Published in final edited form as:

Addict Biol. 2010 July ; 15(3): 299–303. doi:10.1111/j.1369-1600.2010.00212.x.

Ethanol-induced activation of AKT and DARPP-32 in the mouse striatum mediated by opioid receptors

K. Björk*, A. Terasmaa*, H. Sun, W.H. Sommer, and M. Heilig

Laboratory of Clinical and Translational Studies, NIAAA, National Institutes of Health, Bethesda, USA

Abstract

The reinforcing properties of ethanol are in part attributed to interactions between opioid and dopaminergic signaling pathways, but intracellular mediators of such interactions are poorly understood. Here we report that an acute ethanol challenge induces a robust phosphorylation of two key signal transduction kinases, AKT and DARPP-32, in the striatum of mice. Ethanol-induced AKT phosphorylation was blocked by the opioid receptor antagonist naltrexone but unaffected by blockade of dopamine D2 receptors via sulpiride. In contrast, DARPP-32 phosphorylation was abolished by both antagonists. These data suggest that ethanol acts via two distinct but potentially synergistic striatal signalling cascades. One of these is D2-dependent, while the other is not. These findings illustrate that pharmacology of ethanol reward is likely more complex than that for other addictive drugs.

Keywords

ethanol; signal transduction; striatum; dopamine; opioids

Introduction

Converging data from animal and human studies show that interactions between opioid and dopaminergic systems within the mesocorticolimbic reward circuits contribute to the positively reinforcing effects of ethanol. Ethanol-induced neurotransmitter release is followed by a variety of intracellular signal transduction events. Some of these may mediate neuroadaptations that ultimately contribute to the development of alcohol use disorders (Koob 2003; Spanagel 2009; Vengeliene *et al.* 2009).

The pleiotropic signal transduction molecules AKT (also known as phosphokinase B) and DARPP-32 (dopamine and cAMP-regulated phosphoprotein, Mr 32 kDa) are well positioned to play a key role in the development of ethanol-induced neuroadaptations (Beaulieu *et al.* 2005; Svenningsson *et al.* 2005). Their activity is regulated by distinct, stimulus-dependent phosphorylation events. Here, the effects of ethanol on phosphorylation of AKT and DARPP-32 were studied after an acute intraperitoneal administration of 0.75 g/kg ethanol, a dose that has well documented stimulant and reinforcing properties in mice.

Correspondence should be addressed: Markus Heilig, Laboratory of Clinical and Translational Studies, NIAAA/NIH, 10 Center Dr, Bethesda, MD, USA 20892-1108, markus.heilig@mail.nih.gov.

*These authors contributed equally.

Current Addresses: K.B. Translational Neuropharmacology, CMM L8:01, Karolinska Hospital, 171 76 Stockholm, Sweden

A.T. Department of Physiology, University of Tartu, 19 Ravila St., Tartu 50411, Estonia

W.H.S. Molecular Pharmacology, Central Institute of Mental Health, Square J5, 68159 Mannheim, Germany

The authors declare no conflict of interests.

Materials and Methods

Animals

Animal care and handling were performed in accordance with National Institutes of Health guidelines. Three weeks old male C57/BL6 mice were housed with food and water *ad libitum* on a reverse 12 h light/dark cycle (lights on 0600). To habituate them to the experimental procedure, animals were handled and saline-injected on each of three days preceding the experiments.

Animal procedures

To establish a time-course for AKT and DARPP-32 phosphorylation, mice were treated with ethanol (0.75 g/kg i.p.) and sacrificed at 5 different time points (15, 30, 45, 90 and 120 min). Following cervical dislocation, mouse heads were briefly chilled in liquid nitrogen and whole brain was quickly removed and immediately frozen in isopentane at -40°C . Brains were stored at -80°C until further use. Since the time-course analysis revealed peak phosphorylation of AKT and DARPP-32 at 45 and 30 min respectively, these time points were used for additional studies with the opioid and dopamine D2 receptor antagonists. Mice were divided into four groups, in a factorial 2×2 design, in which they received pre-treatment with saline or the respective antagonist, followed by saline or ethanol. Based on preliminary experiments, mice were pre-treated with naltrexone (1mg/kg i.p.) or sulpiride (20 mg/kg) (Tocris Bioscience, Ellisville, MO, USA), 30 minutes prior to ethanol injection. To increase discriminatory power, and more closely approximate doses that produce conditioned place preference for ethanol in C57/BL6 mice, a higher ethanol dose (1.5 g/kg) was used in this experiment. Brains were collected as previously described for the time-course experiment.

Tissue preparation

Brains were dissected in a cryostat at -20°C and striatal samples were collected using a sample corer based on the Paxinos and Watson atlas. Tissue samples were sonicated in lysis buffer (1% SDS, 0.5µl/mL PIC, 1mM PMSF, 2mM orthovanadate and 20mM sodium pyrophosphate). Protein concentration was determined using the DC protein assay (Biorad Biosciences, Hercules, CA, USA). Samples were diluted in 2X sample buffer (Invitrogen, Carlsbad, CA, USA) and boiled in a water bath for 5 minutes. Protein extracts were aliquoted and stored at -80°C until further analysis.

Western blotting

In order to determine the effects of ethanol treatment on the phosphorylation of AKT (Thr-308), and DARPP-32 (Thr-34), individual samples (25 µg, n=6/group) were separated on a 10% bis-Tris gel, and blotted onto nitrocellulose membranes using the Xcell II system (Invitrogen, Carlsbad, CA, USA) exactly as described by (Beaulieu *et al.* 2005). Briefly, following transfer, blots were washed in Tris-buffered saline with 0.05% Tween 20 (TBST) and then blocked with TBST containing 5% nonfat dried milk (NFD). Non-phosphorylated and phosphorylated forms of AKT and DARPP-32 were probed using primary antibodies (Cell Signalling Technology Inc., Beverly, MA, USA) diluted 1:1000, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, diluted 1:10,000 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection and densitometric evaluations were performed using the ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA), the FUJI LAS-3000 system, and the Multigauge Software (FUJIFILM, Tokyo, Japan). Sensitivity of the assays is optimized for detection of pAKT or pDARPP-32. Gels for non-phosphorylated forms were run in parallel with exactly the same protein load. With the exception of AKT all assays are in the

linear range of the saturation curve. Despite using a well established assay that according to the literature is used with up to 50 µg protein load (Beaulieu *et al.* 2008; Beaulieu *et al.* 2007; Beaulieu *et al.* 2005), we do in fact find that detection of non-phosphorylated AKT is at a non-linear part of the saturation curve. Nevertheless, this is unlikely to be an issue, because, while changes in phosphorylation are rapid and occur within the short time window studied, changes in total amount of AKT protein within this brief time interval are unlikely. Furthermore, if such changes did happen, they would obscure any effects on phosphorylation, while we robustly detect these and can also manipulate them pharmacologically. Data are given as ratios of phosphorylated versus non-phosphorylated protein, normalized to the saline control group signal and analyzed using one-way ANOVA and Newman-Keuls post hoc test (STATISTICA, Stat Soft. Inc., Tulsa, OK, USA).

Results

Time-course (Fig 1)

One-way ANOVA for AKT showed a main effect of time ($F[4,31]=7.6$, $p<0.001$), and post-hoc analysis showed a distinct peak of phosphorylation after 45 min (Newman-Keuls post hoc test: $p<0.01$, 45 min vs. all other time points (Fig 1). Similarly, one-way ANOVA for DARPP-32 showed a robust main effect of time ($F[4,33] = 11.7$, $p<0.001$), and more rapid response with peak phosphorylation after 30 min (Newman-Keuls post hoc test: $p<0.001$, 30 min vs. all other time points).

Antagonist treatment (Fig 2)

Two-way ANOVA showed that ethanol treatment (1.5 g/kg i.p.) induced a significant increase of pAKT after 45 min compared to the saline treated group. This effect was abolished by pre-treatment with naltrexone (1mg/kg i.p.) 30 minutes prior to injection of ethanol (main ethanol effect: $F[1,15]=8.9$, $p<0.01$; drug effect: $F[1,15]=5.1$, $p<0.05$; and drug $\times$ ethanol interaction: $F[1,20]=5.1$; $p<0.05$; Newman-Keuls post hoc test $p<0.05$ for the vehicle/ethanol treated group vs. all other groups; Fig 2A). In contrast, pre-treatment with sulpiride (20 mg/kg) failed to suppress ethanol-induced AKT phosphorylation (main ethanol effect: $F[1,18]=14.7$, $p<0.01$; drug effect: $F[1,18] = 0.1$, n.s.; drug $\times$ ethanol interaction $F[1,14]=0.2$, n.s.; Newman-Keuls post hoc test $p<0.05$ for the vehicle/ethanol treated group vs. vehicle/saline and sulpiride/saline groups, $p<0.05$ sulpiride/saline vs. sulpiride/ethanol; Fig 2B). Ethanol treatment (1.5 g/kg i.p.) induced a robust increase of pDARPP-32 after 30 min compared to the saline treated group that was eliminated by pre-treatment with naltrexone (Fig 2C; main ethanol effect $F[1,20]=17.6$, $p<0.001$; drug effect: $F[1,20]=37.6$, $p<0.001$; drug $\times$ ethanol interaction $F[1,20]=37.6$; $p<0.001$; Newman-Keuls post hoc test $p<0.001$ for the vehicle/ethanol treated group vs. all other groups. DARPP-32 phosphorylation was also blocked by sulpiride pretreatment (Fig. 2D; main ethanol effect: $F[1,14]=21.0$, $p<0.001$; drug effect: $F[1,14]=26.5$, $p<0.001$; drug $\times$ ethanol interaction $F[1,14]=26.5$ $p<0.001$; Newman-Keuls post hoc test $p<0.001$ for the vehicle/ethanol treated group vs. all other groups).

Discussion

We found robustly induced phosphorylation of AKT at Thr-308 and of DARPP-32 at Thr-34 in the mouse striatum. The delayed onset of the ethanol-evoked phosphorylation observed here is in line with the concept that the acute behavioral effects of ethanol result from indirect effects on various neurotransmitter systems rather than from ethanol's actions on its primary targets (Spanagel 2009). Phosphorylation of DARPP-32 following an ethanol challenge is in agreement with previous observation of similar effects following administration of several other addictive drugs (Svenningsson *et al.* 2005). For AKT on the

other hand, drug responses seems to be more diverse. In contrast to psychostimulants, which induce a robust dephosphorylation of AKT, ethanol leads to a robust phosphorylation of AKT (Beaulieu *et al.* 2005, Neznanova *et al.* 2009). While distinct from psychostimulants, this profile is similar to that previously described for morphine, a prototypical mu-opioid receptor agonist (Muller & Unterwald 2004). These observations suggest that ethanol mimics the actions of direct mu-opioid agonists by inducing transient AKT phosphorylation, presumably through mu-opioid receptor activation.

We next asked whether ethanol-induced AKT and DARPP-32 phosphorylation is downstream of opioid or dopamine neurotransmission. To this end, we pre-treated mice with either the opioid antagonist naltrexone (1 mg/kg i.p.) or the dopamine D2 receptor antagonist sulpiride (20 mg/kg), 30 min before ethanol administration (1.5 g/kg, i.p.). Ethanol-induced striatal AKT Thr-308 phosphorylation was abolished by naltrexone but not sulpiride (fig. 2A and B). In contrast, phosphorylation of DARPP-32 at Thr-34 was blocked by both antagonists (fig 2C and D).

The finding that a D2 antagonist failed to block ethanol-evoked AKT phosphorylation in the striatum suggests a dopamine independent mechanism. In fact, release of endogenous opioids in response to ethanol has previously also been shown directly within the striatum, and it has long been noted that opioid receptor activation can produce psychostimulant and reinforcing effects in a direct, dopamine independent manner (Marinelli *et al.* 2003; Vaccarino *et al.* 1986). The picture that emerges is thus that ethanol can activate opioid mechanisms at two levels. One of these actions is upstream of the established DA activation in response to ethanol, which ultimately leads to induction of striatal DARPP-32 phosphorylation through activation of D2 receptors. The second, DA-independent component may be induced through direct actions within the striatum, and result in phosphorylation of AKT. Blockade of opioid receptors by naltrexone may act synergistically to prevent both these ethanol actions.

The canonical model for DARPP-32 phosphorylation at Thr-34 posits that D1 activation and subsequent protein kinase A (PKA) activation increase phosphorylation, whereas D2 activation inhibits it (Svenningsson *et al.* 2005). Clearly, this model cannot explain the ability of the D2 antagonist sulpiride to block ethanol induced DARPP-32 phosphorylation observed here. However, ethanol has been shown to facilitate interactions between adenosine A2 and D2 receptors on medium spiny neurons that robustly activate PKA. This would in turn be expected to lead to increased phosphorylation of DARPP-32 (Yao *et al.* 2002). It is possible that D2 antagonism affects phosphorylation through this mechanism. That naltrexone is similarly able to abolish striatal DARPP-32 phosphorylation is likely due to blockade of opioid receptors within the mesencephalon, thus interfering with the ability of endogenous opioids released in response to ethanol to disinhibit dopaminergic neurons.

In conclusion, our results show that reinforcing doses of ethanol increase both DARPP-32 and AKT phosphorylation in the mouse striatum. The differential sensitivity of these effects to naltrexone and sulpiride suggests of two distinct but potentially synergistic striatal signalling cascades that are initiated by actions of ethanol on endogenous opioid systems. One of these is D2-dependent, while the other is not.

Acknowledgments

This study was supported by intramural funding from NIAAA

Reference List

- Beaulieu JM, Gainetdinov RR, Caron MG. The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends in Pharmacological Sciences*. 2007; 28:166–172. [PubMed: 17349698]
- Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, Wetsel WC, Lefkowitz RJ, Gainetdinov RR, Caron MG. A beta-arrestin 2 signaling complex mediates lithium action on Behavior. *Cell*. 2008; 132:125–136. [PubMed: 18191226]
- Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*. 2005; 122:261–273. [PubMed: 16051150]
- Koob GF. Alcoholism: allostasis and beyond. *Alcohol Clin Exp Res*. 2003; 27:232–243. [PubMed: 12605072]
- Marinelli PW, Quirion R, Gianoulakis C. A microdialysis profile of beta-endorphin and catecholamines in the rat nucleus accumbens following alcohol administration. *Psychopharmacology (Berl)*. 2003; 169:60–67. [PubMed: 12759807]
- Muller DL, Unterwald EM. In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther*. 2004; 310:774–782. [PubMed: 15056728]
- Neznanova O, Björk K, Rimondini R, Hansson AC, Hyytiä P, Heilig M, Sommer WH. Acute ethanol challenge inhibits glycogen synthase kinase-3beta in the rat prefrontal cortex. *Int J Neuropsychopharmacol*. 2009; 12:275–80. [PubMed: 19007447]
- Spanagel R. Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol Rev*. 2009; 89:649–705. [PubMed: 19342616]
- Svenningsson P, Nairn AC, Greengard P. DARPP-32 mediates the actions of multiple drugs of abuse. *AAPS J*. 2005; 7:E353–E360. [PubMed: 16353915]
- Vaccarino FJ, Amalric M, Swerdlow NR, Koob GF. Blockade of Amphetamine But Not Opiate-Induced Locomotion Following Antagonism of Dopamine Function in the Rat. *Pharmacology Biochemistry and Behavior*. 1986; 24:61–65.
- Vengeliene V, Celerier E, Chaskiel L, Penzo F, Spanagel R. Compulsive alcohol drinking in rodents. *Addict Biol*. 2009; 14:384–396. [PubMed: 19740366]
- Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, Diamond I. betagamma Dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. *Cell*. 2002; 109:733–743. [PubMed: 12086672]

Figure 1. Time course of striatal AKT (Thr-308) and DARPP-32 (Thr-34) phosphorylation following acute administration of ethanol (0.75g/kg i.p.) in mice

Data for pAKT (solid line) and pDARPP-32 (dashed line) are normalized to the respective total protein levels and expressed as percentage of the sal/sal control group (mean±SEM).

Insets: Representative western blot images from the time point at maximum response.

Statistical analysis was done using 1-way ANOVA and Newman-Keuls post hoc test. For a detailed description of methods and statistics see supplementary information. N = 6/group.

***p<0.001

Figure 2. Ethanol-induced AKT and DARPP-32 phosphorylation is differentially affected by opioid and dopamine D2 receptor blockade

Upper panel: AKT was measured 45 min after either naloxone (A) or sulpiride (B) pre-treatment. *Lower panel:* DARPP-32 was measured 30 min after the same pre-treatments (C and D, respectively). Data are normalized to total AKT or DARPP-32 and expressed as percentage of the vehicle/saline control group (mean±SEM). Data were analyzed using two-way ANOVA and Newman-Keuls post hoc test. For detailed method description and statistics see supplementary material. N=6/group. **p < 0.01, *** p < 0.001, vehicle/ethanol treated group vs. all other groups. #p < 0.05, vehicle/ethanol vs. vehicle/saline and sulpiride/saline treated groups. +p < 0.05, sulpiride/saline vs. sulpiride/ethanol.