Published in final edited form as:

Neuropharmacology. 2018 July 15; 137: 86–95. doi:10.1016/j.neuropharm.2018.04.027.

Pharmacological inhibition of Receptor Protein Tyrosine Phosphatase β/ζ (PTPRZ1) modulates behavioral responses to ethanol

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Abstract

Pleiotrophin (PTN) and Midkine (MK) are neurotrophic factors that are upregulated in the prefrontal cortex after alcohol administration and have been shown to reduce ethanol drinking and reward. PTN and MK are the endogenous inhibitors of Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ (a.k.a. PTPRZ1, RPTPβ, PTPζ), suggesting a potential role for this phosphatase in the regulation of alcohol effects. To determine if RPTPβ/ζ regulates ethanol consumption, we treated mice with recently developed small-molecule inhibitors of RPTPβ/ζ (MY10, MY33-3) before testing them for binge-like drinking using the drinking in the dark protocol. Mice treated with RPTPβ/ζ inhibitors, particularly with MY10, drank less ethanol than controls. MY10 treatment blocked ethanol conditioned place preference, showed limited effects on ethanol-induced ataxia, and potentiated the sedative effects of ethanol. We also tested whether RPTPβ/ζ is involved in ethanol signaling pathways. We found that ethanol treatment of neuroblastoma cells increased phosphorylation of anaplastic lymphoma kinase (ALK) and TrkA, known substrates of RPTPβ/ζ. Treatment of neuroblastoma cells with MY10 or MY33-3 also increased levels of phosphorylated ALK and TrkA. However, concomitant treatment of neuroblastoma cells with ethanol and MY10 or MY33-3 prevented the increase in pTrkA and pALK. These results demonstrate for the first time that ethanol engages TrkA signaling and that RPTPβ/ζ modulates signaling pathways activated by alcohol and behavioral responses to this drug. The data support

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the hypothesis that RPTPβ/ζ might be a novel target of pharmacotherapy for reducing excessive alcohol consumption.

Keywords

ALK; TrkA; alcohol use disorder; binge-drinking; pleiotrophin; midkine

1. INTRODUCTION

Alcohol use disorder (AUD) constitutes a public health crisis. Hazardous use of alcohol is the sixth leading cause of ill health and premature death in high-income countries (Forouzanfar et al., 2016). Approximately 70% of the adults in the WHO European Region drink alcohol and most alcohol is consumed in heavy drinking occasions (60 g of pure alcohol or more on one occasion) which worsen all risks. Binge drinking accounts for over half of the approximately 88,000 deaths yearly that are attributed to alcohol use in the United States, but binge drinkers also put themselves at increased risk for multiple comorbidities (O'Keefe et al., 2007; Petit et al., 2014; Zakhari and Li, 2007). In the United States, recent studies have shown that the prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV AUD has increased significantly (Grant et al., 2017). Thus, new therapeutic approaches to treat AUD are greatly needed.

In an effort to develop new pharmacotherapies to limit alcohol consumption and prevent alcoholism, the identification of novel genes and pathways that may predispose individuals to AUD is key. Two genetic factors that have been recently identified as important regulators of alcohol behavioral effects are Pleiotrophin (PTN) and Midkine (MK). PTN and MK are cytokines important in central nervous system (CNS) functions and repair (Herradon and Perez-Garcia, 2014). Both PTN and MK are also upregulated in different brain areas after administration of different drugs of abuse (Herradon and Perez-Garcia, 2014), suggesting PTN and MK signaling may be important in the regulation of drug-induced addictive behaviors. Accordingly, PTN is found upregulated in the nucleus accumbens after a single administration of amphetamine (Le Greves, 2005) and has been shown to contribute to the extinction of amphetamine-seeking behaviors (Gramage et al., 2010a). Importantly, MK expression is higher in the prefrontal cortex (PFC) of human alcoholics and mice selectively bred for high alcohol consumption (Flatscher-Bader and Wilce, 2008; Mulligan et al., 2006) and PTN is upregulated in the PFC of mice injected with a rewarding dose of ethanol (Vicente-Rodriguez et al., 2014a). Both cytokines have been shown to be potent regulators of behavioral effects induced by ethanol (Chen et al., 2017; Vicente-Rodriguez et al., 2014a; Vicente-Rodriguez et al., 2014b). It has been demonstrated that PTN knockout (PTN−/−) and MK knockout (MK−/) mice are more sensitive to the rewarding effects of alcohol in the conditioned place preference test (Vicente-Rodriguez et al., 2014a; Vicente-Rodriguez et al., 2014b). In contrast, PTN transgenic overexpression in the mouse brain blocks the rewarding effects of alcohol (Vicente-Rodriguez et al., 2014a). Overall, the data suggest that PTN and MK could be used for the treatment of drug addiction disorders including AUD (Alguacil and Herradon, 2015).

Both PTN and MK bind to the Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ (a.k.a. PTPRZ1, RPTPβ, PTPζ), and inactivate its phosphatase activity (Herradon and Ezquerra, 2009). This leads to an increase in tyrosine phosphorylation of substrates critical for the effects of these cytokines such as Fyn kinase (Pariser et al., 2005) and Anaplastic Lymphoma Kinase (ALK) (Perez-Pinera et al., 2007). We hypothesize that PTN and MK actions on substance use disorders can be reproduced with rationally designed small molecule inhibitors of RPTPβ/ζ (Herradon et al., 2009; Herradon and Perez-Garcia, 2014).

Recently, a new series of blood-brain barrier (BBB) permeable molecules designed to mimic the activity of PTN/MK in the CNS were synthesized (Pastor et al., 2018). These compounds exert their actions by interacting with the intracellular domain of RPTPβ/ζ and inhibiting its tyrosine phosphatase activity. The most potent compounds MY10 and MY33-3 (IC_{50} ~ 0.1 μM) significantly increased the phosphorylation of key tyrosine residues of RPTPβ/ζ substrates involved in neuronal survival and differentiation such as ALK. More interestingly, PTN and MK were previously shown to prevent amphetamine neurotoxicity in vivo and in vitro (Gramage et al., 2011; Gramage et al., 2010b) and these RPTPβ/ζ inhibitors have shown similar protective effects against amphetamine-induced toxicity (Pastor et al., 2018).

The purpose of this study was to test inhibitors of RPTPβ/ζ in binge-like drinking, ethanol conditioned place preference, and other behavioral responses to ethanol, and to study the possible interactions of these molecules with ethanol-induced activation of signaling pathways.

2. MATERIALS AND METHODS

2.1. Subjects

Male C57BL/6J mice (8-10 weeks of age) were tested for behavior. Mice were housed under controlled environmental conditions (22±1 °C and a 12-h light/12-h dark cycle) with free access to food and water. All the animals used in this study were maintained in accordance with both the ARRIVE guidelines and the European Union Laboratory Animal Care Rules (86/609/ECC directive) or the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the Animal Research Committee of USP-CEU or the Animal Care Committee of the University of Illinois at Chicago and procedures were used to minimize pain and suffering.

2.2. RPTPβ/ζ inhibitors

MY10 and MY33-3 (Fig. 1) were synthesized as previously described (Pastor et al., 2018). MY10 and MY33-3 were administered at a dose of 60 mg/kg in 10% dehydrated ethanol, 20% polysorbate 80, 70% PEG-300 vehicle. Ethanol in the vehicle results in a dose of less than 0.3 g/kg. Pharmacokinetic studies in mice have shown that 1 h after oral administration the brain to plasma ratio is 3:1 (Pastor et al., 2018). Mice were administered compounds or vehicle by oral gavage in a volume of approximately 0.1 ml.

2.3. Drinking in the dark (DID)

Binge-like drinking was measured using the two-bottle DID procedure as previously described using 20% ethanol (Dutton et al., 2017). Mice were individually housed for 2 weeks in the reverse-dark cycle room prior to testing ethanol consumption. Three days before testing ethanol consumption, mice drank water from two tubes made from 10 ml polycarbonate pipettes connected to stainless steel sipper tubes containing ball-bearings to prevent leakage (Rhodes et al., 2005) for one day, in order to acclimate them to the tubes. Three days later mice were given a choice between 20% ethanol and water in the sipper tubes. Fluid consumption was measured every day for 4 days by measuring the volume of fluid in the tubes. The position of the bottles (left or right) was changed every day to control for side preference. On the first 3 days of testing, mice were given access to the ethanol and water tubes 3 h into the dark cycle for a period of 2 h. On the fourth day, mice were given access to ethanol and water tubes for 4 h and the volume consumed was measured at 4 h. All mice were given vehicle (0.1 ml) by oral gavage on days 1 & 2. On the third and fourth days, mice were administered 60 mg/kg MY10, MY33-3 or vehicle (0.1 ml) by oral gavage 1 hour before the drinking session in the DID test (n = 12/group). Preference score was calculated as the ratio of the volume of ethanol consumed over the volume of total fluid consumed (Chen et al., 2017; Dutton et al., 2017). For the sucrose consumption test, a separate group of mice were tested exactly as in the ethanol consumption test, except that mice were provided with two tubes containing 2% sucrose in water and water instead of 20% ethanol and water.

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 et al., 2006).

2.4. Conditioned Place Preference (CPP)

We used a 2-compartment apparatus; one compartment had a black floor and walls, and the other had a black floor and white walls. The phases included preconditioning (Pre-C, day 1), conditioning (days 2-4) and testing (CPP, day 5). During preconditioning, mice were free to explore the two compartments for a 15-min period; their behavior was monitored by a videotracking system (SD Instruments, San Diego, California, USA) to calculate the time spent in each compartment. The conditioning phase consisted of two conditioning sessions per day (Gramage et al., 2011; Vicente-Rodríguez et al., 2014a). The first session took place in the morning starting at 8 am, in which all mice received a single injection of saline i.p. (10 ml/kg) and were confined to one compartment for 5 min. In the afternoon session, starting at 3 pm, the mice were injected (i.p.) with 2.0 g/kg ethanol, and confined to the other compartment for 5 min. The assignment of the compartments to the ethanol or saline condition was made at random. The procedure used on days 3 and 4 was the same but the order of the treatments (morning/evening) was changed to avoid the influence of circadian variability. Mice were administered the RPTPβ/ζ inhibitor MY10 (60 mg/kg) or vehicle by oral gavage in a volume of approximately 0.1 ml, 1 hour before each of the saline and ethanol conditioning sessions (n = 9-10/group). In the testing phase on day 5, mice received a drug-free, 15-min preference test. In this phase the animals freely moved throughout the

apparatus, as in the preconditioning phase. The time spent in each compartment was also registered. The difference between the time spent in the ethanol-paired compartment in this phase and the time spent in the same compartment in the preconditioning was considered as indicative of the degree of conditioning induced by ethanol.

For the control CPP experiment in which we conditioned with MY10 in the absence of ethanol, compound or vehicle were administered on days 2, 3 and 4 one hour before injecting mice i.p. with saline (10 ml/kg) and confining them to one side of the apparatus for 5 minutes (n = 13/group). In the testing phase on day 5, mice received a compound-free, 15-min preference test and the amount of time the mice spent in the MY10-paired compartment was measured.

2.5. Rotarod test

Mice were trained in 2 sessions on 2 consecutive days by placing them on the rotarod (Panlab, Barcelona, Spain) rotating drum (rod), and allowing them to run/climb under continuous acceleration (2-18 r.p.m) for at least 30 sec. The next day, animals were re-trained, administered 60 mg/kg MY10 or vehicle by oral gavage (0.1 ml) 1 hour before the 2.0 g/kg ethanol administration (n = 8-9/group) and then placed on the rotarod. In a control experiment, animals were administered 60 mg/kg MY10 or vehicle by oral gavage (0.1 ml) 1 hour before saline administration (10 ml/kg, i.p.) (n = 4-5/group). The time to fall was recorded and mice were placed back on the rotarod every 10 minutes until 100 min after ethanol (or saline) administration.

2.6. Loss of the Righting Reflex (LORR)

Mice were administered 60 mg/kg MY10 or vehicle 1 hour before the 3.6 g/kg ethanol administration (n = 8/group), placed on their backs and tested for the ability to right themselves. Mice were considered to have lost the righting reflex if they could not right themselves 3 times within 30 seconds and regained the righting reflex if they could fully right themselves 3 times within 30 sec (Lasek et al., 2011). The duration of LORR was determined as the difference between the time when the reflex was lost and when it was regained.

2.7. Measurement of Blood Ethanol Concentration for ethanol clearance study

Blood ethanol concentration (BEC) was measured in mice used in the LORR experiment one week after the LORR test. Mice were administered 60 mg/kg MY10 or vehicle 1 hour before the 2.0 g/kg ethanol administration (n = 5/group) and 20 μl of blood was obtained via tail vein puncture at 30, 60 and 120 min post-injection. BECs were determined using the alcohol dehydrogenase enzymatic assay (Zapata et al., 2006).

2.8. Cell culture

The human neuroblastoma cell line SH-SY5Y was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and was incubated at 37°C in 5% CO_2. SH-SY5Y cells were cultured in a 1:1 mixture of Eagle's minimum essential medium (EMEM) and F12 medium containing 10% fetal bovine serum (FBS). For treatment with ethanol, cells were cultured to 90% confluence and, three hours before ethanol treatment, medium was

changed to a 1:1 mixture of EMEM and F12 medium without serum in order to serum-starve the cells. For concentration-response assays with ethanol, cells were treated with 10-100 mM ethanol for 15 minutes. For time-response assays with ethanol, cells were treated with 50 mM ethanol for 5, 15 and 30 minutes. For treatment with ethanol and RPTPβ/ζ inhibitors, cells were treated with 1 μM RPTPβ/ζ inhibitor (MY10 or MY33-3) for 5 min prior to 50 mM ethanol for 15 minutes. All determinations were carried out in 3-6 independent experiments.

2.9. Western blots

SH-SY5Y cells were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β–glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and EDTA-free Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA). Lysate protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Separate blots were probed with antibodies to either phosphorylated ALK (pALK, Tyr 1278) or phosphorylated TrkA (pTrkA, Tyr 490) (Cell Signaling Technology, Danvers, MA, USA). Antibodies were diluted in 5% bovine serum albumin in Tris-buffered saline with Tween 20 (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20). Blots were stripped and re-probed with corresponding anti-TrkA (Upstate, Charlottesville, VA) and anti-ALK (Life Technologies, Carlsbad, CA) antibodies to measure total protein levels. To quantify total protein levels of ALK and TrkA, membranes were re-probed with anti-actin antibodies and normalized against the actin signal (Chemicon, Temecula, CA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Santa Cruz, Santa Cruz, CA). The membranes were developed with enhanced chemiluminescence detection reagents (Pierce). Films were scanned and densitometry performed using ImageJ software.

2.10. Statistical analysis

All data were expressed as the mean±SEM and analyzed using Prism software (GraphPad, La Jolla, CA, USA). Rotarod data was analyzed by two-way repeated measures (RM) ANOVA for treatment and time. The DID data was analyzed by Student’s t-test for effects of vehicle vs. compound on day 3 (2 h drinking session) and on day 4 (4 h drinking session) and for effects of day (day 2 vs. day 3) within each compound treatment. The preference score data was analyzed by Student’s t-test for effects of vehicle vs. compound within each compound treatment day (days 3 & 4) and for effects of day (day 2 vs. day 3 and day 2 vs. day 4) within each compound treatment. The BEC and LORR data were analyzed by Student’s t-test. The CPP Data were analyzed by two-way repeated measures (RM) ANOVA for treatment and phase followed by Bonferroni’s post-hoc tests. Protein expression analysis in concentration-response and time-response assays with ethanol was performed by 1-way ANOVA followed by Tukey’s post-hoc tests. Protein expression analysis in cells treated with compounds and ethanol was performed by 2-way ANOVA followed by Tukey’s post-hoc tests, considering compound treatment and ethanol treatment as factors. A p value less than 0.05 was considered a statistically significant difference.

3. RESULTS

3.1. RPTPβ/ζ inhibition attenuates binge-like ethanol consumption

To determine if RPTPβ/ζ inhibition affects binge-like ethanol consumption, we treated mice with the inhibitor MY10 before the drinking sessions on days 3 (2 hour session) and 4 (4 hour session). Fig. 2a shows that MY10-treated mice consumed less ethanol than vehicle-treated mice on day 3 ($t(22)=2.02$, $P = 0.056$) and day 4 ($t(21) = 2.32$, $P =0.03$). In addition, MY10-treated mice consumed significantly less ethanol on day 3 when compared with day 2 (before MY10 treatment, $t(22)=2.57$, $P = 0.018$). Consistent with the decrease in ethanol consumed, mice treated with MY10 showed a reduced preference for the ethanol solution in the 2 h drinking session on day 3 (Fig. 2b; $t(22) = 2.32$, $P =0.03$) and in the 4 h drinking session on day 4 when compared with vehicle-treated mice ($t(21) = 2.68$, $P =0.014$) and on days 3 and 4 when compared with day 2 ($t(22) = 2.81$, $P = 0.01$ and $t(21) = 3.84$, $P = 0.001$, respectively). Consistent with the reduced ethanol consumption, BECs were lower in the MY10-treated group compared with vehicle-treated mice (Fig. 2c; $t(20) = 2.123$, $P = 0.046$) at the end of the 4 h drinking session (day 4). Sucrose drinking was not affected by MY10 treatment (Fig. 2d). Treatment with a second RPTPβ/ζ inhibitor, MY33-3, did not significantly affect ethanol consumption when compared with the vehicle-treated group, but did reduce ethanol consumption when comparing day 2 (before treatment with MY33-3) with day 3 (after treatment with MY33-3, Fig. 2e, $t(22) = 2.31$, $P = 0.03$). In addition, mice treated with MY33-3 on day 3 showed a reduced preference for the ethanol solution when compared to the vehicle-treated mice (Fig. 2f; $t(22) = 2.16$, $P = 0.04$) and a reduced preference when comparing days 2 & 3 ($t(22) =2.45$, $P = 0.02$). However, on day 4, no significant differences between MY33-3 and vehicle-treated mice were observed (Fig. 2f). As expected, BECs at the end of the 4 h drinking session (day 4) were not affected in mice treated with MY33-3 (Fig. 2g). It is important to note that total fluid consumption (water and ethanol combined) was not affected by any of the treatments (Fig. 2h). Collectively, these data indicate that RPTPβ/ζ inhibition reduced ethanol consumption without affecting sucrose drinking and suggest that MY10 appears to be the most effective RPTPβ/ζ inhibitor with potential use in AUD.

3.2. RPTPβ/ζ inhibition attenuates ethanol reward

The reduced ethanol consumption in mice treated with MY10 suggests that RPTPβ/ζ might be involved in the rewarding properties of ethanol. To test this, we performed the ethanol CPP test in mice treated with MY10 or vehicle daily prior to ethanol and saline conditioning sessions (Fig. 3a). ANOVA revealed a significant treatment x phase interaction ($F (1, 17) = 6.69$; $p = 0.02$). Post-hoc comparisons demonstrated that mice treated with vehicle showed a significant CPP (Fig. 3b; $t(17) = 4.89$; $P < 0.001$). In contrast, mice treated with MY10 did not exhibit CPP (Fig. 3b; $t(17) = 1.39$). To test if MY10 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 MY10 (Fig. 3c). Conditioning with MY10 did not alter the time spent in the MY10-paired compartment, indicating that RPTPβ/ζ inhibition in the absence of ethanol does not induce preference or aversion (Fig. 3d). These data suggest that RPTPβ/ζ inhibition attenuates the rewarding properties of ethanol.

3.3. RPTPβ/ζ inhibition has a mild effect on ethanol-induced ataxia and potentiates ethanol sedation

To test the possibility that RPTPβ/ζ could modulate other acute behavioral responses to ethanol we performed experiments to assay the ataxic and sedative effects of ethanol in MY10-treated mice. The motor-incoordinating effects of ethanol were measured using the rotarod test. We tested the recovery from the ataxic effects induced by a rewarding dose of ethanol (2.0 g/kg) known to produce moderate ataxic effects in mice (den Hartog et al., 2013). Acute administration of 2.0 g/kg ethanol produced ataxia in MY10- and vehicle-treated mice illustrated by a reduction in time spent on the rotarod. There were significant effects of time ($F(10,150)=31.22$, $P < 0.0001$; Figure 4a) and treatment ($F(1,15)=5.19$, $P = 0.04$), but no significant time x treatment interaction ($F(1,150)=0.47$, $P = 0.91$). In agreement with studies reported by others (den Hartog et al., 2013), performance improved over time although this improvement seemed to be transiently delayed in MY10-treated mice (Fig. 4a). However, 100 minutes after ethanol injection a similar recovery from ataxia was observed in mice treated with MY10 or vehicle (Fig. 4a). Treatment with MY10 or vehicle one hour before saline injection did not change the amount of time that mice stayed on the rotarod when compared with basal values (Treatment: $F(1,7)=0.04$, $P = 0.84$; time: $F(10,70)=1.53$, $P = 0.15$; Fig. 4b). These data indicate that RPTPβ/ζ has a limited effect on the motor incoordinating effects induced by a rewarding dose of ethanol in mice. We examined BECs at different time points in mice treated with 60 mg/kg or vehicle one hour before an injection of 2.0 g/kg ethanol. We did not observe a difference in the BECs between treatments (Fig. 4c), suggesting that MY10 does not affect clearance of a rewarding and ataxic dose of ethanol in mice.

We also examined MY10- and vehicle-treated mice in a LORR test using a sedating dose of ethanol (3.6 g/kg). The time to lose the righting reflex was similar in all mice (data not shown). However, the data show that the amount of time needed for MY10-treated mice to recover the righting reflex after ethanol injection was significantly higher than that in vehicle-treated mice (Fig. 5; $t(14) = 2.980$, $P = 0.01$). These data suggest that RPTPβ/ζ counteracts the sedative effects of ethanol.

3.4. RPTPβ/ζ inhibitors modulate ethanol-induced activation of ALK and TrkA

In order to study the molecular mechanisms underlying the actions of RPTPβ/ζ inhibitors in the effects of ethanol, we aimed to test the hypothesis that pharmacological inhibition of RPTPβ/ζ regulates ethanol signaling pathways. Previously, it was shown that ALK is a substrate of RPTPβ/ζ (Pérez-Pinera et al., 2007) and that ethanol rapidly activates ALK signaling through phosphorylation of Tyr 1278 in neuroblastoma cells (He et al., 2015). Also, inhibition of RPTPβ/ζ activates TrkA through increased phosphorylation of Tyr 490 (Shintani and Noda, 2008). To determine if ethanol activates TrkA signaling and to confirm the effects of ethanol on ALK signaling, we treated SH-SY5Y neuroblastoma cells with different concentrations of ethanol (10–100 mM) for 15 min (Figs. 6a-e) and with 50 mM ethanol for 5-30 min (Figs. 6f-j). The doses of ethanol used in these studies represent blood alcohol concentrations ranging from intoxicating to sedating. Interestingly, we observed an effect of ethanol treatment on TrkA phosphorylation after incubation of SH-SY5Y cells with 10-100 mM ethanol (Fig. 6b; $F(5,21) = 3.04$; $P = 0.04$) and after 5, 15 and 30 min of 50 mM

ethanol exposure (Fig. 6g; $F(3,14) = 5.02$; $P = 0.02$). We did not detect significant effects of ethanol treatment on total TrkA protein levels (Figs. 6c,h). Post-hoc comparisons showed that ethanol significantly increased phosphorylation of Tyr 490 in TrkA after 15 min of 50 mM exposure (Fig. 6g). In confirmation of previous studies (He et al., 2015), we observed a significant effect of treatment with ethanol on ALK phosphorylation [specifically the 140 kDa isoform of ALK] in SH-SY5Y cells (Fig. 6d; $F(5,21) = 2.87$; $P = 0.048$). Post-hoc comparisons showed significantly increased ALK phosphorylation after incubation with 50 mM ethanol (Fig. 6d, $P < 0.05$). In the time-course assays, we observed a trend of ethanol to increase ALK phosphorylation after 5, 15 and 30 min of 50 mM ethanol exposure (Fig. 6i; $F(3,15) = 3.00$; $P = 0.07$). We did not detect significant effects of ethanol treatment on total ALK protein levels (Figs 6e,j).

We next tested the possibility that treatment of SH-SY5Y cells with the RPTPβ/ζ inhibitor MY10 (1 μM) modulates these signaling pathways (Fig. 7a-e). Analysis of TrkA phosphorylation by 2-way ANOVA indicated that there was not a significant main effect of the compound (Fig. 7b; $F(1,10) = 0.01$, $P = 0.93$), but there was a significant main effect of ethanol (Fig. 7b; $F(1,10) = 5.40$, $P = 0.04$) and a significant MY10 x ethanol interaction (Fig. 7b; $F(1,10) = 8.18$, $P = 0.02$). Post-hoc analyses indicated that ethanol significantly increased phosphorylated TrkA when compared with vehicle (Fig. 7b, $P = 0.03$), whereas the combination of MY10 and ethanol did not increase phosphorylated TrkA when compared with vehicle (Fig. 7b, $P = 0.43$). For phosphorylated ALK, there were no significant main effects of ethanol (Fig. 7d; $F(1,9) = 0.30$, $P = 0.59$) or MY10 (Fig. 7d; $F(1,9) = 0.42$, $P = 0.53$); however, there was a significant MY10 x ethanol interaction (Fig. 7d; $F(1,9) = 19.8$, $P = 0.001$). Post-hoc analyses demonstrated that ethanol alone (Fig. 7d, $P = 0.03$) and MY10 alone (Fig. 7d, $P = 0.02$) significantly increased phosphorylated ALK when compared with vehicle treatment, whereas treatment with the combination of MY10 and ethanol did not increase phosphorylated ALK when compared with vehicle treatment (Fig. 7d, $P = 0.84$). None of the treatments significantly altered total TrkA (Fig. 7c) or total ALK protein levels (Fig. 7e).

We also tested if treatment of SH-SY5Y cells with the RPTPβ/ζ inhibitor MY33-3 (1 μM) modulates these signaling pathways (Fig. 7f-j). For phosphorylated TrkA, there were no significant effects of ethanol (Fig. 7g; $F(1,18) = 0.07$, $P = 0.79$) or compound (Fig. 7g; $F(1,18) = 3.72$, $P = 0.07$); however there was a significant MY33-3 x ethanol interaction (Fig. 7g; $F(1,18) = 13.38$, $P = 0.002$). Post-hoc multiple comparisons indicated that ethanol alone (Fig. 7g, $P = 0.04$) and MY33-3 alone (Fig. 7g, $P = 0.001$) significantly increased phosphorylated TrkA when compared with vehicle treatment. However, treatment with the combination of MY33-3 and ethanol did not increase phosphorylated TrkA when compared with vehicle treatment (Fig. 7g, $P = 0.40$). For phosphorylated ALK, we did not observe significant main effects of ethanol (Fig. 7i; $F(1,12) = 1.00$, $P = 0.33$) or MY33-3 (Fig. 7i; $F(1,12) = 3.11$, $P = 0.10$) but there was a significant MY33-3 x ethanol interaction (Fig. 7i; $F(1,12) = 5.45$, $P = 0.04$). Post-hoc multiple comparisons tests indicated that ethanol alone (Fig. 7i, $P = 0.03$) significantly increased phosphorylated ALK compared with vehicle treatment and that the combination of MY33-3 and ethanol did not significantly increase phosphorylated ALK levels when compared with vehicle treatment (Fig. 7i, $P = 0.19$). None

of the treatments significantly changed total TrkA (Fig. 7h) or total ALK protein levels (Fig. 7j).

4. DISCUSSION

We previously showed that MK−/− and PTN−/− mice are more sensitive to the rewarding effects of ethanol and that these effects are blocked in mice with transgenic PTN overexpression in the brain (Vicente-Rodríguez et al., 2014a). Also, it has been recently shown that MK−/− mice drink more alcohol in the DID and 2-bottle choice ethanol consumption tests than WT mice (Chen et al., 2017). Since PTN and MK are endogenous inhibitors of RPTPβ/ζ, and this phosphatase is preferentially expressed in the CNS (Herradon and Perez-Garcia, 2014), we hypothesized that small-molecule inhibitors of RPTPβ/ζ would mimic MK and PTN actions in the nervous system. Here, we tested the effects of RPTPβ/ζ inhibitors in a battery of behavioral tests in mice treated with ethanol. We found that the RPTPβ/ζ inhibitor, MY10, decreased binge-like drinking in the DID test. MY10 appears to be more effective in reducing ethanol consumption than the other RPTPβ/ζ inhibitor, MY33-3, when given at the same dose. It is possible that a higher dose of MY33-3 might be more effective in reducing ethanol consumption. It is interesting to note that both inhibitors were equally potent in in vitro phosphatase assays, but MY33-3 was less selective since it also inhibited PTP-1B with significant potency (IC50 ~ 0.7 μM) (Pastor et al., 2018). Thus, we cannot rule out the possibility that the inhibition of other phosphatases may trigger signaling events that interfere with this compound's effects.

MY10-treated mice drank less ethanol and exhibited decreased ethanol preference in the DID test, and BECs were correspondingly reduced after the four-hour drinking session on the last day of the test. The effect of MY10 on drinking appears to be specific to ethanol because treatment with MY10 did not affect sucrose consumption. These results suggest that MY10 does not cause general anhedonia. We should point out that mice drank less in our DID test compared to previous studies (Dutton et al., 2017). This may be related to several factors. One is that we performed a 2-bottle choice with water, so mice may have consumed less ethanol when given a choice between water and ethanol compared with access to a single bottle of ethanol. The second may be due to the acute stress of the gavage procedure, or the combination of stress and the availability of water during the drinking test. Nonetheless, MY10 was still effective in reducing ethanol consumption, suggesting that RPTPβ/ζ is potentially a viable therapeutic target to reduce excessive drinking.

To test the possibility that the RPTPβ/ζ inhibitor MY10 decreases ethanol drinking through its ability to block the rewarding effects of ethanol, we tested the effects of MY10 in ethanol place conditioning. The data presented here demonstrate that MY10 reduces ethanol-induced CPP. Importantly, it has been shown that ethanol-induced place preference is blocked in mice with transgenic overexpression in the brain of PTN, an endogenous inhibitor of RPTPβ/ζ (Vicente-Rodriguez et al., 2014a). Treatment of mice with MY10 in the absence of ethanol did not affect place conditioning, indicating that MY10 alone does not affect reward or aversion. This is consistent with our sucrose consumption data, indicating that MY10 may not have a general psychotropic effect, but instead may specifically affect ethanol-related behaviors. Taken together, these data provide evidence that RPTPβ/ζ

inhibition limits alcohol reward. However, these results must be interpreted with caution because performance in the CPP test is dependent on learning of the ethanol-context association. MY10 may have merely blocked this learning process instead of the rewarding properties of ethanol. Future studies are needed to determine the effect of MY10 on learning and memory to distinguish between its effect on ethanol reward vs. learning.

The exact mechanisms by which inhibiting RPTPβ/ζ decreases ethanol consumption and reward are not known and need to be delineated with additional experiments. This receptor is widely expressed in the nervous system (Levy et al. 1993) including relevant areas for ethanol reward such as prefrontal cortex, the nigrostriatal pathway and the ventral tegmental area (Ohyama et al., 1998; Hayashi et al. 2005; Ferrario et al., 2008). ALK, which is a substrate for RPTPβ/ζ, is also expressed in those regions (Bilsland et al., 2008; Iwahara et al., 1997; Vernersson et al., 2006). Notably, knockdown of ALK in the mouse ventral tegmental area leads to decreased binge-like ethanol consumption (Dutton et al, 2017), and treatment of mice with ALK inhibitors reduces drinking and ethanol CPP (Dutton et al, 2017). It is possible that the effects of RPTPβ/ζ on ethanol consumption and reward are through its ability to regulate ALK phosphorylation and activity in the ventral tegmental area.

Here, we found that inhibition of RPTPβ/ζ by MY10 and MY33-3 in neuroblastoma cells increases the phosphorylation of Tyr 1278 in ALK140. We also demonstrated that ethanol treatment increases the phosphorylation of ALK140, in accordance with previously published findings (He et al., 2015). Phosphorylation of Tyr 1278 is an important initial event in the activation of ALK (Guan et al., 2017; Hallberg and Palmer, 2016). Surprisingly, we found that pre-treatment of cells with RPTPβ/ζ inhibitors (particularly MY10) prior to ethanol reduced ethanol-induced phosphorylation of Tyr 1278 in ALK140. It was initially puzzling as to why both ethanol and RPTPβ/ζ inhibitors activate ALK when given separately, yet the combination of the two treatments blocks ALK activation. This might be explained by ALK trafficking. ALK activation causes endocytosis of the receptor and effectively desensitizes it (Mazot et al. 2012). We hypothesize that pre-treatment of neuroblastoma cells with RPTPβ/ζ inhibitors leads to increased autophosphorylation, activation, and endocytosis of ALK, effectively desensitizing ALK prior to ethanol treatment. ALK is then unavailable for ethanol-induced activation through its putative ligand MK (He et al., 2015). A schematic of this is demonstrated in Fig. 8. Because ALK inhibition reduces ethanol consumption, it is tempting to speculate that RPTPβ/ζ inhibitors decrease drinking and reward by promoting ALK endocytosis and interfering with ethanol-induced activation of ALK.

In addition to affecting ethanol drinking and CPP, treatment with MY10 delayed recovery from ethanol-induced ataxia, suggesting that the effects of the inhibitor may potentiate the motor effects of ethanol early after acute administration of the drug. It has to be noted that RPTPβ/ζ is expressed in the cortex and cerebellum (Levy et al. 1993; Tanaka et al., 2003), critical areas for ethanol-induced ataxia (Van Skike et al., 2010). Interestingly, increased activity of PKCγ has been suggested to be responsible for enhanced sensitivity to the motor-impairing effects of ethanol (Van Skike et al., 2010). Inhibition of RPTPβ/ζ by PTN is known to activate different PKC isoforms (Pariser et al., 2005; Herradon and Ezquerra,

2009), suggesting the possibility that inhibition of RPTPβ/ζ by MY10 may potentiate ethanol-induced ataxia in the short term through its ability to modulate the activity of PKCγ.

Here, we also demonstrate that ethanol induces a rapid phosphorylation of Tyr 490 in TrkA in cultured neuroblastoma cells. Phosphorylation of this residue is involved in the activation of the Ras/mitogen-activated protein kinase (MAPK or ERK) and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways triggered by TrkA (Obermeier et al., 1994). Previously, it was shown that neonatal ethanol exposure downregulates the expression of TrkA in different regions of the rat brain (Moore et al., 2004). In contrast, short-term exposure to ethanol resulted in elevated levels of nerve growth factor (NGF) and TrkA mRNA and protein expression in the mouse cerebellum (Wang et al., 2010). However, to the best of our knowledge, the functional consequences of the effects of ethanol on TrkA have not been studied. Here we provide the first evidence that ethanol activates TrkA. The mechanisms by which ethanol engages TrkA signaling are currently unknown and remain an important area for investigation. Note that PTN is upregulated in the mouse PFC after ethanol administration (Vicente-Rodriguez et al., 2014a) and that MK expression is increased in the PFC of alcoholics (Flatscher-Bader and Wilce, 2008) and in neuroblastoma cells incubated with ethanol (He et al., 2015). Both PTN and MK bind to RPTPβ/ζ (Maeda et al., 1999; Meng et al., 2000). RPTPβ/ζ negatively regulates TrkA and ALK signaling pathways by dephosphorylating them (Perez-Pinera et al., 2007; Shintani and Noda, 2008). PTN/MK inactivates RPTPβ/ζ phosphatase activity, thereby activating TrkA and ALK signaling. Thus, it is possible that the ability of ethanol to activate ALK and TrkA is indirect, through MK/PTN binding to and inactivating RPTPβ/ζ.

We also found that MY10 potentiates the sedative effects of ethanol, which may be related to either TrkA or ALK activity. ALK knockout mice exhibit increased ethanol-induced sedation compared with wild-type mice (Lasek et al., 2011), so it is possible that the effect of MY10 on LORR occurs through ALK signaling. TrkA is widely expressed in the CNS including areas such as cortex, thalamus and striatum (Holtzman et al., 1995). In the midbrain, it is known to be expressed in subpopulations of neurons of the interpeduncular nucleus (IPN) (Holtzman et al., 1995) which is part of the limbic system outflow into the brainstem. Interestingly, IPN plays an important role in sleep (Haun et al., 1992), potentially implicating the modulation of the TrkA activity by inhibition of RPTPβ/ζ in the potentiation of ethanol sedative effects caused by MY10. Clearly, additional mechanistic studies are needed to determine how and where in the brain RPTPβ/ζ functions to regulate diverse behavioral responses to ethanol.

5. CONCLUSION

The data support the concept that RPTPβ/ζ may be a novel target of pharmacotherapy for excessive alcohol consumption. Because of its overall effects in the behavioral assays and its ability to interfere with ethanol signaling pathways, we suggest that MY10 is a potential new compound that may be useful for the treatment of alcohol use disorder.

Acknowledgments

We would like to thank Donghong He for advice and assistance in performing the western blots. This work was supported by National Plan on Drug abuse, Ministerio de Sanidad of Spain (MSSSI, grant PNSD001I2015 to G.H.) and by National Institutes of Health (NIAAA, INIA grant U01 AA020912 to A.W.L.). M V-R and R F-C were supported by fellowships from Fundación Universitaria San Pablo CEU.

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Highlights

- We studied the effects of RPTPβ/ζ inhibitors in the regulation of alcohol effects
- RPTPβ/ζ modulates signaling pathways activated by alcohol
- RPTPβ/ζ inhibitors reduce alcohol drinking and reward
- RPTPβ/ζ might be a novel target for excessive alcohol consumption

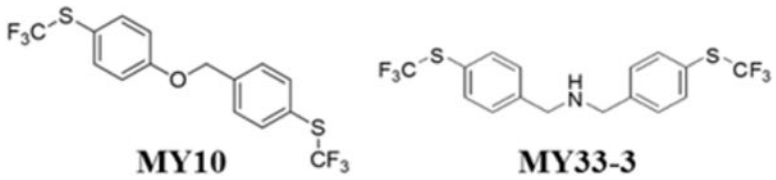

Fig 1.
Structure of the RPTPβ/ζ inhibitors MY10 and MY33-3.

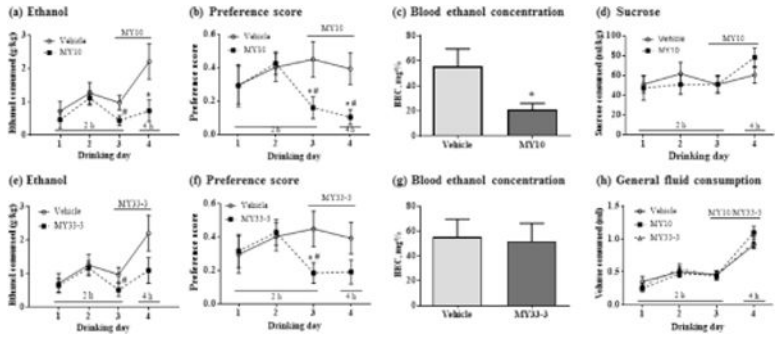

Fig 2. RPTPβ/ζ inhibition attenuates binge-like ethanol consumption

(a) Ethanol consumed (g/kg) during 2 h of drinking on days 1-3 and during 4 h of drinking on day 4. On days 3 and 4, mice were administered 60 mg/kg MY10 or vehicle (n=12/group) 1 hour before each drinking session in the DID test. (b) Ethanol preference in vehicle- and MY10-treated mice, calculated as the ratio of the volume of ethanol consumed over the volume of total fluid consumed. Ethanol preference on days 1-3 was calculated for 2 h-drinking sessions and on day 4 for a 4 h-drinking session. (c) Blood ethanol concentration (BEC, mg%) in vehicle- and MY10-treated mice after the final 4 h-drinking session on day 4. (d) Volume of 2% sucrose solution (ml/kg) consumed during 2 hours of drinking on days 1–3 and 4 hours on day 4 in mice treated with vehicle (n = 5) or MY10 (n = 5). (e) Ethanol consumed (g/kg) during 2 h of drinking on days 1-3 and during 4 h of drinking on day 4. On days 3 and 4, mice were administered 60 mg/kg MY33-3 or vehicle (n=12/group) 1 hour before each drinking session in the DID test. (f) Ethanol preference score in vehicle- and MY33-3-treated mice, calculated as the ratio of the volume of ethanol consumed over the volume of total fluid consumed. Ethanol preference on days 1-3 was calculated for 2 h-drinking sessions and on day 4 for a 4 h-drinking session. (g) Blood ethanol concentration (BEC, mg%) in vehicle- and MY33-3-treated mice after the final 4 h-drinking in the dark session on day 4. (h) Total fluid consumed (water + ethanol, ml) during 2 h-drinking sessions on days 1-3 and the 4 h-drinking session on day 4. On days 3 and 4, mice were administered 60 mg/kg MY10, MY33-3 or vehicle (n=12/group) 1 hour before each drinking session in the DID test. *p < 0.05 vs. Vehicle. #p < 0.05 vs. Day 2.

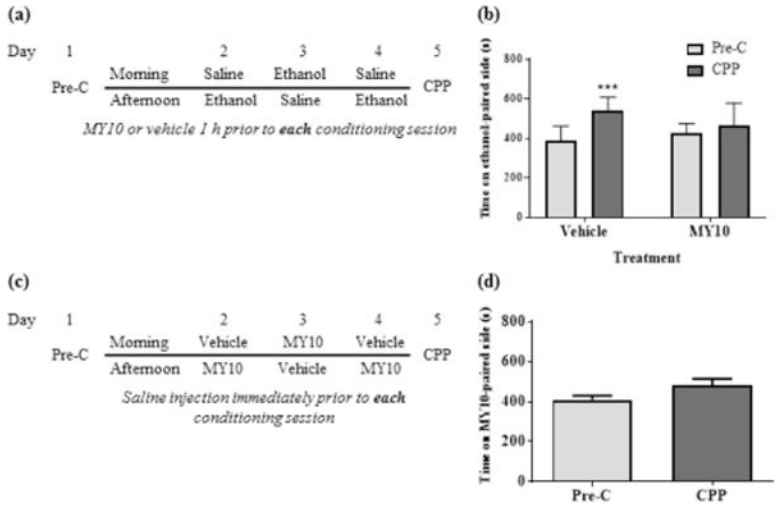

Fig 3. Ethanol (2.0 g/kg)-induced place preference

(a) Scheme showing treatment conditions for the ethanol CPP test (b) Time (seconds) spent on the ethanol-paired side before conditioning (Pre-C) and after conditioning (CPP) in mice treated with vehicle or 60 mg/kg MY10. The data show that MY10 prevents ethanol CPP. (c) Scheme showing the treatment conditions for the MY10 CPP test (in the absence of ethanol). (d) Time (seconds) spent on the MY10-paired side before conditioning (Pre-C) and after conditioning (CPP) in mice conditioned with 60 mg/kg MY10 (n = 13) instead of ethanol. Time spent on the MY10-paired side did not change after conditioning. *** P < 0.001 vs. Pre-C.

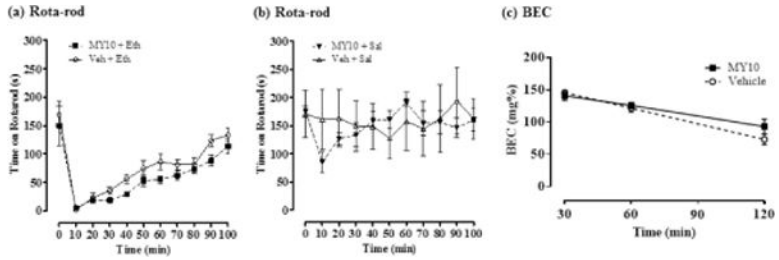

Fig 4. Ethanol (2.0 g/kg)-induced ataxia

(a) Time spent on a rotarod by mice treated with the RPTPβ/ζ inhibitor MY10 (60 mg/kg) or vehicle one hour before injection of 2.0 g/kg ethanol (Eth). (b) Time spent on a rotarod by mice treated with the RPTPβ/ζ inhibitor MY10 (60 mg/kg) or vehicle (Veh) one hour before injection of saline (Sal, i.p.). (c) Blood ethanol concentration (BEC) in mice pretreated with 60 mg/kg MY10 or vehicle and treated with 2.0 g/kg ethanol. Results are presented in mg% over time.

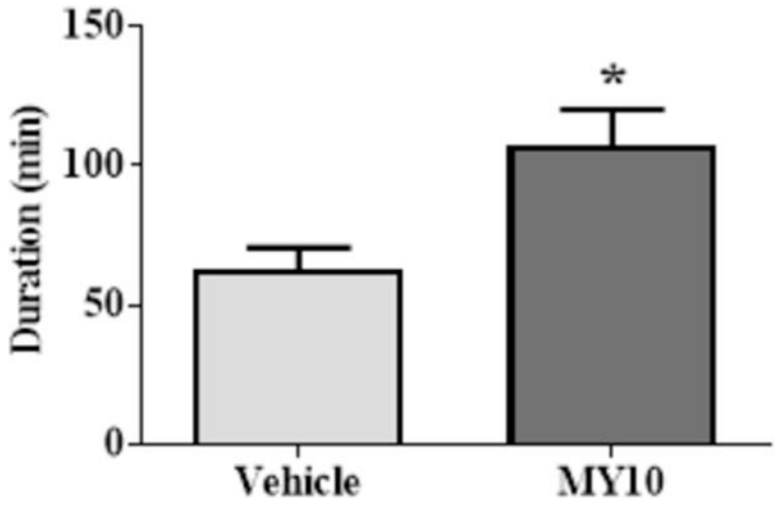

Fig 5. Ethanol (3.6 g/kg)-induced loss of righting reflex (LORR)

Increased duration of LORR induced in mice treated with the RPTPβ/ζ inhibitor MY10 (60 mg/kg) or vehicle one hour before ethanol injection (3.6 g/kg, i.p.) (n = 8). * $P < 0.05$ vs. Vehicle.

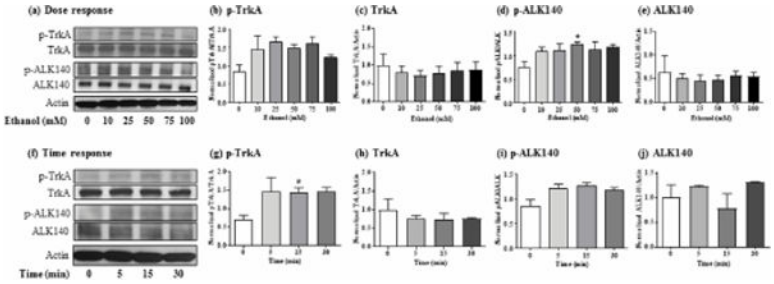

Fig. 6. Ethanol increases phosphorylation of anaplastic lymphoma kinase (ALK) and TrkA in SH-SY5Y cells

(a) Representative western blots showing phosphorylated ALK (pALK, Tyr 1278) 140 and phosphorylated TrkA (p-TrkA, Tyr 490) within 15 min of 0-100 mM ethanol treatment. Total ALK and TrkA western blots are shown below each phosphorylated protein blot for comparison. Both proteins were detected at ~ 140 kDa. (b) Graph shows the ratio p-TrkA/TrkA of optical density measurements corresponding to the p-TrkA and total TrkA protein levels respectively. (c) Graph shows the ratio TrkA/Actin of optical density measurements corresponding to the total TrkA and actin protein levels respectively. (d) Graph shows the ratio p-ALK140/ALK140 of optical density measurements corresponding to the p-ALK140 and total ALK140 protein levels respectively. (e) Graph shows the ratio ALK140/Actin of optical density measurements corresponding to the total ALK140 and actin protein levels respectively. (f) Representative western blots showing phosphorylated ALK (p-ALK) 140 and phosphorylated TrkA (p-TrkA) within 0 to 30 min of 50 mM ethanol treatment. Total ALK and TrkA western blots are shown below each phosphorylated protein blot for comparison. Both proteins were detected at ~ 140 kDa. (g) Graph shows the ratio p-TrkA/TrkA of optical density measurements corresponding to the p-TrkA and total TrkA protein levels respectively. (h) Graph shows the ratio TrkA/Actin of optical density measurements corresponding to the total TrkA and actin protein levels respectively. (i) Graph shows the ratio p-ALK140/ALK140 of optical density measurements corresponding to the p-ALK140 and total ALK140 protein levels respectively. (j) Graph shows the ratio ALK140/Actin of optical density measurements corresponding to the total ALK140 and actin protein levels respectively. Data are presented as the mean ± SEM. * $p < 0.05$ vs. 0 mM ethanol. # $p < 0.05$ vs. 0 min.

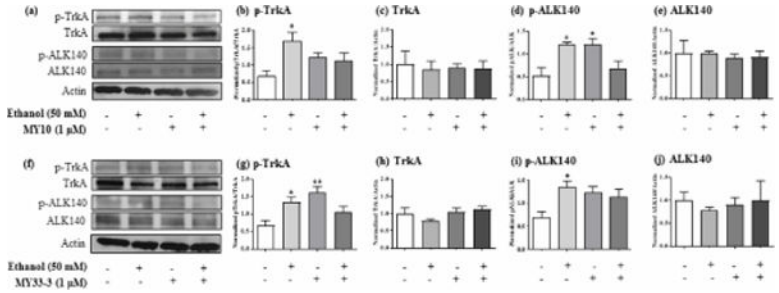

Fig. 7. RPTPβ/ζ inhibitors (MY10 and MY33-3) block ethanol-induced activation of TrkA and ALK in SH-SY5Y cells

(a) Representative western blots showing phosphorylated ALK (p-ALK, Tyr 1278) 140 and phosphorylated TrkA (p-TrkA, Tyr 490) in cells incubated for 5 min with 1 μM MY10 or DMSO (control) and then for 15 min with 50 mM ethanol. Total ALK and TrkA western blots are shown below each phosphorylated protein blot for comparison. Both proteins were detected at ~ 140 kDa. (b) Graph shows the ratio p-TrkA/TrkA of optical density measurements corresponding to the p-TrkA and total TrkA protein levels respectively. (c) Graph shows the ratio TrkA/Actin of optical density measurements corresponding to the total TrkA and actin protein levels respectively. (d) Graph shows the ratio p-ALK140/ALK140 of optical density measurements corresponding to the p-ALK140 and total ALK140 protein levels respectively. (e) Graph shows the ratio ALK140/Actin of optical density measurements corresponding to the total ALK140 and actin protein levels respectively. (f) Representative western blots showing phosphorylated ALK (p-ALK) 140 and phosphorylated TrkA (p-TrkA) in cells incubated for 5 min with 1 μM MY33-3 or DMSO (control) and then for 15 min with 50 mM ethanol. Total ALK and TrkA western blots are shown below each phosphorylated protein blot for comparison. Both proteins were detected at ~ 140 kDa. (g) Graph shows the ratio p-TrkA/TrkA of optical density measurements corresponding to the p-TrkA and total TrkA protein levels respectively. (h) Graph shows the ratio TrkA/Actin of optical density measurements corresponding to the total TrkA and actin protein levels respectively. (i) Graph shows the ratio p-ALK140/ALK140 of optical density measurements corresponding to the p-ALK140 and total ALK140 protein levels respectively. (j) Graph shows the ratio ALK140/Actin of optical density measurements corresponding to the total ALK140 and actin protein levels respectively. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs. unstimulated cells (controls, lane 1).

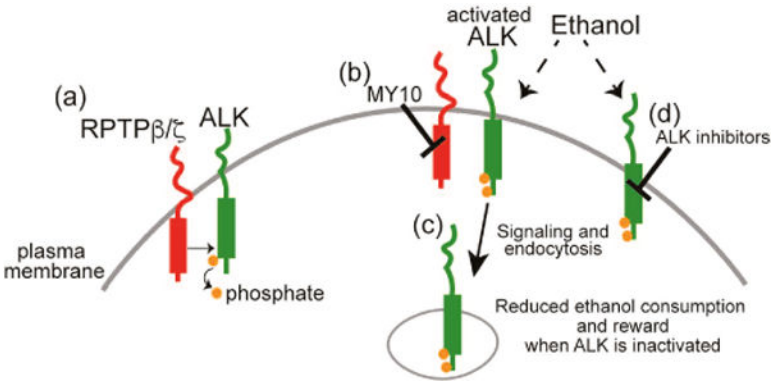

Fig. 8. Schematic showing RPTPβ/ζ and ALK interaction and possible involvement in ethanol consumption and reward

(a) Under normal conditions, RPTPβ/ζ dephosphorylates ALK and maintains ALK in an inactive state. (b) Inhibition of RPTPβ/ζ by inhibitors such as MY10 leads to constitutive ALK phosphorylation and activation. (c) Activated ALK would undergo endocytosis to desensitize the receptor and render it unavailable for ethanol-induced activation. (d) ALK inhibitors also block ethanol-induced activation of ALK. Inactivation of ALK reduces ethanol consumption and reward.