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Midkine in the Mouse Ventral Tegmental Area Limits Ethanol Intake and *Ccl2* Gene Expression

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Abstract

Midkine (MDK) is a cytokine and neurotrophic factor that is more highly expressed in the brains of human alcoholics and in mice predisposed to drink large amounts of ethanol, suggesting that MDK may regulate ethanol consumption. Here we measured ethanol consumption in male and female *Mdk* knockout (−/−) mice using the two-bottle choice and the drinking in the dark (DID) tests. We found that *Mdk* −/− mice consumed significantly more ethanol than wild type controls in both tests. To determine if MDK acts in the ventral tegmental area (VTA) to regulate ethanol consumption, we delivered lentivirus expressing a *Mdk* shRNA into the VTA of male C57BL/6J mice to locally knockdown *Mdk* and performed the DID test. Mice expressing a *Mdk* shRNA in the VTA consumed more ethanol than mice expressing a control non-targeting shRNA, demonstrating that the VTA is one site in the brain through which MDK acts to regulate ethanol consumption. Since MDK also controls the expression of inflammatory cytokines in other organs, we examined gene expression of interleukin-1 beta (*Il1b*), tumor necrosis factor alpha (*Tnf)* and the chemokine (C-C motif) ligand 2 (*Ccl2*) in the VTA of *Mdk* −/− mice and in mice expressing *Mdk* shRNA in the VTA. Expression of *Ccl2* was elevated in the VTA of *Mdk* −/− mice and in mice expressing *Mdk* shRNA in the VTA. These results demonstrate that MDK functions in the VTA to limit ethanol consumption and levels of CCL2, a chemokine known to increase ethanol consumption.

Keywords

Alcohol; addiction; dopamine; chemokine; cytokine; innate immune; *Mdk*; *Ccl2*; *Il1b*; ventral tegmental area

Introduction

Alcohol addiction is a complex psychiatric disorder with genetic and environmental influences. Whole-genome approaches in humans and rodents have aided in the identification of novel genes and pathways that may predispose individuals to alcohol use disorder, with the dual goals of understanding the biological underpinnings of this behavioral disorder and finding potential new therapeutic targets. As an example, transcriptional profiling of mouse brain has yielded thousands of genes whose expression is

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significantly different between strains that consume high *vs.* low amounts of ethanol (Mulligan *et al.*, 2006). The challenge remains to validate individual genes as potential predisposing factors for alcohol addiction. One of these genes, *Mdk*, is expressed at higher levels in the brains of high alcohol-preferring (HAP) mice compared with low-alcohol-preferring (LAP) mice, suggesting that *Mdk* may be involved in the genetic predisposition to alcohol addiction (Mulligan *et al.*, 2006).

Mdk encodes a secreted growth factor and cytokine known as midkine (MDK). In addition to being more abundant in the brains of HAP mice, *Mdk* expression is induced in the brain and in cultured neuroblastoma cells by alcohol exposure (He *et al.*, 2015, Mulligan *et al.*, 2011). MDK protein levels are also higher in the prefrontal cortex of alcoholics, suggesting that expression of this gene is not only responsive to ethanol, but may also contribute to excessive alcohol drinking in humans (Flatscher-Bader *et al.*, 2005, Flatscher-Bader & Wilce, 2006, Flatscher-Bader & Wilce, 2008). Recently, Vicente-Rodriguez *et al.* demonstrated that *Mdk* knockout (−/−) mice, in contrast to wild type mice, develop ethanol conditioned place preference at a low dose of ethanol (1 g/kg), indicating that MDK may act to limit the rewarding properties of ethanol (Vicente-Rodriguez *et al.*, 2014). *Mdk* −/− mice also demonstrate a delayed recovery of ethanol-induced ataxia on the rotarod (Vicente-Rodriguez *et al.*, 2014).

Although *Mdk* modulates these behavioral responses to ethanol, the effect of *Mdk* deletion on ethanol consumption has not been determined. Based on the behavioral phenotypes of *Mdk* −/− mice and the fact that *Mdk* expression is increased in HAP mice, we hypothesized that *Mdk* −/− mice would have altered ethanol consumption compared with wild type mice. Here, we tested ethanol consumption in *Mdk* −/− mice using two paradigms, the two-bottle choice ethanol consumption test, which measures moderate ethanol consumption, and the drinking in the dark (DID) test, which measures binge-like ethanol consumption.

We also performed viral-mediated RNA interference (RNAi) to locally knockdown *Mdk* in a specific neuroanatomical location in the adult mouse. We injected a lentiviral vector expressing a short-hairpin RNA (shRNA) targeting *Mdk* into the ventral tegmental area (VTA) and tested mice for binge-like ethanol consumption. The VTA was chosen because dopamine neurons in this brain region regulate ethanol consumption (Gonzales *et al.*, 2004). Moreover, MDK is a survival factor for dopamine neurons in culture, and *Mdk* −/− mice exhibit decreased dopamine levels in the striatum (Kikuchi *et al.*, 1993, Ohgake *et al.*, 2009, Prediger *et al.*, 2011).

In addition to its effects on the dopamine system, MDK is also an important modulator of inflammatory conditions such as rheumatoid arthritis, diabetic nephropathy, inflammatory bowel disease, and multiple sclerosis (Weckbach *et al.*, 2011). Interestingly, the innate immune system in the brain is activated by chronic alcohol exposure, and deletion of cytokine and chemokine genes and their receptors in mice or treatment of rodents with pharmacological agents that act on neuroimmune targets alters ethanol consumption (Crews & Vetreno, 2015, Robinson *et al.*, 2014). Thus, MDK might also regulate ethanol consumption through an effect on the innate immune response. To test this, we examined transcript levels of the pro-inflammatory cytokine and chemokine genes tumor necrosis

factor (*Tnf*), interleukin 1 beta (*Il1b*), and chemokine (C-C motif) ligand 2 (*Ccl2*) in the VTA of mice with reduced levels of *Mdk*. We found that *Mdk* knockdown in the VTA increased expression of *Ccl2* and *Il1b* and that *Mdk* −/− mice had higher expression of *Ccl2*.

This study establishes MDK as a factor that limits ethanol consumption in mice and suggests that it may also restrict the induction of neuroimmune genes in response to alcohol exposure. Manipulating MDK activity or signaling pathways may be a novel strategy to reduce excessive alcohol drinking and the brain immune response to chronic ethanol exposure.

Materials and methods

Subjects

Mdk −/− mice have been described previously (He *et al.*, 2015, Nakamura *et al.*, 1998). We re-derived *Mdk* −/− mice from frozen embryos obtained from the Riken BioResource Center (Ibaraki, Japan; Riken number RBRC02422, strain name B6.129S2-Mdk$^{<tm1Tmu>}$) at the University of Illinois at Chicago Transgenic Production Service. *Mdk* −/− mice were maintained on a C57BL/6J background (previously backcrossed 2 generations). Heterozygous *Mdk* (+/−) mice were bred to obtain homozygous male and female knockout (−/−) and wild-type (+/+) littermates for behavioral testing. Mice were housed with same-sex littermates and kept in a temperature- (21°C) and humidity-controlled room on a 14 h light/dark cycle with lights on at 6 a.m. and off at 8 p.m., unless otherwise indicated. Male C57BL/6J mice (8 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were tested for behavior between the ages of 10–16 weeks. Food (Harlan Teklad Diet T.7912, Indianapolis, IN, USA) and water were available *ad libitum*. All procedures with mice were conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Illinois at Chicago Animal Care Committee. All efforts were taken to minimize pain and discomfort.

Two-bottle choice experiments

Two-bottle choice experiments for ethanol, saccharin, and quinine preference were performed according to Lasek et al (Lasek *et al.*, 2007) with slight modifications. Mice were individually housed in cages with continuous access to two tubes made from 10 mL polycarbonate pipettes connected to stainless steel sipper tubes containing ball-bearings to prevent leakage (Rhodes *et al.*, 2005). Fluid consumption was measured every day for 4 days at each ethanol concentration 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. Mice drank water from both bottles for the first 4 days and then were given a choice between ethanol and water, starting with 3% (v/v) ethanol and increasing to 6%, 10%, 14%, and 20% ethanol. Ethanol consumption was calculated as g ethanol consumed per kg body weight per day. Preference ratios were calculated as the volume of ethanol consumed over the total volume of fluid consumed (water plus ethanol). Nine male *Mdk* +/+, 7 male *Mdk* −/−, 6 female *Mdk* +/+, and 9 female *Mdk* −/− mice were tested in this experiment. A subgroup of mice was tested for saccharin (0.03 and 0.06%) and quinine (15 and 30 μM) consumption using the same procedure eight days after the ethanol testing was completed.

Drinking in the dark (DID)

Binge-like drinking was measured using the one-bottle DID procedure as previously described using 20% ethanol (Dutton *et al.*, 2016, Rhodes *et al.*, 2005). Briefly, mice were individually housed in a 12 h reversed light/dark cycle room (lights off at 10 a.m. and on at 10 p.m.) for 2 weeks before being tested. For the *Mdk* shRNA experiments (details below), mice were given access to the ethanol solution 3 h into the dark cycle for a period of 2 h on the first 3 days of testing (Monday, Tuesday, and Wednesday). On the fourth day, mice were given access to the ethanol solution for 4 h and the volume consumed was measured at 2 and 4 h. For testing the *Mdk* −/− mice, animals were given access to the ethanol solution for 4 h instead of 2 h on all four days. In addition, the DID procedure was performed for 4 cycles (i.e. for 4 weeks on Monday through Thursday). We tested 6 male *Mdk* +/+, 5 male *Mdk* −/−, 4 female *Mdk* +/+, and 4 female *Mdk* −/− mice.

Lentiviral production and delivery to the ventral tegmental area (VTA)

Replication-deficient lentiviruses expressing a short-hairpin RNA (shRNA) targeting *Mdk* (shMdk) or the non-targeting shRNA control (shScr) were produced as described previously using the lentiviral vector pLL3.7, which expresses the shRNA from the U6 promoter and enhanced green fluorescent protein (EGFP) from the CMV promoter (Lasek *et al.*, 2007). The 19-nucleotide targeting sequence for *Mdk* was 5′-GAGCCGACTGCAAATACAA-3′, corresponding to positions 602–620 on the *Mdk* transcript (Genbank accession number NM_010784.5). Stereotaxic delivery of virus (1 μl of ~3×10^7 pg Gag antigen/ml) bilaterally into the VTA was performed as described previously (Lasek *et al.*, 2007). Briefly, 8-week old male C57BL/6J mice were anesthetized with ketamine and xylazine and placed in a stereotaxic alignment instrument for viral injections (David Kopf Instruments, Tujunga, CA, USA). Coordinates for bilateral infusion of virus were anterior/posterior (in reference to bregma), −3.2 mm; medial/lateral, +/− 0.5 mm; and dorsal/ventral −4.7 mm. Mice recovered for 3 weeks prior to being tested for DID. We tested 16 mice infused with shScr and 17 mice infused with shMdk virus. After behavioral testing, mice were euthanized with pentobarbital (Somnasol) and perfused transcardially with 4% paraformaldehyde. Brains were removed and processed for immunohistochemistry using a GFP antibody to verify injection sites as described previously (Lasek *et al.*, 2007).

Validation of lentiviral vector efficacy in vitro and in vivo

To test *in vitro* knockdown efficacy, Neuro-2a cells (ATCC, Manassas, VA, USA) were cultured according to ATCC recommendations and transfected with lentiviral plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Forty-eight hours after transfection, RNA was isolated, subjected to first strand cDNA synthesis, and quantitative real-time PCR (qPCR) using the GeneJet RNA Purification Kit, Maxima First Strand cDNA Synthesis Kit for RT-qPCR, and the Maxima Probe qPCR Master Mix, respectively (Thermo Fisher Scientific). Taqman primer-probe mixes for *Mdk* and *Actb* were purchased from Thermo Fisher Scientific and reactions were performed using the PikoReal Real-Time PCR System (Thermo Fisher Scientific).

To test *in vivo* knockdown efficacy, 8-week old male C57BL/6J mice were injected in the VTA with lentivirus as described above. We used 9 mice infused with shScr virus and 11 mice infused with shMdk virus. Three weeks after injection, mice were euthanized by CO_2 followed by rapid decapitation. Brains were removed and flash frozen on dry ice. Sections (300 μm) were collected through the VTA and transferred to pre-cooled glass slides on dry ice. Micro-punch sampling of fluorescent tissue was performed on dry ice using a Dual Fluorescent Protein Flashlight (Nightsea, Bedford, MA, USA) and 1 mm-diameter biopsy punches (Integra LifeSciences, Plainsboro, NJ, USA). RNA was isolated and qPCR was performed as described above, except we used *Hprt* as a normalization control instead of *Actb*. Primer sequences are listed in Table S1.

Gene expression

To measure expression of *Ccl2*, *Tnf*, and *Il1b* in the VTA of mice expressing shMdk or shScr in the VTA, the same tissue samples used for verifying *Mdk* knockdown were used (dissection described above). To measure expression of these genes in the VTA of *Mdk* −/− mice, the VTA was dissected from frozen brain sections of male and female mice (12 *Mdk* +/+ and 15 *Mdk* −/− mice), RNA isolated, and cDNA synthesized as indicated above. qPCR was performed with Maxima SYBR green qPCR master mix (Thermo Fisher Scientific). Primers for *Ccl2*, *Tnf*, and *Il1b,* and *Th* are listed in Table S1.

Statistical analysis

Prism software (version 6, Graphpad, La Jolla, CA, USA) was used for statistical analysis. Behavioral tests were analyzed by two-way analysis of variance (ANOVA). Gene expression data was analyzed by student's t-test.

Results

Increased voluntary ethanol consumption by Mdk −/− mice compared with controls

We first tested whether mice deficient in MDK show altered voluntary ethanol consumption using the two-bottle choice test. Male and female *Mdk* +/+ and *Mdk* −/− mice were tested for 4 days at each ethanol concentration, starting with 3% and escalating to 6, 10, 14, and 20% ethanol. There was a significant sex difference in wild type mice, with female mice consuming more ethanol than males (sex: $F_{1, 65} = 81.77$, $P < 0.0001$; concentration: $F_{4, 65} = 50.15$, $P < 0.0001$; interaction: $F_{4, 65} = 6.37$, $P = 0.0002$). As a result, we conducted statistical analyses and show the data separately for each sex. We found that both male and female *Mdk* −/− mice consumed significantly more ethanol than wild type controls (Fig. 1a, b). A two-way ANOVA within each sex demonstrated significant effects of ethanol concentration and genotype (males, genotype: $F_{1, 70} = 9.58$, $P = 0.0028$; males, concentration: $F_{4, 70} = 26.47$, $P < 0.0001$; females, genotype: $F_{1, 65} = 13.14$, $P = 0.0006$; females, concentration: $F_{4, 65} = 58.57$, $P < 0.0001$). *Mdk* −/− mice weighed slightly less than *Mdk* +/+ mice (male *Mdk* +/+, 21.6 ± 0.6 g; male *Mdk* −/−, 20.2 ± 0.8 g; female *Mdk* +/+, 20.7 ± 0.9 g; female *Mdk* −/−, 19.6 ± 0.9 g), however this difference in body weight between genotypes was not significant. The body weight differences between genotypes did not account for the increased ethanol consumption by *Mdk* −/− mice, since volumes of ethanol

consumed (not corrected for body weight) were still significantly higher in *Mdk* −/− mice compared with Mdk +/+ mice (data not shown).

In addition to consuming more ethanol than wild type mice, *Mdk* −/− mice also showed increased ethanol preference in the two-bottle choice test (Fig. 1c, d). Specifically, male *Mdk* −/− had a higher ethanol preference compared with male *Mdk* +/+ mice (genotype: $F_{1, 70} = 4.013$, $P = 0.049$; concentration: $F_{4, 70} = 13.52$, $P < 0.0001$), while there was a trend towards increased ethanol preference by female *Mdk* −/− mice (genotype: $F_{1, 65} = 3.03$, $P = 0.086$; concentration: $F_{4, 65} = 4.44$, $P = 0.0031$). There were no significant genotype differences in water consumption at each ethanol concentration tested in either sex (Fig. 1e, f). Together, these data indicate that a MDK deficiency leads to increased voluntary ethanol consumption.

Normal sweet and bitter taste preference in Mdk −/− mice compared with controls

To determine if the increased ethanol consumption exhibited by *Mdk* −/− mice might be due to a difference in taste preference, we tested *Mdk* −/− and +/+ mice for consumption of sweet (saccharin) and bitter (quinine) solutions. A two-bottle choice test was conducted with 0.03 and 0.06% saccharin *vs.* water and 15 and 30 μM quinine *vs.* water. In the saccharin preference test, both male and female mice consumed significantly more of the 0.06% saccharin solution compared with 0.03% saccharin, but there were no differences between genotypes (Fig. 2a, b; males, genotype: $F_{1, 18} = 0.36$, $P = 0.56$; males, concentration: $F_{1, 18} = 17.77$, $P = 0.0005$; females, genotype: $F_{1, 16} = 0.30$, $P = 0.59$; females, concentration: $F_{1, 16} = 13.83$, $P = 0.0019$). In the quinine taste test, there were no significant differences in preference between *Mdk* −/− and +/+ mice, nor were there differences in consumption between the two quinine concentrations (Fig. 2c, d). This taste test data demonstrates that the increased ethanol consumption by *Mdk* −/− mice is not likely due to inherent changes in sweet or bitter taste preference.

Mdk −/− mice consume more ethanol in a binge-drinking test compared with controls

We next tested whether MDK regulates binge-like ethanol consumption using the DID procedure. Male and female *Mdk* +/+ and −/− mice were given access to one bottle of 20% ethanol for 4 hours, 3 hours into the dark cycle, on Monday-Thursday. Male and female *Mdk* −/− mice consumed significantly more ethanol overall compared with wild type controls during the first 4 days of ethanol exposure (Fig. 3a, b; males, genotype: $F_{1, 64} = 4.0$, $P = 0.050$; males, day: $F_{3, 64} = 1.45$, $P = 0.23$; females, genotype: $F_{1, 48} = 4.28$, $P = 0.044$; females, day: $F_{3, 48} = 3.26$, $P = 0.029$), similar to what we observed in the two-bottle choice experiment, although the magnitude of the change was higher in the two-bottle choice experiment.

We decided to continue the DID experiment for an additional 3 cycles (3 weeks) to determine if *Mdk* deletion might alter ethanol consumption after repeated intermittent exposure to ethanol. Interestingly, there was still a small but significant main effect of genotype over the 4 weeks of DID in male mice (Fig. 3c; genotype: $F_{1, 64} = 4.9$, $P = 0.031$; week: $F_{3, 64} = 1.9$, $P = 0.14$). However, the genotype effect in females was eliminated after the first week, primarily because it appeared that wild type female mice escalated their

intake by the second week of ethanol exposure (Fig. 3d; genotype: $F_{1, 48} = 2.81$, $P = 0.10$; week: $F_{3, 48} = 1.83$, $P = 0.15$). A two-way ANOVA within each genotype by sex and week indicated that wild-type mice escalated their drinking over the 4-week period (week: $F_{3, 56} = 3.65$, $P = 0.018$; sex: $F_{1, 56} = 30.43$, $P < 0.0001$), but *Mdk* −/− mice did not (week: $F_{3, 56} = 0.43$, $P = 0.74$; sex: $F_{1, 56} = 30.1$, $P < 0.0001$). This may be due to a "ceiling effect", such that *Mdk* −/− mice drink the maximum amount of ethanol possible during the 4 hour session initially and therefore can't subsequently increase drinking. Wilcox et al has shown that C57BL6/J males escalate DID over several weeks (Wilcox *et al.*, 2014). Together, these data indicate that MDK may be involved in limiting binge ethanol consumption during the initial exposure to ethanol.

Mdk acts in the VTA to limit binge-like ethanol consumption

MDK promotes the survival of dopamine neurons and *Mdk* −/− mice have decreased levels of striatal dopamine and dopamine receptors (Kikuchi *et al.*, 1993, Ohgake *et al.*, 2009). Since dopamine neurons in the VTA regulate ethanol consumption (Gonzales *et al.*, 2004), we hypothesized that MDK might act in this brain region to regulate ethanol drinking behavior. To test this hypothesis, we developed a lentiviral vector that expresses an shRNA to attenuate *Mdk* expression (Fig. 4a, shMdk). Validation of lentiviral plasmids in cell culture was performed by transfecting plasmids expressing shMdk or the control shRNA (shScr) into Neuro-2a cells. Cells expressing shMdk exhibited a 67% decrease in *Mdk* mRNA levels as measured by qPCR compared with cells expressing shScr (Fig. 4b, $t_4 = 11.27$, $P = 0.0004$). To validate the shMdk lentiviral vector *in vivo*, we injected lentiviruses into the VTA and dissected infected VTA (as visualized by GFP fluorescence) three weeks after infection. *Mdk* expression by qPCR in mice expressing shMdk in the VTA was reduced by 48% compared with *Mdk* expression in mice expressing shScr (Fig. 4c, $t_{18} = 5.43$, $P < 0.0001$).

Having demonstrated the efficacy of the lentiviral vector, we next performed the 4-day DID test with male mice expressing shMdk or shScr in the VTA. Mice expressing shMdk in the VTA consumed more ethanol than controls during the 2-hour drinking sessions on days 1–4 (Fig. 4e; shRNA: $F_{1, 124} = 4.35$, $P = 0.039$; day: $F_{3, 124} = 3.43$, $P = 0.019$). In addition, there was a 16% increase in ethanol consumption by mice expressing shMdk compared to shScr during 4-hours of drinking on day 4 (Fig. 4f; $t_{31} = 3.3$, $P = 0.0025$). Consistent with the increased ethanol consumption by mice expressing shMdk in the VTA after the 4-hour drinking session, blood ethanol concentrations trended to be higher in mice expressing shMdk (156 ± 14.1 mg/dl) compared with mice expressing shScr (122.4 ± 17.9 mg/dl), although this effect was not statistically significant ($t_{31} = 1.48$, $P = 0.149$). We also performed an experiment in which we injected lentiviruses expressing shScr or shMdk into the prefrontal cortex of male mice and tested them in the DID procedure. There was no difference in ethanol consumption between mice expressing shScr and shMdk in the prefrontal cortex, demonstrating anatomical specificity in the effect of the shMdk-expressing lentivirus (Fig. S1). Together, these data indicate that MDK acts in the VTA to limit binge-like ethanol consumption.

Increased expression of innate immune response genes in the VTA of mice with reduced *Mdk*

Levels of the dopamine synthesizing enzyme tyrosine hydroxylase (TH) are decreased in the striatum of *Mdk* −/− mice as measured by immunohistochemistry, and expression of the *Th* gene is higher in aorta from *Mdk* −/− mice compared with wild type mice (Ezquerra *et al.*, 2006, Prediger *et al.*, 2011). This suggests that a deficiency in *Mdk* leads to dysregulation of the dopamine system. To test if *Th* expression is altered in mice with knockdown of *Mdk*, we examined *Th* expression by qPCR in mice expressing shScr or shMdk in the VTA. The same samples used in Fig. 4c were tested. Surprisingly, we found no difference in *Th* expression between mice expressing shScr or shMdk (Fig. 5a).

We next tested the expression of select cytokine and chemokine genes in the VTA of mice expressing shScr and shMdk. MDK is involved in a number of inflammatory conditions and may regulate expression of cytokines and chemokines (Weckbach *et al.*, 2011). Importantly, innate immune response genes are induced in the brain after chronic ethanol exposure and are relevant for ethanol consumption (Blednov *et al.*, 2005, Blednov *et al.*, 2012, Crews & Vetreno, 2015). We examined expression of genes encoding the pro-inflammatory cytokines interleukin 1 beta (*Il1b*) and tumor necrosis factor (*Tnf*), and the chemokine (C-C motif) ligand 2 (*Ccl2*) in the VTA of mice expressing shScr and shMdk. *Ccl2* expression was significantly elevated by 98% in the VTA of mice with knockdown of *Mdk* (Fig. 5b; t_{17} = 2.51, P = 0.023). In addition, *Il1b* expression was significantly increased by 85% in the VTA of mice with knockdown of *Mdk* (Fig. 5c, t_{18} = 2.2, P = 0.041). There was no difference in the expression of *Tnf* between mice expressing shScr and shMdk in the VTA (Fig. 5d).

To confirm these results using an alternative approach, we examined expression of *Th*, *Ccl2*, *Il1b*, and *Tnf* in the VTA of *Mdk* −/− and +/+ mice. Expression of *Ccl2* was increased by 58% in the VTA of *Mdk* −/− compared with *Mdk* +/+ mice (Fig. 5f, t_{25} = 2.31, P = 0.030), similar to what we observed with shRNA-mediated knockdown of *Mdk* in the VTA. Expression levels of *Th*, *Il1b*, and *Tnf* were similar between *Mdk* −/− and +/+ mice (Fig. 5). Collectively, these data indicate that MDK expression in the VTA may limit transcription of *Ccl2*. The increase in *Ccl2* expression in the VTA is potentially one factor responsible for the increased ethanol consumption by *Mdk* −/− mice and mice with *Mdk* knockdown in the VTA (Blednov *et al.*, 2005).

Discussion

Here we provide the first direct demonstration that MDK regulates ethanol consumption. We show that mice with a genetic deletion of *Mdk* consume more ethanol in a test of moderate consumption (two-bottle choice) and in a binge-drinking test (DID) compared with control mice. The results that we obtained with *Mdk* −/− mice are supported by our data showing that mice with lentiviral-mediated RNA interference of *Mdk* in the VTA also consume more ethanol than controls. Together these data strongly suggest that MDK functions in the brain to limit ethanol intake. The effects of *Mdk* deletion on ethanol consumption are not due to alterations in taste sensitivity, as evidenced by normal quinine and saccharin preference in *Mdk* −/− mice compared with *Mdk* +/+ mice. Prior to this study, *Mdk* −/− mice were tested for the rewarding properties of ethanol and found to be more sensitive to ethanol reward in

the conditioned place preference test (Vicente-Rodriguez *et al.*, 2014). It is possible that *Mdk* gene knockout mice consume more ethanol because they find ethanol more rewarding than mice with normal levels of *Mdk*.

One site in which MDK may act to limit ethanol consumption is the VTA. Our data demonstrate that reducing the expression of *Mdk* in the VTA causes increased binge-like ethanol consumption. We also tested mice expressing shMdk in the prefrontal cortex and did not see any difference in ethanol consumption compared with controls, indicating some anatomical specificity in the site of MDK action with respect to alcohol drinking. Interestingly, post-mortem analysis of the frontal cortex of human alcoholics demonstrated that MDK mRNA and protein are overexpressed in alcoholics vs. non-alcoholic controls (Flatscher-Bader & Wilce, 2006, Flatscher-Bader & Wilce, 2008). The VTA was not examined in those studies, so it is possible that MDK expression is also higher in the VTA of alcoholics. Although MDK is highly expressed in the frontal cortex of alcoholics, its expression in this brain region may not be important for regulating alcohol intake, or ethanol has different effects on MDK expression in the VTA compared with the frontal cortex. Alternatively, it is possible that MDK is produced by VTA neurons and secreted into the prefrontal cortex from VTA afferents, where it then acts to regulate ethanol consumption. VTA neurons also project to the nucleus accumbens, a key area of the brain involved in ethanol intake and reward (Gonzales *et al.*, 2004). MDK may also be released into the nucleus accumbens to limit ethanol drinking and reward. Finally, MDK could act in an autocrine fashion through interactions with its receptors, such as ALK, RPTPβ/ζ, or others expressed on VTA neurons, resulting in decreased ethanol consumption. Our shRNA study does not discriminate between the above possibilities, but does indicate that MDK produced by VTA neurons limits ethanol consumption.

We previously found that treatment of neuroblastoma cells in culture with ethanol induces the expression of *Mdk* (He *et al.*, 2015). Since *Mdk* is an ethanol-responsive gene and *Mdk* deletion causes increased intake of ethanol, we hypothesize that the increased expression of MDK in the brain of alcoholics might be an adaptive response to chronic ethanol exposure, meant to maintain homeostasis and curb excessive ethanol consumption. However, it is interesting that mice predisposed to drink high amounts of alcohol (HAP) have higher brain expression of *Mdk* compared with low-alcohol preferring mice (LAP). This would suggest that increased expression of *Mdk* is a predisposing factor for excessive drinking. We measured *Mdk* mRNA expression in the VTA in wild type mice immediately after the fourth drinking session in the DID test and did not observe any difference in *Mdk* expression compared with water-drinking control mice (Chen and Lasek, unpublished results). This suggests that, at least under these ethanol exposure conditions, *Mdk* expression in the VTA does not change. It will be important to determine the expression levels of *Mdk* in the VTA and other brain regions after chronic ethanol exposure and during withdrawal, since the increase in MDK in the frontal cortex of alcoholics may be due to an effect of chronic drinking or withdrawal from alcohol. Additionally future studies will test if increasing the expression of MDK leads to higher vs. lower levels of ethanol intake by directly infusing recombinant MDK protein into the VTA or other brain regions (such as the prefrontal cortex or nucleus accumbens) of ethanol-naïve and ethanol-experienced mice and testing ethanol consumption.

Dopamine neurons in the VTA are important for the rewarding and reinforcing effect of ethanol (Gonzales *et al.*, 2004). MDK is a survival factor for dopamine neurons *in vitro* (Kikuchi *et al.*, 1993) and *Mdk* −/− mice have decreased levels of dopamine and its metabolites in the striatum (Ohgake *et al.*, 2009, Prediger *et al.*, 2011). One study has also shown reduced numbers of TH-positive neurons in the substantia nigra pars compacta of *Mdk* −/− compared with *Mdk* +/+ mice, indicating that the loss of MDK impairs the survival or development of dopamine neurons (Prediger *et al.*, 2011). However, Gramage et al did not observe any difference between *Mdk* −/− and *Mdk* +/+ mice in the innervation of the striatum by dopamine neurons using immunohistochemical staining for TH (Gramage *et al.*, 2011). Here, we did not find any differences in *Th* mRNA expression in the VTA of *Mdk* −/− mice or in mice expressing shMdk in the VTA compared with controls. We also counted the number of TH-positive cells in the VTA in *Mdk* null mice and in mice expressing shMdk and did not find any changes in cell numbers (Chen and Lasek, unpublished data). It is possible, though, that *Mdk* −/− mice do have decreased levels of dopamine in the nucleus accumbens, which could be responsible for the increased ethanol consumption by *Mdk* −/− mice.

In this study we found that expression of *Il1b* and *Ccl2* are increased in the VTA of mice expressing shMdk, and that *Ccl2* expression is increased in the VTA of *Mdk* −/− mice. One caveat to the lentiviral injections is that the lentivirus could potentially activate glia at the site of injection, causing increased cytokine expression (Kunitsyna *et al.*, 2016). Since we found that *Ccl2* expression increased in both the *Mdk* knockout mice and in mice injected with *Mdk* lentivirus, it is likely that this increase is due to a specific effect of decreased *Mdk* levels. Although it is difficult to compare between different qPCR experiments, we found that the qPCR Cq values for *Ccl2* did not differ between wild type, non-injected mice and mice injected with the control shScr lentivirus (data not shown). However, we did find that the Cq values for *Il1b* were significantly lower in the VTA of mice injected with the control lentivirus compared with wild type mice, suggesting that *Il1b* expression may be induced upon lentiviral infection and that the increase of *Il1b* in the VTA of mice expressing shMdk may be due to a non-specific effect of the shRNA or lentivirus.

Neuroimmune signaling has emerged as a potentially important contributor to excessive ethanol consumption (Robinson *et al.*, 2014). Chronic ethanol exposure not only induces genes involved in neuroimmune signaling pathways in the brain, but some of these genes have been validated as important for alcohol consumption using gene knockout mice and pharmacological tools (Blednov *et al.*, 2015, Blednov *et al.*, 2005, Blednov *et al.*, 2012). MDK expression is correlated with the expression of cytokines and chemokines in several different tissues types and plays a role in inflammatory diseases (Weckbach *et al.*, 2011). Increased and decreased CCL2 (also known as MCP-1) protein expression is observed in the kidneys of *Mdk* −/− mice in models of glomerulonephritis and diabetic nephropathy, respectively (Kojima *et al.*, 2013). Here, we found that *Ccl2* gene expression is elevated in *Mdk* −/− mice and in mice expressing shMdk in the VTA. CCL2 is a potent chemoattractant that recruits monocytes and microglia to sites of tissue injury (Yao & Tsirka, 2014) and is induced by chronic ethanol exposure and withdrawal (Harper *et al.*, 2015, Kane *et al.*, 2013, Kane *et al.*, 2014, Knapp *et al.*, 2016, Lippai *et al.*, 2013). CCL2 is also increased in the serum and brain of human alcoholics (He & Crews, 2008, Manzardo *et al.*, 2016).

Female *Ccl2* knockout mice consume less ethanol than control females (Blednov *et al.*, 2005), so an increase in CCL2 expression in *Mdk* −/− mice could potentially be responsible for increased ethanol consumption by these mice. In addition, ethanol-preferring P rats have elevated CCL2 levels in the central nucleus of the amygdala and the VTA compared with non-preferring (NP) rats (June *et al.*, 2015). Neuronal knockdown of *Ccl2* in the VTA of P rats decreases binge drinking (June *et al.*, 2015), and intracerebroventricular infusion of CCL2 protein increases operant ethanol self-administration in rats (Valenta & Gonzales, 2016). Future experiments are necessary to determine whether increased CCL2 in the VTA of *Mdk* −/− mice is responsible for increased ethanol consumption by knocking down *Ccl2* in this brain region and testing drinking behavior.

Interestingly, there is a relationship between CCL2 and dopamine neurotransmission. CCL2 is expressed in dopamine neurons in the adult rat substantia nigra and VTA (Banisadr *et al.*, 2005a, Banisadr *et al.*, 2005b) and promotes dopamine neuron survival and neurite outgrowth in midbrain neuron cultures (Edman *et al.*, 2008). Injection of CCL2 unilaterally into the rat substantia nigra decreases striatal dopamine content and its metabolites 1 hour after injection as measured by high-pressure liquid chromatography, and also rapidly increases dopamine release in the striatum as measured by microdialysis (Guyon *et al.*, 2009). In addition, CCL2 treatment of brain slices containing the substantia nigra increases dopamine neuron firing rates (Guyon *et al.*, 2009). Direct infusion of CCL2 into the VTA also increases the amount of phosphorylated TH (Wakida *et al.*, 2014), which is an active form of the enzyme. Together, these studies provide evidence that CCL2 regulates dopamine neurotransmission. It is possible, then, that the increase in *Ccl2* expression in the VTA of *Mdk* −/− mice impacts both dopamine levels and release in the nucleus accumbens, leading to enhanced ethanol consumption.

How might MDK signal to limit *Ccl2* expression and alcohol drinking? MDK is a ligand for several receptors that are expressed in the central nervous system, such as anaplastic lymphoma kinase (ALK) and the protein tyrosine phosphatase β/ζ (Weckbach *et al.*, 2011). We have found that shRNA-mediated knockdown of *Alk* in the mouse VTA decreases binge-like ethanol consumption (Dutton *et al.*, 2016), which suggests that MDK in the VTA may not be acting through ALK to regulate alcohol drinking, since knockdown of *Mdk* in the VTA increases drinking. It is possible that MDK is a ligand for another receptor in the VTA whose downstream signaling pathways regulate the transcription of *Ccl2* and ethanol drinking behavior. Further work is needed to determine the functional receptor for MDK in the VTA and the downstream signaling pathways activated by MDK.

In addition to MDK playing a role in ethanol reward and drinking, it may also be involved in addiction to other drugs of abuse. *Mdk* −/− mice are more sensitive to the anxiolytic effects of diazepam (Vicente-Rodriguez *et al.*, 2014) and show reduced extinction to cocaine conditioned place preference (Gramage *et al.*, 2013). *Mdk* expression is also induced in the VTA, the nucleus accumbens, and the hippocampus in response to morphine administration (Ezquerra *et al.*, 2007, Garcia-Perez *et al.*, 2015, Garcia-Perez *et al.*, 2014). MDK seems to play an important role in immune responses in the brain in response to amphetamine because *Mdk* −/− mice show alterations in amphetamine-induced striatal astrocytosis and microgliosis (Vicente-Rodriguez *et al.*, 2016). Thus, targeting MDK and its signaling

pathways may be a novel strategy for reducing neuroinflammation and addiction to multiple substances, including alcohol (Vicente-Rodriguez *et al.*, 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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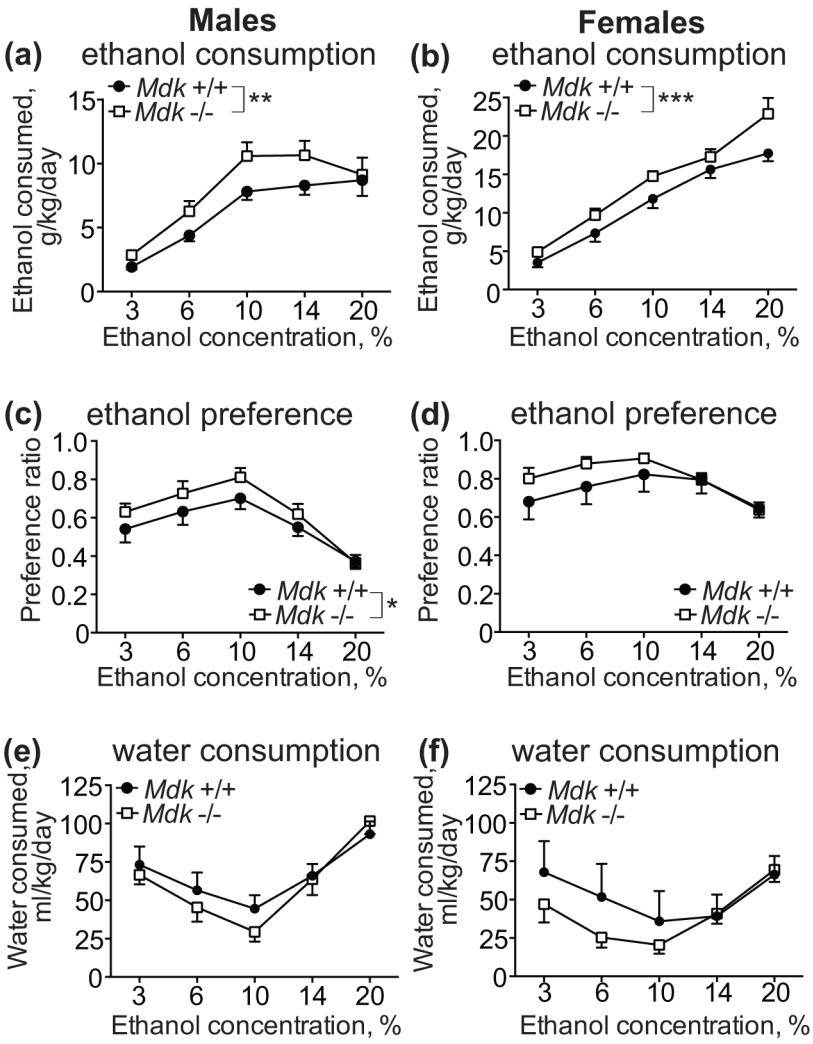

Figure 1. Midkine knockout mice consume more ethanol than controls in a 2-bottle choice ethanol consumption test

Male (a, c, e) and female (b, d, f) wild type (*Mdk* +/+, filled circles) and homozygous midkine knockout (*Mdk* −/−, open squares) mice underwent a 2-bottle choice test for water or 3, 6, 10, 14, and 20% (v/v) ethanol with four days of 24 h access at each ethanol concentration. (a, b) ethanol consumed in g ethanol/kg body weight/day. There was a significant effect of genotype and ethanol concentration in both sexes. (c, d) Ethanol preference, calculated as the ratio of the volume of ethanol consumed over the volume of total fluid consumed. There was a significant effect of genotype and ethanol concentration in males, and a significant effect of ethanol concentration in females. (e, f) Water consumed during the ethanol consumption test at each concentration of ethanol. Data is presented as ml water consumed/kg body weight/day. No significant genotype effect was observed for water consumption in either sex. Male *Mdk* +/+, n = 9; male *Mdk* −/−, n = 7; female *Mdk* +/+, n = 6; female *Mdk* −/−, n = 9. * P < 0.05, ** $P < 0.01$, *** $P < 0.001$ by two-way ANOVA. Data are presented as the mean ± SEM.

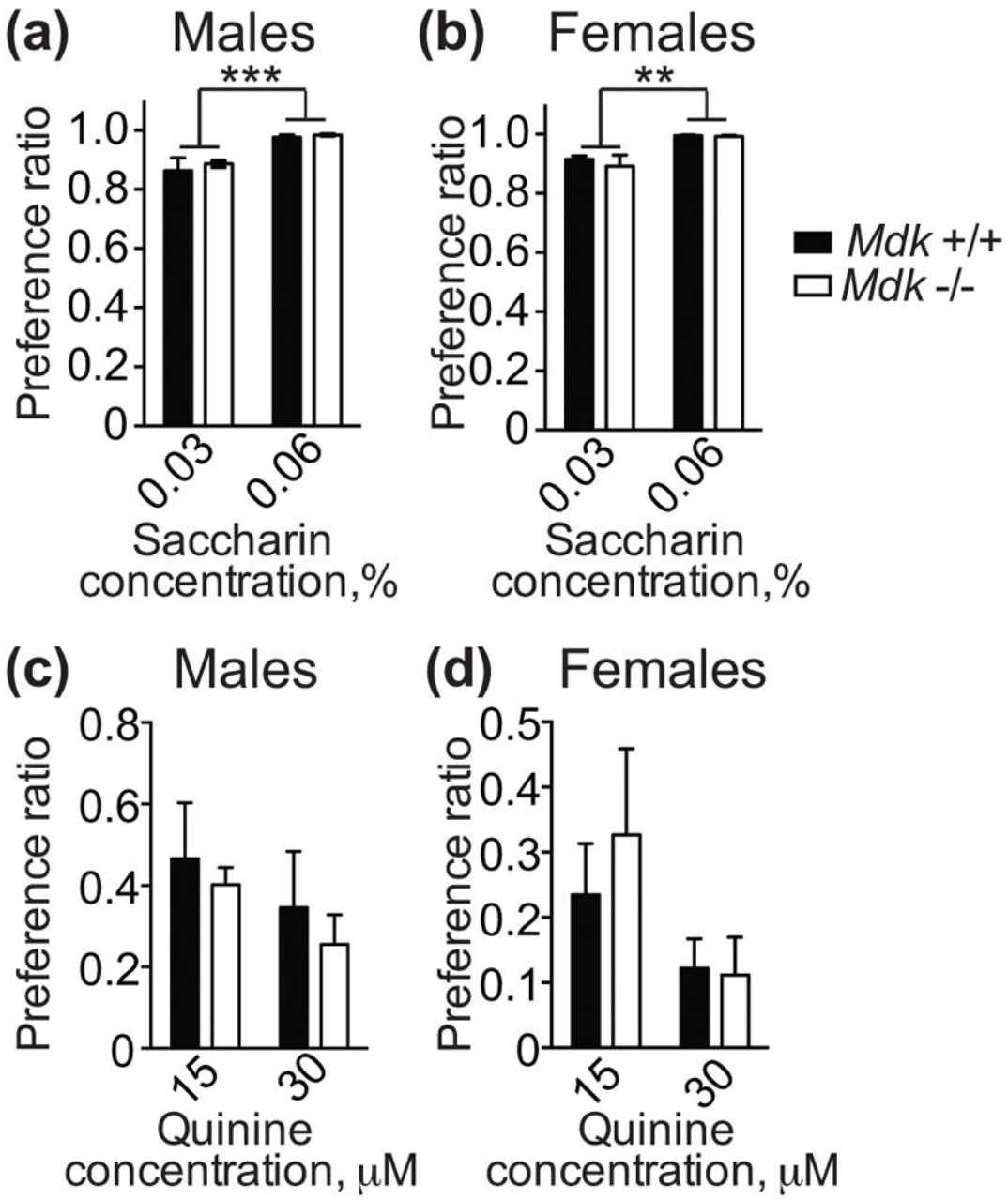

Figure 2. Midkine knockout mice do not differ in saccharin or quinine intake compared with controls

Male (a, c) and female (b, d) wild type (*Mdk* +/+, black bars) and homozygous midkine knockout (*Mdk* −/−, white bars) mice underwent a 2-bottle choice test for water and saccharin (0.03 or 0.06%, w/v) or water and quinine (15 or 30 μM). (a, b) Saccharin consumption plotted as the ratio of the volume of saccharin solution consumed over total fluid consumed. There were no significant effects of genotype, but there were significant main effects of saccharin concentration for both sexes. Male *Mdk* +/+, n = 9; male *Mdk* −/−, n = 7; female *Mdk* +/+, n = 6; female *Mdk* −/−, n = 9. ** $P < 0.01$, *** $P < 0.001$ by two-way ANOVA. (c, d) Quinine consumption plotted as the ratio of the volume of quinine

solution consumed over total fluid consumed. There were no significant differences between genotypes or quinine concentration. Male *Mdk* +/+, n = 6; male *Mdk* −/−, n = 5; female *Mdk* +/+, n = 4; female *Mdk* −/−, n = 6. Data are presented as the mean ± SEM.

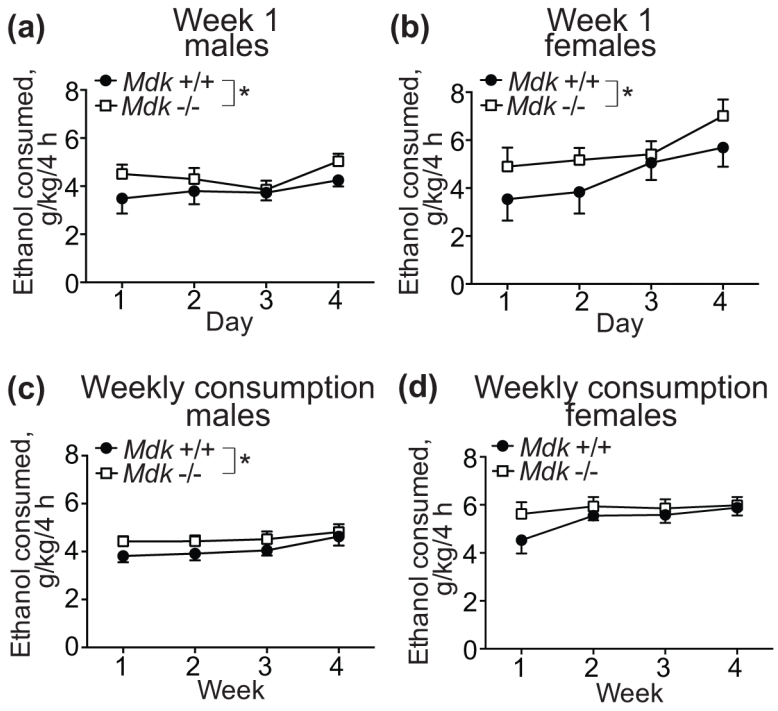

Figure 3. Midkine knockout mice consume more ethanol than controls in the drinking in the dark (DID) test

Male (a, c) and female (b, d) wild type (*Mdk* +/+, filled circles) and homozygous midkine knockout (*Mdk* −/−, open squares) mice underwent a modified DID test, in which they were provided 20% ethanol in water (v/v) for 4 h per day, 4 days per week for 4 weeks. (a, b) Ethanol consumed in g ethanol/kg body weight/4 h during the first week of the DID test. There were significant effects of genotype for males and females and a significant effect of day for females. (c, d) Average ethanol consumed each week, expressed as g ethanol/kg body weight/4 h. There was a significant effect of genotype in males. Male *Mdk* +/+, n = 9; male *Mdk* −/−, n = 9; female *Mdk* +/+, n = 7; female *Mdk* −/−, n = 7. * $P < 0.05$ by two-way ANOVA. Data are presented as the mean ± SEM.

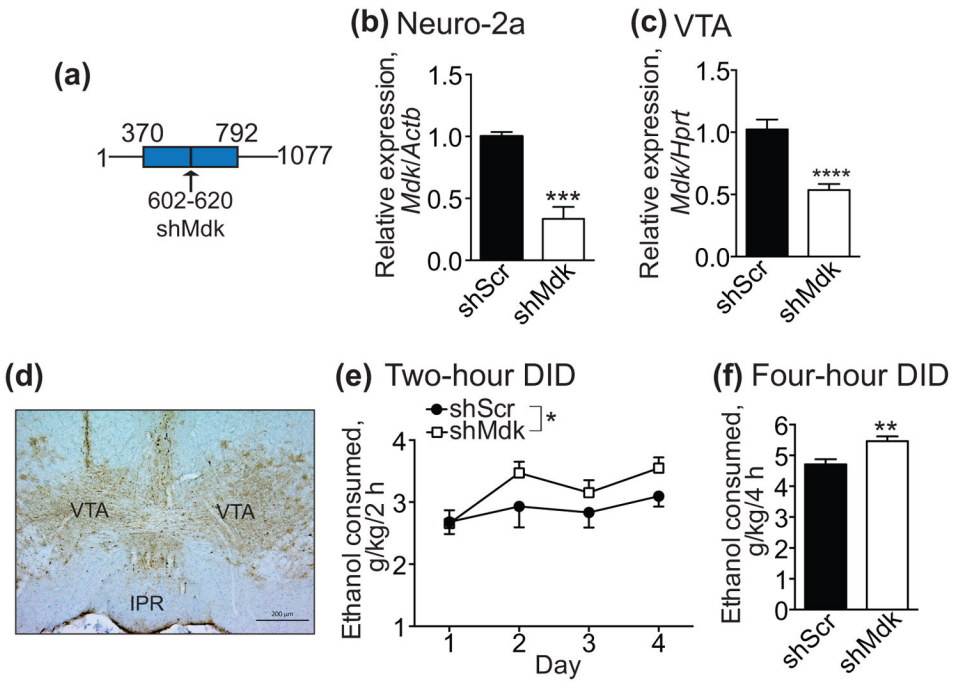

Figure 4. *Mdk* knockdown by RNAi in the ventral tegmental area (VTA) of male mice increases binge-like ethanol consumption

C57BL/6J mice were stereotaxically injected in the VTA with a lentivirus expressing a short hairpin RNA (shRNA) targeting *Mdk* (shMdk) or a control shRNA (shScr) and green fluorescent protein (GFP). Mice were allowed to recover for three weeks and tested for ethanol consumption in the drinking in the dark protocol. (a) Schematic diagram showing the location of the 19 nucleotide targeting sequence on the *Mdk* transcript (transcript variant 1). The blue box indicates the open reading frame. The arrow points to a vertical black line indicating the location of the targeting sequence. Numbers indicate the nucleotide position on the transcript. (b) Demonstration of knockdown efficacy of shMdk in Neuro-2a cells transfected with the lentiviral plasmid encoding shMdk compared with cells transfected with shScr. RNA was isolated from cells 48 h after transfection and analyzed by quantitative real-time PCR (qPCR, n = 3) (c) Demonstration of knockdown efficacy of shMdk in mouse VTA three weeks after lentiviral infection. Infected VTA was dissected from mice 3 weeks after injection, RNA isolated and analyzed by qPCR. shScr, n = 9; shMdk, n = 11. (d) Representative image of lentiviral infection in the VTA. Immunohistochemistry with a GFP antibody was performed on brain sections containing the VTA. IPR, interpeduncular nucleus, rostral. Scale bar, 200 μM. (e) Ethanol consumption in the DID test by mice infected in the VTA with lentivirus expressing shMdk (open squares) or shScr (filled circles). Shown is the amount of ethanol consumed in g ethanol/kg body weight during the 2 h drinking sessions each day. There were significant main effects of shRNA and day. (f) Ethanol consumption in mice infected in the VTA with lentivirus expressing shMdk (white bar) or shScr (black bar) during the 4 h drinking session on day 4 in the DID test, expressed as g ethanol/kg body weight. shScr, n = 16; shMdk, n = 17. $*P < 0.05$ by two-way ANOVA. $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ by student's t-test. Data are presented as the mean ± SEM.

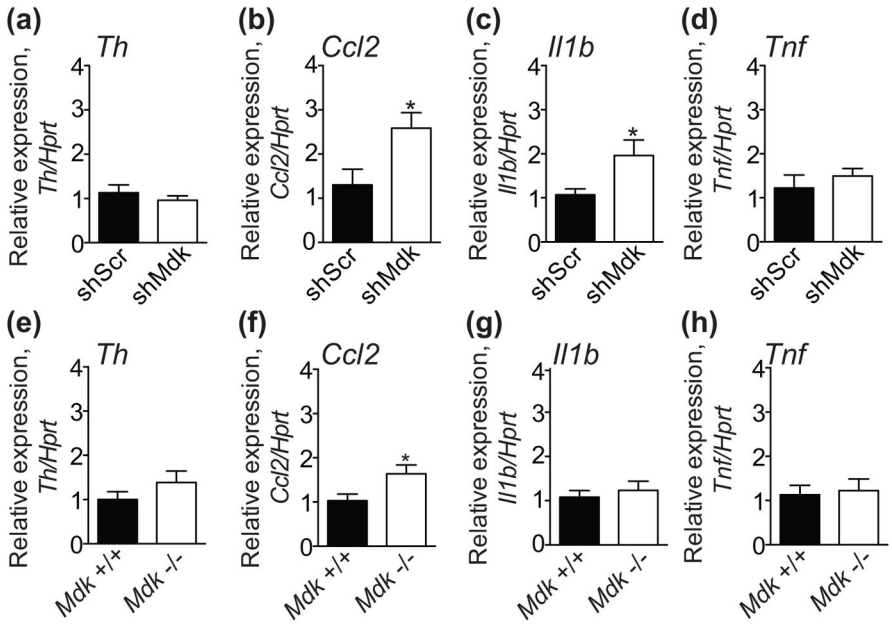

Figure 5. Increased expression of *Ccl2* in the ventral tegmental area (VTA) of mice deficient in midkine

(a–d) VTA RNA samples from mice expressing shScr (black bars, n = 9) or shMdk (white bars, n = 11) in the VTA were analyzed by qPCR for expression of (a) *Th*, (b) *Ccl2*, (c) *Il1b*, and (d) *Tnf*. The same samples from Fig. 4c that were used to test knockdown of *Mdk* in the VTA were used in this experiment. There were significant increases in *Ccl2* and *Il1b* expression in VTA samples from mice expressing shMdk compared with shScr. (e–h) VTA RNA samples from wild type (+/+, black bars, n = 12) and Mdk knockout (−/−, white bars, n = 15) mice were analyzed by qPCR for expression of (e) *Th*, (f) *Ccl2*, (g) *Il1b*, and (h) *Tnf*. There was a significant increase in *Ccl2* expression in the VTA of *Mdk* −/− compared with *Mdk* +/+ mice. $*P < 0.05$ by student's t-test. Data are presented as the mean ± SEM.