Homer2 scaffolds, may be central to the manifestation of excessive alcohol intake.

In support of this assertion, a susceptibility locus for alcohol dependence is indicated on human chromosome 2p21, where the gene for PKC ϵ is mapped (43,44), and genotypic differences exist for basal p(Ser729)-PKC ϵ between inbred mouse strains with divergent alcohol drinking phenotypes (10). Herein, we extend these prior data to selectively bred and transgenic lines of mice varying in binge drinking behavior. When assayed in the entire NAC, SHAC line mice selectively bred for high BACs under SHAC procedures exhibited higher p(Ser729)-PKC ϵ levels, compared to SLAC line animals selected for low BACs under SHAC procedures. Conversely, *mGlu5^{F1128R}* TG mice that manifest low binge alcohol drinking (5) exhibit lower constitutive p(Ser729)-PKC ϵ levels in the NAC than WT littermates that exhibit binge drinking behavior (5). As the *mGlu5^{F1128R}* TG mice employed in the present study exhibit reduced Homer binding to mGlu5 (5), these later results provide novel evidence in support of an important role for mGlu5-Homer interactions in regulating constitutive PKC ϵ priming *in vivo*, as suggested previously (19). However, while HDID-1 mice, selected for high BAC under 4-h DID procedures, exhibited higher indices of CeA p(Ser729)-PKC ϵ than their genetically heterogeneous HS/Npt controls, no differences were observed in putative up-stream signaling molecules in amygdala, nor were differences present for p(Ser729)-PKC ϵ levels in the NAC. As strain differences exist regarding basal NAC levels of p(Ser729)-PKC ϵ (10; Figure 5A), the failure to detect differences in PKC ϵ priming between HDID-1 and HS/Npt controls could simply relate to the genetic heterogeneity of the control line employed in this study. Alternatively, the use of more sensitive mass spectrometry procedures to examine the phosphorylation of Ser729, or of other sites more reflective of enzyme activation [e.g., Thr566 or Ser710 (40)], might provide

more information pertaining to the link between idiopathic or alcohol-induced changes in PKC ϵ activity and alcohol drinking. Further preclinical exploration of this potential link is warranted, particularly considering evidence indicating the functional relevance for NAC Group1 mGluR/Homer2/PI3K/PKC ϵ signaling in excessive alcohol drinking (present study; 5–7,11).

PKC ϵ translocation and binge alcohol consumption

The correlational nature of the aforementioned immunoblotting studies cannot inform as to cause-effect relations between PKC ϵ activity and binge drinking. However, earlier data from studies of constitutive PKC ϵ KO mice argued an active and necessary role for this isozyme in alcoholism-related behaviors (13, 25) and herein, the inhibition of PKC ϵ translocation in either the NACs or the CeA by the local infusion of a Tat- ϵ V1-2 peptide (34) was sufficient to reduce binge alcohol consumption in 2 distinct murine models. To the best of our knowledge, these data are the first demonstration that PKC ϵ translocation specifically in the NACs modulates binge alcohol intake, and further support the CeA as an anatomical site of action of PKC ϵ for excessive alcohol consumption (28). This demonstration of a causal relation between PKC ϵ translocation in at least two AUD-relevant brain regions and binge alcohol intake not only highlights the significance of both our immunoblotting results (10; present study) and those of the genetic mapping studies in humans (43,44), but also provides neuroanatomical insight into the facilitatory role for PKC ϵ in excessive alcohol drinking.

Potential up-stream modulators of PKC ϵ translocation and binge alcohol consumption.

Previously, a signaling pathway initiated upon mGlu5 stimulation, that involved Homers and PI3K activity, was proposed to play an important role in the activation of PKC ϵ underpinning voluntary alcohol intake (19). In partial validation of this hypothesis, we demonstrated previously a critical role for Group1 mGluR/Homer2-mediated activation of both G $\alpha_{q/11}$ and G $\beta\gamma$ signaling in the NACs and the CeA in maintaining binge alcohol drinking (5–7,11). Herein, an intra-NAC infusion of Tat- ϵ V1-2 while effective at reducing alcohol intake in WT mice, did not inhibit alcohol intake in *Homer2* KO mice, implicating Homer2 as an important regulator of both PKC ϵ priming and the facilitation of binge alcohol drinking elicited by PKC ϵ translocation. In the NACs, the inhibitory effects of co-infusion of the Tat- ϵ V1-2 inhibitor with mGlu1, mGlu5, PLC or PI3K antagonists were not different from those produced by PKC ϵ inhibition alone, indicating that at least in this striatal subregion, mGlu1/5, PLC, PI3K and PKC ϵ operate within shared intracellular signaling pathways, perhaps scaffolded by Homer2. While there is no evidence that Homer proteins interact directly with any PKC, our prior work indicated that intact Homer2 expression or Homer coupling to mGlu5 is required in order to observe an attenuation of binge drinking by mGlu1/5 receptor antagonists and inhibitors of PLC and PI3K [6,7,11]. Moreover, the present observation of decreased constitutive PKC ϵ priming in the NAC of *mGlu5^{F1128R}* mice with reduced Homer binding supports some functional relation between mGluR-Homer complexes and PKC ϵ . Thus, the coincident up-regulation in mGlu1/5, Homer2, and PLC expression, the activational state of PI3K and PKC ϵ priming observed in the NACs of binge drinking animals or in animals with a high genetic propensity to binge drink [5,6,11; present study] likely reflects a shared signaling pathway that is permissive to excessive alcohol consumption.

Although binge alcohol drinking up-regulates p(Ser729)-PKC ϵ expression in the CeA and higher constitutive PKC ϵ priming in the CeA is a genetic correlate of excessive alcohol drinking, the specific signaling pathway(s) operating in the CeA to regulate binge alcohol intake appear to be distinct from those operating within the NACs. For one, PI3K inhibition within the CeA does not affect binge alcohol drinking (5,6 vs. 7), arguing that Akt/PI3K hyperactivity in the CeA is not a major mediator of binge alcohol intake and likely not a major contributor to alcohol-induced increases in PKC ϵ priming observed within this region. Moreover, mGlu1 and PLC operate independently within the CeA to influence binge drinking (7) and herein, the effect of an intra-CeA co-infusion of Tat- ϵ V1-2 with an mGlu5 antagonist was greater than that produced by the antagonist alone. Such a result argues against a specific role for CeA mGlu5 in PKC translocation facilitating alcohol intake. However, the inhibitory effect of an intra-CeA co-infusion of Tat- ϵ V1-2 and an mGlu1 antagonist was not different from that produced by the mGlu1 alone, arguing that PKC ϵ operates in the CeA, via some mGluR1-dependent pathway, to influence binge alcohol intake. This mGlu1/PKC ϵ pathway does not involve G $\alpha_{q/11}$ -mediated stimulation of PLC, as the attenuation of binge drinking by co-infusion of PLC and PKC ϵ inhibitors was greater than that produced by PKC ϵ inhibition alone. Thus, the capacity of PKC ϵ priming to regulate binge drinking may be entirely independent of the generation of DAG by PLC. Although evidence indicates that PI3K can regulate PKC ϵ priming (19), our failure to observe alcohol-induced increased PI3K activity in the CeA, coupled with no effect of intra-CeA infusions of different PI3K inhibitors upon binge drinking (7), argue that alcohol-induced or idiopathic increases in PKC ϵ priming observed in this region likely do not involve $\beta\gamma$ -mediated activation of PI3K upon mGlu1 stimulation (7).

What then is the pathway involved in mGlu1/PKC ϵ -dependent signaling that regulate binge drinking in the CeA? mGlu1 activation can elicit cAMP formation *in vitro* (45), raising the possibility that mGlu1-PKC ϵ signaling in the CeA may involve this second messenger. Indeed PKC ϵ activation can be regulated by G α_i -coupled corticotrophin-releasing factor receptor 1 (CRF1) receptors (34) and there is cross-talk between PKA and PKC in opioid-withdrawn animals (46), supporting this possibility. Alternatively, mammalian target of rapamycin complex 2 (mTORC2) is an important upstream regulator of PKC ϵ priming (47). While not yet assessed in brain, mTORC2 activity can be up-regulated by alcohol when assayed in myocytes (48), Group1 mGluR-mediated inhibition of tuberous sclerosis complexes can increase mTORC activation (49), and the capacity of Group1 mGluRs to augment mTORC activity depend upon constitutively expressed Homer proteins (50). Thus, either alcohol-induced or idiopathic increases in mGluR/Homer2-PKC ϵ signaling in CeA may involve mTORC1 and/or mTORC2 as an intermediary, and this possibility requires systematic investigation. Alternatively, both mGlu1 and PKC ϵ can regulate the function of N-type calcium channels, and Homer proteins have been implicated in mGlu1-regulation of channel function (51). While this latter mechanism cannot account for *how* mGlu1 stimulation elicits PKC ϵ translocation in the CeA, genetic or pharmacological interruption of mGlu1, Homer2, N-type calcium channels or PKC ϵ all produce an alcohol-avoiding phenotype in preclinical animal models (5–7,11,23,52). Thus, N-type calcium channels may be an important point of signaling convergence between mGlu1 and PKC to be explored in future studies.

Conclusion

Using a combination of immunoblotting and neuropharmacological approaches, the results of the present study support idiopathic or alcohol-induced increases in PKC ϵ priming/translocation in the NACs and CeA as an important signaling event in the etiology of alcoholism and provide experimental support for the potential utility of targeting PKC ϵ for the pharmacotherapeutic intervention of AUDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Thirty days of binge alcohol consumption under Drinking in the Dark (DID) procedures up-regulates Protein Kinase C epsilon (PKC ϵ) activity in the nucleus accumbens shell (NACs) and central nucleus of the amygdala (CeA)

Summary of the change in protein levels of total PKC ϵ , p-Ser729-PKC ϵ and their relative levels (PKC ϵ ratio) in the NACs (A) and CeA (B) following 24 hr withdrawal from 30 days of alcohol drinking under Drinking in the Dark (DID or D) procedures. Two representative immunoblots for PKC ϵ and p-Ser729-PKC ϵ for each group are included as insets. After normalization for loading, the data are expressed as a percent of the average protein levels of

water-drinking controls (Water or W; n=5–6/gel). Data represent the mean $\pm$ SEM of the number of animals indicated in parentheses in the figure, * p <0.05 (t-tests).

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Figure 2. Line differences in central nucleus of the amygdala (CeA) protein kinase C epsilon (PKC ϵ) activity between selectively bred HDID-1 and genetically heterogeneous HS/Npt control mice

Summary of the protein levels of total PKC ϵ and p-Ser729-PKC ϵ , as well as their relative levels (PKC ϵ ratio) in the nucleus accumbens shell (NACs) (**A**) and CeA (**B**) of mice from S₁₅ of selective breeding under DID procedures (HDID-1) and mice from their HS/Npt founder line (Control). The data are expressed as a percentage of average levels of HS/Npt animals (5–6/gel). Representative immunoblots of PKC ϵ and p-Ser729-PKC ϵ are included as insets (C= HS/Npt founder line control, H= HDID-1 selectively bred line). Data represent

the mean $\pm$ SEM of the number of animals indicated in parentheses the figure, $*p<0.05$ (t-tests).

Figure 3. Inhibition of nucleus accumbens shell (NACs) and central nucleus of the amygdala (CeA) protein kinase C epsilon (PKCε) reduces binge alcohol drinking in C57BL/6J mice
Summary of the effects of an intra-NACs (A) and intra-CeA (B) infusion of the PKCε inhibitor Tat-εV1-2 alone or in combination with the mGlu5 antagonist MTEP (3.0 μg/side), the mGlu1 antagonist JNJ-16259685 (JNJ; 15 pg/side), the PLC inhibitor U-73122 (U; 0.58 fg/side), and the PI3K antagonist LY-294002 (LY; 0.17 ng/side) upon 20% alcohol intake under DID procedures. Control animals were infused with a scrambled peptide (Scrambled). The data represent the mean ± SEM of the number of animals indicated in the figure, * $p < 0.05$ vs. Scrambled, + $p < 0.05$ vs. Tat-εV1-2 (LSD post-hoc tests). Pictograms depicting

the location of microinjector tips in the NACs (**C**) and CeA (**D**) of mice from 1 replicate of the neuropharmacological studies. Numbers represent the anterior-posterior (AP) distance from Bregma (in mm) at which the microinjectors were localized.

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Figure 4. Homer2 is necessary for the attenuating effects of nucleus accumbens shell (NACs) and central nucleus of the amygdala (CeA) protein kinase C epsilon (PKCε) inhibition

Summary of the effects of intra-NACs (A) and intra-CeA (B) infusions of the PKCε inhibitor Tat-εV1-2 or control peptide (Scrambled) upon 20% alcohol intake under Drinking in the Dark (DID) procedures by *Homer2* wild-type (WT) and knock-out (KO) mice. The data represent the mean $\pm$ SEM of the number of animals indicated in the figure, * $p < 0.05$ vs. Scrambled in WT mice (t-tests).

Figure 5. Role for protein kinase C epsilon (PKCε) signaling in the nucleus accumbens (NAC) in binge alcohol consumption under Scheduled High Alcohol Consumption (SHAC) procedures

A, Summary of the change in protein levels of total PKCε p-Ser729-PKCε and their relative levels (PKCε ratio) in the entire NAC following 24 hr withdrawal from 6 bouts of alcohol drinking under Scheduled High Alcohol Consumption (SHAC or S) procedures and water drinking controls (Water or W; 5–6/gel). Representative immunoblots are included as insets.

B, Summary of the line differences in protein levels in the entire NAC of *S*₄ mice selectively bred for high versus low alcohol intake under SHAC procedures (SLAC line or L =

Scheduled Low Alcohol Consumption line; SHAC line or H = Scheduled High Alcohol Consumption line), at 3 months after their final 30 min alcohol-drinking session. The data are expressed as a percent of average levels of the SLAC line (5–6 SLAC mice/gel). Representative immunoblots are included as insets. **C**, Summary of the line differences in protein levels in the NAC of *mGlu5^{F1128R}* transgenic mice (*mGlu5* TG or T) and wild-type littermates (*mGlu5* WT or W), expressed as a percent of average levels of WT animals (5–6 WT mice/gel). Representative immunoblots are included as insets. **D**, Summary of the effects of an intra-NAC shell infusion of the PKC ϵ inhibitor Tat- ϵ V1-2 or control peptide (Scrambled) upon 5% alcohol intake exhibited by B6 mice under SHAC procedures. Data represent the mean $\pm$ SEM of the number of animals indicated in the figure, * p <0.05 (t-test