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Short communication

CaMKII inhibition in the prefrontal cortex specifically increases the positive reinforcing effects of sweetened alcohol in C57BL/6J mice

Sara Faccidomo[a], Grant T. Reid[a], Abigail E. Agoglia[a], Sherifat A. Ademola[a], Clyde W. Hodge[a,b,*]

[a] Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States
[b] Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

HIGHLIGHTS

- CAMKII activity is important for neural plasticity and development of drug addiction.
- Moderate alcohol drinking alters CaMKII expression and phosphorylation in the CNS.
- Pharmacological inhibition of CAMKII in the PFC increases lever-press responding reinforced by sweetened alcohol.
- The PFC may dampen drug use via top-down control of limbic motivational brain regions.

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional enzyme that is required for synaptic plasticity and has been proposed to be a primary molecular component of the etiology of alcohol addiction. Chronic alcohol intake upregulates CaMKIIα protein expression in reward-related brain regions including the amygdala and nucleus accumbens, and CaMKIIα activity in the amygdala is required for the positive reinforcing effects of alcohol, suggesting this system promotes consumption in the early stages of alcohol addiction. Alternatively, the medial prefrontal cortex (mPFC) is known to inhibit limbic activity via CaMKII-dependent excitatory projections and may, therefore, enable top-down regulation of motivation. Here we sought to remove that regulatory control by site-specifically inhibiting CaMKII activity in the mPFC, and measured effects on the positive reinforcing effects of sweetened alcohol in C57BL/6J mice. Infusion of the CAMKII inhibitor KN-93 (0–10.0 μg) in the mPFC primarily increased alcohol+sucrose reinforced response rate in a dose- and time-dependent manner. KN-93 infusion reduced response rate in behavior-matched sucrose-only controls. Importantly, potentiation of operant responding for sweetened alcohol occurred immediately after infusion, at a time during which effects on sucrose responding were not observed, and persisted through the session. These results suggest that endogenous CaMKII activity in the mPFC exerts inhibitory control over the positive reinforcing effects of alcohol. Downregulation of CaMKII signaling in the mPFC might contribute to escalated alcohol use.

Alcohol dependence is a complex neuropsychiatric disorder that afflicts more than 9% of the total US population. Epidemiological studies suggest that a majority of individuals (52%) actively engage in moderate, or sub-dependent, levels of alcohol drinking at amounts that are capable of inducing widespread changes in fundamental neural systems that normally regulate cognition, motivation, and other adaptive functions of the organism [1,2]. For example, recent evidence indicates that moderate drinking launches a cascade of neuroadaptations in the amygdala proteome, linked to calcium/calmodulin-dependent protein kinase II (CaMKII), that mechanistically drive the positive reinforcing effects of alcohol and may serve, therefore, as a molecular pathway from use to abuse [3]. Thus, elucidating the neural mechanisms that regulate alcohol consumption in the non-dependent state is critical for a comprehensive understanding of the etiology of addiction.

CaMKII is a family of Ca^{2+}-activated Ser/Thr protein kinases that mediates many intracellular responses in the brain including regulation of membrane current, neurotransmitter synthesis

* Corresponding author at: Department of Psychiatry, School of Medicine, University of North Carolina at Chapel Hill, CB #7178, Thurston Bowles Building, Chapel Hill, NC 27599, United States. Fax: +1 919 966 5679.
E-mail address: clyde_hodge@med.unc.edu (C.W. Hodge).

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and release, cytoskeletal organization, and synaptic plasticity [4,5]. CaMKII is activated when neuronal depolarization leads to Ca^{2+} entry into the cell through multiple sources including ionotropic glutamate receptors (calcium-permeable AMPA and NMDA receptors), L-type voltage-gated calcium channels, and via release from internal stores. Following activation, CaMKII translocate to the membrane and/or postsynaptic density where it regulates receptor (i.e., NMDA, AMPA) activity [6]. Importantly, a number of CaMKII interacting proteins, including NMDA and AMPA receptors, PSD proteins, CREB, and the MAPKs regulate alcohol-related behaviors including self-administration and relapse (e.g., [7–10]). Thus, CaMKII may represent a molecular point of convergence in the regulation of maladaptive behaviors associated with alcohol abuse.

Emerging evidence indicates that CAMKII activity and function are altered by alcohol in reward-related brain regions. Increases in CaMKII protein expression and activation (e.g., phosphorylation) have been reported in the cortex, nucleus accumbens and amygdala following alcohol dependence as well as by moderate, or non-dependent, levels induced by voluntary drinking or operant self-administration [3,11–13]. The functional importance of CAMKII on alcohol-associated behaviors has been recently studied using both pharmacological and genetic approaches. CaMKIIα autophosphorylation-deficient mice show reduced alcohol consumption and preference and have a blunted response to the locomotor-stimulating effects of alcohol [14]. We have shown that the positive reinforcing effects of sweetened alcohol require CaMKII activity in the amygdala [3] but the complete neural circuitry of CaMKII-dependent regulation of alcohol-seeking behavior remains to be fully characterized.

Behavioral pathologies in addiction, such as exacerbated drug-seeking behavior, represent a dynamic interplay between heightened motivation and cognitive regulatory processes that control goal-directed behaviors. Under normal conditions, the medial prefrontal cortex (mPFC) exerts "top-down" control over limbic motivational systems via CaMKII-positive glutamatergic projections to brain regions, such as the nucleus accumbens and amygdala. However, repeated drug use is thought to result in PFC dysregulation, loss of executive control, and exacerbated drug use due to impaired response inhibition [15]. Although evidence cited above implicates CaMKII signaling in the rewarding and reinforcing effects of alcohol, the role of this critical cell signaling molecule in the PFC remains unknown. To address this gap in knowledge, the objective of the current experiment was to determine if CaMKII activity in the mPFC mechanistically regulates the positive reinforcing effects of alcohol. If the mPFC exerts top-down regulatory control of behavioral pathologies in alcohol addiction via response inhibition, we predicted that blockade of CaMKII activity in the mPFC would increase, or exacerbate, sweetened alcohol self-administration via increased positive reinforcement function. Given the prominence of CaMKII signaling in the development and regulation of new behavior, these findings will provide novel information on how alcohol may gain control over behavior during the early stages of addiction when drug use is primarily driven by positive reinforcement.

Male C57BL/6J mice (10-weeks old; $n = 40$) were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were group-housed and initially handled for 7 days (Fig. 1A). Food and water were freely available unless noted. The vivarium was maintained on a reverse 12:12 light/dark schedule, with average temperature and humidity of 25 °C and 40%, respectively. All protocols were conducted in accordance with the Institutional Animal Care and Use Committee of the University of North Carolina-Chapel Hill and the Guide for the Care and Use of Laboratory Animals.

Self-administration studies were conducted as described [3]. One week after arrival, separate groups of mice were presented with a 9% alcohol (v/v)/2% sucrose (w/v) (Alcohol + Sucrose; Pharmco-AAPER, Shelbyville, KY) or 2% Sucrose (w/v; Sucrose-only) solution, along with water, in their home-cage for 2 weeks (Fig. 1A). Home-cage exposure facilitates operant self-administration training [16]. After home-cage self-administration, mice were water restricted for 20-h and then placed into an operant conditioning chamber for a 16-h overnight session during which lever press responses were reinforced with delivery of either Alcohol+Sucrose or sucrose-only. All conditioning occurred in sound-attenuating chambers (Med Associates; St. Albans, VT) containing retractable levers. Only responding on the active lever was reinforced. After 3 overnight sessions, mice were no longer water restricted and training was shortened to 1-h sessions (Fig. 1A). Throughout the remainder of the experiment, the response requirement was maintained at a fixed-ratio 4 schedule of reinforcement. Blood was collected after surgery on the 87th self-administration session via submandibular bleed and plasma alcohol levels were measured using an AM1 Alcohol Analyzer (Analox Instruments, Ltd., Lunenburg, MA; [7]; Fig. 1A).

After 45 sessions (Fig. 1A), mice were anesthetized with ketamine (120 mg/kg,i.p.) and xylazine (9 mg/kg,i.p.), placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA), and implanted with a 26-gauge guide cannula (Plastics One, Roanoke, VA) unilaterally aimed at either the right or the left mPFC (AP +1.7 mm; ML ±0.4 mm; DV −1.2 mm), from skull surface [3,17]. The guide cannula was secured to the skull with dental cement (Durelon, Butler Schein, Dublin, OH) and a 33-gauge obturator was inserted. After 1 week of recovery, alcohol+sucrose and sucrose-only self-administration sessions resumed. During baseline, alcohol+sucrose self-administering mice consumed pharmacologically significant doses of sweetened alcohol (Mean ± SEM = 0.97 ± 0.15 g/kg). Dose consumed on Day 87 (Mean ± SEM = 1.06 ± 0.26 g/kg) was positively correlated with BAC (34.7 mg/dl ± 14.2) at the end of the 1-h session ($r^2 = 0.749$; Fig. 1B). Alcohol + sucrose and sucrose-only groups were behavior-matched on total responses during self-administration sessions before (data not shown) and after surgery (Fig. 1E and H, aCSF bars) indicating that KN-93 drug effects were not related to basal performance.

The selective CAMKII inhibitor KN-93 (*N*-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulphonamide phosphate; Tocris Bioscience, Bristol, UK) was administered via site-specific microinjection. Mice were habituated to handing, and sham microinjections were conducted prior to sessions until stable levels of responding were observed (ca. 3 sham injections). Next, a within-subjects dose-effect curve for the CAMKII inhibitor, KN-93, was conducted in a counterbalanced design ($n = 9$ for alcohol + sucrose; $n = 11$ for sucrose-only mice). KN-93 (artificial cerebrospinal fluid, aCSF; 0.3–10 μg/injection; 4 injections/mouse) was infused into the mPFC of unrestrained mice (0.5 μl/injection; 0.125 μl/min; 33-gauge injector extended 2 mm beyond the tip of the guide cannula). After the infusion, mice were immediately placed into the operant chamber for a 1-h self-administration session. Injections occurred 2×/week. Immediately after the final session, mice were deeply anesthetized with sodium pentobarbital (150 mg/kg) and intracardially perfused with 0.9% phosphate buffered saline and 4% paraformaldehyde for histological verification of injection site (Fig. 1C). Three mice were excluded from analysis due to missed cannula placement.

Mice that did not reliably self-administer alcohol + sucrose or sucrose-only after surgery were used to test the effects of KN-93 on locomotor activity as an index of non-specific effects ($n = 8$ alcohol + sucrose; $n = 9$ sucrose-only mice). Open-field activity was measured in Plexiglas chambers (27.9 cm^2; ENV-510, Med Associates) that recorded X-Y ambulatory movements. Mice habituated to the open-field for 2-h. One week later, mice were given a sham injection and placed in the open-field for 1-h. Subsequently, KN-93