

Binge-Like Ethanol Drinking Increases *Otx2*, *Wnt1*, and *Mdk* Gene Expression in the Ventral Tegmental Area of Adult Mice

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ABSTRACT: Alcohol use disorder is associated with pathophysiological changes in the dopaminergic system. Orthodenticle homeobox 2 (OTX2) is a transcription factor important for the development of dopaminergic neurons residing in the ventral tegmental area (VTA), a critical region of the brain involved in drug reinforcement. Previous studies have demonstrated that ethanol exposure during embryonic development reduces *Otx2* mRNA levels in the central nervous system. We hypothesized that levels of OTX2 would be altered by binge-like ethanol consumption in adult animals. To test this, *Otx2* mRNA and protein levels in the mouse VTA were measured by quantitative real-time PCR and western blotting, respectively, after mice drank ethanol for 4 days in a procedure that elicits binge levels of ethanol consumption (drinking in the dark). Expression of known and putative OTX2 transcriptional target genes (*Sema3c*, *Wnt1*, and *Mdk*) were also measured in the VTA after ethanol drinking. *Otx2* mRNA and protein levels were elevated in the VTA 24 hours after the fourth drinking session and there was a corresponding increase in the expression of *Mdk* transcript. Interestingly, *Wnt1* transcript was elevated in the VTA immediately after the fourth drinking session but returned to control levels 24 hours later. We next investigated if viral-mediated reduction of *Otx2* in the mouse VTA would alter ethanol or sucrose intake. Lentiviral vectors expressing a shRNA targeting *Otx2* or a control shRNA were injected into the VTA and mice were tested in the drinking in the dark protocol for ethanol and sucrose drinking. Reducing levels of OTX2 in the VTA did not alter ethanol or sucrose consumption. One limitation is that the extent of OTX2 reduction may not have been sufficient. Although OTX2 in the VTA may not play a role in binge-like drinking in adult mice, OTX2 could contribute to ethanol-induced transcriptional changes in this region.

KEYWORDS: Alcohol use disorder, dopamine, ethanol, mouse, *Otx2*, *Wnt1*, *Mdk*

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Significance Statement

- Binge-like ethanol drinking by mice increased *Otx2* mRNA and protein in the ventral tegmental area.
- *Wnt1* in the ventral tegmental area was elevated immediately after the fourth binge drinking session.
- *Mdk* in the ventral tegmental area increased 24 hours after the fourth drinking session.

Introduction

According to the Centers for Disease Control and Prevention (CDC), alcohol misuse is the third major preventable killer worldwide. Alcohol-related damages are very costly to the United States, with 75% of the costs related to alcohol misuse attributed to binge drinking.^{1,2} It is imperative to understand the biological mechanisms that contribute to binge drinking because this could lead to the identification of new therapeutic targets for reducing harmful drinking and the associated health and economic costs. One neuroanatomical substrate regulating binge drinking is the ventral tegmental area (VTA). Dopaminergic neurons in the VTA play a key role in drug reward and reinforcement and are involved binge drinking and the development of alcohol use disorder (AUD).³ Ethanol acutely activates dopaminergic neurons within the VTA,⁴⁻⁶

and chronic ethanol exposure induces maladaptive changes in the dopaminergic system.⁷⁻⁹

Orthodenticle homeobox 2 (OTX2) is a transcription factor that is involved in the differentiation of VTA dopamine neurons during brain development,¹⁰⁻¹⁴ and ethanol exposure during embryonic development alters *Otx2* gene expression in the brain.^{15,16} For example, male offspring of pregnant dams that drank ethanol had a significant reduction in *Otx2* mRNA in the whole brain compared to male offspring of pregnant dams that drank water.¹⁵ *Otx2* gene expression in the mouse brainstem also correlates with the amount of ethanol-induced cell death during embryogenesis.¹⁶ These results raise the possibility that ethanol could alter dopamine neuron development and/or function by affecting *Otx2* gene expression.

Reduced OTX2 protein in the VTA of juvenile mice is associated with vulnerability to stress-induced depression in adulthood,¹⁷ and there is significant comorbidity between AUD and depression.¹⁸⁻²⁰ However, OTX2 is not just expressed during embryonic development; its expression is maintained in dopamine neurons in adult mice.^{21,22} To our knowledge, the effect of alcohol drinking by adult animals on *Otx2* gene and protein levels in the VTA has not been investigated. Therefore, in this study we examined mRNA and protein levels of OTX2 in the



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adult mouse VTA after binge-like levels of alcohol drinking using the well-established drinking in the dark (DID) test.²³ Known targets of OTX2 transcriptional activity within the VTA include *Wnt1* and *Sema3c*.¹⁷ Thus, in addition to measuring the expression of *Otx2*, we also determined if the expression of known and putative OTX2 transcriptional targets in the VTA are altered by binge levels of ethanol drinking.

The results presented here demonstrate that binge-like ethanol drinking by mice leads to an increase in *Otx2* mRNA and protein in the VTA. We also found ethanol-induced increases in *Wnt1* and a putative OTX2 transcriptional target gene, *Mdk*, which we discovered to have OTX2 binding motifs in its promoter. Notably, we previously found that reducing levels of *Mdk* in the VTA resulted in increased ethanol intake by mice.²⁴ We hypothesized that reducing *Otx2* expression would have similar effect as reducing *Mdk* in the VTA. Thus, to determine if OTX2 plays a behaviorally relevant role in ethanol drinking, we reduced levels of *Otx2* in the mouse VTA using a lentiviral vector expressing a short hairpin (sh)RNA to target *Otx2* for RNA interference and tested ethanol drinking in the DID test. Our results provide important new mechanistic insight into the effects of binge levels of ethanol drinking on gene expression changes in the dopaminergic system.

Materials and Methods

Animals

Male and female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 8 weeks of age. Mice were singly housed in a 12 hours reverse dark cycle room (lights off at 10 am) for 2 to 3 weeks prior to experiments and were tested for ethanol consumption beginning at 10 to 11 weeks of age. Mice had access to food (Teklad 7912 diet, Envigo, Indianapolis, IN, USA) ad libitum. Mice also had access to water ad libitum, except during the 2 or 4 hours ethanol and sucrose drinking tests. All procedures were approved by the University of Illinois at Chicago Animal Care Committee (permits 16-144 and 19-132). Animal care adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and all efforts were made to minimize animal suffering.

Drinking in the dark (DID)

The DID test is a mouse model of binge drinking that results in pharmacologically relevant blood alcohol levels (BALs) (≥ 80 mg%).²³ Each mouse was provided with a modified sipper tube in the home cage that contained either 20% ethanol (Decon Labs, King of Prussia, PA, USA) in water or water (for the control group), 3 hours into the dark cycle.²⁴ Mice were acclimated to the sipper tube containing water for one 2 hours drinking session on Friday, 3 days prior to providing them with ethanol or water in the DID procedure. Mice were weighed

4 hours before the start of the DID test. Mice were given 2 hours access to the 20% ethanol solution or water from Monday to Wednesday, 3 hours into the dark cycle, and the amount of fluid consumed was measured at the end of each drinking session. On the fourth day (Thursday), mice had access to the ethanol or water for 4 hours and the amount of fluid consumed was measured at both 2 and 4 hours. For mice that underwent repeated cycles of drinking in the dark (ie, lentiviral experiments), ethanol bottles were replaced with standard water bottles on Thursday after the drinking session and DID was resumed on the following Monday (3 days off, followed by 4 days on ethanol). These same mice were also tested for sucrose consumption 3 days after the completion of the ethanol drinking test and were provided with 2% (w/v) sucrose solution using the same procedures as the ethanol test and were euthanized 1 day (~20 hours) following the completion of the fourth sucrose drinking test.

VTA dissection

Mice were euthanized by rapid decapitation immediately (referred to as the “0 hour” group) or 24 hours after fourth drinking session for measuring gene expression and protein levels. The brain was rapidly removed from the skull and the VTA was dissected by sectioning the brain into 1 mm-thick sections using a brain matrix (Zivic Instruments, Pittsburgh, PA, USA) and punched out of the section using a glass Pasteur pipet with an inner diameter of 1 mm. This dissection method enriched for VTA but likely also includes some surrounding tissue. Tissue punches were transferred to a 1.5 ml centrifuge tube, snap frozen on dry ice, and stored at -80°C until processing for RNA or protein measurements. Trunk blood was obtained immediately after decapitation or was collected from the tail vein (only after the 12th drinking session in the lentivirus experiments) using heparinized capillary tubes right after the 4 hours drinking session in order to measure blood alcohol levels (BALs). Blood samples were stored at -80°C until processing for BALs measurement.

Nicotinamide adenine dinucleotide-alcohol dehydrogenase (NAD-ADH) enzymatic assay

BALs were determined as indicated as described in Zapata et al.²⁵ For cell lysis and deproteinization, 10 μl of whole blood samples and ethanol standards were incubated with 40 μl of 3.4% of perchloric acid and centrifuged at 4°C for 6 minutes at 2000 rpm. The supernatant (7 μl) was removed and plated in triplicate wells within a 96 well plate. Samples were incubated in 0.43 M Tris-HCl buffer (pH 8.8) containing 2.36 $\mu\text{g}/\text{ml}$ of ADH (Sigma-Aldrich, St. Louis, MO) and 0.43 mg/ml β -NAD (Sigma-Aldrich) for 40 minutes at room temperature. Accumulation of β -NADH was measured by reading sample absorbance at 340 nm.

Table 1. Primer sequences.

GENE NAME	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
<i>Hprt</i>	GTTGGGCTTACCTCACTGCT	TCATCGCTAATCACGACGCT
<i>Mdk</i>	CCCTGCAACTGGAAGAAGGAAT	TTGGAGGTGCAGGGCTTAGT
<i>Otx2</i>	TGAGGCCTGCCAAGAAGAAG	GACAAGGGGTCAGACAGTGG
<i>Rp13a</i>	TACCAGAAAGTTTGCTTACCTGGG	TGCCTGTTTCCGTAACCTCAAG
<i>Sema3c</i>	TGGCAAAGGACGATGCTCTT	GCGTCCACAAACATGGGTTC
<i>Th</i>	TCTTGAAGGAACGGACTGGC	GAGTGCATAGGTGAGGAGGC
<i>Wnt1</i>	GATGGTGGGGCATCGTGAA	GATGAACGCTGTTTCTCGGC

Quantitative real-time PCR (qPCR)

RNA was isolated using the miRNeasy Micro Kit (Qiagen, Germantown, MA, USA) as per manufacturer's instructions. Total RNA (80 ng/ μ l) underwent first strand cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for qPCR. *Hprt* and *Rp13a* were used as reference genes. Primer sequences for all genes are shown in Table 1. Relative gene expression was calculated via the $\Delta\Delta$ cycle quantification value (Cq) method. Enrichment for VTA tissue was determined by analyzing expression of *Th*. Sample exclusion criteria were *Th* Cq values that were identified as outliers by Grubbs' test, which would indicate inaccurate VTA dissection. Two out of 96 samples were excluded from analysis because they were outliers. In addition, fewer samples were used to measure *Wnt1* mRNA levels because we ran out of total RNA for some samples.

Western blot

Tissue was homogenized in 50 μ l of RIPA buffer (150 mM NaCl, 1.0% IGEPAL[®] CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) from Cell Signaling Technology (Danvers, MA). RIPA buffer was supplemented with Halt[™] phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific). Protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Equal amounts of protein (20 μ g) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated using the Novex[™] 4% to 12% Tris-Glycine Mini Gels, WedgeWell[™] format, 15-well (Thermo Fisher Scientific), and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS, 25 mM Tris-HCl and 150 mM NaCl) and incubated with primary antibodies overnight at 4°C (OTX2, 1:1000, Proteintech #13497-1-AP, RRID: AB_2157176; TH, 1:10,000, Millipore #MAB318, RRID: AB_2201528; and

β -actin, 1:10,000, Santa Cruz Biotechnology, #sc-47778, RRID: AB_626632). Membranes were then incubated with secondary antibodies conjugated with infrared (IR) dyes at room temperature for 1 hour (LI-COR Biosciences, Lincoln, NE, USA; IRDye 680RD donkey anti-mouse IgG, #925-68072, RRID: AB_2814912; IRDye 800CW donkey anti-rabbit IgG, #925-32213, RRID: AB_2715510). Bands were visualized using the Odyssey Fc system (LI-COR Biosciences) and quantified using Image Studio Lite software (LI-COR Biosciences). Protein levels were normalized to the average density of the β -actin band for each sample. Samples with very low or undetectable TH band intensities were excluded.

Lentivirus production

Replication-deficient lentiviruses expressing a short-hairpin RNA (shRNA) targeting *Otx2* (shOtx2) 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 (GFP) from the cytomegalovirus (CMV) promoter.²⁶ The 23-nucleotide targeting sequence for *Otx2* was 5'-TTGCAAATGATTGATCAAATATA-3', corresponding to positions 1549-1571 on the *Otx2* transcript (Genbank accession number NM_001286481.1).

Stereotaxic surgery

Stereotaxic delivery of the lentiviral vector (1 μ l of 4×10^7 pg/ml p24 gag antigen for shScr and 3×10^7 pg/ml for shOtx2) bilaterally into the VTA was performed as described previously.^{26,27} Briefly, 8 week old male and female C57BL/6J mice were anesthetized with intraperitoneal (IP) ketamine and xylazine (100 and 8 mg/kg, respectively) and placed in a stereotaxic alignment instrument (David Kopf Instruments, Tujunga, CA, USA). Coordinates for bilateral infusion of virus were -3.2 mm anterior/posterior (in reference to bregma), ± 0.5 mm medial/lateral, and -4.7 mm dorsal/ventral. The virus was injected at a rate of 0.2 μ l/min and cannulas remained lowered for an additional 7 minutes to prevent backflow of the virus. After the

infusion step, the scalp was closed with nylon monofilament suture (Ethicon, Somerville, NJ, USA) and tissue glue (Vetclose, Dublin, OH, USA) and the mice injected subcutaneously with 2 mg/kg meloxicam for analgesia. Mice recovered for 3 weeks prior to beginning behavioral testing.

Verifying shRNA-induced depletion of Otx2 in the VTA by qPCR

Three cohorts of mice ($n=18-19$) were used to verify knock-down of VTA *Otx2*. Three weeks after lentiviral infusion into the VTA, mice were euthanized, brains were rapidly removed from the cranium, and frozen at -80°C until sectioning to a thickness of $300\mu\text{m}$ using a cryostat (Microm HM 550, Thermo Fisher Scientific). Sections were mounted on Superfrost microscope slides (Thermo Fisher Scientific). The fluorescent signal from GFP was visualized with a flashlight paired with filter glasses (NightSea, Lexington, MA, USA). The green fluorescent area on the section was punched out with a 1 mm biopsy punch with plunger (Militex, Plainsboro, NJ, USA) and processed for RNA isolation and qPCR.

Immunohistochemistry (IHC)

Mice were euthanized with Somnasol (sodium pentobarbital 390 mg/ml and sodium phenytoin 50 mg/ml, IP) and transcardially perfused with ice-cold 0.01 M PBS and 4% paraformaldehyde. Brains were removed, snap frozen on dry ice, and stored at -80°C until processing for IHC. Snap frozen brains embedded in Optimal Cutting Temperature compound (OCT) were sliced into $40\mu\text{m}$ -thick coronal sections. Free-floating sections were blocked and permeabilized for 1 hour in blocking solution (10% donkey serum [Jackson ImmunoResearch, West Grove, PA, USA], 0.25% Triton X-100, and 0.01 M PBS). Sections were then incubated overnight in antibodies to TH (1:1000, Millipore #MAB318, RRID: AB_2201528), OTX2 (1:1000, ProteinTech #13497-1-AP, RRID: AB_2157176), or GFP (1:1000, Thermo Fisher, #A-11120, RRID: AB_221568 or #G10362, RRID: AB_2536526) diluted in blocking solution (2.5% donkey serum and 0.0625% Triton X-100). Sections were then washed three times with PBS and incubated with donkey anti-mouse IgG conjugated to AlexaFluor 488 (Jackson ImmunoResearch, #715-545-150, RRID: AB_2340846) and donkey anti-rabbit IgG conjugated to Alexa Fluor 594 (Jackson ImmunoResearch #711-585-152, RRID: AB_2340621). Sections were mounted on Superfrost Microscope Slides (Thermo Fischer Scientific) using Vectashield antifade mounting medium (Vector Laboratories #H-1200, Burlingame, CA, USA). A Zeiss LSM 710 confocal microscope was used to acquire images. Three animals were excluded from analysis because of inaccurate viral transduction.

Quantification of OTX2 and TH fluorescence in the VTA

ImageJ software (National Institutes of Health) was used to calculate corrected total cell fluorescence. OTX2 intensity was measured in GFP+ cells. Regions with no infection were used as background signal. The equation used for calculating corrected total cell fluorescence is $\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background readings})$. Quantification of OTX2 intensity in GFP+ cells was performed on a total of 47 cells from 29 sections from 9 mice in the shScr group and 50 cells from 23 sections from 10 mice in the shOtx2 group. Results are reported as the mean OTX2 intensity per mouse.

Statistical analysis

Prism version 8 (GraphPad, San Diego, CA, USA) was used for statistical analysis. Student's *t*-test was used for comparison between two groups. For comparison between two different independent variables, a two-way analysis of variance (ANOVA) or repeated measures (RM) ANOVA was used. Tukey's multiple comparisons tests were performed if a significant interaction was found by ANOVA. A *P*-value less than .05 was considered statistically significant. Data are presented as the mean \pm S.E.M.

Results

Ethanol intake and BALs

Male and female mice underwent the 4 day DID procedure with either ethanol or water (control) and were euthanized immediately after the last drinking session (0 hour group) or 24 hours later and the VTA was dissected for measurements of RNA and protein. Ethanol intakes are shown in Figure 1. Females drank significantly more ethanol than males during the 2 hours drinking sessions, as we have previously observed²⁸ (Figure 1a, 0 hour: sex, $F(1, 45) = 20.72$, $P < .0001$; time, $F(3, 135) = 3.23$, $P = .024$; interaction, $F(3, 135) = 0.71$, $P = .55$; Figure 1b, 24 hours: sex, $F(1, 46) = 7.09$, $P = .011$; time, $F(3, 138) = 9.24$, $P < .0001$; interaction, $F(3, 138) = 3.29$, $P = .023$). Females also consumed more ethanol than males during the 4 hours drinking session on the fourth day (0 hour: $t(46) = 5.41$, $P < .0001$; 24 hours: $t(46) = 5.24$, $P < .0001$). Consistent with higher levels of ethanol drinking by females, BALs were higher in females compared to males (Figure 1c, males, 65.3 ± 16.3 mg/dl; females, 135.0 ± 14.8 mg/dl; $t(45) = 3.16$, $P = .003$). BALs were also significantly correlated with ethanol consumed during the 4 hours drinking session in both sexes (Figure 1d, males, $R^2 = 0.476$, $P = .0002$; females, $R^2 = 0.214$, $P = .026$). These results demonstrate that male and female mice consumed intoxicating levels of ethanol in the DID protocol.

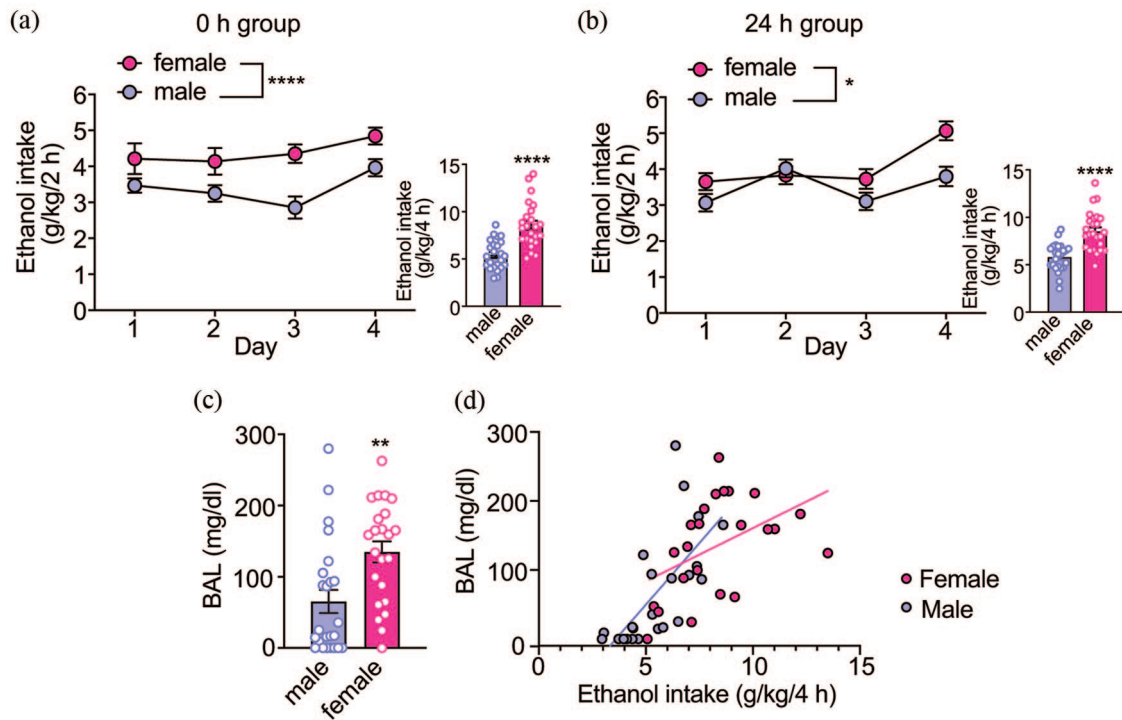


Figure 1. Ethanol intake and blood alcohol levels (BALs) in the drinking in the dark protocol: (a, b) ethanol intake in mice sacrificed at 0 hour (a) or 24 hours (b) after the fourth drinking session. Line graphs show ethanol intake each day during the 2 hours drinking session. Bar graph to the right of each line graph shows ethanol consumed during the 4 hours drinking session on the fourth day, (c) BALs in the 0 hour group, obtained immediately after the 4 hours drinking session on day 4, and (d) correlations between BALs and the amount of ethanol consumed during the fourth drinking session. Data are presented as the mean \pm SEM. Mice in the 0 hour group: male, $n=24$; female, $n=23$. Mice in the 24 hours group: male, $n=24$; female, $n=24$. * $P < .05$. ** $P < .01$. **** $P < .0001$.

OTX2 mRNA and protein levels are elevated in the VTA 24 hours after binge-like ethanol intake

Immediately after the last drinking session, *Otx2* mRNA levels in the VTA were not significantly altered by ethanol drinking when compared with water-drinking controls (Figure 2a, water, $n=9$ per sex, ethanol, $n=9$ per sex; treatment, $F(1, 32) = 3.93$, $P = .056$; sex, $F(1, 32) = 0.19$, $P = .67$; interaction, $F(1, 32) = 0.59$, $P = .45$). However, 24 hours after the fourth drinking session, *Otx2* mRNA levels in the VTA were increased in the ethanol-drinking group compared to the control group (Figure 2b, water, $n=12$ per sex, ethanol $n=12$ for males and $n=11$ for females; treatment, $F(1, 43) = 6.35$, $P = .016$; sex, $F(1, 43) = 5.64$, $P = .022$; interaction, $F(1, 43) = 3.76$, $P = .059$). In addition, *Otx2* expression in the VTA differed by sex, with *Otx2* levels higher in females compared to males. This appeared to be driven by a greater increase in *Otx2* in female mice drinking ethanol, although the sex by ethanol interaction did not quite reach statistical significance.

OTX2 protein levels in the VTA were higher immediately after the drinking session in females that drank ethanol when compared with males that drank ethanol (Figure 2c, male water, $n=12$, male ethanol, $n=10$, female water, $n=11$, female ethanol, $n=11$; treatment, $F(1, 40) = 0.99$, $P = .327$; sex, $F(1, 40) = 2.88$, $P = .097$; interaction, $F(1, 40) = 5.17$, $P = .029$; post hoc Tukey's test, female ethanol vs male ethanol, $P = .043$). At 24 hours after

the fourth drinking session, OTX2 protein levels were higher in the groups that drank ethanol compared to controls, regardless of sex (Figure 2d, male water $n=9$, male ethanol $n=9$, female water $n=8$, female ethanol $n=8$; treatment, $F(1, 30) = 6.11$, $P = .019$; sex, $F(1, 30) = 0.89$, $P = .35$; interaction, $F(1, 30) = 1.27$, $P = .27$). Taken together, these data indicate that binge levels of ethanol drinking lead to increased *Otx2* transcript and protein levels in the mouse VTA, specifically 24 hours after the end of the ethanol drinking session.

Increased expression of Wnt1 and Mdk in the VTA after binge-like ethanol drinking

Next, we investigated whether the expression of two known transcriptional targets of OTX2, *Wnt1* and *Sema3c*, were altered by binge levels of ethanol intake. *Sema3c* transcript levels did not change after ethanol drinking either immediately after the last drinking session or 24 hours later (data not shown). In contrast, *Wnt1* transcript levels were increased immediately after the drinking session (Figure 3a, male water $n=8$, male ethanol $n=7$, female water $n=8$, female ethanol $n=7$; treatment, $F(1, 26) = 7.63$, $P = .01$; sex, $F(1, 26) = 1.26$, $P = .27$; interaction, $F(1, 26) = 0.28$, $P = .60$) and returned to control levels 24 hours later (Figure 3b, male water $n=12$, male ethanol $n=11$, female water $n=10$, female ethanol, $n=9$; treatment, $F(1, 38) = 0.24$, $P = .63$; sex, $F(1, 38) = 0.11$, $P = .74$; interaction, $F(1, 38) = 0.02$, $P = .89$).

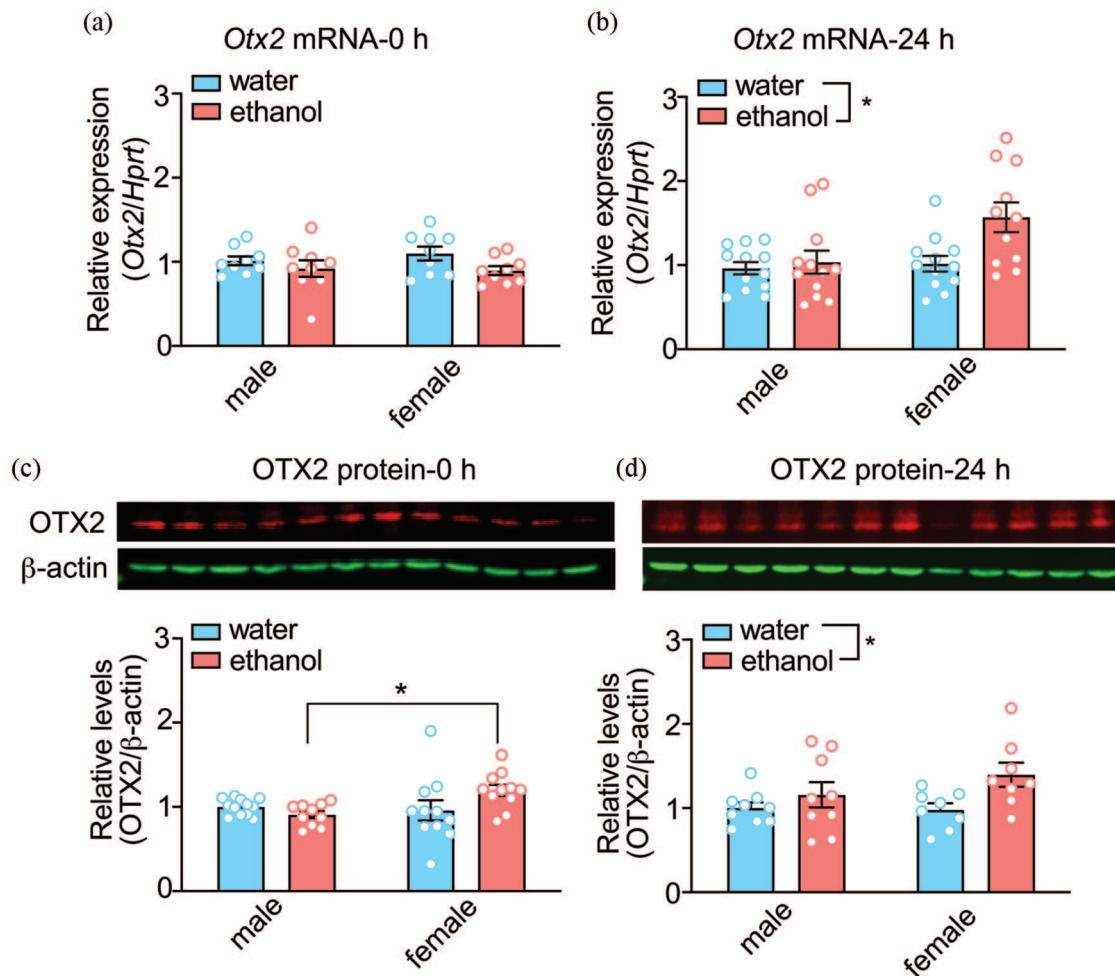


Figure 2. OTX2 mRNA and protein levels are elevated in the VTA 24 hours after binge-like ethanol drinking. Mice had access to water or ethanol in the drinking in the dark protocol. The VTA was dissected from mouse brain immediately (0 hour, a, c) or 24 hours (b, d) after the fourth drinking session and levels of OTX2 mRNA and protein were measured by qPCR and western blot, respectively. (a, b) Relative *Otx2* transcript levels, and (c, d) relative OTX2 protein levels.

Representative images of western blots are shown above each graph. * $P < .05$, main effect of ethanol by two-way ANOVA. Data are presented as the mean \pm SEM, $n = 8$ to 12.

Thus, binge ethanol drinking resulted in increased *Wnt1* gene expression in the VTA immediately after the drinking session.

Next, we determined if binge-like ethanol consumption could impact *Mdk* gene expression in the VTA. MDK is a neurotrophic factor important for the survival of dopaminergic neurons,^{29,30} and we previously demonstrated involvement of *Mdk* in ethanol drinking behavior.²⁴ Notably, using the JASPAR database³¹ we discovered that the promoter of the *Mdk* gene has two consensus binding sites for OTX2, located at 2798 to 2791 (TTAATCCA) and 1063 to 1056 (GTAATCCT) base pairs upstream of the transcriptional start site, suggesting that *Mdk* expression could be transcriptionally regulated by OTX2. Ethanol drinking did not affect *Mdk* mRNA levels immediately after the last drinking session (Figure 3c, male water $n = 9$, male ethanol $n = 9$, female water $n = 9$, female ethanol, $n = 9$; treatment, $F(1, 32) = 0.48$, $P = .49$; sex, $F(1, 32) = 0.082$, $P = .78$; interaction, $F(1, 32) = 1.02$, $P = .32$). However, *Mdk* mRNA levels were elevated in the ethanol-drinking group compared with the control group 24 hours later (Figure 3d,

male water $n = 12$, male ethanol $n = 12$, female water $n = 12$, female ethanol $n = 11$; treatment, $F(1, 43) = 4.14$, $P = .048$; sex, $F(1, 43) = 1.68$, $P = .20$; interaction, $F(1, 43) = 0.037$, $P = .85$).

Finally, we tested whether binge-like ethanol drinking would alter tyrosine hydroxylase (*Th*) gene and protein expression in the VTA, because there is evidence that OTX2 can affect TH protein levels.¹⁴ *Th* gene expression was not changed after ethanol drinking at either time point (Supplemental Figure 1). Consistent with the mRNA results, TH protein levels were also not altered by ethanol consumption (Supplemental Figure 1). These results suggest that binge levels of ethanol drinking (at least for 4 days) does not significantly impact *Th* gene and protein levels.

Reducing Otx2 expression in the mouse VTA does not affect binge-like ethanol drinking

Because OTX2 protein is increased in the mouse VTA after ethanol drinking, we next determined whether OTX2 in the

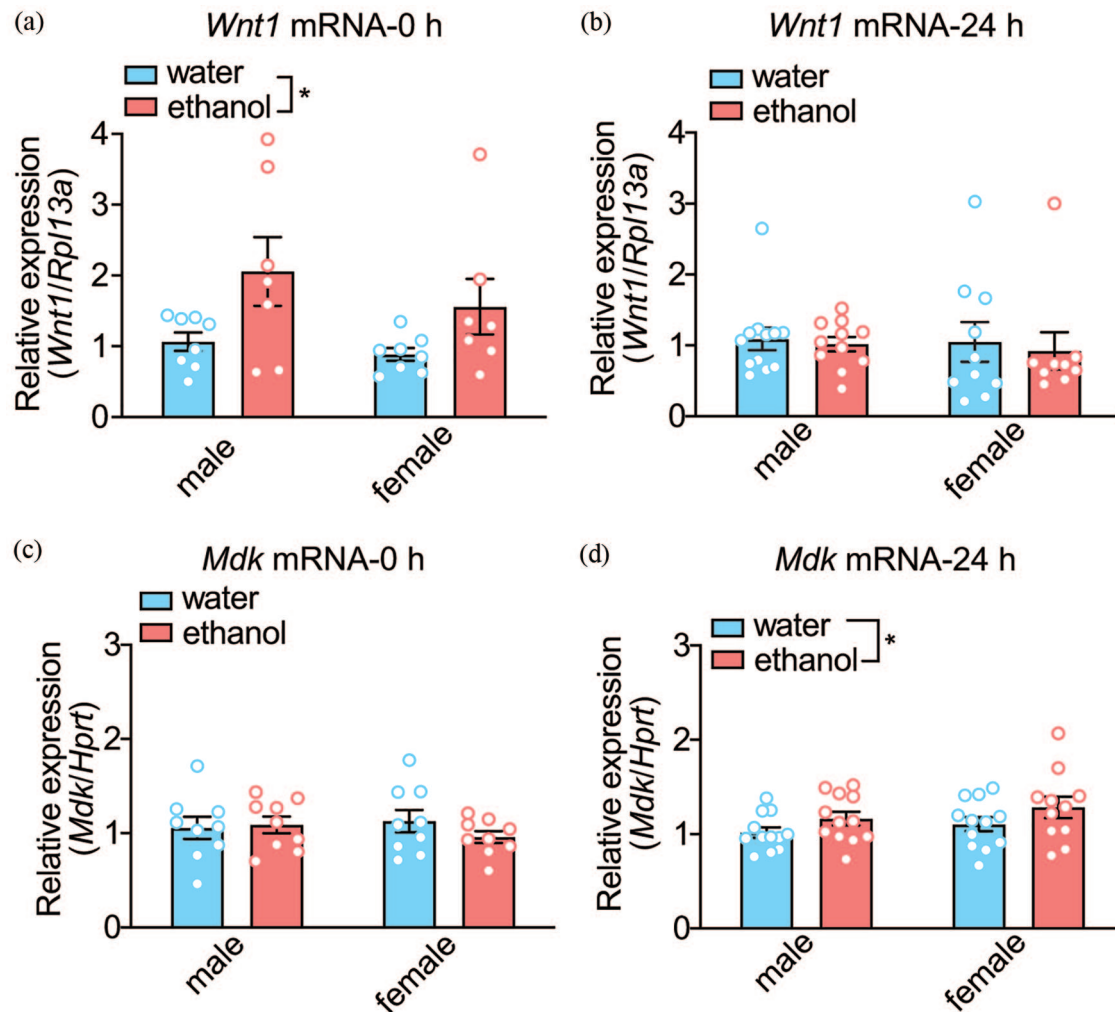


Figure 3. Increased expression of *Wnt1* and *Mdk* in the VTA after binge-like ethanol drinking. Mice had access to ethanol or water in the drinking in the dark protocol and the VTA was dissected either immediately (0 hour, a, c) or 24 hours (b, d) after the fourth drinking session. Levels of *Wnt1* (a, b) and *Mdk* (c, d) mRNA were measured by qPCR.

* $P < .05$, main effect of ethanol by two-way ANOVA. Data are presented as the mean \pm SEM, $n = 7$ to 12.

VTA might play a role in ethanol intake. To do this, we used a lentiviral vector to reduce levels of *Otx2* in the VTA by RNA interference. Mice were microinjected in the VTA with lentivirus expressing either an shRNA targeting *Otx2* (shOtx2) or a control non-targeting shRNA (shScr),²⁶ along with GFP as a reporter for transduced cells. We first measured the levels of *Otx2* transcript in mice 3 weeks after injection to determine if shOtx2 reduced expression of *Otx2* in the VTA. Transduced VTA was dissected from brain sections, RNA isolated, and cDNA analyzed by qPCR for *Otx2* mRNA levels. Expression of shOtx2 in the VTA resulted in an $\sim 20\%$ reduction in *Otx2* transcript when compared to the shScr-expressing group (Figure 4a, female shScr, $n = 9$, female shOtx2, $n = 10$, male shScr $n = 9$, male shOtx2, $n = 9$, $t(35) = 2.31$, $P = .027$). shOtx2 did not significantly alter *Tb* mRNA levels in the same samples (Figure 4b, $t(35) = 0.96$, $P = .342$).

Next, mice were injected in the VTA with lentivirus expressing either shOtx2 or shScr and tested for ethanol intake in the DID protocol 3 weeks after surgery. Ethanol consumed during

the 2 hours sessions did not differ between shOtx2- and shScr-expressing mice (Figure 4c, male shScr, $n = 8$, male shOtx2, $n = 8$; shRNA, $F(1, 162) = 0.95$, $P = .33$; session, $F(11, 162) = 2.88$, $P = .0018$; interaction, $F(11, 162) = 0.51$, $P = .83$; Figure 4h, female shScr, $n = 6$, female shOtx2, $n = 6$; shRNA, $F(1, 116) = 0.003$, $P = .96$; session, $F(11, 116) = 2.51$, $P = .007$; interaction, $F(11, 116) = 0.71$, $P = .72$). We also measured 4 hours ethanol intake on the fourth session of each week. Similar to the 2 hours drinking sessions, the amount of ethanol consumed did not significantly differ between mice expressing shScr or shOtx2 (Figure 4d–f, males, session 4, $t(14) = 0.67$, $P = .51$; session 8, $t(14) = 1.63$, $P = .13$; session 12, $t(14) = 1.09$, $P = .29$; Figure 4i–k, females, session 4, $t(10) = 0.49$, $P = .63$; session 8, $t(10) = 1.26$, $P = .24$; session 12, $t(10) = 1.97$, $P = .077$). Finally, BALs were measured immediately after the 4 hours drinking session on day 12 and there was no difference in the BALs between the shScr- and shOtx2-expressing mice (Figure 4g, males, $t(14) = 0.26$, $P = .80$; Figure 4l, females, $t(10) = 0.57$, $P = .58$). Together these results indicate that reducing *Otx2*

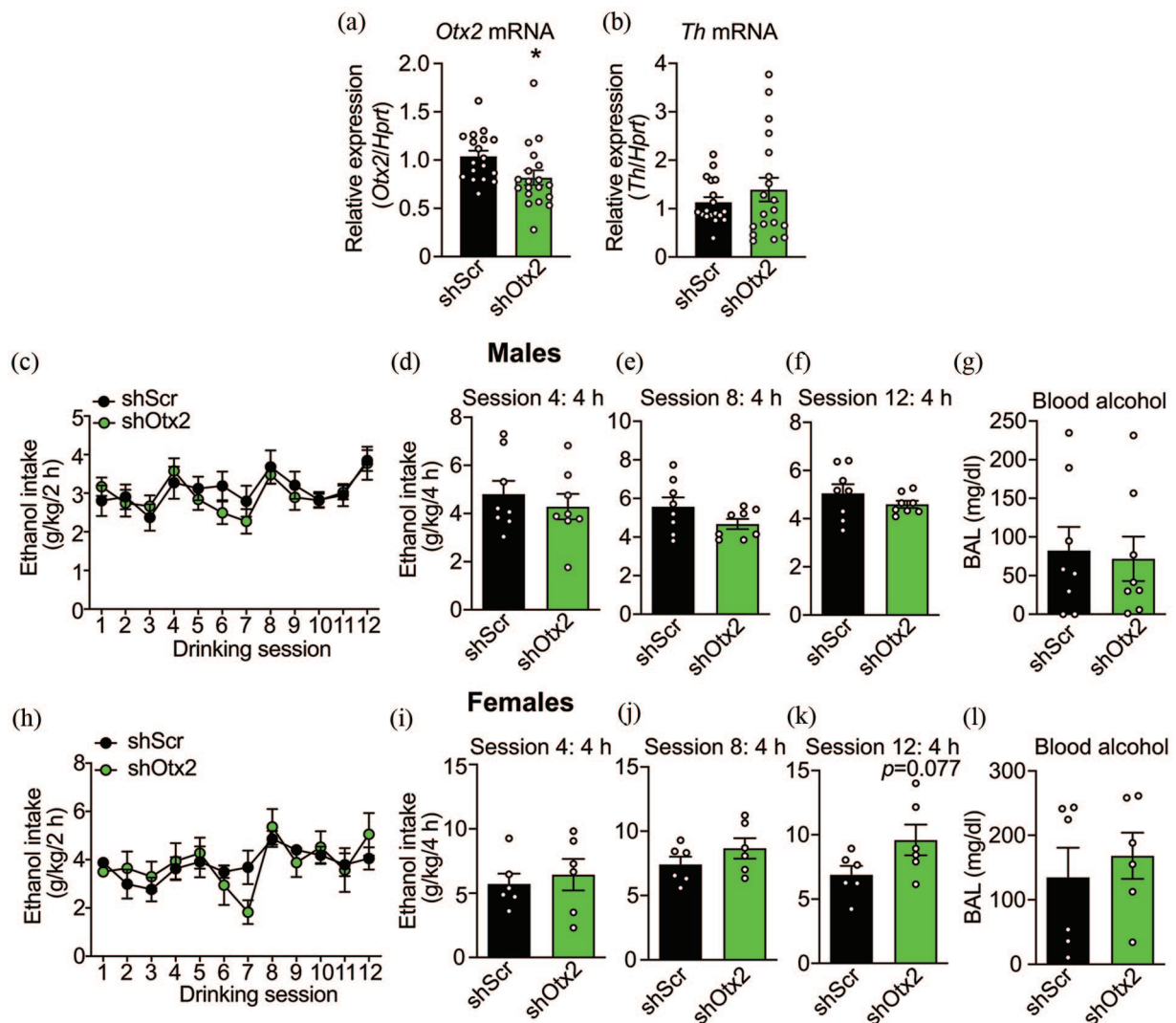


Figure 4. Reducing *Otx2* expression in the mouse VTA does not affect binge-like ethanol drinking: (a) *Otx2* and (b) *Th* transcript levels in the VTA of mice injected with lentivirus expressing a control shRNA (shScr) or a shRNA targeting *Otx2* (shOtx2) 3 weeks after injection. * $P < .05$ by *t*-test. Data are presented as the mean \pm SEM, $n = 18$ to 19, (c, h) daily ethanol intake in 2 hours by (c) male and (h) female mice expressing shScr or shOtx2 in the VTA. Ethanol intake by (d-f) males and (i-k) females during the 4 hours drinking session on day 4 (d, i), day 8 (e, j), and day 12 (f, k), (g, l) BALs after the final 4 hours drinking session on day 12 in males (g) and females (l). Data are presented as the mean \pm SEM, $n = 8$ for males and $n = 6$ for females.

transcript in the mouse VTA does not alter binge-like ethanol intake.

Reducing Otx2 expression in the VTA does not affect sucrose consumption

Although reducing *Otx2* in the VTA did not alter ethanol drinking, we tested whether this might affect intake of sucrose, because *Otx2* in the VTA has been implicated in anhedonia.¹⁷ The same group of mice used for the ethanol drinking test were tested for consumption of a 2% sucrose solution for 4 days, 3 days after completing the ethanol drinking test. There was no difference in the amount of sucrose solution consumed between mice expressing shScr and shOtx2 during the 2 hours daily sessions (Figure 5a, males, shRNA, $F(1, 56) = 0.47$, $P = .50$; day, $F(3, 56) = 4.82$, $P = .0047$; interaction, $F(3, 56) = 0.09$, $P = .97$;

Figure 5c, females, $F(1, 40) = 0.39$, $P = .54$, day, $F(3, 40) = 3.81$, $P = .017$; interaction, $F(3, 40) = 0.31$, $P = .82$). Sucrose intake also did not differ during the 4 hours session on day 4 (Figure 5b, males, $t(14) = 0.08$, $P = .94$; Figure 5d, females, $t(10) = 0.89$, $P = .39$).

Finally, after the completion of the sucrose drinking test, we confirmed viral transduction in the VTA and measured the amount of OTX2 protein levels by immunohistochemistry with antibodies to TH, GFP, and OTX2 (Figure 5e-h). Virus spread was ~ 0.33 mm in the anterior-posterior direction in mice injected with lentivirus expressing shScr and shOtx2, demonstrating equivalent viral transduction in the VTA in both groups. In addition, OTX2 protein was reduced by 66% in GFP-positive cells in the shOtx2 group when compared with the shScr group (Figure 5h, shScr $n = 9$ mice, shOtx2, $n = 10$ mice, $t(17) = 4.2$, $P = .0006$). There was no difference in TH

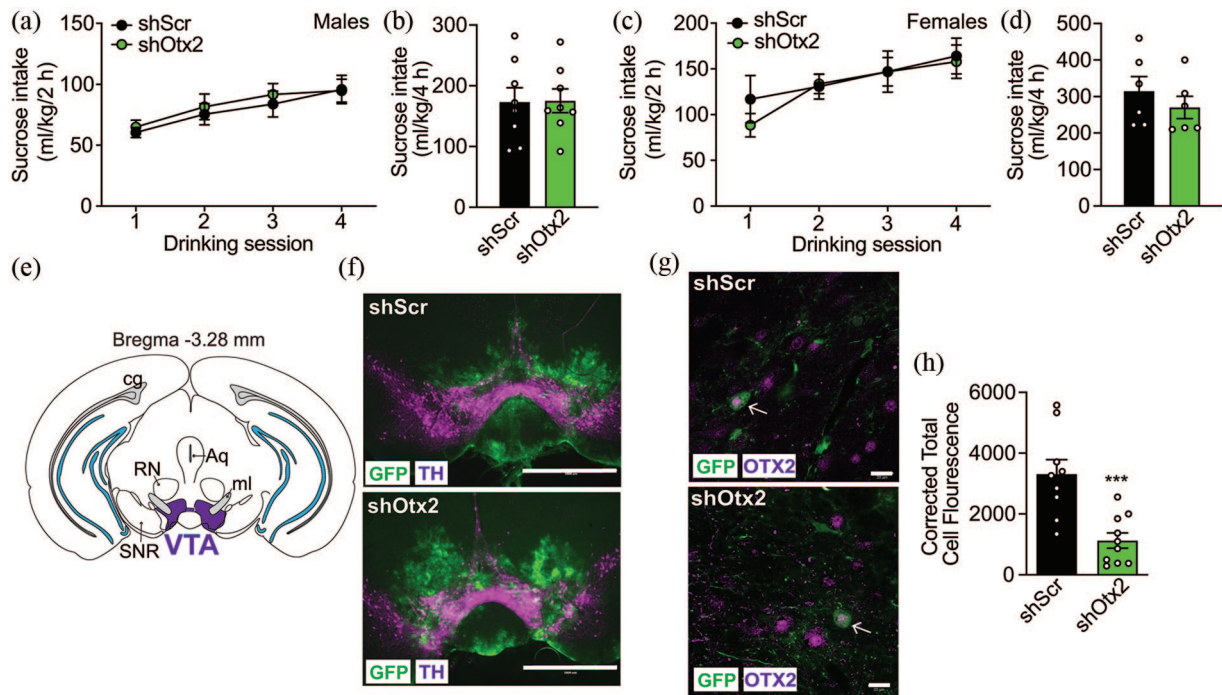


Figure 5. Reducing *Otx2* expression in the VTA does not affect sucrose consumption. Mice were injected with lentivirus expressing shScr or shOtx2. After the completion of the ethanol drinking test, they were tested for consumption of 2% sucrose for 4 days in the drinking in the dark protocol: (a, c) daily sucrose intake in ml/kg in 2 hours by (a) males and (c) females: (b–d) sucrose intake in 4 hours during the fourth drinking session by (b) males and (d) females. Data are presented as the mean \pm SEM, $n=8$ for males and $n=6$ for females, (e) drawing of a coronal section of mouse brain showing the VTA in purple representing the main site of viral transduction, (f) representative images of virally-transduced VTA, with viral transduction indicated by green fluorescence (GFP) and the VTA indicated by tyrosine hydroxylase (TH) immunofluorescence in magenta. Scale bar, 1 mm, (g) representative fluorescent images of transduced cells in the VTA expressing GFP (green) and OTX2 (magenta). Scale bar, 20 μ m. Arrows point to cells expressing GFP and OTX2, and (h) quantification of OTX2 fluorescence in cells expressing GFP. Data are presented as the mean \pm SEM. Each data point represents the mean OTX2 fluorescence in GFP + cells per mouse. For the shScr group, 47 cells from 29 sections from 9 mice were analyzed. For the shOtx2 group, 50 cells from 23 sections from 10 mice were analyzed.

intensity in GFP-positive neurons between the shScr and shOtx2 groups (data not shown), demonstrating the specificity of the effect. These results indicate that the VTA was effectively transduced by lentivirus and resulted in reduced OTX2 protein levels.

Discussion

Here we show that binge-like ethanol drinking increases *Otx2* mRNA and protein, *Wnt1* mRNA, and *Mdk* mRNA levels in the VTA of adult mice. One interesting finding from this study is that many of the transcriptional and protein changes induced by binge-like ethanol drinking occurred in both males and females, even though female mice drank more ethanol than the males. This suggests that a certain threshold dose of alcohol can induce transcriptional changes in the VTA that are independent of sex (at least for the genes that we measured). Although prior studies found that ethanol exposure during embryonic development can affect *Otx2* gene expression in the brain, *Otx2* expression specifically in the VTA of adult mice after binge-like drinking has not been previously examined. The increase in OTX2 protein that we observed 24 hours after the ethanol drinking session suggests that transcription of OTX2 target genes in the VTA may also be altered after ethanol drinking.

We first measured the expression of the known OTX2 target genes, *Wnt1* and *Sema3c*, in the VTA after ethanol drinking. These genes were selected because Peña et al¹⁷ found that maternal separation stress at postnatal days 10 to 20 in male mice resulted in decreased expression, and OTX2 binding at the promoters, of the *Sema3c* and *Wnt1* genes in the VTA, suggesting that the transcriptional activity of these genes is regulated by OTX2. We found that *Sema3c* mRNA levels were not changed after ethanol drinking. However, *Wnt1* transcript was elevated in the VTA of mice that had consumed ethanol immediately after the fourth drinking session. *Wnt1* levels returned to normal by 24 hours after the fourth drinking session. These data indicate that *Wnt1* expression in the VTA is transiently induced during the binge drinking session, presumably leading to increased WNT1 protein. OTX2 protein was not increased immediately after the fourth drinking session, indicating that OTX2 may not contribute to the ethanol-induced increase in *Wnt1* mRNA levels at this timepoint. It is possible instead that WNT1 contributes to the increase in OTX2 in the VTA observed 24 hours after ethanol drinking, because there is evidence of reciprocal regulation of *Otx2* expression by WNT1.³² β -catenin is a downstream mediator of WNT signaling³³ that binds to the promoter region of *Otx2* and regulates its expression,³² and we found that *Otx2*

transcript was increased at 24 hours. Directly testing this hypothesis would require experiments measuring WNT1 protein levels and binding of β -catenin at the *Otx2* promoter after ethanol drinking, and manipulation of WNT1 expression to establish a causal role for it in ethanol-induced changes in *Otx2*. Interestingly, chronic alcohol exposure has been found to suppress the WNT1/ β -catenin signaling pathway in the liver,³⁴ but WNT1 signaling in the adult brain has not been investigated in the context of alcohol use disorder. This would be an excellent area for future studies.

We also found increased *Mdk* mRNA in the VTA 24 hours after the ethanol drinking session. Previous findings from our group showed that a single ethanol exposure in vitro leads to increased *MDK* expression in human neuroblastoma cell lines,³⁵ and that mouse *Mdk* plays a role in preventing excessive ethanol drinking, as *Mdk*^{-/-} mice and mice expressing *Mdk* shRNA in the VTA have higher levels of ethanol intake when compared to control mice.²⁴ We examined if *Mdk* could be an OTX2-regulated gene because both *Mdk* and *Otx2* are important for dopamine neuron development.^{10-14,21,29,30} Using an in silico approach, we discovered predicted OTX2 binding sites in the promoter of the *Mdk* gene, so it is possible that the increase in OTX2 protein expression observed 24 hours after ethanol drinking contributes to increased *Mdk* transcription at this timepoint. It will be important to determine if OTX2 directly associates with the *Mdk* promoter. If this is the case, these data indicate that *Mdk* is a novel ethanol responsive OTX2 target gene in the VTA.

Because reducing *Mdk* in the VTA has been shown to increase ethanol intake,²⁴ we decided to test the hypothesis that reducing levels of *Otx2* would similarly result in increased ethanol consumption. Reducing *Otx2* in the VTA of mice did not affect ethanol or sucrose intake. One simple explanation for this finding is that there was not a sufficient decrease in OTX2 protein in the VTA to elicit a change in behavior. *Otx2* transcript levels were only reduced by 20% by qPCR 3 weeks after viral infusion. We also did not measure the effect of shOtx2 on *Mdk* gene expression. We did, however, quantify OTX2 immunoreactivity after the completion of the behavioral testing in individual cells in the VTA that were transduced with virus and found a 66% reduction in OTX2 protein, indicating that shOtx2 was effective. The other possibility is that viral transduction did not cover a large enough area of the VTA to have an effect, although we have used this lentiviral vector to knockdown expression of other genes in the VTA and found bidirectional effects on alcohol drinking.^{24,26,36} OTX2 protein was also seen by IHC in other non-dopaminergic cells in the VTA, and the lentivirus used in this study can, in theory, transduce all cells. If OTX2 function in different VTA cell types has opposing effects, it may be necessary to knockdown *Otx2* in a specific cell type to reveal a phenotype on ethanol drinking. Knockdown of *Otx2* in the VTA could also have induced compensatory changes that prevented changes in alcohol and sucrose drinking behavior.

Another possibility is that reducing OTX2 levels might render mice more susceptible to binge-like drinking behavior, but that another environmental perturbation (ie, a stressor) would be required to reveal a significant effect on alcohol intake. For example, reducing *Otx2* in the juvenile mouse VTA renders them more susceptible to stress-induced depressive-like behaviors in adulthood.¹⁷ A related issue is that perturbing OTX2 in the adult VTA may not be effective because OTX2 activity may only be required during a specific developmental window¹⁷ or critical period, which is consistent with a known role for OTX2 in critical period plasticity in the visual system.^{37,38} Finally, it is possible that OTX2 in the VTA just doesn't play a role in ethanol intake. *Otx2* is expressed in select regions of the CNS and could presumably be involved in binge-like ethanol drinking through its transcriptional activity in those regions. For example, *Otx2* is highly expressed in the medial habenula and regulates the development of a habenula-interpeduncular circuit³⁹ involved in reward and aversion.⁴⁰ *Otx2* is also strongly expressed in the choroid plexus.⁴¹ OTX2 protein is secreted into the cerebrospinal fluid by choroid plexus ependymal cells and internalized by cortical parvalbumin-expressing interneurons through its association with perineuronal nets,^{41,42} where it can then regulate transcription in a non-cell autonomous manner. OTX2 activity in the cortex could also feasibly impact binge-like drinking. Future studies should explore alternative sites in which OTX2 might act to regulate binge drinking and other behaviors related to AUD.

Author Contributions

Both authors designed experiments, CC performed experiments, and both authors wrote the manuscript.

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Supplemental Material

Supplemental material for this article is available online.

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