Anaplastic lymphoma kinase regulates binge-like drinking and dopamine receptor sensitivity in the ventral tegmental area

John W. Dutton III1,2*, Hu Chen1*, Chang You3, Mark S. Brodie3 & Amy W. Lasek1

Department of Psychiatry, University of Illinois, Chicago, IL USA1, Biologic Resources Laboratory, University of Illinois, Chicago, IL USA2 and Department of Physiology and Biophysics, University of Illinois, Chicago, IL USA3

ABSTRACT

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase associated with alcohol dependence in humans and behavioral responses to ethanol in mice. To characterize the ability of ALK to control ethanol consumption, we treated mice with the ALK inhibitors TAE684 or alectinib before testing them for binge-like drinking using the drinking in the dark protocol. Mice treated with ALK inhibitors drank less ethanol than controls. In addition, TAE684 treatment abolished ethanol conditioned place preference, indicating that ALK regulates the rewarding properties of ethanol. Because the ventral tegmental area (VTA) is a key brain region involved in the rewarding effects of ethanol, we determined if Alk expression in the VTA is important for binge-like ethanol consumption. Mice expressing a short hairpin ribonucleic acid targeting Alk in the VTA drank less ethanol compared with controls. ALK is expressed on dopamine (DA) neurons in the VTA, suggesting that ALK might regulate their firing properties. Extracellular recordings of putative DA neurons in VTA slices demonstrated that ALK inhibition did not affect the ability of ethanol to stimulate, or DA to inhibit, the firing of DA neurons. However, inhibiting ALK attenuated the time-dependent reversal of inhibition produced by moderate concentrations of DA, suggesting that ALK affects DA D2 autoreceptor (D2R) desensitization. Altered desensitization of the D2R changes the firing of DA neurons and is predicted to affect DA levels and alcohol drinking. These data support the possibility that ALK might be a novel target of pharmacotherapy for reducing excessive alcohol consumption.

Keywords Alcohol addiction, ALK, binge-drinking, dopamine receptor, ventral tegmental area.

INTRODUCTION

Binge drinking is a pattern of harmful alcohol use defined by the National Institute on Alcohol Abuse and Alcoholism as drinking an amount of alcohol in a short period of time (2 hours) that is sufficient to produce blood alcohol concentrations ≥0.08 grams percent. Binge drinking accounts for over half of the approximately 80,000 deaths yearly that are attributed to alcohol use. Binge drinkers also put themselves at increased risk for the development of diseases affecting the cardiovascular system, liver and brain, among others (O’Keefe, Bybee & Lavie 2007; Zakhari & Li 2007; Petit et al. 2014). In the United States, the prevalence of binge drinking among adults is approximately 18 percent (Kanny et al. 2013), and binge drinking increases the risk of developing alcohol dependence (Bonomo et al. 2004). New pharmacotherapies aimed at reducing binge drinking would be useful in improving health outcomes and preventing alcoholism.

To find new molecular targets for the treatment of alcohol use disorders (AUDs), extensive efforts are underway to identify genes that regulate behavioral responses to ethanol. Variants in the human ALK gene are associated with alcohol dependence, ratings of subjective high after alcohol consumption and sensitivity to the ataxic effects of alcohol (Wang et al. 2011; Lasek et al. 2011b).

*These two authors contributed equally to the work.
In addition, Alk knockout (Alk\(^{KO}\)) mice exhibit altered behavioral responses to ethanol (Lasek et al. 2011b). The Alk gene encodes a receptor tyrosine kinase that is expressed throughout the developing and adult brain with enriched expression in the olfactory bulbs, frontal cortex, striatum, hippocampus, thalamus and midbrain nuclei (Iwahara et al. 1997; Vernersson et al. 2006; Bilsland et al. 2008). ALK appears to regulate neurogenesis and associative learning, because Alk\(^{KO}\) mice show altered neurogenesis and subtle enhancements in spatial learning (Bilsland et al. 2008; Weiss et al. 2012). Alk\(^{KO}\) mice also display less anxiety-like and depression-like behaviors (Bilsland et al. 2008; Weiss et al. 2012). Finally, inhibition of ALK activity with TAE684 decreases cocaine sensitization and conditioned place preference (CPP) (Lasek et al. 2011a). Together, these studies indicate that ALK is involved in learning and memory processes and behaviors related to psychiatric disorders such as addiction. Given the clinical and preclinical evidence suggesting that ALK regulates behaviors related to alcohol abuse, ALK is a potentially novel therapeutic target for the treatment of AUDs.

Our goal was to determine if the potent ALK inhibitors, TAE684 and alectinib, alter binge-like drinking in mice. We used the drinking in the dark (DID) model to examine the effect of ALK inhibition on binge drinking. In this model, C57BL/6 mice consume intoxicating amounts of alcohol, achieving blood ethanol concentrations in excess of 0.1 grams percent (Rhodes et al. 2005). We also tested TAE684 for the ability to regulate the rewarding effects of ethanol using the CPP test, because binge-drinking behavior is linked to heightened sensitivity to ethanol reward (Green & Grahame 2008; King et al. 2011; King et al. 2014).

For ALK to alter alcohol-related behaviors, it must be involved in alcohol-sensitive pathways important for those behaviors. One such pathway is the mesolimbic DA system, which consists of DA neurons in the VTA that project to the nucleus accumbens (Acb). This system is clearly important for ethanol consumption and reward (Gonzales, Job & Doyon 2004). ALK acting in the mesolimbic system to regulate alcohol drinking could alter physiological responses to ethanol or interfere with neuronal adaptations upon alcohol exposure. We therefore tested for the effect of ALK inhibition on physiological responses of VTA DA neurons. The present study provides a novel mechanistic link between ALK activity, responses of VTA neurons to DA and binge drinking.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6J mice were used for the DID test, immunohistochemistry and electrophysiology. Male DBA/2J mice were used for ethanol CPP. All mice were 8 weeks old when purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and 9–12 weeks old during behavioral testing. Mice were group-housed in a temperature–and humidity–controlled environment under a 14-hour light/dark cycle (lights on at 6 AM and off at 8 PM), unless they underwent the DID test (described later). Animals had access to food and water ad libitum and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures performed on animals were approved by the University of Illinois at Chicago (UIC) Animal Care Committee.

**Drinking in the dark test**

The 4-day ethanol DID test was performed as described previously (Rhodes et al. 2005). Briefly, mice were individually housed in a 12-hour reversed light/dark cycle room (lights off at 10 AM and on at 10 PM) for 2 weeks prior to behavioral testing. The DID test was performed by replacing the water bottle 3 hours into the dark cycle with a single sipper tube containing 20 percent ethanol in water. On the first 3 days (Monday, Tuesday and Wednesday), mice were given access to the ethanol solution for 2 hours. On the fourth day, mice were given access to the ethanol solution for 4 hours, and the volume consumed was measured at 2 and 4 hours. Blood samples (20 μl) were collected immediately after the 4-hour drinking session on day 4 to measure blood ethanol concentrations (BECs). Blood was collected in heparinized capillary tubes via tail vein puncture. BECs were determined using a nicotinamide adenine dinucleotide-alcohol dehydrogenase enzymatic assay (Zapata, Gonzales & Shippenberg 2006). To test sucrose consumption, mice underwent a DID test identical to the ethanol consumption test, except the sipper tube contained 2 percent sucrose in water instead of ethanol.

**Ethanol conditioned place preference**

Conditioned place preference training and testing were conducted in a modified mouse open field apparatus with 48-channel infrared beam detectors and Activity Monitor software (Med Associates, St. Albans, VT USA). Modifications of the open field apparatus to create a 2-chambered setup for CPP have been described previously (Hilderbrand & Lasek 2014). On the first day of testing, mice were placed in the CPP apparatus and allowed access to both sides of the chamber for 30 minutes to measure baseline preference. Mice were then assigned to the non-preferred side for ethanol injections. Mice were conditioned over the next 8 weekdays with an intraperitoneal (i.p.) injection of 2 g/kg ethanol (20 percent v/v in
Anaplastic lymphoma kinase inhibitor treatment in vivo

TAE684 and alectinib were purchased from Selleck Chemicals (Houston, TX, USA). TAE684 was administered at a dose of 10 mg/kg in 90 percent PEG 300, 10 percent 1-methyl-2-pyrrolidinone vehicle. This dose is effective in inhibiting the growth of ALK-dependent tumors and attenuating behavioral responses to cocaine in mice (Galkin et al. 2007; Lasek et al. 2011a). Pharmacokinetic studies in mice have shown that maximal plasma levels of TAE684 are achieved 7 hours after oral administration; the elimination half-life is approximately 12 hours; bioavailability is 60–70 percent, and the brain to plasma ratio is 2:1 (Galkin et al. 2007; Zhang et al. 2012). Alectinib was administered at a dose of 60 mg/kg in 0.02 N HCl, 10 percent dimethyl sulfoxide (DMSO), 10 percent Cremophor EL, 15 percent PEG400 and 15 percent 2-hydroxypropyl-β-cyclodextrin vehicle (Sakamoto et al. 2011). This dose regresses brain tumor xenografts in mice (Kodama et al. 2014). Pharmacokinetic studies in mice have shown that maximal plasma levels of alectinib are achieved approximately 7 hours after oral administration; the half-life is approximately 9 hours, and bioavailability is approximately 70 percent (Sakamoto et al. 2011). In rats, alectinib levels peak in the brain at about 8 hours (Kodama et al. 2014). Mice were administered compounds or vehicle by oral gavage in a volume of approximately 0.1 ml. TAE684, alectinib or vehicle was administered 5 hours before each drinking session in the DID test (10–12 mice per group) or 5 hours before each of the saline and ethanol conditioning sessions in the CPP test (13–17 mice per group). For the control CPP experiment in which we conditioned with TAE684 in the absence of ethanol, TAE684 was administered on days 2, 4, 6 and 8, and vehicle was administered on days 3, 5, 7 and 9. 5 hours before injecting mice i.p. with saline (10 ml/kg) and confining them to one side of the apparatus for 5 minutes. The timing of ALK inhibitor administration in these experiments was chosen so that maximal plasma or brain levels would be achieved during behavioral testing (Galkin et al. 2007; Sakamoto et al. 2011).

Stereotaxic surgery and lentiviral injections

Replication-deficient lentiviruses expressing shScr or shAlk were produced as described previously (Lasek et al. 2011a). The vector backbone was pLL3.7, which contains shAlk or shScr under the control of the U6 promoter, and EGFP from a CMV promoter (Addgene nos. 59299 and 59297, Cambridge, MA, USA). For stereotaxic viral injections, mice were anesthetized with ketamine/xylazine, placed in a stereotaxic alignment system (David Kopf Instruments) and injected with 1.0 μl of virus solution bilaterally. VTA coordinates were: AP – 3.2 mm, ML +/- 0.5 mm, DV – 4.7 mm. Viral titers were approximately 3 × 10^7 pg p24 gag antigen/ml. Mice were allowed to recover for 3 weeks prior to commencing the DID experiment. Viral infection was confirmed by histology after the completion of the DID test. We used 12 mice per group and removed 1 mouse from each group in the analysis because of incorrect localization of viral infection. Acb injections were performed as previously described (Lasek et al. 2011a).

Immunofluorescent staining of brain sections

Mice were euthanized with pentobarbital followed by transcardial perfusion with cold phosphate-buffered saline and 4 percent paraformaldehyde. Brains were processed as described previously (Kharazia, Jacobs & Prince 2003). Primary antibodies for green fluorescent protein (GFP) and tyrosine hydroxylase (TH) detection were: rabbit anti-TH (Millipore, Temecula, CA) and mouse anti-GFP (Life Technologies, Carlsbad, CA, USA). Primary antibodies for TH and ALK detection were: mouse anti-TH (Millipore) and rabbit anti-ALK, no. 3815 (Weiss et al. 2012). Images of lentiviral infection in the VTA were captured using an EVOS Fl inverted fluorescence microscope with a 4X objective lens (Life Technologies) and images of ALK and TH staining in the VTA were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY, USA). For quantification of knockdown of ALK in the VTA after lentiviral infection, the intensity of ALK staining in the infected VTA was quantified from 2 to 3 sections per mouse and 9 mice per group (shScr, shAlk) 3 weeks after lentiviral infection. Intensity was measured using the National Institutes of Health ImageJ software.

Preparation of brain slices for electrophysiology

Brain slices containing the VTA were prepared as described (Brodie et al. 1999). Coronal sections (400 μm thick) were cut on a vibratome in chilled cutting solution, then placed in artificial cerebrospinal fluid (aCSF, 35°C) and maintained for at least 1 hour before recording. Recordings were made up to 6 hours after the slices were placed in the recording chamber. The composition of the aCSF in these experiments was (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.24,
Calculated over 1 minute intervals during the experiments: peak drug-induced changes in firing rate were expressed as the percentage change from the control firing rate. For the effects of ALK inhibitors on ethanol concentration-response curves, ethanol (40, 80, 120 mM) was tested before and after addition of the ALK inhibitor (100 nM TAE684 or alectinib in DMSO) to the extracellular medium. For the effects of ALK inhibitors on responses to acute DA, short application (5 minutes) of single concentrations of DA (1, 2, 5 or 10 μM) was added to slices in the presence of 0.1 percent DMSO (control), TAE684 or alectinib. A washout period of at least 10 minutes was used between concentrations to permit the firing rate to return to baseline. To test reversal of DA inhibition (DIR or desensitization), neurons of the VTA were incubated with DA for 40 minutes, and firing was measured every 5 minutes over the 40-minute period. DA concentrations were adjusted for each neuron so that inhibition at 5 minutes was between 50 and 95 percent (Nimittivilai & Brodie 2010). This method controlled for differences in sensitivity between neurons but also sometimes resulted in the mean concentrations of DA slightly differing between groups.

Statistical analysis
All data were expressed as the mean ± SEM and analyzed using Prism software (GraphPad, La Jolla, CA, USA). DID data from the 2-hour drinking sessions over 4 days was analyzed by two-way repeated measures (RM) ANOVA for treatment and time. The 4 hours DID, BEC and ethanol preference scores from CPP were analyzed using a Student’s t-test. Electrophysiology data were analyzed using a two-way RM ANOVA with post-hoc Tukey’s multiple comparisons tests.

RESULTS
Anaplastic lymphoma kinase inhibition attenuates binge-like ethanol consumption
To examine how ALK inhibition might affect binge-like ethanol consumption, we treated mice with the ALK inhibitor TAE684 and found that they consumed significantly less ethanol during the 2-hour drinking sessions over the 4-day DID experiment when compared with mice treated with vehicle [Fig. 1a; two-way RM ANOVA, treatment \( F_{(1, 22)} = 5.33, \ P = 0.0311 \)]. Mice treated with TAE684 consumed 37 percent less ethanol overall when compared with mice treated with vehicle. We also examined ethanol intake after 4 hours of drinking on day 4 and observed a trend toward decreased ethanol consumption (a 31 percent reduction) \( \left[ t_{(22)} = 1.87, \ P = 0.076 \right] \). Finally, we measured BECs after the 4-hour drinking session. Consistent with decreased ethanol consumption in mice treated with TAE684, BECs were lower in mice treated with TAE684 \{vehicle, 62.9 ± 29.7 mg percent; TAE684, 2.4 ± 2.4 mg percent. \( t_{(10)} = 2.02, \ P = 0.076 \}\}. Treatment of mice with TAE684 did not affect sucrose drinking (Fig. 1b). We performed additional experiments to determine if TAE684 alters the acute sedative response to ethanol or ethanol clearance from the blood. TAE684 did not affect recovery time from the sedative effect of ethanol in the ethanol-induced loss of the righting reflex test, nor did TAE684 alter ethanol clearance from the blood (Fig. S1). Together, these data suggest that inhibition of ALK affects binge-like ethanol drinking without altering ethanol metabolism or the consumption of natural rewards such as sucrose.

To extend these findings to a new clinically available ALK inhibitor, we treated mice with alectinib and found that they consumed significantly less ethanol over the 4 days of DID testing compared with vehicle-treated mice [Fig. 1c; two-way RM ANOVA, treatment \( F_{(1, 18)} = 19.3, \ P = 0.0004 \], time \( F_{(3, 54)} = 2.44, \ P = 0.074 \], treatment x time interaction \( F_{(3, 54)} = 3.21, \ P = 0.031 \}]. Overall, mice treated with alectinib consumed 56 percent less ethanol over the four 2-hour drinking sessions when compared with vehicle-treated mice. Post-hoc Holm–Sidak’s multiple comparisons test demonstrated a significant difference between vehicle- and alectinib-treated mice on days 2, 3 and 4. Ethanol consumption was also significantly reduced by 61 percent in alectinib-treated mice after 4 hours of drinking on day 4 \( t_{(18)} = 6.25, \ P < 0.0001 \). Similar to what we observed in mice treated with TAE684, alectinib did not affect consumption of 2 percent sucrose (Fig. 1d). We also examined two-bottle choice drinking in mice treated with alectinib to determine if ALK inhibition alters ethanol preference. Mice were given the choice between sipper tubes filled with water or 20 percent ethanol in a 4-day DID experiment. We found that mice treated with alectinib drank significantly less ethanol in this procedure, similar to what we observed in the one bottle DID experiment (Fig. S2). The overall reduction in ethanol consumption over 4 days was 78 percent. Importantly, mice treated with alectinib demonstrated significantly reduced preference for ethanol (Fig. S2, a 75 percent reduction overall), indicating that alectinib did not affect general fluid drinking without altering ethanol metabolism or the consumption of natural rewards such as sucrose.
Collectively, these data indicate that ALK inhibitors reduce ethanol consumption without affecting sucrose consumption, ethanol-induced sedation or ethanol clearance.

Anaplastic lymphoma kinase inhibition attenuates ethanol reward

The reduced ethanol consumption in mice treated with ALK inhibitors suggests that ALK might be involved in the rewarding properties of ethanol. To test this, we performed ethanol CPP in DBA/2J mice treated with TAE684 or vehicle. DBA/2J mice were chosen because this inbred mouse strain develops robust CPP for ethanol (Cunningham et al. 1992). DBA/2J mice were treated with TAE684 or vehicle daily prior to ethanol and saline conditioning sessions (Fig. 2a). TAE684 treatment decreased the ethanol place preference score by 76 percent \( (t_{(27)} = 2.02, P = 0.053) \). Prior TAE684 treatment had no effect on the distance traveled on test day (Fig. 2c). We also analyzed the locomotor activity data from the saline and ethanol conditioning days. TAE684 treatment did not affect the distance traveled in mice injected with saline or ethanol (Fig. 2d and e). Interestingly, we found that DBA/2J mice sensitized to the locomotor-stimulating effect of ethanol during the conditioning procedure, as reported previously (Phillips, Dickinson & Burkhart-Kasch 1994), but mice treated with TAE684 sensitized to the same extent as vehicle-treated mice (Fig. 2e; two-way RM ANOVA, time \( F_{(3, 72)} = 24.78, P < 0.0001 \)). To test if TAE684 is rewarding or aversive on its own (i.e., in the absence of ethanol), we performed a control experiment in which mice were place-conditioned with TAE684 (Fig. 2f). Conditioning with TAE684 did not alter the time spent on the TAE684-paired side of the apparatus, indicating that ALK inhibition in the absence of ethanol does not induce preference or aversion (Fig. 2g). These data suggest that ALK inhibition attenuates the rewarding properties of ethanol without affecting ethanol-induced locomotor sensitization.

Anaplastic lymphoma kinase is expressed in dopamine neurons and in non-dopamine cells in the ventral tegmental area

The VTA mediates the rewarding properties of ethanol, and several groups have demonstrated that the VTA plays a role in binge-like ethanol consumption in mice (Gonzales et al. 2004). As a result, the VTA is one region where ALK might act to control ethanol consumption. We analyzed ALK expression in the VTA using immunohistochemistry. Antibodies to ALK and TH (an enzyme
involved in the biosynthesis of DA) were incubated on naïve mouse brain slices containing the VTA and detected using fluorescent secondary antibodies and confocal microscopy. ALK immunoreactivity was evident in the soma and processes of several cell types in the VTA (Fig. 3), including cells expressing TH, indicating that ALK is expressed in DA neurons in the VTA. However, ALK staining was not restricted to DA neurons. The presence of ALK in DA neurons and non-DA cells in the VTA supports a role for ALK in modulating VTA activity.

Anaplastic lymphoma kinase expression in the ventral tegmental area regulates binge-like ethanol consumption

To determine if ALK functions in the VTA to affect alcohol drinking, we employed a lentiviral-delivered shRNA targeting Alk (shAlk) to site-specifically knockdown Alk expression using an shRNA that we previously validated to reduce Alk mRNA in the Acb by 30 percent (Lasek et al. 2011a). Mice underwent stereotaxic surgery and were injected in the VTA with lentivirus expressing shAlk or a control shRNA (shScr). Mice expressing shAlk in the VTA consumed less ethanol during the 2-hour drinking sessions over the 4-day DID test compared with mice expressing shScr {Fig. 4a; two-way RM ANOVA, shRNA \[EF(1,20) = 8.68, P = 0.008\], time \[EF(3, 60) = 3.71, P = 0.016\]]. Ethanol consumption was also significantly reduced during the 4-hour drinking session on day 4 \[t(20) = 2.51, P = 0.021\]. Consistent with reduced ethanol consumption on day 4, BECs were decreased in mice expressing shAlk compared with shScr \[shScr, 178.8 ± 22.8 mg percent; shAlk, 82.3 ± 26.9 mg percent\].
After the completion of the DID test, targeting of lentivirus to the VTA was verified by immunohistochemistry using antibodies to TH and GFP, which is expressed by the lentivirus (Fig. 4b). In a separate group of animals, we analyzed ALK expression by immunohistochemistry in infected VTA to determine the effectiveness of knockdown by shAlk (Fig. 4c). ALK expression was quantified from confocal images using ImageJ. The intensity of ALK staining was reduced by 55 percent in mice expressing shAlk compared with shScr \{shScr, 100 ± 14; shAlk, 45 ± 7.4 [t(39) = 3.41, P = 0.0015]\}. These results indicate that ALK acts in the VTA to regulate binge-like ethanol consumption.

The Acb receives DA inputs from the VTA and also mediates binge-like ethanol consumption in mice (Kasten & Boehm 2014; Cozzoli et al. 2015). We previously found that knockdown of Alk in the Acb decreases cocaine sensitization (Lasek et al. 2011a), so it seemed feasible that ALK might function in the Acb to regulate ethanol consumption. To test this, mice were injected in the Acb with lentivirus expressing shAlk or shScr and tested in the DID procedure. Mice that received shAlk and shScr lentiviruses consumed the same amount of ethanol, despite robust viral infection in the Acb (Fig. 4d and e). Together, these results indicate that ALK functions in the VTA, but not the Acb, to regulate binge-like ethanol consumption.

Anaplastic lymphoma kinase inhibitors attenuate dopamine inhibition reversal in dopamine neurons

Because downregulation of ALK in the VTA decreases binge-like drinking and ALK is expressed in DA neurons, we hypothesized that pharmacological inhibition of ALK alters the physiology of VTA DA neurons. Neurons within the VTA were identified as DA according to electrophysiological criteria and sensitivity to DA, as we have reported (Nimitvilai, Arora & Brodie 2012a). We first examined the response of VTA DA neurons to ethanol in the presence of TAE684 or alectinib, because we previously showed that ethanol administered acutely to slices increases the firing rate of DA neurons in the VTA.

Figure 3 Anaplastic lymphoma kinase (ALK) is expressed in dopamine neurons and in non-dopamine cells in the ventral tegmental area. Confocal microscope images showing (a) nuclear (DAPI) staining in blue, (b) immunofluorescence to tyrosine hydroxylase (TH) in green, (c) immunofluorescence to ALK in red and (d) a merged image of DAPI, TH and ALK immunofluorescence. Yellow arrows point to TH neurons expressing ALK and white arrowheads indicate expression of ALK in non-TH expressing cells. Scalebar, 50 μm.
Treatment with TAE684 or alectinib did not change the ethanol-induced excitation of VTA DA neurons compared with controls (Fig. 5a and b). We next examined the effect of ALK inhibitors on the inhibitory response to DA. DA inhibits the firing of VTA DA neurons because of the action of DA at the D2R autoreceptor (Lacey, Mercuri & North 1987). Acute inhibition of VTA DA neurons by DA was not altered in the presence of TAE684 or alectinib (Fig. 5c). These data indicate that inhibition of ALK does not change the acute response of VTA DA neurons to stimulation by ethanol or inhibition by DA.

Inhibition of DA neurons by DA subsides over time in the continued presence of DA due to desensitization of the D2R, a process we have termed DA inhibition reversal (DIR) (Nimitvilai & Brodie 2010; Nimitvilai et al. 2012a,c). We assessed DIR in VTA slices treated with TAE684, alectinib or vehicle by examining the DA-induced reduction in spontaneous firing rate at 5 minute intervals over a 40-minute period (Fig. 5d–g). The concentration of DA administered to control neurons was 6.5 ± 1.4 μM; the concentration of DA administered to neurons in the presence of TAE684 was 7.9 ± 1.5 μM, and the concentration of DA administered to neurons in the presence of alectinib was 6.75 ± 1.3 μM. After 5 minutes of exposure, DA produced an initial inhibition of /C0 88.4 ± 5.4 percent in controls, /C0 86.8 ± 6.9 percent in TAE684-treated and /C0 82.5 ± 7.7 percent in alectinib-treated slices. In control slices, DA neuron firing rate recovered to /C0 47.5 ± 7.4 percent by 40 minutes, indicating DIR. In contrast, in TAE684-treated slices, the firing rate at 40 minutes was /C0 59.0 ± 10.3 percent, and in alectinib-treated slices, the firing rate at 40 min was /C0 79.0 ± 7.6 percent (two-way ANOVA, treatment [F(2, 22) = 1.90, P = 0.173], time [F(7, 154) = 7.0, P < 0.0001], treatment x time interaction [F(14, 154) = 1.42, P = 0.149]). Closer examination of the data using Tukey’s multiple comparisons test showed that the effect of time was driven primarily by DIR in the control slices. There were significant differences between the 5-minute time point and the 15 (P = 0.041), 20 (P = 0.005), 25 (P = 0.0004), 30, 35 and 40-minute time points.
(\(P < 0.0001\)) in the controls. In the TAE684-treated slices, there were significant differences between the 5- and 25-minute (\(P = 0.02\)), 35-minute (\(P = 0.035\)) and 40-minute (\(P = 0.012\)) time points, suggesting a delayed DIR. In contrast, there were no significant differences over time in the alectinib-treated slices, indicating a complete antagonism of DIR. These results indicate that ALK inhibitors can delay or disrupt D2R desensitization in VTA DA neurons.

**DISCUSSION**

Our results indicate that ALK regulates binge-like drinking and the rewarding properties of ethanol in mice. Polymorphisms in the human ALK gene are associated with alcohol dependence and measures of subjective high and motor-incoordination following alcohol intake (Wang et al. 2011; Lasek et al. 2011b), suggesting that ALK may be a viable therapeutic target for the treatment of AUDs. Here, we tested two ALK inhibitors, TAE684 and alectinib. We found that both inhibitors decrease binge-like drinking in the DID test. Alectinib appears to be more effective than TAE684 in reducing ethanol consumption (Fig. 1). This could be due to differences in the doses we used (10 mg/kg TAE684 versus 60 mg/kg alectinib). We chose these doses based on published reports that they cause tumor regression in mouse models, including brain tumors in the case of alectinib (Galkin...
ALK than TAE684 (Galkin et al. 2007; Davis et al. 2011; Sakamoto et al. 2011; Zhang et al. 2012) and is currently approved in Japan for the treatment of non-small cell lung cancer and is being tested in clinical trials in the United States. Although all kinase inhibitors potentially have off-target effects by inhibiting multiple kinases, we believe that the reduction in ethanol consumption is due to inhibition of ALK, because alectinib and TAE684 are in different chemical classes. In addition, we have demonstrated that an shRNA targeting ALK also reduces ethanol consumption. Because of its selectivity, potency, ability to penetrate the blood–brain barrier, and positive clinical development, alectinib is a potential new compound that may be useful for the treatment of alcohol use disorders.

In our DID experiments, there was a discrepancy between the amount of ethanol consumed and BECs in the DID test between mice that were treated by gavage versus infected with lentiviral-delivered shRNAs in the VTA. Control mice treated with vehicle in the inhibitor studies (by oral gavage) consumed 20 percent less ethanol compared with mice treated with the control shRNA in the VTA. This is probably due to the acute stress of the gavage procedure. However, the BECs in the control gavage group were half that of the shRNA control group. This may be due to the episodic nature of drinking in the DID test. Wilcox et al. have shown that mice consume nearly half of their ethanol in the DID test in the first 30 minutes of the drinking session and that BECs correlate better with the rate of drinking during the early part of the drinking session (Wilcox et al. 2014). It is possible that the kinetics of drinking in the DID test are altered in mice treated by gavage. Moreover, it must be taken into consideration that alcohol is metabolized during the drinking session and can affect BECs, which are obtained at the end of the 4-hour session. Others have also found a large discrepancy between the magnitude of changes in ethanol consumption and BECs (Lowery et al. 2010; Melon & Boehm 2011).

One brain region in which ALK appears to regulate alcohol consumption is the VTA. We found that knockdown of Alk in the VTA reduces binge-like drinking in the DID test. In contrast, we previously demonstrated that AlkKO mice drink more ethanol in a limited, intermittent-access binge drinking test (Lasek et al. 2011b). Increased drinking in AlkKO mice may be due to compensatory effects of eliminating ALK during development. In support of this, we found that phosphorylation of MEK (an upstream regulator of ERK signaling) is increased in the brains of AlkKO mice (Lasek et al. 2011b). Because ALK activates ERK (Hallberg & Palmer 2013), increased MEK phosphorylation in AlkKO mice suggests that compensatory changes have occurred. Over-activation of ERK is associated with increased ethanol consumption. Selectively bred mice with high ethanol preference over-express several genes in the ERK pathway (Mulligan et al. 2006). The difference between phenotypes observed with ALK suppression in adults and in AlkKO mice indicates significant compensatory developmental mechanisms. Interestingly, we found anatomical specificity with regard to the regulation of ethanol consumption by ALK. Knockdown of Alk in the Acb was ineffective in reducing ethanol intake, despite robust infection in the Acb and our demonstration previously that this same lentiviral construct injected into the Acb reduces cocaine sensitization (Lasek et al. 2011a). ALK therefore appears to be important for ethanol consumption through its activity in the VTA and for behavioral responses to cocaine through its activity in the Acb. Although ALK inhibition attenuates cocaine sensitization, it does not appear to affect ethanol sensitization (Fig. 2), indicating a molecular difference between these two drugs with regard to sensitization.

Two potential ligands for ALK that may be relevant for ALK in promoting ethanol consumption are the growth factors pleiotrophin and midkine (Fig. 6) (Stoica et al. 2001, 2002). Midkine expression is higher in the prefrontal cortex of human alcoholics and mice selectively bred for high alcohol consumption (Mulligan et al. 2006; Flatscher-Bader & Wilce 2008) and pleiotrophin expression is regulated by ethanol (Vicente-Rodriguez et al. 2014a). In addition, both factors regulate behavioral responses to ethanol, including CPP (Vicente-Rodriguez et al. 2014a, 2014b). We found that ethanol activates ALK through midkine (He et al. 2015), suggesting that midkine might be a viable ligand for ALK, however, there is a controversy as to whether midkine and pleiotrophin are true in vivo ligands for ALK. Pleiotrophin and midkine are also ligands for a phosphatase, RPTPβζ, that regulates ALK activity (Maeda et al. 1999; Meng et al. 2000; Perez-Pinera et al. 2007). In addition, other ligands for ALK have been described, including FAM150A, FAM150B and heparin (Guan et al. 2015; Murray et al. 2015).

Our results provide one potential mechanism by which ALK regulates binge drinking and ethanol reward. ALK alters the physiology of VTA DA neurons by promoting desensitization of D2R. We found that inhibition of
ALK decreased desensitization but did not alter ethanol-stimulated firing or the initial inhibition of firing by DA in VTA DA neurons. Regulation of D2R desensitization is a completely novel function for ALK. A proposed model for this is illustrated in Fig. 6. ALK may facilitate the endocytosis and/or trafficking of D2R after prolonged DA exposure but does not change the acute inhibitory response to DA. Receptor tyrosine kinases (RTKs) such as ALK are trans-activated by stimulation of G-protein coupled receptors (GPCRs) such as D2R (Delcourt, Bockaert & Marin 2007; Cattaneo et al. 2014). For instance, stimulation of D2R by quinpirole activates the RTKs epidermal growth factor receptor and platelet-derived growth factor receptor (Kotcheva et al. 2002; Wang et al. 2005). We hypothesize that long-term DA stimulation of D2R may trans-activate ALK signaling, leading to internalization and degradation of complexes containing both D2R and ALK. Activation of ALK by an agonist antibody causes ALK internalization and degradation, while inhibition of ALK activity by TAE684 blocks this process (Mazot et al. 2012). Similarly, D2R is internalized and degraded after agonist stimulation (Bartlett et al. 2005). Cross talk between GPCRs and RTKs can promote receptor internalization (Gavi et al. 2007). We have shown that D2R desensitization in VTA DA neurons is mediated by the phosphatidylinositol system, protein kinase C and the endocytic protein dynamin (Nimitvilai et al. 2012a,c; Nimitvilai, McElvain & Brodie 2013). ALK can activate phospholipase C-γ, an upstream activator of PKC (Bai et al. 1998) and therefore may promote D2R trafficking via PKC signaling. Investigation of ALK-activated signaling pathways that promote D2R trafficking is an exciting area for future research.

It is currently not clear how altered desensitization of D2R in VTA DA neurons affects ethanol binge drinking and reward, because there are no studies that have specifically addressed this issue. However, we predict that attenuating desensitization of D2R by ALK inhibitors would sustain inhibitory signaling by D2R, decreasing DA neuronal activity and thereby reduce DA release in VTA projection targets such as the Acb, amygdala and prefrontal cortex. We hypothesize that this would decrease ethanol consumption, because DA is released upon ethanol exposure and contributes to ethanol reinforcement and reward. A model for how ALK inhibition would affect the firing of DA neurons and subsequent DA release and ethanol consumption is illustrated in Fig. 6. Control of DA output plays an important role in ethanol consumption, as blocking DA neurotransmission in the Acb reduces ethanol consumption (Gonzales et al. 2004). Likewise, inhibition of VTA DA neurons by the D2R agonist quinpirole decreases operant ethanol administration and intra-VTA ethanol self-administration (Hodge et al. 1993; Rodd et al. 2004), and D2R knockout mice show altered ethanol consumption and reward (Cunningham et al. 2000; Risinger et al. 2000; Bulwa et al. 2011; Delis et al. 2013).

We previously demonstrated that acute application of ethanol to VTA slices blocks DIR (Nimitvilai et al. 2012b), similar to the effect of the ALK inhibitors on DIR. This result was counterintuitive, because ALK inhibitors also decrease binge-like ethanol consumption. Perhaps, the inhibition of D2R desensitization by acute ethanol is a homeostatic, negative regulatory response of DA neurons that maintains autoreceptor inhibition of neuronal activity in the face of excessive activation of DA neurons. Decreased DA release would be expected to curb excessive alcohol drinking. Inhibition of ALK has the same effect, effectively ‘substituting’ for the pharmacological effect of alcohol, engaging this negative regulatory mechanism on DA neurons and consequently making continued alcohol consumption undesirable. Alternatively, ALK inhibition may make alcohol aversive. Interestingly, we found that TAE684 blocked ethanol CPP but was not rewarding or aversive in the absence of ethanol, indicating a specific interaction between ALK inhibition and the rewarding properties of ethanol.
The prolonged effects of ethanol on ALK signaling and D2R desensitization are not known. This is important to investigate, because chronic ethanol exposure likely results in neuroadaptations in DA neurons and a dysregulation of D2R signaling. Examining the role of D2R desensitization after chronic ethanol exposure will be important to understand mechanisms that promote chronic alcohol abuse. However, our findings suggest a novel and potentially critical link between ALK signaling, D2R desensitization and binge drinking and provide an exciting new area of research aimed at preventing harmful drinking.

Acknowledgements

This study was supported by the National Institute on Alcohol Abuse and Alcoholism (AA016654, AA020912, and AA022538 to AWL; AA09125 and AA05846 to MSB) and the National Institutes of Health Office of the Director (OD010914). We thank Drs. Liquan Xue and Stephen Morris for the gift of the anti-ALK antiserum, Donghong He for producing lentivirus and Maureen A. McElvain for assistance with electrophysiology. We also thank the Confocal Microscopy Facility at the Research Resources Center at UIC.

Disclosure

The authors declare no competing financial interests.

Authors Contribution

A.W.L. and M.S.B. conceived of and designed the research. A.W.L., J.W.D., H.C. and C.Y. performed the experiments. All authors analyzed the data and assisted in interpretation. A.W.L. and M.S.B. wrote the manuscript. All authors critically reviewed content and approved the final version for publication.

References


Nimitvilai S, McElvain MA, Arora DS, Brodie MS (2012c) Reversal of quinpirole inhibition of ventral tegmental area neurons is linked to the phosphatidylinositol system and is induced by agonists linked to Gi(q). J Neurophysiol 108:263–274.


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Figure S1 The ALK inhibitor TAE684 does not affect ethanol-induced loss of righting reflex (LORR) or ethanol clearance from the blood.
Figure S2 The ALK inhibitor alectinib decreases ethanol consumption and preference in a two-bottle choice drinking in the dark (DID) procedure.