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JNK Pathway Activation Is Controlled by Tao/TAOK3 to Modulate Ethanol Sensitivity

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Abstract

Neuronal signal transduction by the JNK MAP kinase pathway is altered by a broad array of stimuli including exposure to the widely abused drug ethanol, but the behavioral relevance and the regulation of JNK signaling is unclear. Here we demonstrate that JNK signaling functions downstream of the Sterile20 kinase family gene tao/taok3 to regulate the behavioral effects of acute ethanol exposure in both the fruit fly Drosophila and mice. In flies tao is required in neurons to promote sensitivity to the locomotor stimulant effects of acute ethanol exposure and to establish specific brain structures. Reduced expression of key JNK pathway genes substantially rescued the structural and behavioral phenotypes of tao mutants. Decreasing and increasing JNK pathway activity resulted in increased and decreased sensitivity to the locomotor stimulant properties of acute ethanol exposure, respectively. Further, JNK expression in a limited pattern of neurons that included brain regions implicated in ethanol responses was sufficient to restore normal behavior. Mice heterozygous for a disrupted allele of the homologous Taok3 gene (Taok3Gt+) were resistant to the acute sedative effects of ethanol. JNK activity was constitutively increased in brains of Taok3Gt+/ mice, and acute induction of phospho-JNK in brain tissue by ethanol was occluded in Taok3Gt+/- mice. Finally, acute administration of a JNK inhibitor conferred resistance to the sedative effects of ethanol in wild-type but not Taok3Gt/+ mice. Taken together, these data support a role of a TAO/TAOK3-JNK neuronal signaling pathway in regulating sensitivity to acute ethanol exposure in flies and in mice.

Introduction

Alcohol is a highly popular psychoactive drug that humans have manufactured and consumed since prehistoric times. However, alcohol abuse is widespread and it imposes high social and monetary costs on society [1]. The efficacy of behavioral and medical interventions for alcohol abusers may be improved by understanding the basic biological mechanisms by which alcohol coopts normal body functions to lead to maladaptive behaviors. Previous studies demonstrated that a low level of response to the inebriating effects of alcohol is associated with an increased risk for alcohol abuse [2,3]. This implies that genes that govern responding to acute alcohol exposure may contribute to alcohol abuse. Understanding the genetic basis of the pharmacological actions of alcohol may thus provide new approaches to treatment of alcohol use disorders. The development of sophisticated assays that model aspects of alcoholism in genetically tractable organisms such as the mouse and the fruit fly Drosophila has greatly accelerated the identification of genes that impact alcohol responses, and some of these studies have led to preclinical and clinical trials for new treatments [4,5,6].

Mitogen activated protein kinases (MAPKs) comprise multi-tiered signal transduction cascades involved in sensing diverse extracellular stimuli including growth factors, cytokines and environmental stressors. MAPK activation induces changes in transcription, cellular morphology, differentiation, proliferation, and in cell death [7,8]. Three partially overlapping MAPK pathways exist that are defined by the p38, extracellular signal regulated kinase (ERK), and c-jun N-terminal kinase (JNK) families [9,10,11].

Ethanol modulates MAPK signaling in multiple tissues, including the brain [9,12,13,14]. In the fruit fly Drosophila and in mice, inhibition of the EGFR/ERK pathway increases sensitivity to ethanol-induced sedation [4,15], and ERK also regulates ethanol sensitivity of the camouflage response in zebrafish [16,17]. Chronic ethanol consumption and ethanol withdrawal induces ERK-dependent changes in synaptic plasticity in the rat dorsolateral striatum [18], and pharmacological inhibition of ERK signaling in mice can alter operant responding for ethanol [19]. Additionally, both ERK and JNK are activated in the basolateral amygdala in response to ethanol-associated cues [20]. Long-term exposure to ethanol can also induce JNK and p38 activity in the...
Drosophila tao promotes sensitivity to the locomotor stimulating effects of acute ethanol exposure [22]. tao encodes a serine-threonine kinase of the GCK-VIII subfamily of the Ste20p family, characterized by a highly conserved N-terminal kinase domain and a C-terminal region that regulates cytoskeletal dynamics [23,24,25,26,27,28]. Fly TAO is homologous to mammalian TAOK1, TAOK2 and TAOK3 [23,29,30]. Cell culture experiments support a role for mammalian TAOK in MAPK signaling through the p38 and/or JNK pathways. All three TAOK proteins can activate p38 [31,32,33]. Further, JNK signaling can be activated by TAOK1 and TAOK2, whereas it can be inhibited by TAOK3 [23,27,30,31].

To identify TAOK signaling pathways we searched for genetic interactions between fly TAO and key constituents of candidate MAPK pathways. Here we demonstrate that JNK is negatively regulated by TAO/TAOK3 in vivo, and we provide evidence for a neuronal role for JNK signaling in the behavioral effects of acute ethanol exposure. Support for these findings come from two evolutionarily divergent model organisms, the mouse and the fruit fly, suggesting that the relationship between TAO/TAOK3 and JNK in ethanol behavioral responses is conserved.

Materials and Methods

Ethics Statement

All animal protocols were approved by the Ernest Gallo Clinic and Research Center (EGCRC) Institutional Animal Care and Use Committee (approval number 09.11.197).

Drosophila Strains and Behavior

Flies were maintained on standard cornmeal/molasses/yeast media at 25°C and 70% relative humidity with an approximately 16 hr/8 hr light/dark schedule. Mutant strains were obtained from the NIG Kyoto Stock Center (pucNP20), the Harvard Exelixis collection (bsK610715), and the Bloomington Drosophila Stock Center. All strains were outcrossed for at least five generations to the w1118 Berlin genetic background. Behavior experiments used groups of 20 genetically identical male flies, and experiments were repeated on multiple days. Ethanol-induced locomotion assays were performed as described previously [34,35]. Flies were exposed to 33% ethanol vapor (50:100 arbitrary flow units of ethanol:humidified air at a total flow of 5.5 L/min). Movies of ethanol-exposed flies were captured using Quantime, and analyzed using a modified version of DIAS motion tracking software. The average speed of a population of 20 flies was determined at 14 intervals of 15 sec duration over the course of a 21 min exposure, and plotted versus time of exposure. Ethanol-induced locomotor activity was quantified as the area under the curve during the hyperactive phase. Ethanol absorption was measured in populations of 20 male flies, exposed to 33% ethanol vapor for 21 minutes. Flies were flash frozen, homogenized, and assayed for ethanol content using an alcohol dehydrogenase-based spectrophotometric assay (Diagnostic Chemicals, Ltd).

Drosophila Immunohistochemistry

Brains were dissected in PBS, fixed for 30 min at room temperature in PBS with 4% formaldehyde, washed twice in PBS with 0.3% triton X-100 (PBT), and blocked in PBT with 5% normal goat serum. Samples were incubated with primary antibody overnight, diluted as follows in PBT with 5% normal goat serum: anti-Fasciclin II 1:200 (1D4), anti-repo 1:10 (8D12, Developmental Studies Hybridoma Bank, Iowa), anti-GFP 1:1000 (Invitrogn), anti-TH 1:100 (Immunostar). After treatment with primary antibody, samples were washed three times in PBT and incubated overnight with fluorescently labeled secondary antibody diluted 1:500 (Molecular Probes). Samples were washed three times in PBT before mounting in Vectashield (Vector Labs).

Generation of Taok3 genetrap (Taok3Gt) Mice

C57BL/6j-derived blastocysts were injected with the RKK451 genetrap line ES cells, obtained from Bay Genomics (www.genetrap.org), according to standard protocols [36]. The genetrap line was generated on a 129/OlaHsd (agouti) background. Several chimeras were crossed to wild-type C57BL/6j mice and germline transmission of the targeted allele in progeny was confirmed by identifying agouti pups. Backcrosses of the resulting F1 mice (Taok3Gt129/4B6 × C57BL/6j) for 5 or more generations were done to generate Taok3Gt+/+ heterozygous and wild-type controls for all studies, with two exceptions: studies to address the effects of Sg600129 on ethanol consumption and clearance were performed on C57BL/6j mice obtained from Jackson Laboratory (Bar Harbor, ME), and locomotor stimulation experiments were performed on a C57BL/6j X 129/SvF1 background. Ethanol has been reported to weakly stimulate activity in C57BL/6j mice [37]. For this reason, Taok3Gt+/4B6 were crossed to 129/SvF1 mice and the resulting F1 progeny were used for ethanol-induced locomotor stimulation experiments.

Mouse Genotypic Analysis

Mice were genotyped by PCR with DNA isolated from tail biopsies using standard protocols. The following primers were used to amplify a fragment of the genetrap insertion: forward: 5’- TTATCGATGAGCGTGGTTATGC-3’; reverse: 5’- GCGCGTACACTCGGCAATAATTC-3’.

Quantitative RT-PCR (qPCR)

Total RNA was isolated from whole brain tissue using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) 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, 1 x 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. Taqman probes used in this study were Taok3 (spanning exons 10–11): Mm001195800_m1, rodent Gapdh: 4352932E, Drosophila puc: Dm02135504_m1, Drosophila RpL32: Dm021351827_g1 (Applied Biosystems). Data were analyzed using the comparative C_T method ('Applied Biosystems user bulletin #2).

Protein Analysis

Protein extracts were derived from whole brain tissue in RIPA buffer containing phosphatase inhibitor (Sigma Aldrich, Inc., St. Louis, MO, USA) using standard techniques. For Western analysis, 10 μg protein samples were electrophoresed on NuPAGE 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes (Invitrogen). Primary antibodies were goat anti-TAOK3 (ab21205, ABCAM, Inc., Cambridge, MA) 1:1000, rabbit anti-phospho-
Mouse Ethanol Self-administration

Oral alcohol self-administration was examined using a limited access, drinking in the dark (DID) 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% v/v ethanol during the same time of day, on alternate days for 12 days (6 days of ethanol access) and ethanol consumption was calculated. For pharmacological studies, subjects received a subcutaneous vehicle injection (Tween 80/0.9% NaCl) 10 min prior to ethanol availability on the first three days of ethanol access to acclimate subjects to injections. On subsequent ethanol access days subjects in the experimental group received a 5 mg/kg subcutaneous injection of inhibitor JNK II/Tween 80/0.9% NaCl, whereas control subjects continued to receive vehicle.

Results

Mutation of JNK Pathway Genes Suppresses the Brain Structural Defects of tao Mutants

Because both fly tao and mouse Taok3 were identified as negative regulators of the JNK MAP kinase signaling in cultured cells [23,27,30,31,42], we hypothesized that tao may act as a negative regulator of JNK signaling in the fly brain. Mutation of fly tao results in strong resistance to the locomotor stimulant properties of ethanol, and also results in highly penetrant structural defects in key neuropils of the central brain, including the mushroom bodies [22]. As a first test, we determined whether mutations in JNK pathway genes suppressed or enhanced the mushroom body structural defects of loss of function allele taoEP1455, in which axons of mushroom body α/β neurons fail to form lobes [22], (Fig. 1A, D).

We examined the morphology of mushroom body α/β lobes in the brains of flies mutant for tao combined with loss-of-function alleles of the lone Drosophila JNK MAP kinase gene basket (bsk). Heterozygous hypomorphic bsk alleles had little to no effect on the mushroom body morphology defects in male flies hemizygous for taoEP1455 (data not shown). However, a viable combination of two hypomorphic alleles, bsk+kb, that did not affect mushroom body morphology (Fig. 1B), almost completely restored normal mushroom body morphology in taoEP1455 mutants (Fig. 1C).

We further examined the brain morphology of these bsk mutants by immunostaining with anti-tyrosine hydroxylase (TH), which marks dopaminergic neurons, specific sets of which control ethanol-induced hyperactivity [43], and observed no defects in cell number or anatomical projection (data not shown). To confirm that the JNK pathway was responsible, we tested mutations in the upstream JNK MAPK kinase hemipterus (hep). For this test we used females because hemizygous strong loss-of-function muta-
Mutations in the JNK MAPK Gene basket Affect Ethanol-induced Hyperactivity and Interact Genetically with tao

Most mutations result in strong resistance to the locomotor stimulant effects of acute ethanol exposure in flies [22]. To determine if decreased JNK signaling affects sensitivity to acute ethanol exposure, we tested male flies heterozygous for conventional loss-of-function mutations in bsk [14,45]. bsk2/+ flies showed normal ethanol-induced hyperactivity, but bsk2/+ and bsk2/+ heterozygotes as well as flies heterozygous for bskEP1455, a P-element insertion in bsk that failed to complement the hypomorphic bsk+ allele for lethality displayed increased ethanol-induced hyperactivity (Fig. 2A–D), suggesting that reduced JNK signaling increased sensitivity to the locomotor stimulant effects of ethanol. These mutants showed normal kinetics of ethanol accumulation, indicating that the bsk phenotype is not due to altered ethanol absorption or metabolism (Fig. 2 legend).

To test whether the negative genetic interaction between tao and JNK signaling extends to behavior, we assayed the effects of acute ethanol exposure on double mutant flies that were hemizygous for taoEP1455 and heterozygous for bskEP1455. The double mutant flies displayed increased ethanol-induced locomotion relative to flies singly mutant for tao (Fig. 2E), indicating that decreased JNK signaling was sufficient to restore normal hyperactivity to tao, and consistent with a negative regulatory interaction between tao and JNK signaling for ethanol behavior. Interestingly, heterozygous bskEP1455 did not suppress the MB morphology defect caused by taoEP1455 (Fig. 2F,G); these flies completely lacked MB α/β lobes. These data indicate that the increase in hyperactivity observed in the bsk mutant is independent of any structural effect on the MB and suggest that JNK signaling may be required in one or more brain loci that compensate for the loss of the MB α/β lobes caused by the taoEP1455 mutation.

JNK Phosphatase Puckered Promotes Ethanol-induced Hyperactivity

Our finding that bsk mutants exhibit increased sensitivity to the locomotor stimulant effects of acute ethanol exposure suggested that the JNK signaling pathway is important for this behavior. Expression of the MAPK phosphatase Puckered (Puc) is induced by Bsk activation, and subsequently Puc dephosphorylates and inactivates Bsk in a negative feedback loop [46,47]. Consequently, loss of puc function increases JNK activation in response to upstream signals. Microarray studies indicate that puc expression is induced in response to acute ethanol exposure, with kinetics suggesting that the activation of the JNK pathway might play a role in ethanol-induced behavior [35].

To confirm ethanol-induction of puc transcription, we exposed wild-type male flies to ethanol vapor or air and measured puc RNA levels in whole head extracts using quantitative PCR analysis. We found that puc levels were increased approximately two-fold in ethanol-treated flies, consistent with activation of JNK signaling by acute ethanol exposure (Fig. 3A). We next tested whether decreased puc expression affected ethanol-induced hyperactivity. Flies heterozygous for puc+, a strong loss-of-function allele of puc, showed greatly reduced ethanol-induced hyperactivity (Fig. 3B,C), indicating that reduced puc activity and increased activation of the JNK pathway reduced this behavior, consistent with the increased hyperactivity observed in bsk mutants. As with bsk mutants, we examined brain morphology in puc+/+ flies using anti-FasII, and anti-TH staining, and observed no defects (Fig. 3D, data not shown). Further, puc+/+ flies had normal kinetics of ethanol accumulation (Fig. 3 legend). We conclude that increased JNK signaling decreases sensitivity to the locomotor stimulant properties of ethanol in Drosophila. While a developmental role for JNK...
expression system to test if neuron-specific expression of puc could rescue the ethanol phenotype of puc+/+ flies. We crossed flies carrying puc and a UAS-puc transgene to the pan-neuronal driver line elavG4, and compared the ethanol-induced hyperactivity of the progeny to single-transgenic controls. We found that ethanol-induced hyperactivity was restored in puc+/+ mutants with elavG4 and UAS-puc, showing that the behavioral defect of puc+/+ flies can be rescued with neuron-specific expression of puc (Fig. 3E). elavG4 did not affect ethanol-induced hyperactivity of puc+/+ flies in the absence of the UAS-puc transgene, indicating that expression of Gal4 alone in neurons was not responsible for the rescue of the puc+/+ phenotype (Fig. 3F). Thus expression of puc solely in neurons is sufficient to promote ethanol-induced hyperactivity.

To define the site of action for puc for ethanol-induced hyperactivity, we examined the expression pattern of NP1311, a Gal4 enhancer trap transposon inserted in the third intron of the puc locus. We asked whether expression of puc specifically in this limited pattern was sufficient to rescue the behavioral phenotype of puc+/+ mutants. Flies heterozygous for NP1311 displayed normal levels of ethanol-induced hyperactivity, and restricted expression of puc in the NP1311 pattern was sufficient to restore ethanol-stimulated locomotion to control levels in puc+/+ mutants (Fig. 4D). These results indicate that puc expression in the pattern of NP1311 expression is sufficient to promote the locomotor stimulant properties of ethanol.

To define the expression pattern of NP1311, brains of NP1311+/;UAS-CD8:GFP/+ and NP1311+/;UAS-nls:GFP/+ flies were dissected and GFP expression visualized. NP1311 drove GFP expression strongly in a number of adult brain loci, including the α/β neurons of the mushroom bodies (Fig. 4B,C). However, GFP expression was absent from neurons of the ellipsoid body, a brain region that is required to promote ethanol-stimulated locomotion [43], (Fig. 4D). Expression was seen in a number of dopaminergic neurons, including cells in the PPL1 (Fig. 4F), PPL2 (Fig. 4G), and PAL (Fig. 4H) clusters. Some expression was seen also in the PPM3 cluster (Fig. 4I), which also affects ethanol-induced locomotion. No expression was seen in the PPM1/2 (Fig. 4J) or PAM (Fig. 4K) dopaminergic clusters. No expression was observed in glia (Fig. 4E). The NP1311 enhancer trap likely captures a significant portion of the endogenous expression pattern of puc [48].

Disruption of Taok3 in Mice Alters Acute Sensitivity to Ethanol

Because tao interacts genetically with the JNK pathway and because JNK pathway activation affects ethanol-induced behavior in Drosophila we investigated whether these relationships were conserved in ethanol-dependent behaviors in mice. The Tao kinase family is represented in vertebrates by three orthologous genes, Taok1, Taok2 and Taok3 [24,32]. Based on our observation that Drosophila tao interacts negatively with the JNK MAPK genes bsk and hep, we chose to investigate Taok3, the only TAOK that can inhibit JNK activity in cultured cells [30]. We generated Taok3 genetrap mice using the cell line RRK451 from the International Gene Trap Consortium (http://www.genetrap.org/). This cell line contains a genetrap insertion in the eighth intron of the Taok3 gene and is predicted to result in a fusion transcript of exons 1–8 of Taok3 with the gene encoding β-galactosidase, truncating the endogenous Taok3 transcript within sequence encoding a portion of the kinase domain (Fig. 5A). Mice heterozygous for the Taok3 genetrap allele (Taok3Gt/+ ) were viable and normal in appearance. However, we failed to recover homozygous Taok3Gt/Gt mice, suggesting that disruption of both copies of Taok3 results in

Neuronal Expression of puc Rescues Ethanol-induced Hyperactivity in puc Mutants

Tao activity is both necessary and sufficient in neurons in flies for increasing sensitivity to the locomotor stimulant properties of ethanol [22], and our results indicate that JNK signaling acts downstream of Tao. To investigate whether JNK acts in neurons to regulate ethanol responses, we used the Gal4-UAS heterologous

signaling in ethanol-induced behavior cannot be ruled out, the induction of JNK pathway activity by ethanol exposure suggests that the pathway more directly modulates ethanol-induced behaviors.

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Figure 2. Genetic interaction between tao and bsk. A, Flies exposed to a continuous stream of ethanol vapor (black bar on x-axis) increase locomotor activity over time. A, bsk+/+ B, bsk27/+ and C, bsk27/+. (bSk2/Y is bsk(Y1915) mutants show enhanced ethanol-stimulated locomotion. Locomotor speed measured just prior to ethanol exposure is indicated at minute 1 for each graph. D, Quantification of traces in A–C measured as the distance traveled during the hyperactive phase. E, Mutation of bsk suppressed the decreased ethanol-induced locomotor stimulation of tao2/Y flies. F, bsk(Y1915) did not affect mushroom body morphology. Anterior projections of adult male wholemount brains stained with anti-FasIII. α/β lobes are indicated. G, Lack of suppression of mushroom body structural defects in tao2/Y, bsk(Y1915)/+ double mutant. Asterisk indicates posterior axon mass characteristic of tao2/Y flies. Ethanol absorption was unaltered in bsk mutants compared to the genetic background control strain (p = 0.5229, one-way ANOVA). Genotypes were compared using one-way ANOVA with Bonferroni correction. **p < 0.01, ***p < 0.0001. Scale bars = 15 μm.

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Neuronal TAO/JNK Controls Ethanol Sensitivity

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embryonic lethality and that the Taok3Gt allele may be hypomorphic or null.

To further characterize Taok3 gene disruption by the genetrap allele, we measured Taok3 transcript levels in brain tissue obtained from adult Taok3Gt/+ mice by quantitative PCR and observed a 40% reduction of endogenous Taok3 transcript (Fig. 5B). Similarly, we observed a 50% reduction in TAOK3 protein levels in brain tissue from Taok3Gt/+ mice by Western analysis (Fig. 5C,D), indicating that the genetrap insertion effectively disrupted the Taok3 gene. We also observed wild-type levels of TAOK1 and TAOK2 in brain tissue from Taok3Gt/+ mice (data not shown), indicating that disruption of one copy of the Taok3 gene did not result in compensatory changes in protein levels of TAOK1 or TAOK2.

Because disruption of the Drosophila tao gene strongly reduced the locomotor stimulant properties of acute ethanol exposure [22], we assessed the behavioral response of Taok3Gt/+ mice to ethanol in a battery of tests. We first assessed Taok3Gt/+ and wild-type control mice for hyperactivity induced by a 1 g/kg dose of ethanol. We observed a normal response (Fig. 5E), indicating that disruption of one copy of the Taok3 gene was not sufficient to affect ethanol-stimulated locomotion.

In contrast, when we tested Taok3Gt/+ mice in the loss-of-righting reflex (LORR) assay, a test of the sedative-hypnotic effects of ethanol, we observed a significant decrease in recovery time (Fig. 5F). We assessed ethanol metabolism/clearance of the same 4 g/kg dose ethanol in a drug-naïve group of animals and failed to detect a difference between genotypes (Fig. 5G), suggesting that the resistance of Taok3Gt/+ mice to the sedative properties of ethanol was not the result of altered ethanol pharmacokinetics.

Studies in humans and in rodent models suggested that initial sensitivity to ethanol may predict subsequent ethanol consumption [2,49,50,51,52,53,54,55,56]. However, using a limited access paradigm in which mice gained access to a single 20% w/v solution of ethanol for 4 hours during the dark or active phase of their circadian cycle, we did not detect a genotypic difference in ethanol consumption (Fig. 5H). Thus, reduction of Taok3 expression conferred resistance to the sedative effects of ethanol, but it did not alter ethanol consumption or ethanol-stimulated locomotion.

Figure 3. puc is required in neurons for ethanol-induced locomotor stimulation. A. Quantitative PCR detection of puc expression levels in fly head extracts following 30 min ethanol or air control exposure. **p=0.0024 t test. B. Reduced ethanol-induced locomotor stimulation in puc1/+ flies. C. Quantification of traces shown in B. ***p<0.0001 t test. Ethanol absorption of puc1/+ was unaffected (p = 0.9607 t test). D. Mushroom body morphology of puc1/+ mutants was normal. Scale bar = 10 μm. E. Decreased ethanol-induced locomotor stimulation of puc1/+ mutants rescued by neuron-specific expression of puc. puc1/+ mutants carrying a UAS-puc transgene were crossed to the elavC155-Gal4 to restore puc expression levels specifically in neurons. F. elavC155-Gal4 did not increase ethanol-induced locomotor stimulation in puc1/+ mutants.

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Figure 4. Expression pattern of a *puc* reporter line in the central brain. A, Rescue of decreased *puc*+/ ethanol-induced locomotor stimulation in the NP1311 expression pattern. Rescue genotypes were compared using one-way ANOVA with Bonferroni correction. *p<0.05,
**p < 0.01. B–K Expression pattern of NP1311 puc enhancer trap detected with UAS-GFP. B, Confocal projection of the central brain, with NP1311 expression visualized with membrane-bound GFP (UAS-CD8.GFP, counterstained with NC82 to visualized neuropil. C, Projection showing mushroom body s/β and y lobes, counterstained with anti-FasII. Pt, pars intercerebralis. D, Projection showing lack of expression in the ellipsoid body (EB), counterstained with anti-FasII. E, NP1311 is neuron-specific. Individual cells in NP1311/+; UAS-nls.GFP/+ brains counterstained with anti-Repo to mark glia. F–K NP1311 expression in dopaminergic neuron clusters. Arrowheads indicate cells with overlapping nls.GFP (green) and tyrosine hydroxylase (TH, magenta) staining. F, PPL1 cluster, G, PPL2, H, PAL, and I, PPM3. No overlap was observed in J, PPM1/2, or K, PAM. Scale bars = 20 μm (B), 10 μm (CD), or 5 μm (LE, FK).
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JNK is Negatively Regulated by TAOK3 in Mice and is Activated in the Brain by Ethanol

Mammalian cell culture experiments demonstrated that TAOK3 is a negative regulator of JNK activity; indeed, TAOK3 initially was named “JIK” for “JNK Inhibitor Kinase” [30]. To determine if TAOK3 inhibits JNK in vivo, we performed Western analysis using total brain extracts from Taok3Gt/+ and control mice. We found that phospho-JNK levels were constitutively increased in brains of Taok3Gt/+ mice, suggesting that TAOK3 negatively regulates JNK in vivo (Fig. 6A,B).

JNK activity in the brain might also be regulated by ethanol exposure [21,57,38,59,60,61,62,63,64,65]. We examined whether JNK activation was affected by treating mice with an acute, sedating dose of ethanol (4 g/kg). We observed an increase in phospho-JNK upon ethanol treatment in the brains of wild-type mice compared to saline-treated controls, indicating that acute ethanol treatment increased JNK activity in the brain (Fig. 6A,B). However, acute ethanol treatment did not increase the level of phospho-JNK in brains of Taok3Gt/+ mice relative to controls (Fig. 6A,B). Thus, while reduced TAOK3 levels increased constitutive JNK pathway activity in the brain, it prevented increased JNK activation that was elicited by acute ethanol exposure.

JNK Inhibition Confers Resistance to Ethanol-induced Sedation in Mice

Based on our data suggesting that TAOK3 promotes ethanol-induced sedation and that ethanol increases activated JNK in a TAOK3-dependent manner, we predicted that acute inhibition of the JNK pathway might affect ethanol-induced sedation. We tested this hypothesis by administering to wild-type mice 5 mg/kg of SP600125, which acts as a reversible ATP-competitive inhibitor of JNK MAP kinases [66], 30 minutes prior to the LORR assay. We found that SP600125 treatment conferred resistance to the sedating effects of ethanol in wild-type mice (Fig. 7A). In contrast, we found that SP600125 treatment failed to alter the duration of ethanol-induced sedation in Taok3Gt/+ mice (Fig. 7A). The same dose of JNK inhibitor did not alter clearance of 4 g/kg ethanol (Fig. 7B), suggesting that the effect of SP600125 on ethanol-induced sedation was not the result of altered ethanol pharma-

Figure 5. Characterization of Taok3Gt/+ mice. A, Schematic of the Taok3 locus with the genetrap insertion located between exons 8 and 9 of the gene. Exon numbers are indicated. “β geo” = β galactosidase/neomycin resistance markers within the genetrap insertion. B, Quantitative PCR data showing reduced Taok3 transcript in brain tissue of Taok3Gt/+ mice compared to controls. * p < 0.05 t test. C, D Western blot analysis of brain lysates from Taok3Gt/+ and wild-type control mice showing reduced TAOK3 protein levels in mutants. * p < 0.05 t test. E, Taok3Gt/+ mice exhibit normal basal activity and locomotor stimulation in response to 1 g/kg ethanol, p = 0.27 ANOVA. F, Taok3Gt/+ mice are resistant to ethanol-induced sedation in the LORR assay following treatment with 4 g/kg ethanol. * p < 0.05 t test. G, Ethanol absorption/clearance is unaffected by the Taok3Gt allele, BEC = blood ethanol content. p = 0.07, repeated measures ANOVA. H, Voluntary ethanol consumption is normal in Taok3Gt/+ mice. p = 0.85, repeated measures ANOVA.
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Neuronal TAO/JNK Controls Ethanol Sensitivity

Interaction experiments allow us to propose a signaling framework for ethanol behavioral responses that includes TAO/TAOK3, JNK, and the kinase Par-1.

While manipulation of JNK pathway signaling caused ethanol-induced behavioral phenotypes in both flies and mice, the behaviors affected were different in these organisms. In Drosophila, JNK signaling may affect ethanol-induced locomotion specifically. A previous study did not detect changes in acute ethanol sedation sensitivity in flies that overexpressed wild-type, activated, and dominant negative JNK pathway proteins in the nervous system [4]. We similarly observed no effect on sedation sensitivity of the JNK pathway hypomorphic mutations (data not shown). Greater reductions in puc and bsk expression did result in altered ethanol sedation sensitivity, however these mutants also exhibited deficits in general locomotion, complicating the interpretation of ethanol behavioral phenotypes. RNAi directed against bsk has been reported to reduce sleep by increasing locomotion [68]. We believe, however, that the increase in ethanol-induced locomotion is mechanistically distinct, because in two of the three bsk alleles we tested we did not observe increased pre-exposure locomotor activity.

Conversely, mouse Taok3 mutants with constitutively activated JNK showed altered ethanol-induced sedation, but showed no effect on ethanol-induced locomotor activation. Acute disruption of JNK signaling in mice by the inhibitor SP600125 also affected ethanol-induced sedation, though we have not yet tested its effects on ethanol-induced locomotor activation. Gene family diversification may explain the species specific phenotypes: in mammals TAOK1 or TAOK2 may functionally compensate for TAOK3 with respect to ethanol-stimulated locomotion. As an initial test of this hypothesis, we observed unaltered ethanol-stimulated locomotion in mice in which both copies of the Taok1 or Taok2 gene were disrupted [69]. The analysis of compound Taok1/Taok2/Taok3 mutants may help clarify to what extent, if any, these genes are functionally redundant with respect to ethanol-stimulated locomotion. The JNK and other MAPK signaling pathway gene families also increase in complexity from flies to mice, such that additional positive and negative signaling activities may mask behaviorally relevant actions of specific JNK isoforms. Alternatively, the TAO/JNK signaling pathway may be used differently in different organisms.

We identified the MAPK phosphatase Puc as a key mediator of JNK pathway effects on ethanol-induced behavior in Drosophila. puc expression was induced upon ethanol exposure, decreased puc expression conferred resistance to the locomotor stimulating effects of ethanol.

**Figure 6. JNK activity is induced by ethanol.** A, B. Western blot analysis of brain lysates from Taok3Gt/+ and wild-type control mice showing induction of phospho-JNK by ethanol in wild-type mice and increased phospho-JNK levels in brains of saline-treated Taok3Gt/+ mice relative to controls. *p<0.05 t test. Ethanol induction of phospho-JNK is occluded in Taok3Gt/+ mice.

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**Figure 7. JNK activity modulates behavioral sensitivity to ethanol in mice.** A. Pharmacological inhibition of JNK confers resistance to ethanol-induced sedation in wild-type but not Taok3Gt/+ mice. Mice were treated subcutaneously with vehicle or 5 mg/kg JNK inhibitor SP600125, 30 minutes prior to the LORR assay. **p<0.01, t test. B. SP600125 (5 mg/kg) does not alter ethanol pharmacokinetics. p = 0.94 repeated measures ANOVA. BEC = blood ethanol content. C SP600125 (5 mg/kg) does not affect voluntary ethanol consumption. p = 0.73 repeated measures ANOVA. All mice received vehicle injections on days 1–3 before assignment to control or experimental groups.

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of ethanol, and the behavioral actions of puc localized to the nervous system. Importantly, *puc* expression induction indicates active JNK signaling [47], and is consistent with the increased phosho-JNK observed in the brains of ethanol treated mice. Combined with the behavioral phenotypes of *bsk/JNK* and *hep/JNK*, the *puc* findings indicate that JNK activity normally promotes ethanol-stimulated locomotion in *Drosophila*. Puc is related to the DUSP family of mammalian MAPK phosphatases that are capable of dephosphorylating MAP kinases of all three families [70,71,72]. The modulation of JNK activity in mammals may be accomplished by multiple regulatory phosphatases in a more complex manner than in flies.

We also found that expression of Puc in a limited set of neurons is sufficient to promote sensitivity to the locomotor stimulant properties of ethanol. The expression pattern of the *puc* *NP1311* Gal4 enhancer trap used for genetic rescue includes the MB α/β neurons, and the function of these neurons is required for promoting ethanol-stimulated locomotion [22]. However, *bsk* mutations can restore ethanol-induced hyperactivity in *tau* mutants lacking the MB α/β axon lobes, implying that the MB are not the primary locus at which JNK signaling affects ethanol-stimulated locomotion. Instead, JNK signaling may act in a separate neural locus that can compensate for function of the MB in this behavior. The R2/4 neurons of the ellipsoid body are also required for normal ethanol-stimulated locomotion [43], however these neurons are not included in the *NP1311* expression pattern. The *NP1311* pattern does encompass some dopaminergic neurons, including a small number of neurons in the PPM3 cluster, which are known to mediate ethanol-stimulated hyperactivity [43]. *NP1311* also expresses strongly in neurons of the pars intercerebralis, which affect ethanol-induced sedation sensitivity [73]. However, the circuitry governing ethanol-induced behaviors is poorly defined, and more detailed mapping of the requirement for JNK signaling using newer Gal4 enhancer traps that more sparsely label defined, and more detailed mapping of the requirement for JNK signaling may act in a separate neural locus that can compensate for function of the MB in this behavior.

In mice both pharmacological inhibition of the JNK pathway and a 50% reduction in *Taok3* expression that increases activated JNK levels speeded recovery from ethanol-induced sedation. While these experiments clearly implicate JNK signaling in ethanol sensitivity in mice, a simple relationship between the level of activated JNK in the mouse brain and ethanol-induced sedation may not exist. However, *Taok3* mutant mice also failed to increase levels of activated JNK upon exposure to ethanol. It may be, therefore, that ethanol-induced behavior is affected by the net increase in activated JNK level over baseline, rather than by its absolute level. Consistent with this idea is the observation that *Taok3Gt/+* mice are resistant to the effects of SP600125 on ethanol-induced sedation and provides additional evidence that TAOK3 signals through JNK rather than parallel pathways to regulate ethanol-induced sedation. Alternatively, it is possible that *Taok3* mutations specifically affect the activation state of particular JNK isoforms, while acute inhibition of all JNK isoforms by SP600125 has a different aggregate effect on behavior.

Repeated high level (binge) or chronic ethanol intake induces oxidative stress that is linked to tissue damage in organs including the liver, and it is well established that oxidative stress induces JNK signaling [75]. Similar ethanol exposure conditions can also lead to neuronal death through less well defined mechanisms that may include oxidative stress, excitotoxicity, or thiamine deficiency [76,77]. The ethanol concentrations that resulted in *tao/Taok3* and JNK pathway activation and behavioral phenotypes in mutants to a single acute exposure were too low to result in tissue damage [67], thus it is likely that a distinct JNK signaling mechanism is engaged. It remains possible, however, that acute ethanol exposure results in a neuronal oxidative stress response that is below the threshold for tissue damage, but that is sufficient to affect JNK signaling and behavior. Oxidative stress has been proposed to contribute to both normal synaptic development and synaptic plasticity [70]. Further, evidence in flies suggests that JNK signaling can affect both neuronal development and synaptic morphology [79,80,81].

Decreased *Drosophila* Par-1 expression also suppresses the structural and ethanol behavioral phenotypes of *tau* mutants [22]. This raises the possibility that Par-1 and the JNK pathway may define a signaling pathway with TAO. Both Par-1 and JNK are implicated in the planar polarity (PP) pathway that controls oriented growth of cells in tissue morphogenesis [92,93,94]. The core constituents of the PP pathway also control multiple steps of neuronal development, including neurite outgrowth, axon path-finding, and axon branching [85,86,87]. Accordingly, the PP pathway is required for the appropriate development of the *Drosophila* mushroom bodies [97]. Furthermore, behavioral roles were recently found for specific genes in the core PP pathway, including susceptibility to seizures and to social defeat stress [98,99], and core PP pathway genes are expressed in the adult nervous system. Members of the TAO kinase family interact with both PAR-1 and the JNK pathway [23,27,30,31,90], however a direct connection of TAO kinases to the PP pathway has not been established. At a cellular level Par-1, TAO kinases, and the JNK pathway all regulate the structural dynamics of the cytoskeleton [91,92], and may exert these effects through the microtubule binding protein Tau [22,93,94,95]. Indeed, the cytoskeleton is markedly rearranged following acute ethanol exposure [96,97]. Although we failed to detect gross morphological defects in brain structure of *Taok3Gt/+* mice by immunohistological analysis (data not shown), we can not exclude the possibility that anomalies in brain morphology and/or neuronal connectivity underlie the ethanol sensitivity phenotype of these mice.

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Author Contributions

Conceived and designed the experiments: DK IK MEZ JPL FWW. Performed the experiments: DK IK UH FWW. Analyzed the data: DK IK 11111. Epub ahead of print].

References

1. Nutt DJ, King LA, Phillips LD, Independent Scientific Committee on Drugs (2010) Drug harms in the UK: a multicriteria decision analysis. Lancet 376: 1538-1563.
2. Schuekht MA (1994) Low level of response to alcohol as a predictor of future alcoholism. Am J Psychiatry 151: 184–189.
3. Morean ME, Corbin WR (2010) Subjective response to alcohol: a critical review of the literature. Alcohol Clin Exp Res 34: 385–395.
4. Corl AB, Berger KH, Ophir-Shohat G, Gesch J, Simms JA, et al. (2009) Happyhour, a Ste20 family kinase, implicates EGFR signaling in ethanol-induced behaviors. Cell 137: 949–960.
5. Davies DL, Bortolato M, Finn DA, Ramaker MJ, Barak S, et al. (2012) Recent advances in the discovery and preclinical testing of novel compounds for the prevention and/or treatment of alcohol use disorders. Alcohol Clin Exp Res. Doi:10.1111/j.1530-0277.2012.01946.x. [Epub ahead of print].
Hutchison M, Berman KS, Cobb MH (1998) Isolation of TAO1, a protein kinase that inhibits JNK and is negatively regulated by ethanol. J Biol Chem 273: 28622–28629.

Johne C, Matenia D, Li XY, Timm T, Balusamy K, et al. (2008) Spred1 and Spred2 are negative regulators of ethanol-induced JNK activity. J Biol Chem 283: 20553–20562.

Kapfhamer D, Bettinger JC, Davies AG, Eastman CL, Smail EA, et al. (2008) TOLLIP regulates c-Jun and p38MAPK signaling in vivo. Hum Mol Genet 17: 2593–2603.

Lee YK, Jeong J, Hwang J, Yoo YJ, et al. (2007) Ethanol-induced oxidative stress is mediated by p38 MAPK pathway in mouse hippocampal neurons. J Neurosci 27: 11977–11984.

Anoor AR, James TT, Jackson DE, Shukla SD (2010) Differential Changes in Amygka. Neuropharmacology 53: 1832–1836.

Valles SL, Blanco AM, Pascaul M, Guerri C (2005) Involvement of TLR4/type 1 receptor in ethanol-induced cell death in the brain and in astrocytes. Brain Pathol 15: 365–371.

Vaunsens P, Brang Y, Mornet B, Orlowski T, Soria J, et al. (2000) Ethanol-stimulated extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) activities in the rat brain. Alcohol Clin Exp Res 24: 716–726.

Wang SJ, Li J, Xie GQ, Zhou R, et al. (2011) Alteration of synaptic transmission in the basolateral amygdala by chronic ethanol treatment. Neuropsychopharmacology 33: 1835–1846.

Wang F, Wu J, Cheng L, Yang J, Xie G, et al. (2003) Comparative studies of a new subfamily of human Ste20-like kinases. Biochem Pharmacol 65: 456–467.

Wolff FW, Rodan AR, Tsai LT, Hebeisen U (2002) High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci 22: 11035–11044.

Kong EC, Allouche L, Chapot PA, Vranizan K, Moore MS, et al. (2010) Ethanol contributes to genetic differences in ethanol sensitivity and rapid tolerance in Drosophila. Alcohol Clin Exp Res 34: 302–316.

Hogan B, Beddington R, Costantini F, Lacy E (1994) Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Downing C, Rodi¹-Hendricks KK, Fialhert L, Dudek BC (2005) Genetic analysis of the psychomotor stimulant effect of ethanol. Genes, Brain, Behav 4: 140–151.

Gao Y, Signore AP, Yin W, Cao G, Yin XM, et al. (2003) Neuroprotection against focal ischemic brain injury by inhibition of c-jun NH2-terminal kinase and attenuation of the mitochondrial apoptosis-signaling pathway. J Cereb Blood Flow Metab 23: 694–712.

Mitsunaga K, Tsutsumi O, Matsuy S, Harada K, Tomiyasu N, et al. (2006) Activation of c-jun NH2-terminal kinase (JNK) signaling in experimentally induced gastric lesions in rats. Clin Exp Immunol 145: 24–29.

Takamura M, Matsuda Y, Yamagawa S, Tamura Y, Honda Y, et al. (2007) An upstream inhibitor of c-jun NH2-terminal kinase, SP600125, protects mice from D-galactosamine/lipopolysaccharide-induced hepatic failure by modulating BH3-only proteins. Life Sci 80: 1335–1344.

Naassila M, Pierrefiche O, Ledent C, Daoust M (2004) Decreased alcohol self-administration in rats following chronic ethanol treatment. Alcohol Clin Exp Res 28: 1459–1468.

Palmer AA, Sharpe AL, Burkhart-Kasch S, McKinnon CS, Coste SC, et al. (2004) Corticotropin-releasing factor overexpression decreases ethanol drinking and increases sensitivity to the sedative effects of ethanol. Psychopharmacology (Berl) 176: 396–407.

Boyce-Rustay JM, Wierdlom LM, Millstein RA, Carroll J, Murphy DL, et al. (2006) Ethanol-related behaviors in serotonin transporter knockout mice. Alcohol Clin Exp Res 30: 1957–1965.

Newton PM, Mesing RO (2007) Increased sensitivity to the aversive effects of ethanol in PKC epsilon null mice revealed by place conditioning. Behav Brain Res 121: 439–442.

Kapfhamer D, Bettinger JC, Davies AG, Eastman CL, Smail EA, et al. (2008) Spred1 and Spred2 are negative regulators of ethanol-induced JNK activity. J Biol Chem 283: 20553–20562.

McAlhany RE Jr, West JR, Miranda RC (2000) Gcl-derived neurotrophic factor (GDNF) prevents ethanol-induced apoptosis and JNK activation. Neuropharmacology 41: 209–216.

de Lange RM, Bunton SM, Gaju P, Templeton D, Huang E, et al. (2007) Partial rescue of ethanol-induced neuronal apoptosis by growth factor activation of phosphoinositol-3-kinase. Alcohol Clin Exp Res 31: 1241–1249.
61. Han JY, Jeong JY, Lee YK, Roh GS, Kim HJ, et al. (2006) Suppression of survival kinases and activation of JNK mediate ethanol-induced cell death in the developing rat brain. Neurosci Lett 398: 113–117.

62. Han JY, Jeong EY, Kim YS, Roh GS, Kim HJ, et al. (2008) C-Jun N-terminal kinase regulates the interaction between 14-3-3 and Bad in ethanol-induced cell death. J Neurosci Res 86: 3221–3229.

63. Villela SN, Njane B, Linden R, Carri NG (2006) Glial-derived neurotrophic factor (GDNF) prevents ethanol (EtOH) induced B2/B glial cell death by both PI3K/AKT and MEK/ERK signaling pathways. Brain Res Bull 71: 116–126.

64. Young C, Strako ML, Johnson SA, Creeley C, Ohyes JW (2008) Ethanol causes and lithium prevents neuroapoptosis and suppression of pERK in the infant mouse brain. Neurobiol Dis 31: 353–360.

65. Ren J, Bahnock SK, Li Q, Huff AF, Li SY, et al. (2009) Aldolase dehydrogenase-2 transgene ameliorates chronic alcohol ingestion-induced apoptosis in cerebral cortex. Toxicol Lett 187: 149–156.

66. Bennett BL, Sasaki DT, Murray BW, O’Leary EC, Sakata ST, et al. (2001) SP600125, an anaphosphorazole inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 98: 13681–13686.

67. French RL, Heberlein U (2009) Glycogen synthase kinase-3/Shaggy mediates ethanol-induced excitotoxic cell death of Drosophila olfactory neurons. Proc Natl Acad Sci U S A 106: 20924–20929.

68. Takahama K, Tomita J, Ueno T, Yamazaki M, Kume S, et al. (2012) Pan-neuronal knockdown of the c-Jun N-terminal Kinase (JNK) results in a reduction in sleep and longevity in Drosophila. Biochem Biophys Res Commun 417: 807–811.

69. Kaplhammer D, Taylor S, Zou ME, Lin JP, Kharazia V, et al. (2012) TAO2 controls behavioral response to ethanol in mice. Genes Brain Behav [Epub ahead of print].

70. Lang R, Hammer M, Mages J (2006) DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. J Immunol 177: 7497–7504.

71. Jeffrey KL, Camps M, Rommel C, Mackay CR (2007) Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. Nat Rev Drug Discov 6: 391–403.

72. Patterson KL, Brummer T, O’Brien PM, Daly RJ (2009) Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J 418: 475–489.

73. Corb AL, Rodan AR, Heberlein U (2005) Insulin signaling in the nervous system regulates ethanol intoxication in Drosophila melanogaster. Nat Neurosci 8: 18–19.

74. PirriFF BD, Ngo TT, Hibbard KL, Murphy C, Jennet A, et al. (2010) Refinement of tools for targeted gene expression in Drosophila. Genetics 186: 735–755.

75. Das SK, Vasudevan DM (2007) Alcohol-induced oxidative stress. Life Sci 81: 177–187.

76. Fadda F, Rossetti ZL (1998) Chronic ethanol consumption: From neuroadaptation to neurodegeneration. Progress in Neurobiology 56: 385–431.

77. Gibson GE, Zhang H (2002) Interactions of oxidative stress with thiamine homeostasis promote neurodegeneration. Neurochem Int 40: 493–504.

78. Milton VJ, Sweezy ST (2012) Oxidative stress in synapse development and function. Dev Neurobiol 72: 100–110.

79. Collins CA, Wairark VP, Johnson SL, DiAntonio A (2006) Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron 51: 57–69.

80. Sraha M, Leyssen M, Choi GM, Fradin LG, Noordermeer JN, et al. (2006) A signaling network for patterning of neuronal connectivity in the Drosophila brain. PLoS Biol 4: e348.

81. Milton VJ, Jarrett HE, Gowers C, Chalak S, Briggs L, et al. (2011) Oxidative stress induces overgrowth of the Drosophila neuromuscular junction. Proc Natl Acad Sci U S A 108: 17521–17526.

82. Weber U, Paricio N, Mollick M (2000) Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. Development 127: 3619–3629.

83. Sun TQ, Lu B, Feng J, Reinhard C, Jan YN, et al. (2001) PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. Nat Cell Biol 3: 629–636.

84. Goodrich LV (2008) The plane facts of PCP in the CNS. Neuron 60: 9–16.

85. Gao FB, Brennan JE, Jan LY, Jan YN (1999) Genes regulating dendritic outgrowth, branching, and routing in Drosophila. Genes Dev 13: 2349–2351.

86. Sanchez-Alvarez L, Visanuvimol J, McEwan A, Su A, Imai JH, et al. (2011) VANG-1 and PRKL-1 cooperate to negatively regulate neurite formation in Caenorhabditis elegans. PLoS Genet 7: e1002257.

87. Ng J (2012) Wnt/PCP proteins regulate stereotypical axon branch extension in Drosophila. Development 139: 165–177.

88. Tao H, Manak JR, Sowers L, Mei X, Kyonari H, et al. (2011) Mutations in prickle orthologs cause seizures in flies, mice, and humans. Am J Hum Genet, 88: 139–149.

89. Wilkinson MB, Dias C, Magda J, Mazzi-Robison M, Lobo M, et al. (2011) A novel role of the WNT-dishevelled-GSK3beta signaling cascade in the mouse nucleus accumbens in a social defeat model of depression. J Neurosci 31: 9084–9092.

90. Wang JW, Imai Y, Lu B (2007) Activation of PAR-1 kinase and stimulation of tau phosphorylation by diverse signals require the tumor suppressor protein LKB1. J Neurosci 27: 574–581.

91. Xia Y, Karin M (2004) The control of cell motility and epithelial morphogenesis by Jun kinases. Trends Cell Biol 14: 94–101.

92. Insalera R, Chen S, Shi SH (2011) Par proteins and neuronal polarity. Dev Neurobiol 71: 483–494.

93. Biernat J, Wu YZ, Timm T, Zheng-Fischhofer Q, Mandelkow E, et al. (2002) Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. Mol Biol Cell 13: 4013–4029.

94. Biernat J, Wu YZ, Timm T, Zheng-Fischhofer Q, Mandelkow E, et al. (2002) Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. Mol Biol Cell 13: 4013–4029.

95. Srahna M, Leyssen M, Choi CM, Fradkin LG, Noordermeer JN, et al. (2006) A novel role of the WNT-dishevelled-GSK3beta signaling cascade in the mouse nucleus accumbens in a social defeat model of depression. J Neurosci 31: 9084–9092.

96. Offenhauser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, et al. (2006) Genetics 186: 2549–2561.

97. Goodrich LV (2008) The plane facts of PCP in the CNS. Neuron 60: 9–16.

98. Sahara N, Murayama M, Lee B, Park JM, Lagalwar S, et al. (2008) Active c-jun N-terminal kinase induces caspase cleavage of tau and additional phosphorylation by GSK-3beta is required for tau aggregation. Eur J Neurosci 27: 2897–2906.

99. Wezel MK, Naska S, Lahlbette CL, Rymar VV, Fujitani M, et al. (2008) Pathways regulate neurodegeneration and phospho-tau accumulation during aging and Alzheimer’s disease. Neuro 59: 708–721.

100. Offenhauser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, et al. (2006) A novel role of the WNT-dishevelled-GSK3beta signaling cascade in the mouse nucleus accumbens in a social defeat model of depression. J Neurosci 31: 9084–9092.