

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

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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 signaling 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 Dopamine, ethanol, opioids, signal transduction, striatum.

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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 protein kinase 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, Nairn & Greengard 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.

MATERIALS AND METHODS

Animals

Animal care and handling were performed in accordance with National Institutes of Health guidelines. Three-week-old male C57/BL6 mice were housed with food and water *ad libitum* on a reverse 12-hour 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.

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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 five different timepoints (15, 30, 45, 90 and 120 minutes). 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 minutes, respectively, these timepoints 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 (1 mg/kg i.p.) or sulpiride (20 mg/kg) (Tocris Bioscience, Ellisville, MO), 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% sodium dodecyl sulfate, 0.5 $\mu\text{l}/\text{mL}$ protease inhibitor cocktail, 1 mM phenylmethanesulfonyl fluoride, 2 mM orthovanadate and 20 mM sodium pyrophosphate). Protein concentration was determined using the detergent compatible protein assay (Biorad Biosciences, Hercules, CA). Samples were diluted in $2\times$ sample buffer (Invitrogen, Carlsbad, CA) 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 , $n = 6/\text{group}$) were separated on a 10% bis-Tris gel, and blotted onto nitrocellulose membranes using the Xcell II system (Invitrogen) 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. Non-phosphorylated and phosphorylated forms of AKT and DARPP-32 were probed using primary antibodies (Cell Signalling Technology Inc., Beverly, MA) 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), the FUJI LAS-3000 system and the Multigauge Software (Fujifilm, Tokyo, Japan). Sensitivity of the assays was 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 were 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.* 2005; Beaulieu, Gainetdinov & Caron 2007; Beaulieu *et al.* 2008), 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 the 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).

RESULTS

Time course

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 minutes (Newman-Keuls *post hoc* test: $P < 0.01$, 45 minutes versus all other timepoints (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 minutes (Newman-Keuls *post hoc* test: $P < 0.001$, 30 minutes versus all other timepoints).

Antagonist treatment

Two-way ANOVA showed that ethanol treatment (1.5 g/kg i.p.) induced a significant increase in pAKT after 45 minutes compared with the saline-treated group. This effect was abolished by pre-treatment with naltrexone (1 mg/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-ethanol interaction: $F[1,20] = 5.1$; $P < 0.05$; Newman-Keuls *post hoc* test $P < 0.05$ for the vehicle/ethanol-treated group versus

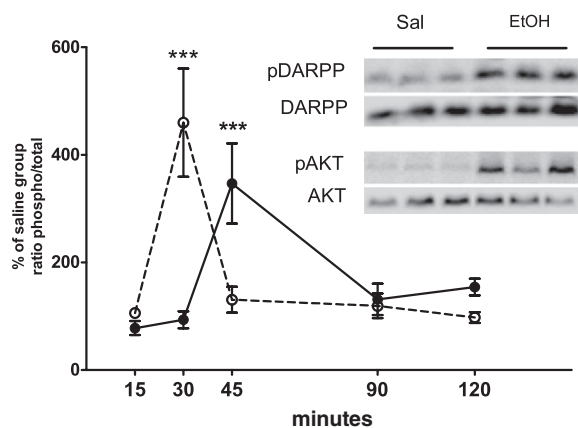


Figure 1 Time course of striatal AKT (Thr-308) and DARPP-32 (Thr-34) phosphorylation following acute administration of ethanol (0.75 g/kg i.p.) in mice. Data for pAKT (solid line) and pDARPP-32 (dashed line) are normalized to the respective total protein levels and expressed as percentage of the sal/sal control group (mean \pm SEM). *Insets:* Representative Western blot images from the timepoint at maximum response. Statistical analysis was carried out using one-way ANOVA and Newman–Keuls *post hoc* test. For a detailed description of methods and statistics, see supplementary information. $n = 6/\text{group}$. *** $P < 0.001$

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–ethanol interaction $F[1,14] = 0.2$, n.s.; Newman–Keuls *post hoc* test $P < 0.05$ for the vehicle/ethanol-treated group versus vehicle/saline and sulpiride/saline groups, $P < 0.05$ sulpiride/saline versus sulpiride/ethanol; Fig. 2b). Ethanol treatment (1.5 g/kg i.p.) induced a robust increase in pDARPP-32 after 30 minutes compared with the saline treated group. This increase 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–ethanol interaction $F[1,20] = 37.6$; $P < 0.001$; Newman–Keuls *post hoc* test $P < 0.001$ for the vehicle/ethanol-treated group versus all other groups). DARPP-32 phosphorylation was also blocked by sulpiride pre-treatment (Fig. 2d; main ethanol effect: $F[1,14] = 21.0$, $P < 0.001$; drug effect: $F[1,14] = 26.5$, $P < 0.001$; drug–ethanol interaction $F[1,14] = 26.5$, $P < 0.001$; Newman–Keuls *post hoc* test $P < 0.001$ for the vehicle/ethanol-treated group versus 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 minutes before ethanol administration (1.5 g/kg, i.p.). Ethanol-induced striatal AKT Thr-308 phosphorylation was abolished by naltrexone but not by 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 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 (Vaccarino *et al.* 1986; Marinelli, Quirion & Gianoulakis 2003). The picture that emerges is thus that ethanol can activate opioid mechanisms at two levels. One of these actions is upstream of the established dopamine activation in response to ethanol, which ultimately leads to induction of striatal DARPP-32 phosphorylation through activation of D2 receptors. The second, dopamine-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 increases phosphorylation, whereas D2 activation inhibits it (Svenningsson *et al.* 2005). Clearly, this model cannot explain the ability

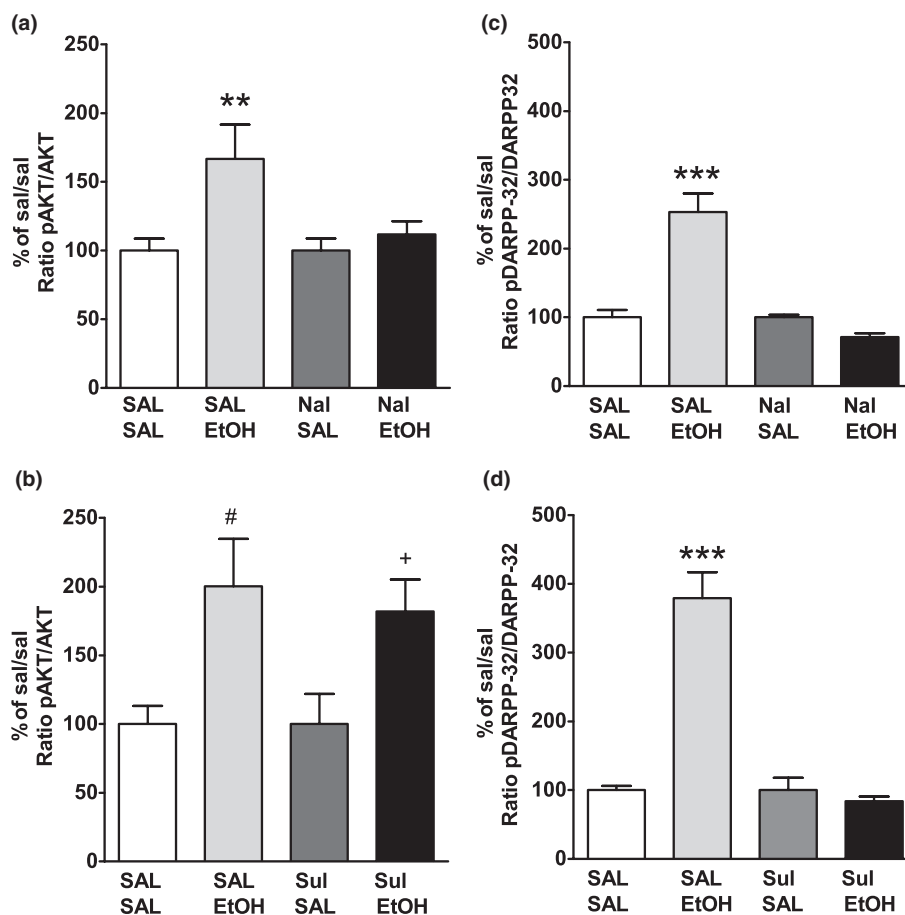


Figure 2 Ethanol-induced AKT and DARPP-32 phosphorylation is differentially affected by opioid and dopamine D2 receptor blockade. *Upper panel:* AKT was measured 45 minutes after either naltrexone (a) or sulpiride (b) pre-treatment. *Lower panel:* DARPP-32 was measured 30 minutes after the same pre-treatments (c and d, respectively). Data are normalized to total AKT or DARPP-32 and expressed as percentage of the vehicle/saline control group (mean \pm SEM). Data were analyzed using two-way ANOVA and Newman-Keuls *post hoc* test. For detailed method description and statistics, see supplementary material. $n = 6/\text{group}$. ** $P < 0.01$, *** $P < 0.001$, vehicle/ethanol-treated group versus all other groups. # $P < 0.05$, vehicle/ethanol versus vehicle/saline and sulpiride/saline-treated groups. + $P < 0.05$, sulpiride/saline versus sulpiride/ethanol

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 two distinct but potentially synergistic striatal signaling cas-

cases that are initiated by actions of ethanol on endogenous opioid systems. One of these is D2-dependent, while the other is not.

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Disclosure/Conflict of Interest

The authors declare no conflict of interests.

Authors' Contribution

Authors jointly conceived and designed the experiments, AT, KB and HS carried them out and all authors jointly drafted the manuscript.

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