Genetic inactivation of midkine modulates behavioural responses to ethanol possibly by enhancing GABA(A) receptor sensitivity to GABA(A) acting drugs

Marta Vicente-Rodríguez[1], Carmen Pérez-García[1], María Haro[2], María P. Ramos[2], Gonzalo Herradón[1,*]

[1]Pharmacology lab, Department of Pharmaceutical and Health Sciences. Facultad de Farmacia. Universidad CEU San Pablo, Madrid, Spain.

[2]Biochemistry lab, Department of Chemistry and Biochemistry. Facultad de Farmacia. Universidad CEU San Pablo, Madrid, Spain.

*Corresponding author:
Gonzalo Herradon, PhD
Lab. Pharmacology
Faculty of Pharmacy
Universidad CEU San Pablo
Urb. Montepríncipe
28668 Boadilla del Monte, Madrid, Spain.
Tel: 34-91-3724700 (Ext. 4840)
Fax: 34-91-3510475
e-mail: herradon@ceu.es

Abbreviations:

ALDH: Aldehyde dehydrogenase

ALK: Anaplastic Lymphoma Kinase

COMT: Catechol-O-methyltransferase

CPP: Conditioned Place Preference

GABA: gamma-aminobutyric acid

LORR: Loss of the Righting Reflex

MK: Midkine

PFC: Prefrontal cortex

RPTP: Receptor Protein Tyrosine Phosphatase

PTN: Pleiotrophin

ABSTRACT

Midkine (MK) is a cytokine with important functions in dopaminergic neurons that is found upregulated in the prefrontal cortex of alcoholics. We have studied the behavioural effects of ethanol in MK genetically deficient (MK-/-) and wild type (MK+/+) mice. A low dose of ethanol (1.0 g/kg), unable to cause conditioned place preference (CPP) in MK+/+ mice, induced a significant CPP in MK-/- mice, suggesting that MK prevents the rewarding effects of low doses of ethanol. However, this difference between genotypes is lost when a higher, rewarding, dose of ethanol (2.0 g/kg) is used. Accordingly, the anxiolytic effects of 1.0 mg/kg diazepam, other GABA(A) acting drug, were significantly enhanced in MK-/- mice compared to MK+/+ mice; however, 2.0 mg/kg diazepam caused increased anxiolytic effects in MK+/+ mice. In addition, MK-/- mice showed a significant delayed recovery from ethanol (2.0 g/kg)-induced ataxia whereas the sedative effects induced by ethanol (3.6 g/kg), tested in a loss of righting reflex paradigm, were found to be similar in MK-/- and MK+/+ mice. The data indicate that MK differentially regulates the behavioural responses to ethanol. The results suggest that differences in the sensitivity of GABA(A) receptors to GABA(A) acting drugs caused by genetic inactivation of MK could underlie the different behavioural responses to ethanol in MK-/- mice. Overall, these results suggest that MK may be a novel genetic factor of importance in alcohol use disorders, and that potentiation of MK signalling pathway may be a promising therapeutic strategy in the treatment of these disorders.

Keywords: Conditioned place preference, pleiotrophin, fyn, addiction, ALK, alcohol.

1. **Introduction**

Alcoholism is a devastating condition with serious health and social consequences. Evidence suggests a strong genetic component for predisposition to alcoholism [1]. Ethanol is known to exert many of its biochemical and behavioural effects through an interaction with the gamma-aminobutyric acid (GABA) receptor system [2]. Although several genes including GABA(A) receptor subunits have been demonstrated to be involved in ethanol dependence and behavioural responses to ethanol [3, 4], the genetics of ethanol dependence is poorly understood and likely involves many genes. Two genetic factors that are regulated in different brain areas after administrations of different drugs of abuse are pleiotrophin (PTN) and midkine (MK) [5]. PTN and MK share important neurotrophic effects on dopaminergic neurons [6, 7]. It has been previously shown that PTN knockout (PTN-/-) and MK knockout (MK-/-) mice are more vulnerable to amphetamine and cocaine conditioning effects respectively [8-10], suggesting that regulation of expression of PTN and/or MK in the brain after administration of drugs of abuse is involved in the regulation of drug-induced rewarding effects [11]. In addition, it has been shown that PTN and MK counteract amphetamine and cocaine neurotoxic effects [12-16].

We have recently shown that PTN, a cytokine highly redundant in function with MK [6, 7], counteracts the rewarding and sedative effects of ethanol [17]. Interestingly, MK gene expression is known to be upregulated in the prefrontal cortex (PFC) of long-term alcoholic subjects [18]. Since alcoholism is associated with reduction in the number of neurons and dendritic arbors in the PFC which correlate with cognitive defects in alcoholics and MK is known to be a survival factor for neurons, upregulation of MK expression has been suggested to occur in order to counteract ethanol-induced

cell damage and apoptotic processes in this brain area [19]. However, a possible regulation of ethanol rewarding effects by this neurotrophic factor should not be discarded because PFC is also known to play a pivotal role in the mesocorticolimbic dopaminergic system [20]. To test the possibility that MK is involved in the modulation of the behavioural responses to ethanol we have tested the effects induced by ethanol in MK-/- and wild type (MK+/+) mice.

2. **Methods**

2.1. Animals

MK-/- mice were kindly provided by Dr. Thomas F. Deuel (The Scripps Research Institute, La Jolla, CA). MK-/- mice were generated as previously described by using a basic vector to target a part of exon 1, intron 1 and a part of exon 2 of MK [21]. Male MK-/- and MK+/+ mice on a C57BL/6J background were used at 8-10 weeks of age (20-25 g). Animals were carefully distributed so the average of the animal's age was similar in every experimental group. The genotypes of the MK-/- mice were confirmed with the polymerase chain reaction using as primers 5'-ATC GGT TCC AAG TCC TCC CTC CGT C-3' forward and 5'-CAC CTT CCT CAG TTG ACA AAG ACA AGC-3' reverse to generate from genomic DNA extracted from tails of MK-/- and MK+/+ mice a cDNA of ~0.7 kb.

All the animals used in this study were maintained in accordance with European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols were approved by the Animal Research Committee of USP-CEU.

2.2. Conditioned Place Preference (CPP)

A biased apparatus was used and consisted of two Plexiglas square compartments of the same size (20 cm long x 14 cm high x 27 cm wide). One compartment had black plexiglas floor and walls, and the other had black plexiglas floor and white walls. The procedure consisted of a 5-day schedule with three phases: 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 behaviour was monitored by a videotracking system (San Diego, California,

USA) to calculate the time spent in each compartment. We previously used this `biased` apparatus and subject assignment, i.e., mice were paired with the drug in the non-preferred compartment, to study genotype differences in amphetamine- and cocaine-induced CPP [8, 9]. Confirming those studies, the compartment with white walls in the present work was the non-preferred compartment by all mouse genotypes (~ 30 % stay of total time in the preconditioning phase).

We performed a 3-day conditioning phase with double conditioning sessions. The first one involved a morning session starting at 8 am, in which all animals received a single injection of saline i.p. (10 ml/kg) and were immediately confined to the initially preferred compartment for 5 min. In the evening session starting at 3 pm, the animals were injected (i.p.) with 1.0 g/kg ethanol (n = 21-22/group) or 2.0 g/kg ethanol (n = 17-18/group), or 10 ml/kg saline (n = 6-7/group) as a conditioning control, and immediately confined to the initially non-preferred compartment for 5 min. On the following two days, the procedure used was the same but the order of the treatments (morning/evening) was changed to avoid the influence of circadian variability.

In the testing phase on day 5, mice received a drug-free, 15-min preference test. The percentage of time-spent (stay) in the non-preferred (drug-paired) compartment was calculated in all cases and the difference between the time spent in this compartment in this phase (day 5, CPP) and the time spent in the same compartment in the preconditioning (day 1, Pre-C) was considered as indicative of the degree of conditioning induced by ethanol.

2.3. Rotarod test

We used a rotarod apparatus (Panlab, Barcelona, Spain) to assess the effects of ethanol administration on motor coordination and balance in MK+/+ (n = 8) and MK-/-

(n = 11) mice. Mice were trained in 2 sessions in 2 consecutive days by placing them on the rotarod 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 injected with 2.0 g/kg ethanol and then placed on the rotarod. The time to fall was recorded and mice were placed back on the rotarod every 10 minutes until 100 min after ethanol administration.

2.4. Loss of the Righting Reflex (LORR)

MK+/+ (n = 8) and MK-/- (n = 9) mice were injected i.p. with 3.6 g/kg ethanol. After injection each mouse was placed on its back and tested for the ability to right itself. The mouse was determined to have lost the righting reflex if it could not right itself 3 times within 30 sec. and regained the righting reflex if it could fully right itself 3 times within 30 sec. The duration of LORR was determined as the difference between the time when the reflex was lost and when it was regained.

2.5. Elevated plus maze

The apparatus consists of a black Plexiglas apparatus with four arms (16 cm long x 5 cm wide) set in cross from a neutral central square (5 cm x 5 cm). Two opposite arms are delimited by vertical walls (closed arms), while the two other opposite arms have unprotected edges (open arms). The maze is elevated 60 cm above the ground. 30 min before the observation session, MK+/+ and MK-/- mice received a single administration of saline (10 ml/kg, i.p.), 1.0 mg/kg diazepam (n = 8-10/group) or 2.0 mg/kg diazepam (n = 12-13/group). At the beginning of the 5 min observation session, each mouse was placed in the central neutral zone, facing one of the open arms.

The total numbers of visits to the closed and open arms, and the time spent in the closed and open arms, were then recorded.

2.6. Statistical analysis

CPP data were analysed by 2-way ANOVA with repeated measures followed by Bonferroni's post-hoc tests, considering experimental phase and genotype as variables. Rotarod data were analysed by 2-way ANOVA with repeated measures followed by Bonferroni's post-hoc test, considering genotype and time as variables. LORR data were analysed by student's t test. In the elevated plus maze, the number of entries to the open arms and the time spent in the open arms by every experimental group were compared by 2-way ANOVA followed by Bonferroni's post-hoc tests, considering treatment and genotype as factors. A p value less than 0.05 was considered a statistically significant difference. All statistical analyses were performed using Graphpad prism 5 program (La Jolla, CA, USA).

3. **Results**

We first used a low dose of ethanol (1.0 g/kg, i.p.) which has been reported to be unable to induce CPP in mice or to produce moderate conditioning effects [22]. Accordingly, we found that 1.0 g/kg ethanol did not cause a significant CPP in MK+/+ mice (Fig. 1A). In contrast, 1.0 g/kg ethanol caused a significant CPP in MK-/- mice (Fig. 1A) suggesting an important role of MK in the modulation of ethanol conditioning effects. However, a higher dose of ethanol (2.0 g/kg) that has been consistently shown to induce CPP in mice [22] tended to induce CPP more efficiently in MK+/+ mice compared to MK-/- mice (Fig. 1B). Saline conditioning did not show significant changes on place preference compared to preconditioning values of both genotypes (Fig. 1C). The data confirm an important role of MK in the regulation of the rewarding effects of ethanol at low doses.

To test the possibility that MK could modulate other acute behavioural responses to ethanol we performed experiments to assay the ataxic and sedative effects of ethanol in both genotypes. The motor incoordinating effects of ethanol were measured using the rotarod test. Acute administration of 2.0 g/kg ethanol produced motor ataxia in both genotypes of mice illustrated by a significant reduction in time spent on the rotarod (Fig. 2). In agreement with studies reported by others [23], performance improved over time in MK+/+ mice (Fig. 2). However, MK-/- mice showed a significant delayed recovery from ethanol-induced ataxia (Fig. 2). In addition, we examined MK-/- and MK+/+ mice for their behavioural response to ethanol in a LORR test using a sedating dose of ethanol (3.6 g/kg). The data show that the amount of time needed for MK-/- and MK+/+ mice to recover the righting reflex after ethanol injection was effectively similar ($P = 0.1630$; Fig. 3). These data indicate that endogenous MK is not key for the sedative effects of ethanol.

To examine the possibility that differences in the sensitivity of GABA(A) receptors to GABA(A) acting drugs could underlie the different behavioural responses to ethanol in MK-/- mice, we studied the effects of another GABA(A) receptor agonist, diazepam, in both genotypes. Doses of 1.0 and 2.0 mg/kg diazepam which exert acute anxiolytic but no sedative effects [24] were used in the elevated plus maze test. 1.0 mg/kg diazepam increased the time spent in the open arms by mice from both genotypes (Fig. 4A); however, this increase was significantly higher in MK-/- mice compared to MK+/+ mice (Fig. 4A). As expected, 2.0 mg/kg diazepam significantly increased the time spent in open arms by MK+/+ mice compared to 1.0 mg/kg (Fig. 4B vs. 4A). In contrast, the time spent in open arms by MK-/- mice treated with 2.0 mg/kg diazepam was significantly reduced compared with MK+/+ mice (Fig. 4B) and with MK-/- mice treated with the lower dose (1.0 mg/kg) of diazepam. We did not find significant differences between genotypes in the number of entries in open arms (data not shown). The total number of entries in all arms was also similar between genotypes indicating similar locomotor activity.

4. **Discussion**

Genetic predisposition, as well as adaptive changes in expression of drug-sensitive genes, is thought to contribute to alcohol dependence [25]. One of the genes significantly upregulated in the PFC of patients after chronic alcohol intake is MK [18, 19]. MK is also found upregulated after administration of other drugs of abuse [5]. Interestingly, MK-/- mice show increased vulnerability to the neurotoxic effects of amphetamine and cocaine [13, 14, 16] and deficits in the extinction of cocaine-induced CPP [8]. Therefore, we hypothesized that upregulation of MK after administration of drugs of abuse constitutes a protective mechanism against drug-induced neurotoxic and neuroadaptative effects [26]. Whether or not this is the case of other drugs known to increase the expression of MK in the brain, such as ethanol, remained to be studied.

In the present work, the conditioning effects of ethanol have been tested in CPP assays in which previous cues acquire secondary reinforcing properties when paired with a primary reinforce. The CPP paradigm is being increasingly used to study neuroadaptations and to identify novel genetic factors possibly underlying drug seeking behaviours, particularly in genetically modified mouse models [22]. We now demonstrate that genetic deletion of MK is key for ethanol to induce CPP at a low dose in mice. However, this difference between genotypes is lost when a higher, rewarding, dose of ethanol (2.0 g/kg) is used. It has to be noted that we used in our experiments a CPP biased design and that mice from both genotypes started the CPP procedure with a similar degree of initial preference to the compartment with black walls. The use of a biased design is feasible in place conditioning studies and the outcome can be interpreted in terms of rewarding drug effects, as long as an absolute preference for the drug-paired compartment is produced with a well-established rewarding dose of ethanol

(2.0 g/kg) in the control (MK+/+) subjects [22]. Thus, although we cannot discount the possibility of a conditioned reduction in aversion to the non-preferred compartment due to the known anxiolytic effects of alcohol, the data presented here more likely reflect the rewarding effects of ethanol in our CPP paradigm.

Interestingly, we found a similar pattern with another GABA(A) receptor agonist. The anxiolytic effects of diazepam were significantly enhanced in MK-/- mice treated with the lower dose of the drug; however, these effects were significantly increased in MK+/+ mice treated with the higher dose of diazepam compared with MK-/- mice in which the anxiolytic effects of 2.0 mg/kg diazepam were almost absent. The data suggest that MK-/- mice might be more sensitive to the effects of GABA(A) acting drugs and behave as high responders to low doses of these drugs, but this difference can only be seen in the low dose and is then lost because of ceiling effects.

It is important to note that the modulatory role of MK on the rewarding effects of ethanol cannot be generalized to all types of behavioural responses to ethanol. The data obtained in the rotarod test clearly indicate that genetic inactivation of MK in mice causes a significantly prolonged delay in recovery from the ataxic effects induced by ethanol. However, the data collected in the LORR test suggest that the sedative effects induced by ethanol are not modulated by MK.

The development of alcohol dependence is accompanied by changes in the expression of genes involved in the regulation of the innate immune response [27]. Induction of innate immune genes has been proposed to contribute to neuroplastic adaptations in the brain and negative affective states that promote behavioural changes leading to addiction [28]. It has been established that immune signalling contributes significantly to changes in the rewarding pathways of the brain induced by drugs of abuse [28], suggesting that endogenous modulators of immune response could be

considered as novel pharmacological targets in addictive disorders. Very interestingly, MK is a known regulator of innate immune response [29]. MK is known to modulate expression of proinflammatory cytokines and the expansion of regulatory T-cells [30]. Accordingly, in different animal models of autoinmune diseases, it has been shown that CD4+ T-cells from MK-/- mice express lower amounts of IFN-γ among other cytokines that are critically involved in the immune response [30]. It has to be noted that specific changes in the signal transduction of IFN-γ underlie some of the previously described changes in immune function associated with chronic alcohol abuse [27]. Our data strongly support the need of additional studies to define the possible connection of the regulation of immune response by MK and the changes in the behavioural responses to ethanol in MK-/- mice.

The role of MK in the regulation of the behavioural responses to ethanol might be related to its mechanism of action. MK binds the Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ [31]. After ligand receptor interactions of RPTPβ/ζ with MK [6], tyrosine phosphorylation is increased in signalling molecules such as Anaplastic Lymphoma Kinase (ALK) [32] and Fyn kinase [33] which are molecules known to play important roles in behavioural responses to ethanol. It has been shown that increased ALK expression in the brain negatively correlates with ethanol consumption in mice [34]. On the other hand, Fyn kinase is part of a signalling pathway that is specifically activated during alcohol exposure and contributes to the molecular mechanisms underlying the maintenance of alcohol self-administration [35]. Also, it has to be noted that phosphorylation of GABA(A) receptors is an important mechanism for dynamically modulating inhibitory synaptic function in the mammalian brain. Phosphorylation of residues within GABA(A) receptors has been shown to play an essential role in the assembly, trafficking, and cell surface stability of these receptors [36] which can

influence the pharmacological effects of GABA(A) acting drugs. Interestingly, Fyn kinase contributes to tyrosine phosphorylation of the GABA(A) receptor [37], suggesting the possibility that an impaired MK-RPTPβ/ζ-Fyn axis in MK-/- mice could underlie the different behavioural responses of MK-/- mice to pharmacological activation of GABA(A) receptors by affecting the phosphorylation status of these receptors. In any case, additional studies are needed to dissect the possible contribution of ALK and/or Fyn kinase to the mechanisms triggered by MK in the modulation of the behavioural responses to ethanol and other GABA(A) acting drugs.

5. **Conclusion**

The data demonstrate a differential regulation of specific behavioural responses to ethanol by MK. The results suggest that differences in the sensitivity of GABA(A) receptors to GABA(A) acting drugs caused by genetic inactivation of MK could underlie the different behavioural responses to ethanol in MK-/- mice. The data suggest that MK may be a novel genetic factor of importance in alcohol use disorders and support potentiation of the MK signalling pathway as a promising therapeutic strategy in the treatment of alcohol use disorders.

Acknowledgements

This work has been supported by grants SAF2009-08136 and CENIT CEN-20101023 from Ministerio de Ciencia e Innovación of Spain.

References

[1] Kos MZ, Glahn DC, Carless MA, Olvera R, McKay DR, Quillen EE, et al. Novel QTL at chromosome 6p22 for alcohol consumption: Implications for the genetic liability of alcohol use disorders. Am J Med Genet B Neuropsychiatr Genet 2014;165:294-302.

[2] Chester JA, Cunningham CL. GABA(A) receptor modulation of the rewarding and aversive effects of ethanol. Alcohol 2002;26:131-43.

[3] Ducci F, Goldman D. Genetic approaches to addiction: genes and alcohol. Addiction 2008;103:1414-28.

[4] Melroy WE, Stephens SH, Sakai JT, Kamens HM, McQueen MB, Corley RP, et al. Examination of Genetic Variation in GABRA2 with Conduct Disorder and Alcohol Abuse and Dependence in a Longitudinal Study. Behav Genet 2014;44:356-67.

[5] Herradon G, Ezquerra L, Gramage E, Alguacil LF. Targeting the pleiotrophin/receptor protein tyrosine phosphatase beta/zeta signaling pathway to limit neurotoxicity induced by drug abuse. Mini Rev Med Chem 2009;9:440-7.

[6] Muramatsu T. Structure and function of midkine as the basis of its pharmacological effects. Br J Pharmacol 2014;171:814-26.

[7] Herradon G, Ezquerra L. Blocking receptor protein tyrosine phosphatase beta/zeta: a potential therapeutic strategy for Parkinson's disease. Curr Med Chem 2009;16:3322-9.

[8] Gramage E, Perez-Garcia C, Vicente-Rodriguez M, Bollen S, Rojo L, Herradon G. Regulation of extinction of cocaine-induced place preference by midkine is related to a differential phosphorylation of peroxiredoxin 6 in dorsal striatum. Behav Brain Res 2013;253:223-31.

[9] Gramage E, Putelli A, Polanco MJ, Gonzalez-Martin C, Ezquerra L, Alguacil LF, et al. The neurotrophic factor pleiotrophin modulates amphetamine-seeking behaviour and amphetamine-induced neurotoxic effects: evidence from pleiotrophin knockout mice. Addict Biol 2010;15:403-12.

[10] Martin YB, Gramage E, Herradon G. Maintenance of amphetamine-induced place preference does not correlate with astrocytosis. Eur J Pharmacol 2013;699:258-63.

[11] Gramage E, Herradon G. Connecting Parkinson's disease and drug addiction: common players reveal unexpected disease connections and novel therapeutic approaches. Curr Pharm Des 2011;17:449-61.

[12] Gramage E, Alguacil LF, Herradon G. Pleiotrophin prevents cocaine-induced toxicity in vitro. Eur J Pharmacol 2008;595:35-8.

[13] Gramage E, Herradon G, Martin YB, Vicente-Rodriguez M, Rojo L, Gnekow H, et al. Differential phosphoproteome of the striatum from pleiotrophin knockout and midkine knockout mice treated with amphetamine: correlations with amphetamine-induced neurotoxicity. Toxicology 2013;306:147-56.

[14] Gramage E, Martin YB, Ramanah P, Perez-Garcia C, Herradon G. Midkine regulates amphetamine-induced astrocytosis in striatum but has no effects on amphetamine-induced striatal dopaminergic denervation and addictive effects: functional differences between pleiotrophin and midkine. Neuroscience 2011;190:307-17.

[15] Gramage E, Rossi L, Granado N, Moratalla R, Herradon G. Genetic inactivation of pleiotrophin triggers amphetamine-induced cell loss in the substantia nigra and enhances amphetamine neurotoxicity in the striatum. Neuroscience 2010;170:308-16.

[16] Vicente-Rodriguez M, Gramage E, Herradon G, Perez-Garcia C. Phosphoproteomic analysis of the striatum from pleiotrophin knockout and midkine knockout mice treated with cocaine reveals regulation of oxidative stress-related proteins potentially underlying cocaine-induced neurotoxicity and neurodegeneration. Toxicology 2013;314:166-73.

[17] Vicente-Rodríguez M, Pérez-García C, Ferrer-Alcón M, Uribarri M, Sánchez-Alonso MG, Ramos MP, et al. Pleiotrophin differentially regulates the rewarding and sedative effects of etanol. J Neurochem 2014, in press.

[18] Flatscher-Bader T, van der Brug M, Hwang JW, Gochee PA, Matsumoto I, Niwa S, et al. Alcohol-responsive genes in the frontal cortex and nucleus accumbens of human alcoholics. J Neurochem 2005;93:359-70.

[19] Flatscher-Bader T, Wilce PA. Impact of Alcohol Abuse on Protein Expression of Midkine and Excitatory Amino Acid Transporter 1 in the Human Prefrontal Cortex. Alcohol Clin Exp Res 2008;32:1849-58

[20] Gass JT, Chandler LJ. The Plasticity of Extinction: Contribution of the Prefrontal Cortex in Treating Addiction through Inhibitory Learning. Front Psychiatry 2013;4:46.

[21] Nakamura E, Kadomatsu K, Yuasa S, Muramatsu H, Mamiya T, Nabeshima T, et al. Disruption of the midkine gene (Mdk) resulted in altered expression of a calcium

binding protein in the hippocampus of infant mice and their abnormal behaviour. Genes Cells 1998;3:811-22.

[22] Tzschentke TM. Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 2007;12:227-462.

[23] den Hartog CR, Beckley JT, Smothers TC, Lench DH, Holseberg ZL, Fedarovich H, et al. Alterations in ethanol-induced behaviors and consumption in knock-in mice expressing ethanol-resistant NMDA receptors. PLoS One 2013;8:e80541.

[24] Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, et al. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 2000;290:131-4.

[25] Rietschel M, Treutlein J. The genetics of alcohol dependence. Ann N Y Acad Sci 2013;1282:39-70.

[26] Herradon G, Perez-Garcia C. Targeting midkine and pleiotrophin signaling pathways in addiction and neurodegenerative disorders: Recent progress and perspectives. Br J Pharmacol 2014;171:837-48.

[27] Beech RD, Qu J, Leffert JJ, Lin A, Hong KA, Hansen J, et al. Altered expression of cytokine signaling pathway genes in peripheral blood cells of alcohol dependent subjects: preliminary findings. Alcohol Clin Exp Res 2012;36:1487-96.

[28] Hutchinson MR, Watkins LR. Why is neuroimmunopharmacology crucial for the future of addiction research? Neuropharmacology 2014;76 Pt B:218-27.

[29] Cohen S, Shachar I. Midkine as a regulator of B cell survival in health and disease. Br J Pharmacol 2014;171(4):888-95.

[30] Weckbach LT, Muramatsu T, Walzog B. Midkine in inflammation. ScientificWorldJournal 2011;11:2491-505.

[31] Sakaguchi N, Muramatsu H, Ichihara-Tanaka K, Maeda N, Noda M, Yamamoto T, et al. Receptor-type protein tyrosine phosphatase zeta as a component of the signaling receptor complex for midkine-dependent survival of embryonic neurons. Neurosci Res 2003;45(2):219-24.

[32] Perez-Pinera P, Zhang W, Chang Y, Vega JA, Deuel TF. Anaplastic lymphoma kinase is activated through the pleiotrophin/receptor protein-tyrosine phosphatase beta/zeta signaling pathway: an alternative mechanism of receptor tyrosine kinase activation. J Biol Chem 2007;282:28683-90.

[33] Pariser H, Ezquerra L, Herradon G, Perez-Pinera P, Deuel TF. Fyn is a downstream target of the pleiotrophin/receptor protein tyrosine phosphatase beta/zeta-

signaling pathway: regulation of tyrosine phosphorylation of Fyn by pleiotrophin. Biochem Biophys Res Commun 2005;332:664-9.

[34] Lasek AW, Lim J, Kliethermes CL, Berger KH, Joslyn G, Brush G, et al. An evolutionary conserved role for anaplastic lymphoma kinase in behavioral responses to ethanol. PLoS One 2012;6:e22636.

[35] Wang J, Carnicella S, Phamluong K, Jeanblanc J, Ronesi JA, Chaudhri N, et al. Ethanol induces long-term facilitation of NR2B-NMDA receptor activity in the dorsal striatum: implications for alcohol drinking behavior. J Neurosci 2007;27:3593-602.

[36] Comenencia-Ortiz E, Moss SJ, Davies PA. Phosphorylation of GABA receptors influences receptor trafficking and neurosteroid actions. Psychopharmacology (Berl) 2014;in press.

[37] Jurd R, Tretter V, Walker J, Brandon NJ, Moss SJ. Fyn kinase contributes to tyrosine phosphorylation of the GABA(A) receptor gamma2 subunit. Mol Cell Neurosci 2010;44:129-34.

Figure Legends

Fig 1. Ethanol-induced place preference. (A) Results are presented as the mean ± SEM of the percentage of the time spent by MK+/+ (n = 22) and MK-/- (n = 21) in the ethanol (1.0 g/kg)-paired (least-preferred) compartment during preconditioning (Pre-C, day 1) and testing phases (CPP, day 5). 2-way repeated measures ANOVA showed a significant effect of phase ($F(1,41) = 15.16$, $P < 0.001$). **(B)** Results represent the percentage of the time spent by MK+/+ (n = 18) and MK-/- (n = 17) mice in the ethanol (2.0 g/kg)-paired compartment during preconditioning (Pre-C, day 1) and testing phases (CPP, day 5). 2-way repeated measures ANOVA revealed a significant effect of genotype ($F(1,33) = 4.904$, $P < 0.05$) and phase ($F(1,33) = 34.47$, $P < 0.001$). **(C)** Results from control experiments in which MK+/+ (n = 6) and MK-/- (n = 7) mice were only treated with saline during conditioning. *** $P < 0.001$ vs. Pre-C. ** $P < 0.01$ vs. Pre-C.

Fig 2. Ethanol (2.0 g/kg)-induced ataxia. Time (mean ± SEM) spent on a rotarod following injection of 2.0 g/kg ethanol in MK-/- and MK+/+ mice. At time point 0 min, basal values of time spent on the rotarod by mice from both genotypes are represented. 2-way repeated measures ANOVA revealed a significant effect of time ($F(10,170) = 32.73$, $P < 0.001$), of genotype ($F(1,170) = 6.324$, $P < 0.05$) and a significant interaction genotype x time, $F(10,170) = 2.422$, $P < 0.05$).

Fig 3. Ethanol (3.6 g/kg)-induced sedation. Loss of righting reflex (LORR) test in MK-/- and MK+/+ mice at 3.6 g/kg ethanol. Results are presented as the mean ± SEM of the time to recovery from sedation.

Figure 4: Behavioural performance of diazepam-treated MK+/+ and MK-/- mice in the Elevated Plus Maze. (A) Figure shows mean ± SEM of time spent in the open arms by MK+/+ and MK-/- mice treated with diazepam (1.0 mg/kg; n = 8-10/genotype) or saline (n = 8-9/genotype). 2-way ANOVA revealed a significant effect of treatment ($F(1,31) = 29.73$, $P < 0.001$) and of genotype ($F(1,31) = 4.757$, $P < 0.05$). (B) Figure shows mean ± SEM of time spent in the open arms by MK+/+ and MK-/- mice treated with diazepam (2.0 mg/kg; n = 12-13/genotype) or saline (n = 12-16/genotype). 2-way ANOVA revealed a significant effect of treatment ($F(1,49) = 10.84$, $P < 0.01$) and a significant interaction treatment x genotype ($F(1,49) = 4.083$, $P < 0.05$). *** $P < 0.001$ vs. saline. ** $P < 0.01$ vs. saline. * $P < 0.05$ vs. saline. # $P < 0.05$ vs. MK+/+.