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# *Taok2* Controls Behavioral Response to Ethanol in Mice

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## Abstract

Despite recent advances in the understanding of ethanol's biological action, many of the molecular targets of ethanol and mechanisms behind ethanol's effect on behavior remain poorly understood. In an effort to identify novel genes, the products of which regulate behavioral responses to ethanol, we recently identified a mutation in the *dtao* gene that confers resistance to the locomotor stimulating effect of ethanol in *Drosophila*. *dtao* encodes a member of the Ste20 family of serine/threonine kinases implicated in MAP kinase signaling pathways. In the present study, we report that conditional ablation of the mouse *dtao* homolog, *Taok2*, constitutively and specifically in the nervous system, results in strain-specific and overlapping alterations in ethanol-dependent behaviors. These data suggest a functional conservation of *dtao* and *Taok2* in mediating ethanol's biological action and identify *Taok2* as a putative candidate gene for ethanol use disorders in humans.

### Keywords

TAOK2; MAP Kinase; ethanol sensitivity; ethanol consumption; conditioned place preference

## Introduction

An estimated 12.5% of the U.S. population suffers from alcohol dependence (Hasin *et al.* 2007), exacting immense individual and societal costs. Despite the high prevalence of alcohol use disorders, the biological effects of ethanol remain poorly understood. In contrast to many drugs of abuse, ethanol acts on multiple biological targets, including neurotransmitter systems, cellular membrane components and intracellular signaling pathways (Buck and Harris, 1991; Chandler *et al.* 2003; Harris and Hitzmann, 1981; Moonat *et al*. 2010; Newton and Messing, 2006).

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Emerging evidence suggests that mitogen activated protein kinase (MAPK) signaling cascades may play an important role in the action of ethanol and other drugs of abuse (Arror and Shukla, 2004; Corominas *et al.* 2007; Herbert and O'Callaghan, 2000). MAPK signaling transduces such diverse extracellular stimuli as growth factors, cytokines, and environmental stressors via a three-tiered intracellular signaling pathway to induce changes in gene transcription, cellular morphology, differentiation, proliferation and death (Krishna and Narang, 2008; Seger and Krebs, 1995). Specifically, MAP kinase kinase kinases (MAP3Ks) phosphorylate and activate MAP2Ks, which in turn phosphorylate and activate three classes of MAP kinases: p38, ERK, and JNK (Davis, 1994; Sugden and Clerk, 1997; Takeda and Ichijo, 2002). Meta-analysis of expression studies has revealed that brain transcript levels of various MAPK signaling components are altered in response to ethanol, cocaine, and other drugs of abuse (Daniels *et al.* 2002; Freeman *et al.* 2010; Hassan *et al.* 2003; Konu *et al.* 2001). Furthermore, ethanol treatment increases phosphorylation and activation of p38, ERK and JNK in both liver and brain (Aroor and Shukla, 2004; Aroor *et al.* 2010; Jung *et al.* 2010; Ku *et al.* 2007).

In a screen for *Drosophila melanogaster* mutants with abnormal ethanol-induced hyperactivity, we recently identified a loss-of-function mutation in the *dtao* gene that almost completely abolishes flies' hyperactivity response to ethanol (King *et al.* 2011). *dtao* encodes a putative MAP3K of the GCK-VIII subfamily of Ste20p (sterile 20 protein) kinases (Dan *et al.* 2001). These proteins, also known as TAO (thousand-and-one amino acid) kinases, are characterized by a highly conserved serine/threonine kinase domain, which regulates MAP signaling (Chen and Cobb, 2001; Hutchison *et al.* 1998; Tassi *et al.* 1999; Yasuda *et al.* 2007). In addition to their catalytic function, TAO kinases regulate cytoskeletal organization through interaction with actin and tubulin, via a structurally divergent C-terminal tail region (Johne *et al.* 2008; Mitsopoulos *et al.* 2003; Moore *et al.* 2000; Timm *et al.* 2003; Zihni *et al.* 2006).

In mammals, *dtao* is represented by three orthologous genes: *Taok1*, *Taok2* and *Taok3* (Yustein *et al.* 2003). TAOK2, in addition to putative roles in the regulation of actin and tubulin dynamics, regulates neuronal plasticity via activation of p38 and trafficking of cell adhesion molecules arcadlin and N-cadherin (Yamagata *et al.* 1999; Yasuda *et al.* 2007), indicating that it may also regulate neuroadaptive changes associated with ethanol exposure. Based on these observations, we hypothesized that disruption of the *Taok2* gene in mice would alter the behavioral response to ethanol.

## Methods

### Generation of *Taok2tm1fl* mice

The *Taok2tm1* targeting construct was generated using an approach similar to that reported by Aoyama *et al.* 2005. Briefly, a C57BL/6J-derived bacterial artificial chromosome clone (RP23-142A14) comprising the *Taok2* gene was obtained from Invitrogen (Carlsbad, CA, USA), digested with BamHI and BsrBI (New England Biolabs, Ipswich, MA, USA) and shotgun subcloned into the BamHI/SmaI sites of vector pBSDT-AII (Aoyama *et al.* 2005). Plasmid-transformed *E.coli* colonies were screened by PCR using the following primers: forward: 5'GCTGAGGCTACCTCCTCCTT, reverse: 5'TGCTGCTTATGCAGTTGGAC to

identify an 8.7 Kb genomic clone containing exons 1–7 of *Taok2* and flanking intronic sequence. DNA from insert-containing plasmids was incubated with plasmid pMODloxZeoΔamp3 (Aoyama *et al.* 2005) in the presence of EZ:Tn transposase (Epicentre Biotechnologies, Madison, WI, USA) to introduce two lox P sites flanking a zeocin selectable marker. The position and orientation of this insertion was determined by sequencing. Plasmid DNA from this clone was incubated with Cre recombinase (New England Biolabs) to remove the zeocin selectable marker and combine the two lox P sites into a single site. To introduce a second lox P site into the construct, plasmid DNA from this clone was incubated with plasmid pGPS21loxFRTNeo (Aoyama et al. 2005) in the presence of TnsABC transposase (New England Biolabs). pGPS21loxFRTNeo contains two Tn7-derived transposable elements flanking two lox P sites, two FRT sites and the neomycin phosphotransferase gene (Neo) placed downstream of the EM7 and PGK promotors to confer kanamycin and G418 resistance in *E. coli* and mouse embryonic stem (ES) cells, respectively. Removal of the Neo cassette and one of the lox P sites may be performed either in ES cells or mice with Flpe-mediated recombination. Position and orientation of the insertion into the genomic fragment was determined by sequencing, and a clone with both lox P sites in the same orientation was selected for electroporation in ES cells. Sequence analysis of the entire insert of the resulting targeting construct verified insertion of lox P sites within the first and seventh introns of the *Taok2* gene and confirmed that the remaining genomic sequence was left intact. Mouse ES cells and transgenic mice targeting *Taok2* were generated using standard methods by the Transgenic Core Facility at the Ernest Gallo Clinic and Research Center (EGCRC, Bradley 1987, Hogan *et al.*1994). Briefly, DNA from plasmids containing the *Taok2*-targeted locus was linearized at the PmeI (New England Biolabs) site in the multiple cloning site of the vector and electroporated into C57BL/6-derived ES cells (Primogenix, Laurie, MO, USA). PCR analysis was performed on the resulting G418-resistent clones using a forward primer spanning part of the PGK promotor (5'-GGGGAACTTCCTGACTAGGG) and a reverse primer recognizing mouse genomic sequence immediately 3' of that included in the targeting construct (5'-AGGGCCTAGGGCAAAATAGA) to assess homologous recombination. Prior to blastocyst injection, the integrity of these targeted ES cell clones was verified by sequence analysis of the targeted region. Cells were injected into C57BL/6J$^{tyrc\text{-}2J}$-derived blastocysts (Jackson Laboratory, Bar Harbor, ME, USA) and implanted in pseudopregnant CD1 dams to generate chimeric mice. C57BL/6J$^{tyrc\text{-}2J}$mice carry a recessive point mutation in the tyrosinase gene resulting in a white coat color, allowing the distinction of targeted cells that confer a black coat color. Chimeric pups obtained from these lines were crossed with C57BL/6J$^{tyrc\text{-}2J}$mice to test for germline transmission and stabilization of the line, designated, *Taok2tm1*. Subsequently, *Taok2tm1/+* mice were crossed with Flpe mice (Jackson Laboratory) to remove the Neo marker and yield the *Taok2tm1fl* allele.

## Mouse crosses

*Taok2tm1fl/+* mice were intercrossed to generate *Taok2tmfl/fl* homozygous animals for subsequent crosses. Additionally, *Taok2tm1fl/+* mice were mated with *B6.Cg-Tg(Nes-cre)1Kln/J/+* mice (Jackson Laboratory). *B6.Cg-Tg(Nes-cre)1Kln/J/+* mice, abbreviated "*Nes-cre/+*", express CRE recombinase in the nervous system under control of the rat nestin promotor (Tronche *et al.*1999). *Taok2tm1fl/+;Nes-cre/+* mice were crossed to *Taok2tm1fl/fl*

mice to generate *Taok2tm1fl/fl;Nes-cre/+* mice and littermate controls (*Taok2tm1fl/+* mice) for expression analysis and behavioral experiments. In the course of analyzing these crosses, we observed "leaky" germline expression of CRE recombinase in a small subset of progeny. These animals were bred to C57BL/6J mice to generate *Taok2+/−* mice, which were subsequently intercrossed to generate *Taok2−/−* mice and controls for behavioral experiments.

## Genotypic Analysis

Mice were genotyped using DNA isolated from tail biopsies using standard protocols. The following primers were used to genotype progeny from the above described crosses by PCR analysis: forward primer 5'-CCAAGGACCAGACATCCACT and reverse primer 5'-ACCAGTCCTCGTTTTTGCTG were used to detect presence of the wild-type and *Taok2tm1fl* alleles; forward primer 5'-GATGCAACGAGTGATGAG and reverse primer 5'-TCGGCTATACGTAACAGG were used to detect presence of the *Nes-cre* transgene; forward primer 5'-TGCAGGGTTTGACAGTTGAT and reverse primer 5'-ACTCTGCCTCAGGAGTCCAA were used to detect presence of the null allele.

## Immunohistochemistry

Mice were deeply anesthetized with 100mg/kg of Euthasol® (Virbac, Forth Worth, TX, USA) and intracardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate buffer, (PB) pH 7.4. Brains were removed, post-fixed overnight in the same fixative at 4 °C, incubated in 30% sucrose for 48 hours for cryoprotection. Frozen sagittal, 50 μm-thick sections were cut on a cryostat (Leica Instruments, Nussloch, Germany). Free-floating sections were incubated in 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min, then in 50% alcohol for 10 min, rinsed in PBS, incubated in 10% normal donkey serum in PBS (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min, and incubated overnight in the goat polyclonal antibody recognizing TAOK2 (sc-47447, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000). On the next day sections were rinsed in PBS incubated in 2% normal donkey serum in PBS for 10 min and incubated in biotinylated donkey anti-goat (1:500, Jackson ImmunoResearch) for 3 hours, rinsed in PBS and incubated in ExtrAvidin-peroxidase complex (1:2,500, Sigma-Aldrich, St. Louis, MO, USA) for 2 hours. Peroxidase was histochemically visualized with diaminobenzidine (Sigma-Aldrich). Sections were mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohols, cleared with xylene, and mounted with DPX mounting media (Sigma-Aldrich). Images were taken examined in a Nikon Eclipse E600 microscope equipped with Spot 2 CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). In control experiments the omission of primary antibody resulted in lack of immunostaining.

## Quantitative PCR (QPCR)

Total RNA was isolated from whole brain tissue using Trizol® reagent (Invitrogen) according to the manufacturer's instructions and was treated with RNase-free DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. cDNA was synthesized from 1 μg of total RNA using reverse transcription reagents from Applied

Biosystems (Foster City, CA, USA). Following synthesis, cDNA was diluted 1:10 in water. TaqMan QPCR was performed using standard thermal cycling conditions on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Amplification reactions contained 5 μl of cDNA template, 1x Universal PCR Master Mix, 100 nM each of forward and reverse primers, and 200 nM of FAM-labeled probe in a final volume of 10 μl. The *Taok2* 5'probe and primers set spanning exons 4–5 was Applied Biosystems catalog # Mm001139118. Rodent GAPDH probe and primers (Applied Biosystems) were used as a control for the PCR reactions. Data was analyzed using the comparative Ct method (Applied Biosystems user bulletin #2).

## Western Analysis

Protein extracts were isolated from whole brain tissue in RIPA buffer using standard techniques. 10 μg protein samples were electrophoresed on NuPAGE 4–12% SDS-polyacrylamide gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). Primary antibodies included goat anti-TAOK2 (sc-47447, Santa Cruz Biotechnology, Inc) 1:500 and mouse anti-GAPDH (Fisher Scientific, Pittsburgh, PA, USA), 1:5000. Western blots were incubated with either HRP-linked donkey anti-goat or HRP-linked sheep anti-mouse secondary antibody (GE Healthcare, Biosciences, Piscataway, NJ, USA), 1:5000, and processed with ECL Plus Western Blotting Detection System (GE Healthcare, Biosciences). Chemiluminescence was visualized and quantified using the Storm 660 phosphorimager system (Molecular Dynamics, Sunnyvale, CA, USA).

## Behavioral Testing

Behavioral testing was performed on male mice (to avoid potential behavioral effects of the estrus cycle on behavior) that were 3–6 months of age at time of testing. Behavior was performed as follows: group 1 (*Taok2*−/− and control animals): open field activity, stationary dowel test, LORR test and drinking in the dark with one week rest period between each behavioral test. Group 2 (*Taok2*−/− and control animals): ethanol metabolism and clearance. Group 3: (*Taok2*−/− and control animals): ethanol CPP. Group 4: (*Taok2fl/fl:Nes-cre* and control mice): LORR test. Group 5: (*Taok2fl/fl:Nes-cre* and control mice): drinking in the dark. Group 6: (*Taok2fl/fl:Nes-cre* and control mice): ethanol metabolism and clearance. All animal protocols were approved by the EGCRC institutional animal care and use committee.

## Stationary dowel test of acute functional tolerance (AFT)

A stationary wooden dowel (1.5 cm diameter and 30-cm long) was suspended between two Plexiglas walls 50 cm above a cushioned surface. Mice were trained to remain on the stationary dowel for 5 minutes. Each mouse was then given an intraperitoneal injection of 1.5 g/kg of ethanol (10% w/v in saline) and placed on the dowel. At this dose, subjects were unable to balance on the dowel. After a subject fell from the dowel, it was retested for regain of balance at 5 minutes intervals until it was able to remain on the dowel for 1 minute. Immediately following regain of balance, a blood sample was taken by tail vein puncture (BEC1), the mouse was given a booster injection of 1.5 g/kg ethanol and placed back on the dowel. The time at which the second period of ataxia began was recorded and when the

subject fell from the dowel. Recovery was again tested at 5 minute intervals until the subject was able to remain on the dowel for 1 minute, at which point a second blood sample was taken (BEC2). AFT was calculated as the difference between BEC2 and BEC1. The rate at which AFT developed was calculated as (BEC2-BEC1)/duration of ataxia following the booster injection.

### Loss-of-righting reflex (LORR) assay

Ethanol (10% v/v in saline) was administered IP at a dose of 4g/kg. After injection, mice were placed on their backs and tested for loss of the righting reflex. The mouse was judged to have lost the righting reflex at the time when it could not right itself three times within 30 seconds. When the animal was able to right itself three times within 30 seconds it was deemed to have recovered. The duration of the loss of the righting reflex was calculated as the difference between when the reflex was lost and when it was recovered. For *Taok2−/−* and control mice, blood ethanol content at time of recovery was measured by tail vein bleed.

### Drinking in the dark (DID) assay

Oral alcohol self-administration was examined using a limited access, drinking in the dark assay. One week prior to data collection, mice were singly housed and transferred to a reverse light-dark cycle. On day 8 of the experiment, water intake (ml) was measured for the 4-hour period beginning 4 hours after lights off and body weight (g) was recorded. Subsequently, mice were given access to a single bottle of 20%w/v ethanol during the same time of day, on alternate days for 10 days (i.e., 5 days of data collection) and ethanol consumption was calculated. For *Taok2−/−* and control mice, water consumption was also measured on alternate days during the same 4 hour time period for the duration of the study.

### Two-bottle choice assay for taste preference

Taste preference for saccharin (sweet) and quinine (bitter) was examined using a two-bottle choice protocol. Mice were singly housed for 4 days in double-grommet cages with continuous access to two water bottles under standard 12 hour light/12 hour dark conditions. On day 5, the left water bottle was replaced with a 0.06% w/v solution saccharin in water. On the following day, the amount of saccharin solution and water consumed was determined and the position of the bottles was switched. The next day, saccharin and water consumption were again determined and the procedure was repeated using a 0.03 mM quinine solution (in water) and water.

### Conditioned place preference assay and ethanol-induced locomotor stimulation

Ethanol-induced conditioned place preference (CPP) was performed using an apparatus (Med Associates, St. Albans, VT, USA) consisting of two open field chambers (each 27.3×27.3 cm) separated by a central guillotine door and characterized by custom acrylic floors that are contextually distinct. Ethanol-naïve mice were allowed free access to both chambers of the CPP apparatus for 30 minutes on day 1 of the experiment to habituate to the apparatus, assess general activity level and initial side-preference. Subjects were then assigned to either of two experimental groups in a counterbalanced design in which ethanol was paired with the right chamber or left chamber. On day 2, subjects received either an

intraperitoneal injection of 2g/kg ethanol or saline prior to placement in one of the two chambers for 5 minutes. On day 3, subjects that received an ethanol injection on day 2 were given a saline injection and allowed access to the opposite chamber for 5 minutes. Subjects were thus trained with alternating ethanol and saline treatments for a total of 8 days. On the test day, subjects were allowed free access to both chambers for 30 minutes, during which the time spent in each chamber was recorded. For baseline activity and ethanol-induced locomotor stimulation, the horizontal activity of subjects on the first day of saline treatment (either day 2 or day 3) was compared to activity on the first day of ethanol treatment (day 2 or day 3).

### Open field activity

Locomotor activity measurements were performed in Plexiglas locomotor activity chambers (43 cm X 43 cm, Med Associates, St. Albans, VT, USA), located in sound-attenuated cubicles equipped with a 2.8 watt house light and exhaust fans to mask external noise. The chambers contained two sets of 16 pulse-modulated infrared photobeams on opposite walls to record *x, y* ambulatory movements and are computer interfaced for data sampling at 100 ms resolution. Mice were placed in chambers for 60 minutes and the distance traveled (cm) was measured in 5 minute bins.

### Ethanol metabolism and clearance

Ethanol-naive mice were injected with 4g/kg of ethanol and tail blood samples (10 μl) were obtained at 10, 30, 60, 90, 120 and 180 minutes to measure blood alcohol levels. Blood ethanol content was assessed from serum using the Analox AM1 Analyzer (Analox Instruments, North Yorkshire, UK).

### Statistical Analysis

Data were analyzed using SigmaStat 3.1 software (Systat Software, San Jose, CA, USA) with appropriate post-hoc comparisons performed as indicated by SigmaStat. Statistical tests were performed as follows: mRNA and protein quantification in *Taok2tm1fl/fl;Nes-cre/+* mice was normalized to control littermates and GAPDH signal and compared by student's t-test . Water consumption data for *Taok2tm1fl/fl;Nes-cre/+* and control mice were analyzed by student's t-test and for *Taok2−/−* and control mice during DID experiments by 2-way repeated-measures ANOVA for genotype X day. AFT and LORR data were analyzed by student's t-test. Ethanol consumption data during DID experiments were analyzed by 2-way repeated-measures ANOVA for genotype X day. Similarly, ethanol clearance data were analyzed by 2-way repeated-measures ANOVA for genotype X time. CPP data were analyzed by 2-way repeated measures ANOVA for genotype X time spent in the ethanol-paired chamber on day 1 (habituation) and day 10 (test). Open field activity data were analyzed by 2-way repeated-measures ANOVA for genotype X time. Ethanol-induced locomotor stimulation data were also analyzed by 2-way repeated measures ANOVA for genotype X treatment. Data are presented as mean ± standard error of the mean.

## Results

### Generation and characterization of *Taok2−/−* and *Taok2tm1fl/fl;Nes-cre* mice

To generate a *Taok2* allele in which the gene is conditionally disrupted, we used a modified approach described by Aoyama *et al.* 2005 and Seong *et al.* 2004. This approach simplifies construction of a targeting vector by the insertion of lox P sites and selectable markers in a region of interest using *in vitro* transposition (Aoyama *et al.* 2005). The targeting construct was made using C57BL/6J-derived genomic DNA, a strain for which fully sequenced bacterial artificial clones (BACs) are commercially available, obviating the need to screen a BAC library and facilitating *in silico* design of the targeting construct. The resulting C57BL/6J-derived embryonic stem cells were injected into $C57BL/6J^{tyr}$-derived blastocysts. $C57BL/6J^{tyr}$ mice carry a recessive mutation in the tyrosinase gene that results in a white coat color, allowing for the identification of $C57BL/6J^{Taok2tm1fl}$; $C57BL/6J^{tyr}$ chimeric pups and germline transmission of the *Taok2tm1fl* allele in the subsequent generation. An important advantage of this approach over standard mouse transgenesis methods is that the targeted allele is generated on an isogenic background as opposed to a mixed C57BL/6J X 129-derived background, which may complicate phenotypic analysis (Seong *et al.* 2004).

We targeted the kinase domain of the *Taok2* gene by insertion of lox P sites within introns 1 and 7 to generate the *Taok2tm1* allele. In the presence of FLPe recombinase, the neomycin resistance selectable marker was removed to yield the *Taok2tm1fl* allele (Fig. 1a). This allele, in the presence of a CRE recombinase-expressing transgene, is expected to excise sequences containing exons 2–7 of the *Taok2* locus to generate the deleted or *Taok2* null allele (*Taok2−/−*, Fig. 1a).

To disrupt the *Taok2* gene specifically in the nervous system, we crossed *Taok2tm1fl/fl* mice with *B6.Cg-Tg(Nes-cre)1Kln/J/+* mice (abbreviated *Nes-cre/+*). *Nes-cre/+* mice express CRE recombinase under control of the rat nestin promotor (Tronche *et al.* 1999). *Taok2tm1fl/fl;Nes-cre/+* mice were viable and normal in appearance. In addition, gross brain morphology of *Taok2tm1fl/fl;Nes-cre/+* mice, as determined by immunohistological analysis, was indistinguishable from that of control animals (Fig. 1b,c). In wild-type mice, we observed abundant TAOK2 expression throughout the brain, including cortex, striatum and hippocampus (Fig. 1b). In contrast, we confirmed near absence of TAOK2 protein in brains of *Taok2tm1fl/fl;Nes-cre/+* mice (Fig. 1c).

We assessed the efficiency of *Nes-cre* mediated *Taok2* transcript disruption in brains of *Taok2tm1fl/fl;Nes-cre/+* mice by quantitative PCR (qPCR). QPCR analysis using a 5’ primer/probe set internal to the lox P sites revealed nearly 100% excision of the intervening sequence in *Taok2tm1fl/fl;Nes-cre/+* mice compared to control animals ($t_{(4)} = 22.40$, $P < 0.001$, Fig. 1d). These data indicate that the *Nes-cre* transgene excises exons 2–7 with high efficiency in brains of *Taok2tm1fl/fl;Nes-cre/+* mice.

In addition to disrupting most of the kinase domain, deletion of exons 2–7 is also expected to remove the translational start site of the *Taok2* transcript. To determine if the *Nes-cre* transgene effectively knocks down TAOK2 protein in brains of *Taok2tm1fl/fl;Nes-cre/+* mice, we performed Western blot analysis using a polyclonal antibody raised against a

peptide mapping to an internal region of the human TAOK2 protein and cross-reacting to mouse TAOK2. This analysis showed nearly complete loss of TAOK2 protein in brain tissue isolated from *Taok2tm1fl/fl;Nes-cre/+* mice ($t_{(2)} = -15.38$, $P < 0.01$) and absence of an aberrantly translated product (Fig. 1e,f). These data suggest that *Taok2tm1fl/fl;Nes-cre/+* mice are effectively null for the TAOK2 protein in brain tissue.

## Behavioral characterization

A loss-of-function mutation in the *Drosophila* homolog of *Taok2*, *dtao*, confers resistance to the locomotor activating effects of ethanol (King *et al.* 2011). To determine if loss of *Taok2* in the mouse alters ethanol-related behaviors, we tested *Taok2*-deficient and control mice for ethanol-induced ataxia, acute functional tolerance, sedation, ethanol consumption, conditioned place preference and ethanol-induced hyperactivity.

To assess sensitivity to the ataxic affects of ethanol and acute functional tolerance, we tested *Taok2−/−* and control mice in the stationary dowel assay. *Taok2−/−* mice recovered their balance more quickly from both the first ($t_{(19)} = -2.49$, $P < 0.05$, Fig. 2a) and second ($t_{(19)} = -2.16$, $P < 0.05$, Fig. 2c) injections of ethanol in this assay, suggesting that they may be acutely resistant to the ataxic effects of ethanol. To further characterize the acute response of *Taok2−/−* mice to ethanol in this assay, we measured blood ethanol content (BEC) at the time of recovery from both injections and observed that *Taok2−/−* mice exhibited significantly reduced BEC upon recovery from the first ethanol injection ($t_{(19)} = -3.06$, $P < 0.05$, Fig. 2b), whereas BEC values in null mice were comparable to control animals following recovery from the second ethanol injection ($t_{(19)} = -0.83$, $P = 0.42$, Fig. 2d). These data indicate a trend toward increased acute functional tolerance to the ataxic effects of ethanol in *Taok2−/−* mice ($t_{(19)} = 1.94$, $P = 0.07$, Fig. 2e).

We next assessed *Taok2−/−* mice for sensitivity to the sedating effects of ethanol in the loss-of righting-reflex (LORR) assay. After administration of a 4g/kg injection of ethanol intraperitoneally, we measured the time it took for subjects to recover the righting reflex. Compared with controls, *Taok2−/−* mice exhibited a trend toward shorter recovery time from ethanol-induced sedation ($t_{(19)} = -1.69$, $P = 0.11$, Fig. 2f). At the time of recovery, *Taok2−/−* mice showed reduced BEC ($t_{(19)} = -2.54$, $P < 0.05$, Fig. 2g).

The observation that *Taok2−/−* mice recover more quickly with reduced BEC levels from both ataxia and sedation-inducing doses of ethanol suggested that *Taok2−/−* mice may absorb and/or metabolize ethanol more quickly than controls. To address this possibility, we measured BEC levels in *Taok2−/−* and control mice following a 4g/kg intraperitoneal dose ethanol across multiple time points and observed a significant main effect of strain, with *Taok2−/−* mice exhibiting decreased BEC values ($F_{(1, 13)} = 4.56$, $P < 0.05$, Fig. 2h).

Studies in humans and several mouse genetic models have established a negative correlation between sensitivity to the acute effects of ethanol and ethanol consumption or propensity for abuse (Boyce-Rustay *et al.* 2006; Crabbe *et al.* 1996; Fee *et al.* 2004; Kapfhamer *et al.* 2008; Naassila *et al.* 2002, 2004; Newton and Messing 2007; Palmer *et al.* 2004; Schuckit 1994). To assess ethanol consumption in *Taok2−/−* and control mice, we tested subjects in a limited-access, “drinking-in-the dark” (DID) assay. This assay involves acclimating subjects

to a reverse light-dark schedule and providing access to ethanol for several hours during the middle of the dark period when mice are most active. In contrast to standard, continuous access models, it has been shown that mice in the DID assay typically consume pharmacologically relevant (intoxicating) levels of ethanol, resulting in the blood alcohol levels in excess of 1.0mg/ml (Rhodes *et al*. 2005). We observed a significant strain effect for ethanol consumption in the DID assay, with *Taok2−/−* mice consuming increased amounts of ethanol compared to control animals (main effect of genotype: $F_{(1, 14)} = 5.96$, $P < 0.05$, genotype X time interaction: $F_{(4, 56)} = 2.44$, $P = 0.06$, Fig. 3a). Following the final ethanol session, we measured BEC and found a trend toward increased BEC in *Taok2−/−* mice ($t_{(14)} = 1.73$, $P = 0.11$, Fig. 3b). We measured taste preference for a 0.06% w/v saccharin solution and a 0.03 mM quinine solution and did not observe a significant main effect of genotype for either tastant ($t_{(16)} = 0.82$, $P = 0.43$, $t_{(16)} = 1.49$, $P = 0.16$, for saccharin and quinine, respectively), indicating the increased ethanol consumption by *Taok2−/−* mice is likely not due to altered gustation. We also measured water consumption on alternate days during the course of the study and found a trend toward increased consumption (main effect of genotype: $F_{(1, 14)} = 2.56$, $P = 0.13$, genotype X time interaction: $F_{(4, 56)} = 2.16$, $P = 0.09$, Fig. 3c), however water consumption toward the end of the study (days 5, 7 and 9) was comparable between strains, in contrast to ethanol consumption during this time period (days 6, 8 and 10).

Based on our data showing that *Taok2−/−* mice consume more ethanol than control mice, we investigated whether disruption of the *Taok2* gene results in enhanced ethanol-induced conditioned place preference (CPP), a commonly used assay of the rewarding or motivational effects of drugs of abuse (Chester *et al.* 1998; Liu *et al.* 2008). On day 1 of the study (habituation to the chamber), both genotypes exhibited a slight preference for the right side of the apparatus (control: 16.6 ± 0.6 minutes versus *Taok2−/−*: 15.9 ± 1.0 minutes), however the genotypes did not differ significantly from one another ($t_{(22)} = -0.59$, $P = 0.56$). We trained mice using a 2g/kg dose ethanol that did not produce ethanol-induced locomotor sensitization in either genotype in our study (effect of day: $F_{(3, 22)} = 1.28$, $P = 0.29$, Fig. 4a) and observed that expression of ethanol-CPP was completely blocked in *Taok2−/−*?mice (main effect of genotype: $F_{(1, 22)} = 8.50$, $P < 0.01$; main effect of day: $F_{(1, 22)} = 12.75$, $P < 0.05$; genotype X day interaction: $F_{(1, 22)} = 6.91$, $P < 0.05$, Fig. 4b).

In addition to place preference, the CPP assay measures general locomotor activity and ethanol-induced hyperactivity. Both wild-type and *Taok2−/−*?mice increased activity in response to ethanol compared to saline treatment ($F_{(1, 22)} = 45.9$, $P < 0.001$; Fig. 4c). However, *Taok2−/−*?mice were generally more active than controls ($F_{(1, 22)} = 9.54$, $P < 0.005$), such that we failed to detect a significant genotype X treatment interaction ($F_{(1, 22)} = 0.41$, $P = 0.53$; Fig. 4c). These data indicate that *Taok2−/−*?mice exhibit normal ethanol-induced hyperactivity when normalized for an increased baseline activity level, in contrast to *dtao* mutant flies. We further characterized the locomotor hyperactivity phenotype of *Taok2−/−* and control mice by measuring locomotor activity in an open field apparatus and observed a significant main effect of genotype ($F_{(1, 20)} = 13.2$, $P < 0.05$), time ($F_{(11, 20)} = 23.1$, $P < 0.001$), and genotype X time interaction: ($F_{(11, 220)} = 2.00$, $P < 0.05$), indicating that *Taok2−/−* mice do not habituate normally to the open field (Fig. 4d).

Based on our observations that *Taok2−/−* mice exhibit multiple ethanol-dependent behavioral phenotypes that may result, wholly or in part, from increased ethanol metabolism, we investigated whether nervous system-specific disruption of the *Taok2* gene would result in similar ethanol-dependent phenotypes. To assess sensitivity to the sedating effects of ethanol, we tested *Taok2tm1fl/fl;Nes-cre/+* and control mice in the loss-of-righting assay. Compared with control mice, *Taok2tm1fl/fl;Nes-cre/+* mice exhibited a significantly longer recovery time from ethanol ($t_{(12)} = 4.10$, $P < 0.001$; Fig. 5a), indicating that nervous system-specific loss of TAOK2 in mice confers increased sensitivity to the sedative effects of ethanol. The increased sensitivity to ethanol observed in *Taok2tm1fl/fl;Nes-cre/+* mice does not appear to result from altered ethanol absorption and/or clearance, as a separate cohort of subjects did not show a significant strain effect for blood ethanol content across a series of time points following treatment with 4g/kg ethanol (2-way repeated measures ANOVA, effect of genotype: $F_{(1, 9)} = 4.33$, $P > 0.05$; genotype X time interaction: $F_{(5, 45)} = 0.18$, $P > 0.05$; Fig. 5b).

To assess ethanol consumption in *Taok2tm1fl/fl;Nes-cre/+* and control mice, we tested subjects in the DID assay. We observed a significant strain effect for ethanol consumption in the DID assay, with *Taok2tm1fl/fl;Nes-cre/+* mice consuming more ethanol than control mice (effect of genotype: $F_{(1, 20)} = 4.65$, $P < 0.05$, Fig. 5c). BEC levels between strains at the end of the study were comparable ($t_{(20)} = 0.49$, $P = 0.63$, Fig. 5d), as was expected since both mutants and control animals consumed similar amounts of ethanol on the last day of the study (Fig. 5c). The increased ethanol consumption of *Taok2tm1fl/fl;Nes-cre/+* mice was not a result of an overall increase in fluid consumption as control and mutant mice consumed comparable amounts of water during the habituation period ($t_{(12)} = 1.77$, $P = 0.09$, Fig. 5e).

## Discussion

We have generated a conditionally-disrupted allele of the *Taok2* gene in mice using the Cre/loxP system and demonstrate that loss of *Taok2* affects multiple behaviors following exposure to ethanol. Specifically, disruption of *Taok2* constitutively results in earlier recovery from the ataxic and sedative effects of ethanol, increased ethanol consumption, and reduced ethanol-induced conditioned place preference. By comparison, nervous system-specific disruption of *Taok2* increases recovery time from the sedative effects of ethanol and increases ethanol consumption, indicating that global versus nervous system-specific disruption of *Taok2* results in specific and overlapping ethanol-dependent phenotypes.

The earlier recovery time from ethanol-induced ataxia and sedation we observe in *Taok2−/−* mice occurs at lower blood ethanol contents, suggesting that these phenotypes may result from altered ethanol absorption and/or clearance. In fact, we observe altered pharmacokinetics of a 4g/kg dose ethanol in *Taok2−/−* mice, which may explain the ethanol sensitivity phenotypes in the stationary dowel and loss-of-right-reflex tests. Although TAOK2 is most abundantly expressed in the brain, it is present in other tissues, including liver (http://biogps.org/#goto=genereport&id=9344, and data not shown), the primary site of ethanol metabolism by alcohol dehydrogenase (Lands, 1997). Acute ethanol exposure activates JNK (c-JUN NH2-terminal kinase) and mitogen-activated protein kinase (MAPK) p38 in hepatocytes (Aroor and Shukla 2004; Aroor *et al.* 2010; Chen *et al.* 1998), two

signaling pathways also engaged by TAOK2 activation (Calderon de Anda et al. 2012; Chen and Cobb 2001; Yasuda et al. 2007). If and how TAOK2-dependent MAPK signaling may affect ethanol metabolism merits further investigation.

Similarly, an increased rate of ethanol metabolism may underlie the increased ethanol consumption and reduced ethanol-induced CPP phenotypes of *Taok2*−/− mice. We also observed an increase in water consumption of *Taok2*−/− mice relative to controls during the beginning of the drinking in the dark study. Interestingly, water consumption among mutant mice normalized to control levels later in the study, when ethanol consumption increased. Since water and ethanol consumption were measured on alternate days, a straightforward interaction between both is not clear. It will be important in future studies to investigate water and ethanol consumption concomitantly using a 2-bottle choice paradigm.

Global disruption of *Taok2* impairs ethanol-induced conditioned place preference, but fails to affect locomotor hyperactivity, in contrast to disruption of the *Drosophila* homolog, *dtao* (King *et al.* 2011). The ethanol-induced hyperactivity defect of *dtao* mutants has been shown to be mediated through regulation of the downstream target Par-1, a kinase that has been implicated in the activation of TAU protein and microtubule destabilization. In mammals, PAR1 activity is dependent on TAOK1 (Timm *et al.* 2003), however the regulation of PAR1 by TAOK2 or TAOK3 has not been investigated. The possible functional redundancy among mammalian TAOK proteins in regard to PAR1 regulation may explain why we fail to observe altered ethanol-induced hyperactivity defects in *Taok2* mutant mice.

In addition to the ethanol ataxia, sedation and consumption phenotypes of *Taok2*-disrupted mice, we observe that *Taok2*−/− mice exhibit reduced ethanol-induced CPP. It should be noted that several confounding factor may have affected the behavior of *Taok2*−/− mice in this assay: as previously mentioned, altered ethanol pharmacokinetics may have affected the formation of ethanol-induced CPP in this line. In addition, *Taok2*−/− mice are generally hyperactive and show impaired habituation to an open field. Hyperactivity has been reported to disrupt expression of ethanol-induced CPP (Gremel and Cunningham, 2007) and we observed a significant negative correlation between CPP score and activity during the test session ($r = -0.479$, $P < 0.05$).

The CPP assay measures the rewarding aspects of a drug of abuse through an associative learning process in which the subject learns to associate the subjective effects of a drug with a specific environment (Schechter and Calcagnetti, 1993). Functionally, TAOK2 is involved in the trafficking of cell adhesion molecules NCAM and Arcadlin (Yasuda *et al.* 2007), which may alter neuroplasticity and, in the case of NCAM disruption, cause spatial learning deficits (Becker *et al.* 1996; Bukalo *et al.* 2004; Cremer *et al.* 1994; Moy *et al.* 2009; Tang *et al.* 1998; Venero *et al.* 2006; Yamagata *et al.* 1999). Furthermore, ethanol may directly interact with cell adhesion molecules to affect their function (Aravelo *et al.* 2008; Dou *et al.* 2011). Taken together, these observations suggest a model whereby altered cell adhesion molecule trafficking and/or function in *Taok2* mutant mice may impair ethanol-induced CPP by disrupting normal associative learning processes. Such a model might assume that under normal conditions, the interaction of ethanol with cell adhesion molecules somehow

facilitates the associative learning that occurs during CPP training and this process is impaired when TAOK2 is absent, perhaps through its role in cell adhesion molecule trafficking. Additional experiments will be important to determine if *Taok2* mutants are deficient in other spatial learning tasks and to what degree trafficking of cell adhesion molecules may be involved. It is interesting to note that mice in which the cell adhesion molecule CD81 has been disrupted are impaired in cocaine-induced CPP (Michna *et al.* 2001), underscoring the importance of cell adhesion in this behavior.

Perhaps our most interesting finding is that although ethanol metabolism is altered in *Taok2−/−* mice, ethanol metabolism appears normal in mice in which the *Taok2* gene has been disrupted specifically in the nervous system, yet *Tao2fl/fl;Nes/cre* mice consume increased amounts of ethanol and in contrast to *Taok2−/−* mice, are sensitive to ethanol in the LORR assay. These data suggest that the nervous system-specific effect of TAOK2 on ethanol sensitivity is masked by peripheral disruption of *Taok2* in *Taok2−/−* mice and that the increased consumption of ethanol in *Taok2−/−* mice may be independent of the gene's effect on ethanol pharmacokinetics. To further characterize the function of TAOK2 in the nervous system in regulating behavioral sensitivity to ethanol, it will be important to measure BEC levels in *Tao2fl/fl;Nes/cre* mice upon LORR recovery as well as assessment of acute functional tolerance to ethanol.

Previous studies in humans and rodent models have suggested a negative correlation between initial sensitivity to ethanol and ethanol consumption (Boyce-Rustay et al., 2006; Crabbe *et al.* 1996; Fee *et al.* 2004; Kapfhamer *et al.* 2008; Naassila *et al.* 2002, 2004; Newton and Messing 2007; Palmer *et al.* 2004; Schuckit 1994). Based on our observation that nervous system-specific disruption of *Taok2* confers greater sensitivity to ethanol in the LORR assay, we might predict that *Taok2* mutants would consume less ethanol. Instead, we observe that *Taok2* mutants actually consume more ethanol than control mice. It may be noted that the LORR assay measures sensitivity to the sedative/hypnotic effects of ethanol, which may be mechanistically distinct from the ataxic effects of lower doses of ethanol and a more accurate predictor of ethanol consumption (Shuckit, 1994; Thiele *et al.* 2002). It will therefore be interesting to examine the ataxic effects of ethanol in *Taok2fl/fl;Nes/cre* mice.

Cell culture studies have also established a role for TAOK2 in the regulation of the actin cytoskeleton (Moore *et al.* 2000), a function that may also be critical for neuroplasticity (Huntley *et al.* 2002; Lisman *et al.* 2003; Matus 2000). A growing body of literature suggests that ethanol exposure may alter actin organization both pre- and post-synaptically (Funk *et al.* 2007; Mulholland and Chandler, 2007; Offenhäuser *et al.* 2006). Mice lacking the *Eps8* gene, which encodes an EGFR substrate and regulator of actin dynamics, are resistant to the acute intoxicating effects of ethanol and show increased ethanol consumption (Offenhäuser *et al.* 2006), supporting the notion that not only are actin dynamics ethanol-sensitive, but alterations in the cytoskeleton may affect behavioral responses to ethanol. Future experiments should address if altered actin cytoskeleton dynamics underlie the abnormal ethanol responsiveness of *Taok2* mutant mice.

Our data provide, to our knowledge, the first evidence that a Ste20p family kinase functions to regulate ethanol-dependent behaviors in mammals. Specifically, TAOK2 regulates

ethanol-induced ataxia, sedation, consumption and conditioned place preference. Previously, we have shown that the fly homolog, *dtao*, is required for normal ethanol-induced hyperactivity (King *et al.* 2011) and mutations in a related Ste20p family kinase gene, *happyhour*, confers resistance to ethanol-induced sedation in *Drosophila* (Corl *et al*. 2009). These data support the conservation of intracellular signaling through Ste20p family kinases in the regulation of behavioral responses to ethanol in flies and mammals and identify novel candidate genes for ethanol use disorders in humans.

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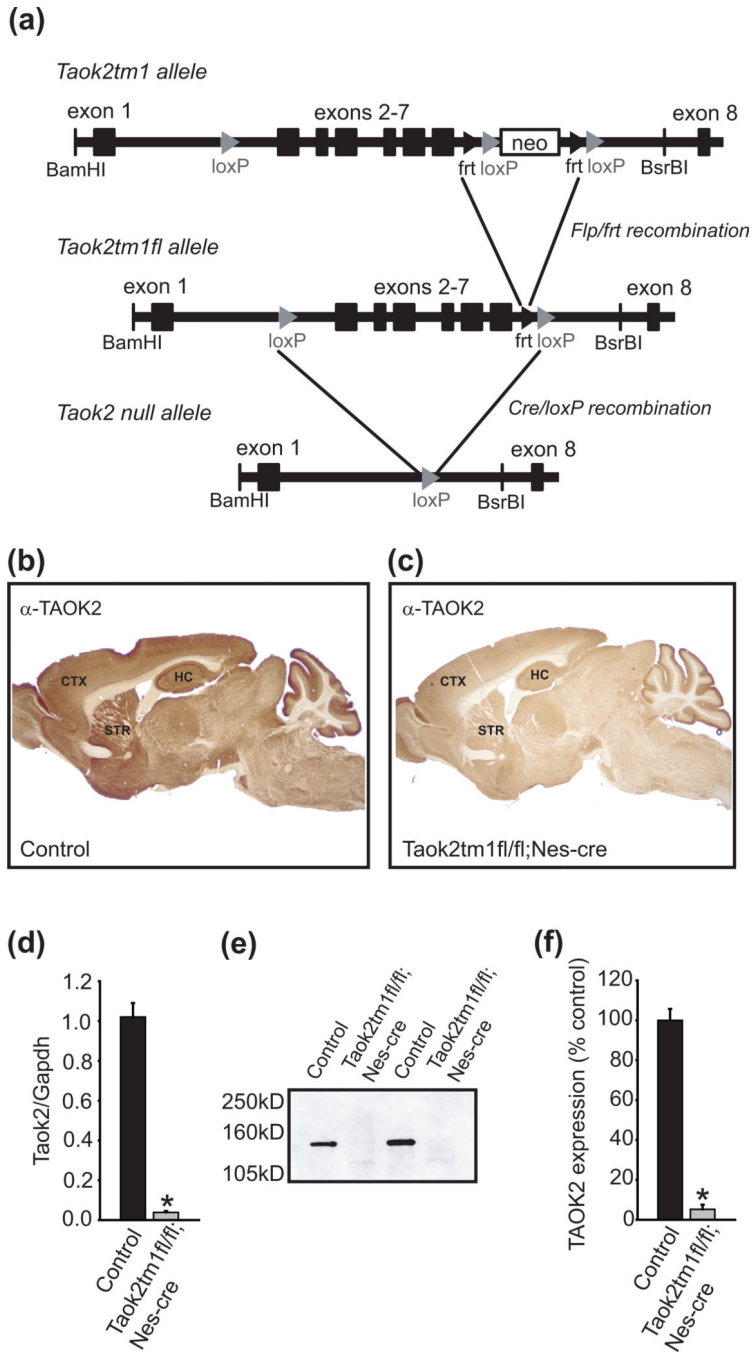


**Figure 1. Targeted disruption of the *Taok2* gene in mice**

(a) Schematic of the conditionally targeted *Taok2* allele. The *Taok2tm1* allele was generated by introduction of lox P sites flanking exons 2 though 7 of the *Taok2* gene. Within intron 7 is a neomycin resistance gene flanked by lox P and frt sites. Upon crossing this line to mice expressing Flpe recombinase, the neomycin selectable marker is removed along with one of the lox P sites to generate the *Taok2tm1fl* allele. In the presence of Cre recombinase, the remaining lox P sites are recombined into a single lox P site, removing exons 2 though 7 and the translational start site to generate the *Taok2tm1*Δ allele. (b) Brain sagittal section of a control mouse shows strong expression of TAOK2 by immunohistological analysis in all areas, especially in cortex, hippocampus and striatum. (c) Immunohistological analysis shows reduced TAOK2 expression in *Taok2tm1fl/fl;Nes-cre* brain.

(d) Quantitative PCR analysis of total mouse brain extracts shows near absence of *Taok2* transcript in *Taok2tm1fl/fl;Nes-cre* brain ($n = 4$) compared to control mice ($n = 2$). (e) Western blot showing comparative loss of TAOK2 protein in total brain lysates of *Taok2tm1fl/fl;Nes-cre* mice relative to controls. (f) Quantification of anti-TAOK2 signal from (e) comparing *Taok2tm1fl/fl;Nes-cre* mice ($n = 2$) and controls ($n = 2$). Lox P sites are denoted by grey chevrons, frt sites by black chevrons. Neo = neomycin resistance gene. The positions of restriction sites BamH1 and BsrB1 used in the generation of the targeting construct are indicated. CTX = cortex; HC = hippocampus; STR = striatum. Error bars are mean ± SEM. Asterisks indicate level of significance (* $P < 0.05$).

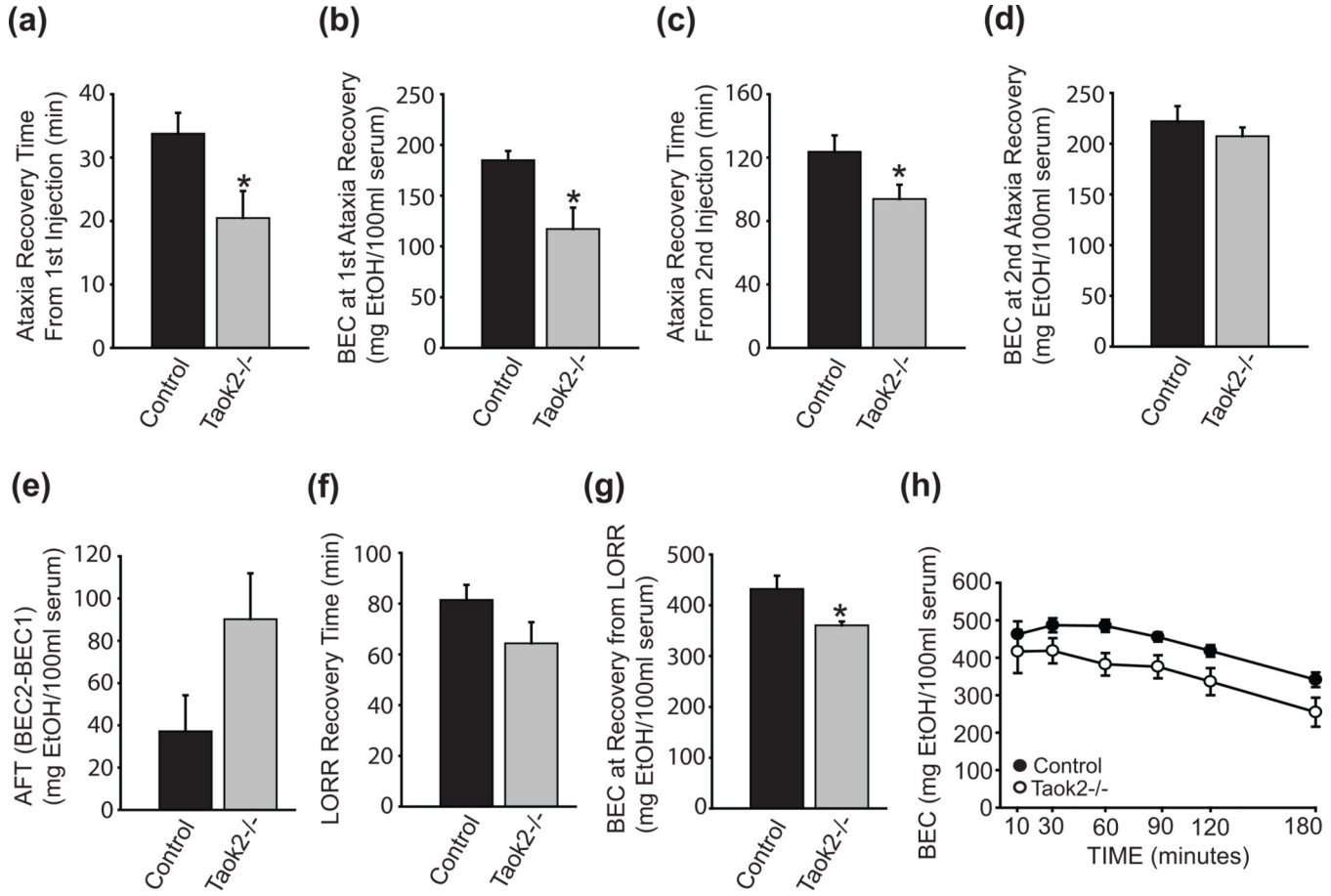


**Figure 2. Constitutive loss of *Taok2* in mice confers resistance to the acute ataxic and sedative effects of ethanol**

(a) *Taok2−/−* mice (*n* = 10) recover more quickly from the ataxic effects of an initial 1.5g/kg dose ethanol than control mice (*n* = 11). (b) BEC1 is reduced in *Taok2−/−* mice upon recovery from the ataxic effects of ethanol. (c) *Taok2−/−* mice recover more quickly from the ataxic effects of a second 1.5g/kg injection of ethanol (d) BEC2 is comparable among control and *Taok2−/−* mice upon recovery from a second injection of ethanol. (e) *Taok2−/−* mice show a trend toward increased ethanol-induced AFT. (f) *Taok2−/−* mice show a trend toward resistance to the sedative effects of ethanol in the LORR assay. (g) BEC levels are significantly decreased upon recovery of the ethanol-induced loss of righting reflex in *Taok2−/−* mice compared to control subjects. (h) Altered metabolism of a 4g/kg dose of ethanol is observed in *Taok2−/−* mice (*n* = 7) compared to control animals (*n* = 8). Error bars are mean ± SEM. Asterisks indicate level of significance (* $P < 0.05$).

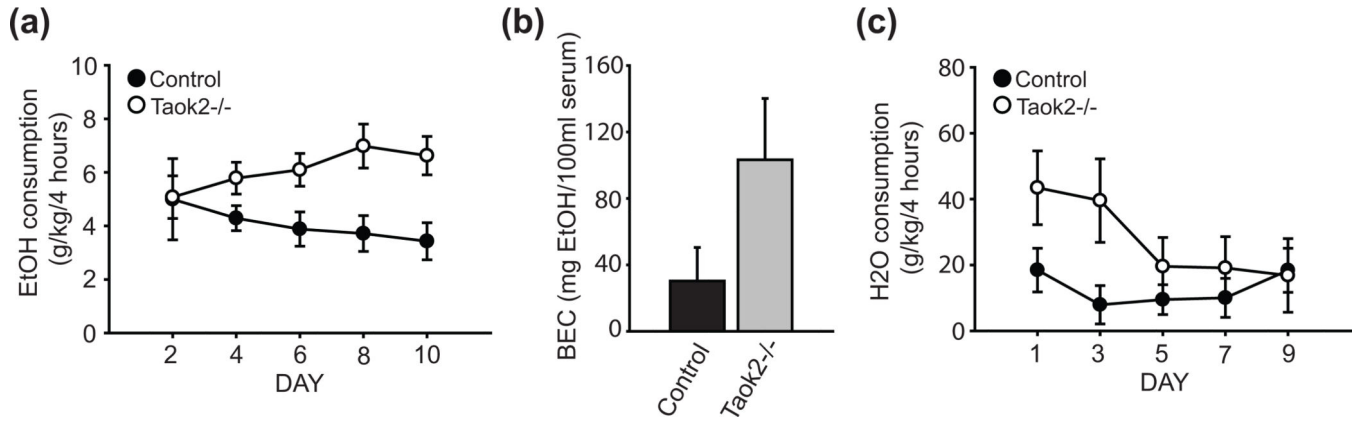


**Figure 3. Constitutive loss of *Taok2* increases ethanol consumption**

(a) *Taok2−/−* mice (*n* = 8) show increased ethanol consumption in the DID assay relative to controls (*n* = 8). (b) BEC values in *Taok2−/−* and control mice on the last day of ethanol access. (c) Water consumption on alternate days during the DID assay. Error bars are mean ± SEM.

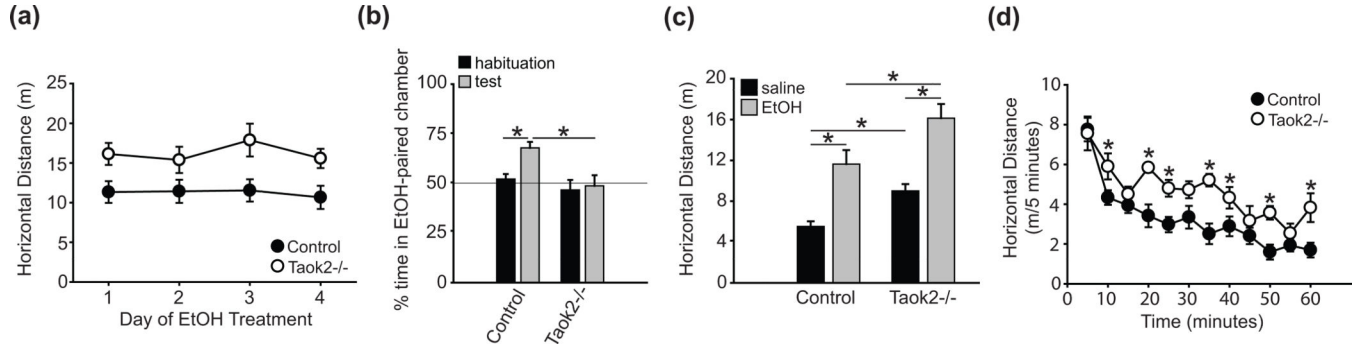


**Figure 4. Constitutive loss of *Taok2* in mice impairs ethanol-induced conditioned place preference without affecting ethanol-induced locomotor hyperactivity**

(a) 2g/kg ethanol dose used in CPP training does not result in locomotor sensitization (b) Ethanol-induced CPP is blocked in *Taok2*−/− mice ($n$ = 8); controls: ($n$ = 16). (c) Control and *Taok2*−/− mice show similar ethanol-induced locomotor stimulation in response to 2g/kg ethanol. Note the increased baseline activity in *Taok2*−/− mice. (d) *Taok2*−/− mice ($n$ = 10) show increased activity and impaired locomotor habituation to an open field apparatus compared to controls ($n$ = 12). Error bars are mean ± SEM. Asterisks indicate level of significance (* $P < 0.05$).

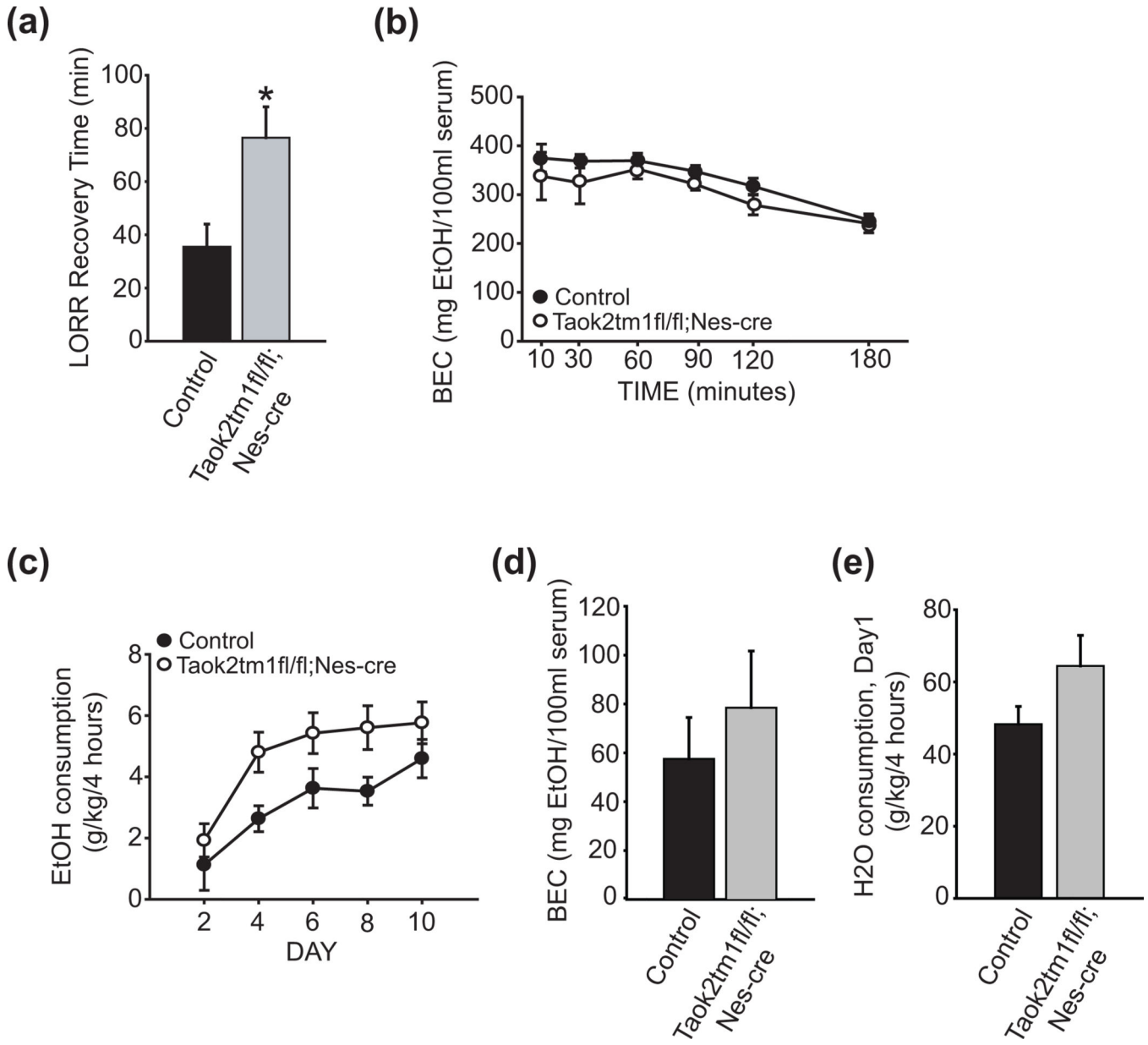


**Figure 5. Nervous system-specific loss of *Taok2* in mice alters ethanol-induced sedation and consumption**

(a) *Taok2tm1fl/fl;Nes-cre* mice ($n$ = 8) show increased recovery time relative to control animals ($n$ = 6) to 4g/kg ethanol in the loss of right reflex (LORR) assay. (b) Normal metabolism of a 4g/kg dose of ethanol is observed in *Taok2tm1fl/fl;Nes-cre* mice ($n$ = 5) compared to controls ($n$ = 6). (c) *Taok2tm1fl/fl;Nes-cre* mice ($n$ = 12) consume more ethanol than controls ($n$ = 9) in a limited-access, drinking in the dark (DID) assay. (d) BEC levels are comparable between *Taok2tm1fl/fl;Nes-cre* and control mice on the final day of ethanol access (e) $H_20$ consumption is normal in *Taok2tm1fl/fl;Nes-cre* mice. BEC = blood ethanol content. Error bars are mean ± SEM. Asterisks indicate level of significance (* $P < 0.05$).