



OPEN

Neuropeptidergic regulation of compulsive ethanol seeking in *C. elegans*

Chinnu Salim^{1,2}, Ann Ke Kan^{1,2}, Enkhzul Batsaikhan¹, E. Clare Patterson¹ & Changhoon Jee¹✉

Despite the catastrophic consequences of alcohol abuse, alcohol use disorders (AUD) and comorbidities continue to strain the healthcare system, largely due to the effects of alcohol-seeking behavior. An improved understanding of the molecular basis of alcohol seeking will lead to enriched treatments for these disorders. Compulsive alcohol seeking is characterized by an imbalance between the superior drive to consume alcohol and the disruption or erosion in control of alcohol use. To model the development of compulsive engagement in alcohol seeking, we simultaneously exploited two distinct and conflicting *Caenorhabditis elegans* behavioral programs, ethanol preference and avoidance of aversive stimulus. We demonstrate that the *C. elegans* model recapitulated the pivotal features of compulsive alcohol seeking in mammals, specifically repeated attempts, endurance, and finally aversion-resistant alcohol seeking. We found that neuropeptide signaling via SEB-3, a CRF receptor-like GPCR, facilitates the development of ethanol preference and compels animals to seek ethanol compulsively. Furthermore, our functional genomic approach and behavioral elucidation suggest that the SEB-3 regulates another neuropeptidergic signaling, the neurokinin receptor orthologue TKR-1, to facilitate compulsive ethanol-seeking behavior.

Alcohol use disorder (AUD) is a chronic neurobehavioral disorder. A chronic exposure leads to the development of tolerance contributing to increased consumption^{1–3}. Subsequently, during the withdrawal or abstinence, alcohol craving and seeking are reinstated^{4–6}. Since these three phases model has provided a inferred understanding of the development of AUD⁷, development of preference and compulsive seeking, known to be involved in progress of alcohol dependence, have been identified as a pivotal and defining characteristic of AUD and other substance use disorders^{8–10}. Furthermore, recent Human genome-wide association study (GWAS) demonstrate that heavy drinking and increased consumption are not sufficient causes of AUD, although binge drinking and increased consumption are key risk factors for AUD¹¹. Hence, the neural substrates underlying compulsive seeking despite catastrophic consequences are crucial components of AUD and comorbidities, but the molecular mechanism remains largely elusive. The compulsive seeking in AUD is characterized by an imbalance between superior drive to alcohol and disruption in control of alcohol use^{12,13}. In order to model this highly complex neuromodulation, we addressed sophisticated behavioral paradigms in the simplest and most completely defined connectome with the advantage of the straightforward genetic, behavioral, and neurophysiological investigation, *C. elegans*^{14–16}. In a comparative proteomics study, 83% of the worm proteome was found to have human homologous genes and recent meta-analysis of orthology-prediction methods, about 52.6% of human protein-coding genome has recognizable worm orthologues^{17,18}, allow the worm a suitable model organism for functional validation of human genes.

Caenorhabditis elegans also has been shown to be a powerful and a deployable genetic tool to study AUD^{19–25}. Worms showed comparable physiological effects in similar human blood alcohol levels and develop acute functional tolerance in the presence of ethanol. Remarkably, worms also develop ethanol withdrawal symptom of tremor, which is reduced and abolished by replenishment of ethanol²². Previously, we have demonstrated and characterized a form of ethanol-preference behavior. Ethanol preference is elicited by prolonged exposure to ethanol in *C. elegans*, like mammals²². In this study, we further characterized the ethanol preference and demonstrated that *C. elegans* progresses compulsive ethanol seeking behavior. To evaluate the compulsive seeking, the repetitive/obsessive aspects and aversion-resistant seeking scale were applied, which has been used in human genetic studies for reliable assessing alcohol craving and dependence^{6,26,27}. Subsequently, we discovered that *seb-3* (Secretin family Class B GPCR), a corticotropin-releasing factor (CRF) receptor-like GPCR in *C. elegans*,

¹Department of Pharmacology, Addiction Science and Toxicology, College of Medicine, University of Tennessee Health Science Center (UTHSC), 71 S. Manassas St., Suite 217, Memphis, TN 38103, USA. ²These authors contributed equally: Chinnu Salim and Ann Ke Kan ✉email: cjee1@uthsc.edu

facilitates the development of ethanol preference and progress to compulsive ethanol seeking behavior. A CRF receptor has long been known to have roles in brain stress response and been implicated in the pathophysiology of anxiety and AUD^{28–31}. Additionally, its function has been implicated in compulsive drug self-administration and stress-induced reinstatement of drug seeking in mammalian studies³². Furthermore, a recent GWAS identified CRFR1 loci is associated with habitual alcohol intake³³. Despite strong evidence of the crucial role of the CRF system in the development of alcohol dependence, few successful clinical trials in humans have yet^{34,35}. Thus, we investigate the multiple neuropeptide systems and their potential coordination to progress compulsive ethanol seeking at the systems level. We have analyzed differentially expressed genes in a gain-of-function variant of *seb-3*, that facilitates faster development of preference and enhances compulsive seeking of ethanol. Here, we suggest TKR-1, neurokinin receptor orthologue, is upregulated by potentiation of SEB-3 to progress maladaptive compulsive ethanol seeking affecting susceptibility to AUD.

Materials and methods

All strains were maintained on nematode growth media (NGM) plates with *Escherichia coli* (OP50) at 20 °C³⁶ and the hermaphrodite was used for behavioral analysis. The wild-type animals used for the experiment were the Bristol N2 strain. The strains *tkr-1(ok2886)*, *tkr-2(ok1620)* and *tkr-3(ok381)* were obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA), which is supported by the National Institutes of Health—Office of Research Infrastructure Programs (P40 OD010440). *seb-3(eg696)gfp* previously isolated from a genetic screening and the *seb-3(tm1848)lf* strain was obtained from S. Mitani (Tokyo Women's Medical University, Tokyo, Japan) and backcrossed twice with N2²². Double and triple mutant strains were generated by PCR, taking advantage of the feature that *tkr-1(ok2886)* and *tkr-2(ok1620)* are deletion alleles³⁷; *tkr-1(ok2886)* III; *seb-3(eg696)* X, *tkr-2(ok1620)* IV; *seb-3(eg696)*X, and *tkr-1(ok2886)*; *tkr-2(ok1620)* IV; *seb-3(eg696)*X.

Transgenic strains include the following: *tkr-1(ok2886)* chjEx101 [(pCJ201 *ptkr-1::tkr-1*(WT)); *pofm-1::gfp*, and *pha-1(e2123)* chjEx102 [(pCJ210 *ptkr-1::gfp*); *pha-1* (+)].

Plasmid construction and microinjection. pCJ201 plasmid with *tkr-1* were made by PCR amplification of a genomic DNA fragment that included about 3.3 kb of upstream regulatory sequence. The 3.3 Kb *tkr-1* promoter region was PCR amplified from genomic DNA and recombined into a Gateway Entry Vector pDG15, using BP Clonase II (Life Technologies, Grand Island, NY), to generate pCJ202 to make pCJ210 [*Pseb-3::gfp*], pCJ202 was recombined with pCJ7, which encodes a GFP reporter³⁸ using LR Clonase II (Life Technologies). pCJ210 [*Pseb-3::gfp*] was microinjected (Narishige's MO-202U microinjector) into the *pha-1(e2123)*. The concentration of the final injection mixtures was 150 ng μl^{-1} . *pha-1(+)*, was co-injected at 50 ng μl^{-1} into *pha-1(e2123)* as an injection marker. using *pha-1* (+) as an injection marker as described³⁸. The pCJ210 was injected at 50 ng μl^{-1} . Transformed animals were screened by keeping the injected animals at 25 °C. Multiple lines of extrachromosomal arrays were maintained, and transformed animals were mounted on 7% agarose pad with Polybead Microspheres (Polysciences, Inc.) and then viewed under Leica DM6B Upright Compound Microscope with Epi-fluorescence attachment. High end camera with Hamamatsu ORCA Flash4.0 sCMOS records the fluorescent images. The fluorescent images were also obtained using Zeiss 710 confocal microscope.

The 7976 bp of genomic DNA fragment of *tkr-1* gene was PCR amplified from purified genomic DNA of WT animals and recombined into a Gateway Entry Vector pDG15³⁹, using BP Clonase II (Life Technologies, Grand Island, NY), to generate pCJ201. About 800 bp of flanking sequences are sequenced by M13 Forward and backward primers. pCJ201 plasmid was injected into the germline of *tkr-1(ok2886)* with *pofm-1::gfp* construct as an injection marker. The concentration of the final injection mixtures was 150 ng μl^{-1} . A *Pofm-1::gfp* plasmid was co-injected at 50 ng μl^{-1} . The pCJ201 was injected at 5–50 ng μl^{-1} . Multiple lines of extrachromosomal arrays were maintained, and L4 animals expressed GFP in coelomocyte were selected as transgenic animals. 16 h later, aversion-resistant Ethanol seeking Assay was performed to evaluate rescue experiment.

Trajectory analysis of *C. elegans* locomotion in the development of ethanol preference. WT animals were allowed to move freely on the assay plate, which contains ethanol in only one of the 4 well (1.9 cm²) of the culture dish and recorded locomotion of animals were analyzed. NGM was added to each well of 4 well tissue culture dish (1.9 cm²) to fill all the wells equally to the top. Outside of well also filled up with NGM and the boundary were slightly covered by disruption of surface tension when NGM was not solidified, which contained a gradient of ethanol but an allowance of free moving of worms on the surface. Out of the 4 wells, one well was selected and the glass Pasteur pipette (2 ml volume) punctured to make a hole for adding ethanol up to 300 Mm. The plate was sealed with parafilm after adding ethanol then 1 h later used. 1 day adult animals were washed twice in S-basal (100 mM NaCl, 50 mM potassium phosphate (pH 6.0),⁴⁰) and once in distilled water. After a final wash, 10 worms (Naïve or Ethanol treated) were introduced to the mid-region of 4-well plate by gentle picking with a platinum wire. Seal with parafilm and recorded the locomotion for 30 min in Wormlab (MBF Bioscience). The trajectory and time spent in the distinct area were analyzed by Wormlab software (MBF Bioscience). For the control speed and travel distance, 10 Naïve or Ethanol treated animals were placed on NGM plate with OP50 lawn (food) then 15 min locomotion was recorded and analyzed with Wormlab (MBF Bioscience).

Aversion-resistant ethanol seeking assay. The aversion assay measures the ability (or willingness) of the animals to cross an aversive barrier, in this case copper, which the nematodes will initially avoid. However, ethanol seeking animals will cross the barrier to reach alcohol.

The ethanol pre-exposure plates were prepared as previously described^{19,21}. Preexposure plates were 6-cm NGM plates that had been seeded with bacteria on half of the plate and were dried for 2 h. Ice-cold ethanol was added up to final concentration of 300 mM ethanol (from our previous publication;²¹) and worms were

introduced to ethanol plates for 4 h pre-exposure with parafilm sealing. Briefly, well-fed naïve or ethanol pre-treated animals (300 mM) were washed twice with S-buffer [100 mM NaCl, 50 mM potassium phosphate (pH 6.0)] and once in distilled water. Then animals were placed on mark above B (arrow) at the chemotaxis assay plate^{41,42}. Animals were allowed to move freely on an assay plate with different concentrations of CuSO₄ barrier to see the compulsive alcohol seeking behavior. The concentration of copper barrier was taken from the previous publication of Hilliard et al.⁴³, starting from a lower concentration of 2–20 mM. Before making the chemical barrier, allow the chemotaxis plates to dry for 1 h. To make a chemical barrier, pre-cut (3 mm thickness) Whatman filter paper (cat No-3030-335) was placed at the center of the 100 mm chemotaxis assay plate dividing the plate into two equal halves across the midline, as marked aversive barrier. A 100 µl of CuSO₄ (each concentration; 2–20 mM) was added to this filter paper barrier as per the experiment, from one edge to the other end. Briefly after adding CuSO₄ solution, the filter paper was gently removed using forceps. Meanwhile, 1 µl of 1 M NaN₃ was added to the point marked A and 30 µl of ethanol (200 proof) on top of it. Immediately after the chemical compound was absorbed into the 10 cm chemotaxis plate, about 100–150 washed animals were placed in the area marked in B section using glassware micropipette. The excess liquid was removed with a Kimwipe for animals not to be clumped at the origin. 40 min later with parafilm sealing, the number of animals in each section marked (A, B, C and D) was counted to calculate the Seeking index. The index was calculated by [(number of animals in A – number of animals in B)/Total number of animals [Seeking index SI = (A – B)/Total(A + B + C + D)]. To measure the copper sensitivity, 20 naïve or Ethanol-pretreated animals were placed on the aversion-resistant assay plate with 5 mM CuSO₄ barrier then the locomotion was recorded for 30 min. Time to respond to the copper barrier, which was stopping forward locomotion and backward then turn to avoid it, was measured in each animal's first encounter with the copper barrier. To assess with different types of aversive stimuli than copper, 5 mM or 10 mM Denatonium benzoate was applied to the assay plate in the same way to create an aversive barrier. SI in each trial was obtained from the population assay of 100–150 animals. Avoidance assay with drop test (0.1 mM, 1 mM, and 5 mM CuSO₄) was conducted as shown in previous study^{43,44}, to measure the sensitivity to nociceptive stimuli of WT and *seb-3(eg696)* gf animals. Single L4 worms were transferred to each NGM plate with abundant OP50, and 15 h later the assay was conducted. Assays were conducted under blind conditions and were not performed more than once on any individual animal. The avoidance index (AI) in (b) is the number of positive responses divided by the total number of trials. Mean latency to respond was calculated for all positive responses.

Sequence alignment. TKR-1 protein sequences (NP_499064.2) were analyzed by database similarity search⁴⁵ and the multiple protein sequences were simultaneously aligned using the COBALT, a constraint based alignment tool⁴⁶. The transmembrane helix domains were predicted using the conserved domain database (CDD)⁴⁷. For the phylogenetic tree analysis, Worm, human, rat, and mouse neurokinin receptor sequences used for alignment: worm TKR-1 (NP_499064.2), h_TACR1/NK1R (NP_001049.1), h_TACR2/NK2R (NP_001048.2), h_TACR3/NK3R (NP_001050.1), r_TACR1/NK1R (NP_036799.1), r_TACR2/NK2R (NP_542946.1), r_TACR3/NK3R (NP_058749.1), m_TACR1/NK1R (XP_006505926.1), m_TACR2/NK2R (NP_067357.1), and m_TACR3/NK3R (NP_033340.3). The phylogenetic tree was constructed by COBALT using minimum evolution method.

Statistical analysis. The mean and standard error of the mean (SEM) were determined for all experimental parameters. The data were analyzed employing the Chi-square test or Mann–Whitney test (Graph pad prism version 8.0.1). Values below 0.05 were considered as significant.

Microarray analysis. Isolation of nucleic acid and microarray followed by analysis of differentially expressed gene profiling was done in Molecular Recourse center of excellence (MRC), UTHSC. Briefly, synchronized 1 day adult population of WT and *seb-3gf(eg696)* where used for total RNA isolation. Approximately 10 one day adult animals were allowed to lay eggs per 15 NGM plates seeded with OP 50 for 8–10 h. After 10 h, the adult animals were removed and embryo were allowed to grow at 20°. After 72 h, 1-day adult animals from each plate were washed out with M9 buffer and washed the worms three times before collected in 1.5 ul nuclease free sterile Eppendorf tubes and stored at –80 with RNA later until use. Isolation of nucleic acid (total RNA) was done in Qi cube unit using Qiagen RNeasy mini kit according to manufacturer's instruction and standard protocol. Quantification of nucleic acid was done by Agilent2100 bioanalyzer, nanodrop and qubit. Individual sample (n = 3) was run for Affymetrix microarray focusing on individual response. Analysis was done using Affymetrix Genotyping Console and Affymetrix Transcriptome Analysis Console software.

For the population of germ cell production inhibited, 5'-fluorodeoxyuridine (FUdR) was treated from late L4 till the collection for the RNA preparation. The total RNA was isolated from 3 replicates of synchronized 1-day adult population of WT, *seb-3gf(eg696)*, FUdR-treated WT, and FUdR-treated *seb-3gf(eg696)*, respectively. Since FUdR affects some physiological function such as osmotic stress related life span extension⁴⁸, gene expression profiling from germ cell production inhibited was verified by excluding overlapping hits through cross-validation through mutual comparison. 1 candidate gene that changed in meaningful direction and amount by FUdR treatment was removed from the final differentially expressed genes candidates. RNA was reverse transcribed and labeled using GeneChip™ Whole Transcript (WT) Pico kit and hybridized to GeneChip™ *C. elegans* Gene 1.0 ST Array (Thermo Fisher Scientific) for whole-genome expression profiling. Text files were retrieved from UTHSC Molecular Resource Center after normalization performed by Affymetrix Expression Console. Quality assurance was checked against reference probes to ensure quality of data. Gene names, accession numbers, and expression were mined from each text file for each sample. All non-annotated information was removed from the file leaving only annotated gene expression. A student *t* test was run for pairwise interactions in order to

obtain p values for significance. Only genes with a p value < 0.05 were considered significant. The mean, variance, standard deviation, and fold change were calculated for each pairwise comparison. Benjamini Hochberg false discovery rate method was applied in order to obtain the adjusted p value for each gene⁴⁹. Heatmaps were created to visualize the expression of the significant genes of each pairwise comparison. Only genes with a p value < 0.05 were considered significant⁴⁹. Gene ontology enrichment analysis was conducted in the GO resource (GO; <http://geneontology.org>) and WormBase (<https://wormbase.org/tools/enrichment/tea/tea.cgi>) and verified through cross-validation through mutual comparison^{50–54}. Additionally, a comprehensive comparison of the results performed in Tissue Enrichment Analysis (TEA), Phenotype Enrichment Analysis (PEA) provided by WormBase was used to investigate genes predicted them to function in the same cellular pathway for further functional evaluation^{53,54}.

Quantitative RT-PCR. RNA extraction was performed as described in⁵⁵. 10 worms of FUDR-treated WT, and FUDR-treated seb-3gff(eg696), respectively, were transferred to a drop of ~ 20 μ l of S-basal buffer to remove bacteria. Washed worms were transferred into 2 μ l of worm lysis buffer (5 mM Tris pH 8.0 (Sigma–Aldrich), 0.5% Triton X-100 (Sigma–Aldrich), 0.5% Tween 20 (Fisher), 0.25 mM EDTA (Fisher) and 1 mg/ml proteinase K in the 0.2 ml PCR tube to extract the RNA. The cDNA synthesis was performed using transcriptor first strand kit (Roche). Quantitative RT-PCR was performed as described previously⁵⁶. SYBR-Green real-time PCR experiments were performed with a 1:4 dilution of cDNA using a LightCycler 480 system (Roche Life Science) following the manufacturer's instructions. The relative expression level were analyzed with the 2(-Delta Delta C(T)) Method⁵⁷ using the geometric mean of *tba-1* and *pmp-3* as an endogenous control^{58,59}. The data from three biological replications were represented in Fig. 7c. The primer sequences used for qRT-PCR have been shown in Sup. 4. We also performed qRT-PCR using isolated total RNA. Total RNA was isolated as described above for microarray analysis. 1 μ g of total RNA was used for cDNA synthesis then 1:8 diluted cDNA was used for qRT-PCR as described. The qRT-PCR from purified total RNA also showed consistency, which is the upregulation of only the *tkr-1* in the TKR family (data not shown).

Results

An ethanol preexposure elicits ethanol preference in wild-type (WT) *C. elegans*. Previous quadrant-plate assay successfully quantified that Ethanol pretreated animals preferentially accumulated in the ethanol area for 30 min of permission to move freely, whereas naïve animals accumulated more in the non-ethanol area at first experience²¹. Here, we further investigated the comprehensive response of *C. elegans* to the ethanol including initial response. The ethanol concentration was determined based on the behavioral and physiological response to various concentrations shown in previous studies^{19–21}. WT animals forcibly housed exposed to 400 mM ethanol plates have been shown an internal ethanol concentration of approximately 50 mM⁶⁰. We treated and tested animals in 300 mM ethanol, which was shown to induce ethanol preference of WT animals within 4 h while being rapidly sobered from intoxication when treated chronically²¹. The trajectory, exploring patterns, of an individual animal exhibited a distinct difference of response to ethanol between naïve and pretreated animals (Fig. 1). Naïve animals kept exploring the area with and without ethanol when the animal is introduced to the assay plate which is only one of the four wells contains ethanol. When animals first encountered ethanol and moved in the ethanol area, the speed of locomotion was reduced which was corresponding to the behavioral characteristic of intoxication. Then, they left the ethanol zone and kept moving, exploring in the non-ethanol zone more time (Fig. 1a,c; 0% appearance in the ethanol area of the assay plate for some of the naïve animals such as #4, #5). In contrast, ethanol-pretreated WT animals headed straight to the ethanol zone and stayed in the ethanol zone (Fig. 1b,d). The naïve animals stayed longer in the non-ethanol area (66% of time spent) whereas ethanol pretreated animals spent 89% of the time in the ethanol area (Fig. 1d). First, we addressed the question of whether the change of locomotion behavior is due to an inability to keep moving. Ethanol pretreated (300 mM) animals were placed on the non-ethanol NGM and their locomotion was analyzed. The ability to move was not defective after ethanol pretreatment. Ethanol preexposure produces a reversible change in the locomotion resulting in a progressive decrease in the speed¹⁹. Subsequently, a decrease of speed is recovered in the presence of ethanol that was shown to be corresponding to intoxication and acute functional tolerance in other systems²⁰. While the animals were exposed to 300 mM exogenous ethanol for pretreatment, their movements initially decreased because they were exposed to exogenous ethanol concentrations that made the worms initially intoxicated. However, no difficulty was observed in the movement of the pretreated animals after recovery for 4 h of pretreatment (Fig. 2). Besides, pretreated animals represented an increase of speed and travel distance upon ethanol withdrawal (Fig. 2c), comparable to behavioral stimulation induced by ethanol withdrawal in other system⁶¹. To further address how preexposure to ethanol might lead animals to stay in the ethanol area although ethanol pretreated animals had an increased exploring and enhanced active behavior, we established the measurement for the motivational strength of ethanol preference progressed by ethanol pretreatment (Fig. 3a). We exploited two distinct behavioral programs in conflict, an ethanol-seeking and avoidance of aversive stimuli blocking ethanol-seeking simultaneously. The motivational strength for the ethanol was indexed by exposing the animals to the ethanol and aversive stimuli at the same time after the pretreatment of ethanol. The nociceptive stimuli of Cu^{2+} set up as an aversive barrier to interfere with chemotaxis to ethanol (Fig. 3a–c). *Caenorhabditis elegans* makes use of polymodal sensory neurons to detect a wide range of aversive and painful stimuli including water soluble chemical repellents such as quinine, denatonium benzoate, and Cu^{2+} ^{43,44} and to let them to avoid it. An animal who detects the aversive stimulus stop its forward locomotion and turn away from the source of stimulus resulting in avoidance. Increased attempt to cross over and endurance of seeking ethanol was observed in the pretreated animals (Fig. 3a). Average trials of single animal (counted out of 103 worms) for 5 min was 0.56 in naïve whereas 2.28 in pretreated (counted out of 80 worms). These repeated trials of pretreated animals resulted in crossing over the chemical barrier and heading

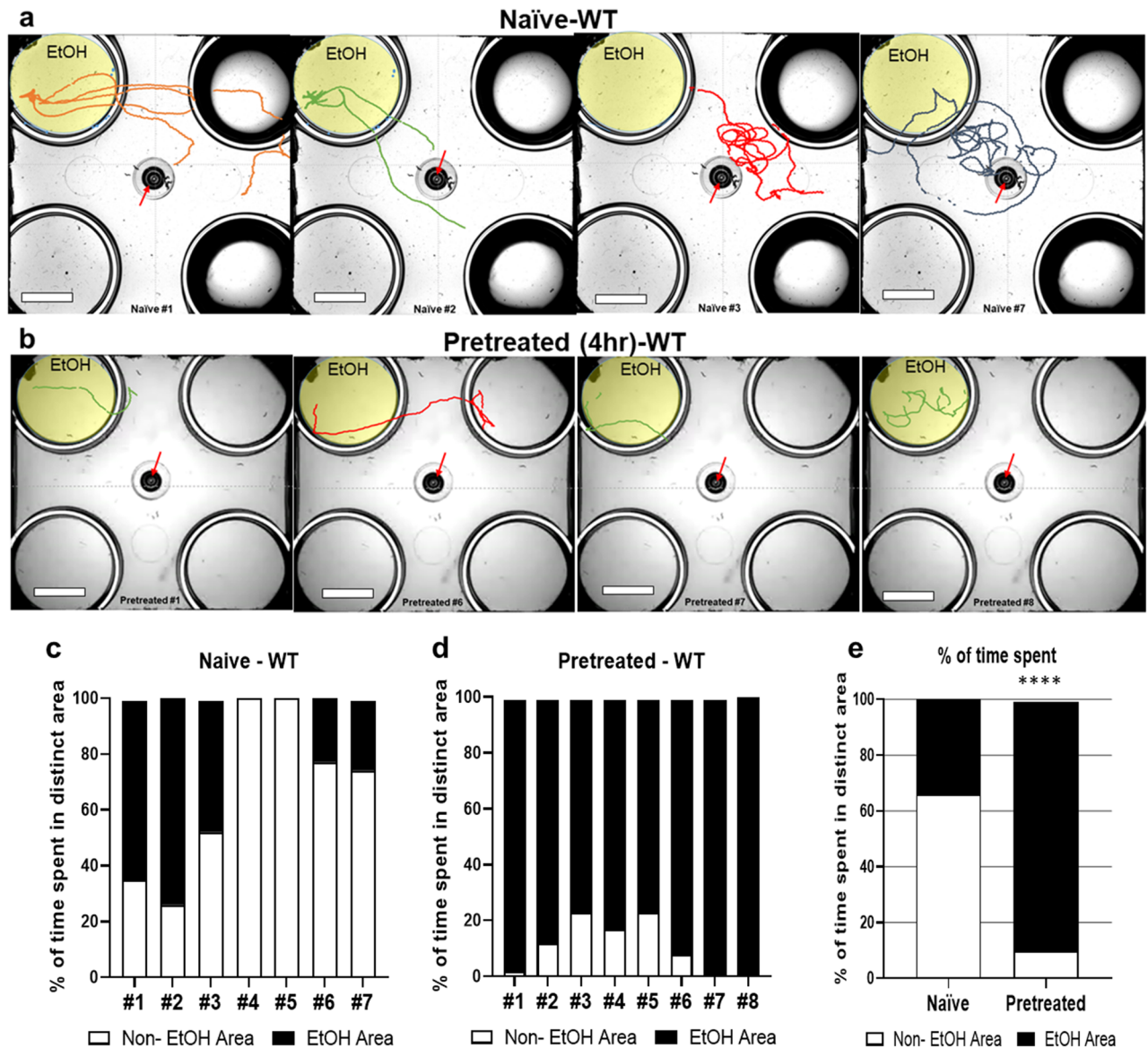


Figure 1. Ethanol-pretreated WT animals headed straight to the ethanol area and remained stayed. (a,b) Trajectory of individual WT animal (Naïve or Ethanol pretreated). The naïve (a) or ethanol pretreated animals (b), respectively, were placed in the middle of assay plate that contains ethanol (300 mM) only in the left top well. All wells are marginally covered by media that allows free moving between the area. Ethanol-pretreated WT animals (4 h; 300 mM) headed straight to ethanol area and stayed, whereas naïve wild type animals explored around. The arrow indicates initial points, where a worm was placed, and the trajectory represents the locomotion analysis for 30 min. During the short process of preparing for recording after placing, naïve animals explore near the initial point thus, the tracking path starts from the vicinity of the initial point, whereas the ethanol-treated animals have already moved toward the ethanol area consequently, the beginning of the tracking path is away from the initial point. Additional trajectories for individual animals were shown in Sup. 1. Scale bar = 10 mm. (c,e) Behavioral quantification of individual animal (c; Naïve, d; 4 h-Ethanol pretreated) and average of total percentage time spent in the distinct area (e). The ethanol pretreated animals spent more time in ethanol area. The data were analyzed employing Chi-square test. chi-square test indicated; df 60.67, $1 z$ 7.789, **** p < 0.0001.

to the ethanol area. The 23.08% of the trials in pretreated animals resulted in successful crossing to seek ethanol whereas only 3.45% of the trials in naïve animals. These repetitive attempting and crossing over the aversive barrier were hypothesized to be proportional to motivational compulsion to seek ethanol since ethanol pretreatment does not interfere with the ability of worms to detect Cu^{2+} barrier. The sensitivity of ethanol-pretreated animals to aversive stimuli was not altered (Fig. 3b) consistent with previous findings that ethanol pretreatment did not affect attraction/aversion to volatile odorants²¹. Subsequently, we quantified the animals successfully reached to ethanol over aversive Cu^{2+} barrier in time and Seeking Index (SI) was obtained as shown in (Fig. 3a). We tested what range of Cu^{2+} concentration naïve or pretreated WT animals could cross over to reach the ethanol area. The drive of pretreated animals to seek ethanol superseded its aversive Cu^{2+} response in a concentration-dependent

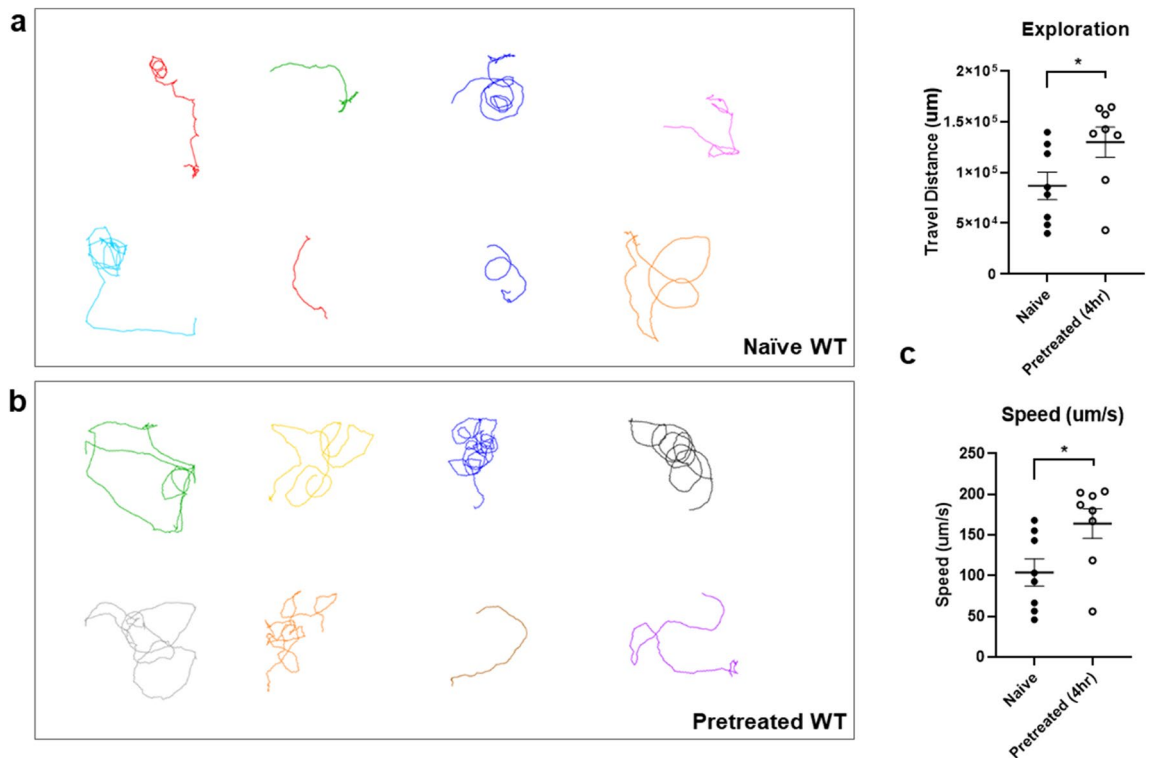


Figure 2. Ethanol pretreated animal is not defective in the locomotion. The locomotion trajectories of naïve (a) or ethanol-pretreated (b) animals on non-ethanol plate (with food, OP50) for 15 min are shown. (c) The stimulation of locomotion, increase of speed and travel distance, was observed in ethanol pretreated WT animals.

manner (Fig. 3c). The ethanol preference developed by pretreatment enabled WT animals to cross the Cu^{2+} barrier in low concentration (2 mM, 5 mM), aversive stimuli that Naïve animal does not cross. However, while going from lower concentration to higher concentration of barrier (10 mM, 20 mM), ethanol pretreated animals failed to overcome the Cu^{2+} barrier. 4 h pretreatment of ethanol was not enough to enable WT animals to cross the aversive Cu^{2+} barrier to reach the ethanol area. Evidently, this change is strengthened with longer ethanol exposures (data not shown). Then we also introduced another aversive stimulus, denatonium benzoate as a barrier. Without pretreatment of ethanol, all the tested concentrations of denatonium barrier successfully block to interfere the naïve animals' chemotaxis to ethanol. However, pretreated animals showed endurance against the denatonium barrier to reach the ethanol area and crossed the low concentration of obstacle (Fig. 3d). Since the compulsivity is defined as the urge to perform and persist to take a substance that escapes control despite serious negative consequences^{62–64}, these results suggest that aversion-resistant ethanol seeking approach can be used as a proxy measure for the compulsive ethanol seeking behavior.

Neuropeptide signaling orchestrates many complex behaviors in the brain, including compulsive behavior. Previously, we demonstrated the functional conservation of the neuropeptide corticotropin-releasing factor (CRF) system in responses to stress and ethanol in worms and mice²². We reported that *seb-3*, CRF (corticotropin-releasing factor) receptor-like G protein-coupled receptor of *C. elegans* facilitated the development of acute tolerance to ethanol and ethanol withdrawal symptom²². Since the CRF (corticotropin releasing factor) receptor function has been implicated in compulsive drug self-administration and stress-induced reinstatement of drug seeking in mammalian studies, we hypothesized *seb-3(eg696)* gain of function (gf) animals would also be able to exhibit enhanced alcohol seeking against the higher concentration of Cu^{2+} barrier. A *seb-3(eg696)*, dominant mutation, was suggested and functionally evaluated as a gain of function mutation due to its identity of mutation, which was single amino acid change in a conserved residue at the third intracellular loop, the binding region for G proteins^{22,65–67}. First, ethanol preference of *seb-3(eg696)* animals and *seb-3(tm1848)* Knockout animals due to its deletion²² was tested in the preference assay on ethanol-containing quadrant plates (Fig. 4a). Animals were allowed to move freely for 30 min between two control quadrants without ethanol and two ethanol quadrants. The naïve animals of WT, *seb-3(eg696)*, and *seb-3(tm1848)* accumulated on non-ethanol regions. No significant ethanol preference was observed in naïve animals of all WT, *seb-3(eg696)*, and *seb-3(tm1848)* (Fig. 4b). Remarkably, *seb-3(eg696)* gf animals represented much faster and greater ethanol preference after ethanol pretreatment. Significant ethanol preference was observed in animals pretreated with ethanol for 2 h whereas WT animals did not (Fig. 4c). Additionally, greater development of preference was observed in *seb-3(eg696)* animals after 4 h ethanol pretreatment. Moreover, impaired development of ethanol preference was observed in ethanol pretreated *seb-3(tm1848)* animals (Fig. 4c) suggesting SEB-3 facilitated the ethanol preference. Subsequently, we conducted aversion-resistant ethanol seeking assay with *seb-3* mutant animals. Quantification of *seb3(eg696)* gf mutant animals' motivational drive to seek ethanol showed enhanced aversion-resistant ethanol seeking behavior compared

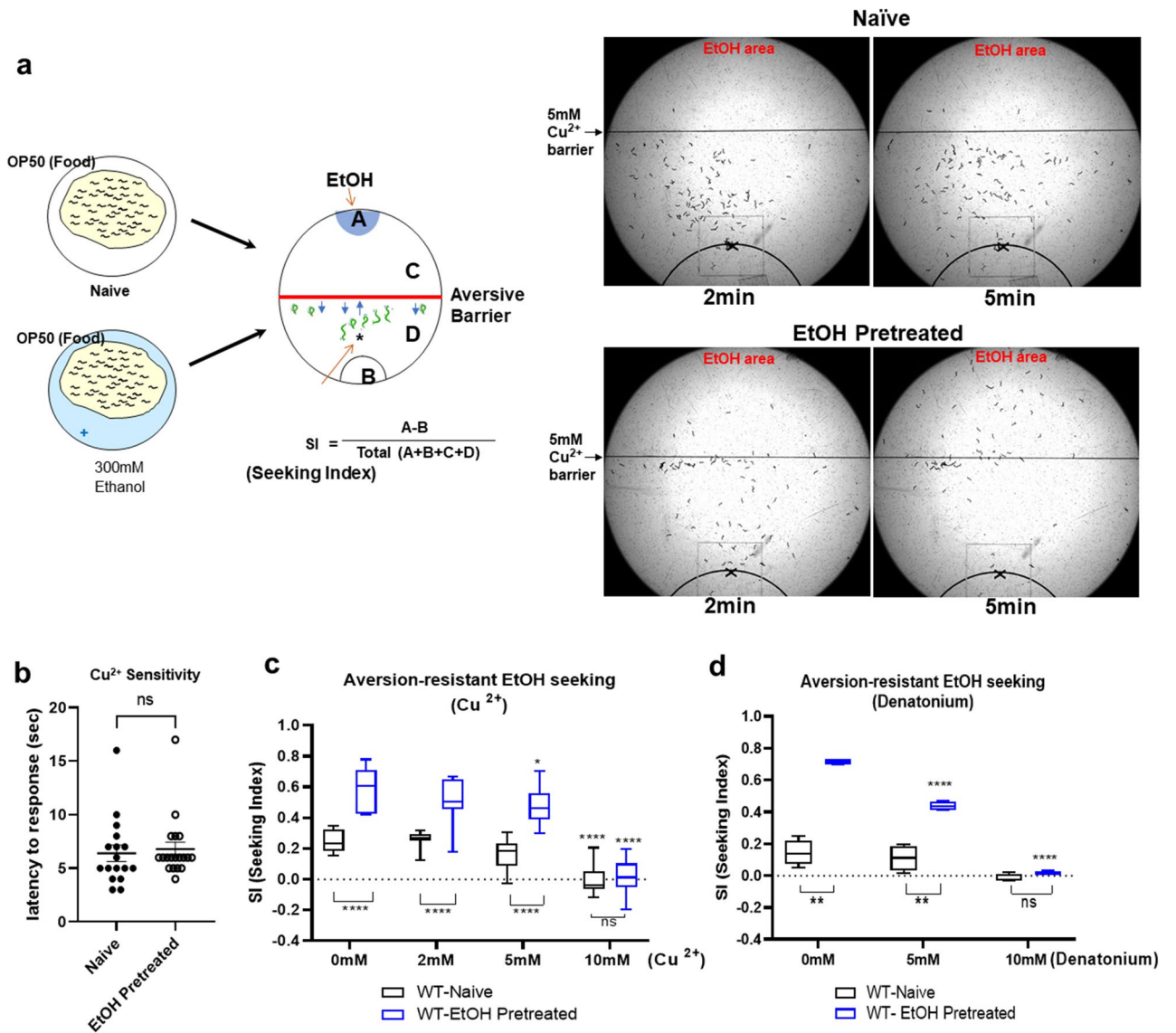


Figure 3. Behavioral quantification of compulsive ethanol seeking after exposure to ethanol against aversive chemical barrier. **(a)** Diagrammatic representation exhibited experimental design to quantify aversion-resistant ethanol seeking. The different concentrations of Cu²⁺ created aversive barrier without mechanical obstacles to quantify the motivational strength of seeking ethanol behavior in animals. **(b)** Cu²⁺ sensitivity of WT animals are not altered after ethanol exposure. **(c)** EtOH pretreated animals demonstrates more animals cross over the Cu barrier for ethanol (aversion-resistant seeking), in low concentration (2 and 5 mM), than does Naive animals [$F_{EtOH\ pretreated}(1, 18) = 46.44, p < 0.0001$; $F_{Concentration}(4, 72) = 99.01, p < 0.0001$; $F_{pretreated \times Concentration}(4, 72) = 11.57, p < 0.001$]. Moving to higher concentration of copper (10 mM), ethanol pretreated WT animals failed to overcome the aversive barrier. A two-way ANOVA comparison of the animal status over concentrations of barrier showed significant differences based on EtOH pretreated, concentrations, and the interaction of the two. Significant post hoc differences (Bonferroni's test) between naive and EtOH pretreated animals at no barrier, 2 mM, 5 mM, and 10 mM is shown. Comparison with 0 mM is also shown ($p < 0.0001, ****$). Values are mean \pm SEM. N = 10 in each conc. **(d)** EtOH pretreated animals demonstrates more animals cross over the Denatonium barrier for ethanol (aversion-resistant seeking), in low concentration, than does Naive animals [$F_{EtOH\ pretreated}(1, 6) = 598.8, p < 0.0001$; $F_{Concentration}(1, 712, 10.27) = 119, p < 0.0001$; $F_{pretreated \times Concentration}(2, 12) = 45.81, p < 0.0001$]. A two-way ANOVA comparison of the animal status over concentrations of barrier showed significant differences based on EtOH pretreated, concentrations, and the interaction of the two. Significant post hoc differences (Bonferroni's test) between naive and EtOH pretreated animals at no barrier, 5 mM, and 10 mM is shown ($p < 0.01, **$). Comparison between genotype is also shown ($p < 0.0001, ****$). Values are mean \pm SEM. N = 4 in each conc.

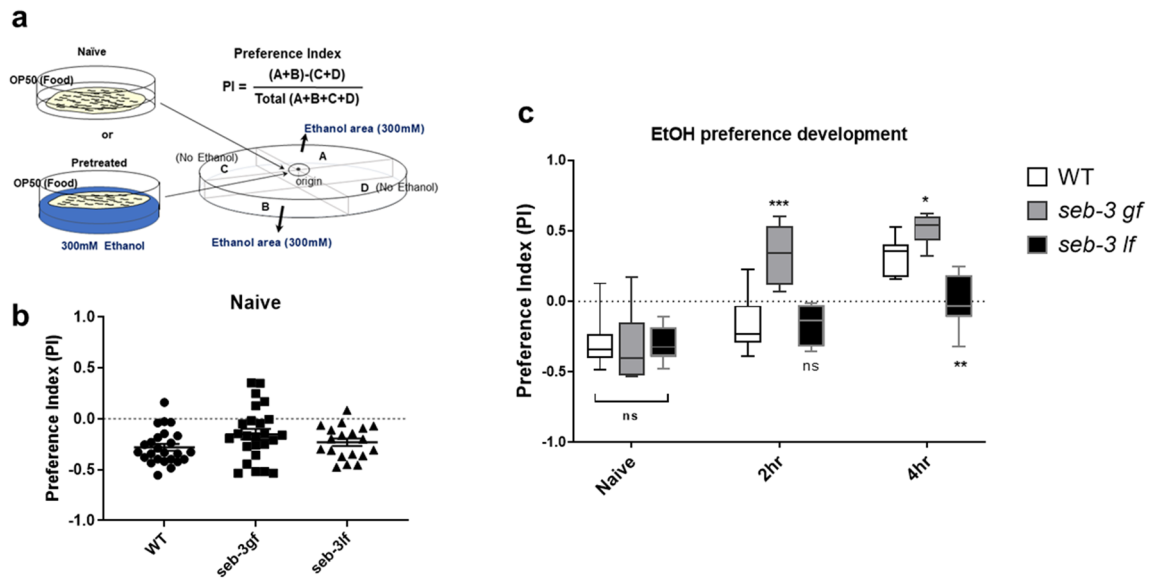


Figure 4. SEB-3 facilitates the development of ethanol preference. (a) Diagrammatic representation of ethanol preference assay. Naïve or ethanol pretreated animals remain free to explore on the quadrants plate before counting. (b) Naïve animals accumulate primarily in the non-ethanol region. One-way ANOVA, $p > 0.05$, $F(2, 66) = 2.539$, ns compared to WT in post hoc multiple comparison test; Dunnett's. (c) Ethanol preference developed more rapidly and greater in *seb-3gf* animals, whereas impaired in *seb-3lf* animals. [Fgenotype(2, 27) = 15.76, $p < 0.0001$; Ftime(1.871, 37.42) = 72.56, $p < 0.0001$; FGenotype \times time(4, 40) = 9.982, $p < 0.001$]. A two-way ANOVA comparison showed significant differences based on genotype, time, and the interaction of the two. Significant post hoc differences (Bonferroni's multiple comparison test) between the genotypes (WT vs. *seb-3gf*) or (WT vs. *seb-3lf*) at naïve, 2 h, and 4 h are shown ($p < 0.05$, *; $p < 0.01$, **; $p < 0.0001$, ****).

to WT animals in overall concentrations of barrier including higher concentrations (10 mM and 20 mM). The ethanol pretreated *seb-3(eg696) gf* animals gathered at the barrier in the middle of assay plate and the repetitive attempting led animals to cross over the aversive barrier. The pretreated *seb-3(eg696) gf* animals exhibited significantly high SI values not only in lower but also in the higher concentration of Cu^{2+} (Fig. 5a). Nevertheless, sensory perception of aversive Cu^{2+} stimuli was not defective in *seb-3(eg696) gf* animals (Fig. 5b–e) represented enhanced ethanol preference, which could override the interference of noxious stimuli. Although *seb-3(eg696) gf* animals are slightly more sensitive to nociceptive stimuli⁽²²⁾, Fig. 5c), crossed the aversive barrier for ethanol. Additionally, *seb-3(tm1848)* Knockout animals did not develop chemotaxis to ethanol after pretreatment as much as WT animals nor cross both low and high concentrations (2 mM, 5 mM, 10 mM) of Cu^{2+} barrier for ethanol (Fig. 5f). Taken together with ethanol preference, we conclude SEB-3 also contribute to the progress of compulsive ethanol seeking.

Having defined the progress of the compulsive ethanol seeking, we next investigated a gene expression profile of *seb-3(eg696) gf* animals to identify the genes and pathways underlying the enhancement of ethanol preference and progress of compulsive ethanol seeking. The total RNA was isolated from synchronized young adult populations of which germ cell production was pharmacologically inhibited to improve the chance of identification of differentially expressed genes in somatic cells including neuronal tissue, then applied to the Affymetrix gene chip array. Microarray analysis revealed 716 transcripts that are differentially expressed in *seb-3(eg696) gf* animals ($\geq 1.5\times$, Fig. 6a) compared to WT animals. Subsequent Gene Ontology (GO) enrichment analysis (see “Methods” section) identified total 16 GO terms in upregulated and downregulated gene clusters (Fig. 6b,c, Sup. 2, and Sup. 3). The most prominent enrichment in biological processes includes the immune system process, defense response, and response to biotic stimulus, which is in line with the role of the stress response for survival and reproduction in adverse conditions.

Remarkably, we identified the upregulated *tkr-1* in Neuropeptide signaling pathway (GO:0007218, Fig. 6b, Sup. 2). A *tkr-1* is predicted to encode a G protein-coupled receptor (GPCR), which is a human orthologue of Tachykinin Receptor also known as neurokinin receptor¹⁸. Tachykinins/Neurokinins and their receptors are neuropeptides signaling conserved from invertebrates to mammals^{68,69}. In mammals, tachykinins are widely distributed in the nervous systems and gastrointestinal tract. The three genes encoding precursors of tachykinins give rise to nine distinct tachykinins, which interact with three different receptors such as neurokinin 1 receptor (NK1R), neurokinin 2 receptor (NK2R), and neurokinin 3 receptor (NK3R)⁷⁰. TKR-1 shows 51% similarity with human NK1R in overall 406 amino acids and 52% similarity with human NK3R. TKR-1 represents the putative ligand-binding pocket defined according to the NMR evidence of Substance P (SP) docking to the NK1R pocket (Fig. 7a,⁷¹). The crystal structure of NK1R in complex with clinically used antagonist also endorse the important role of this region, that is a ligand bind deeper within the receptor core⁷². Tachykinin/Neurokinin system is functionally pleiotropic and has been reported to be involved in the stress responses, anxiety, pain, inflammation, immune response, and sensory processing^{70,73–78}. We confirmed the upregulation of *tkr-1* observed in the

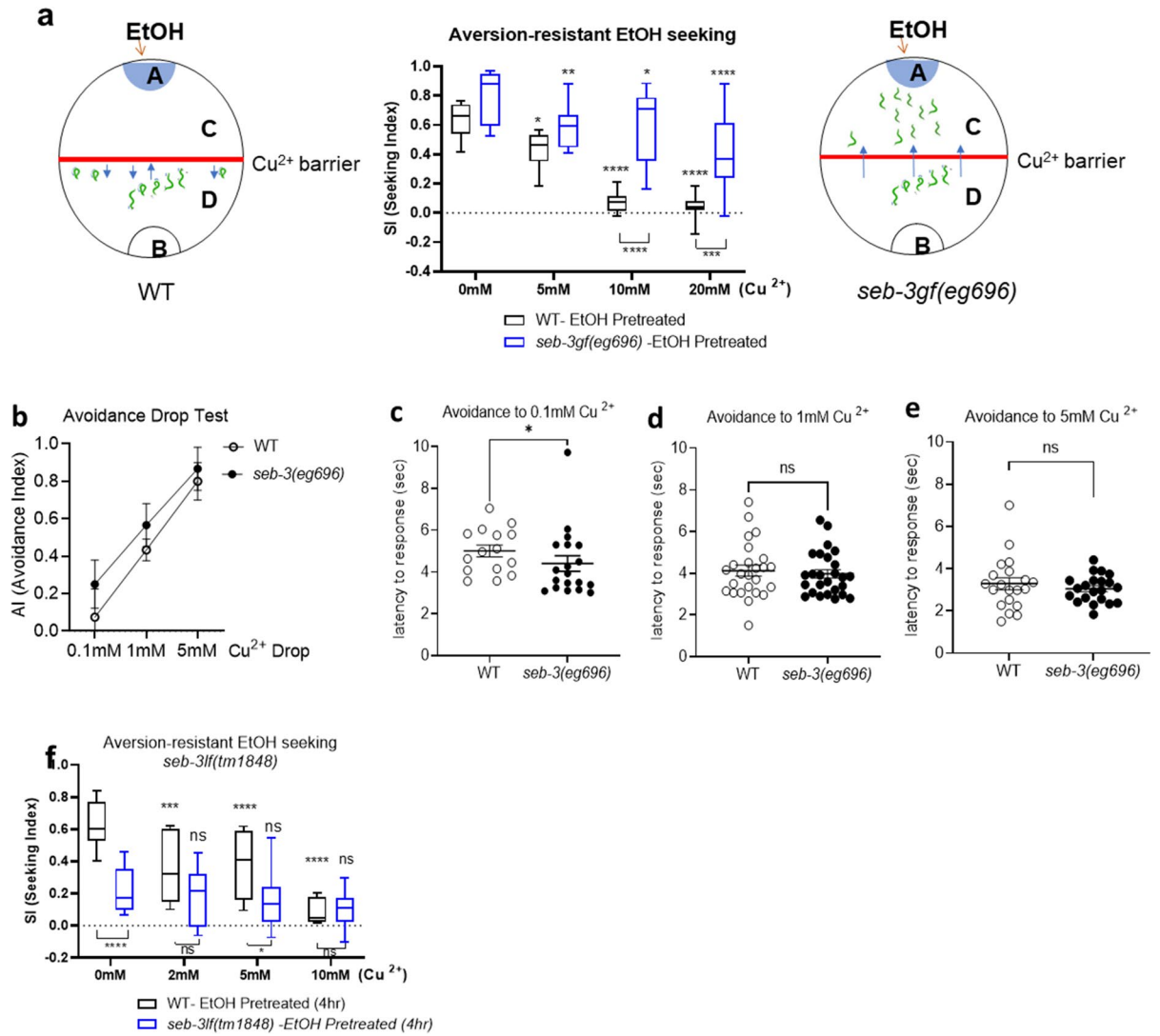


Figure 5. The ethanol pretreated *seb-3gf* animals for 4 h surmount a stronger aversive barrier to seek ethanol. **(a)** Strength of ethanol seeking is represented by the SI under different concentration of copper barrier (no barrier, 10 mM and 20 mM). A *seb-3gf* strain demonstrates more animals cross over the barrier for ethanol (aversion-resistant seeking), in overall concentrations, than does the WT strain [$F_{\text{Genotype}}(1, 18) = 35.78, p < 0.0001$; $F_{\text{Concentration}}(3, 53) = 40.32, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 53) = 6.586, p < 0.001$]. A two-way ANOVA comparison of the strains over concentrations of barrier showed significant differences based on genotype, concentrations, and the interaction of the two. Significant post hoc differences (Dunnnett's test) between no barrier versus 5 mM, 10 mM, or 20 mM in each genotype (WT and *seb-3gf* animals) is shown ($p < 0.05, *$; $p < 0.01, **$; $p < 0.0001, ****$). Comparison between genotype is also shown ($p < 0.001, ***$; $p < 0.0001, ****$). Box and whisker represent minimum to maximum of 10 trials of population assay ($N = 10$). **(b–e)** Avoidance assay with drop test (0.1 mM, 1 mM, and 5 mM CuSO_4). The avoidance index (AI) in **(b)** is the number of positive responses divided by the total number of trials. The latency to stop forward and initiate backward movement was measured. Data were obtained from 10 or more animals and mean values from 3 or more trials were analyzed by one-way ANOVA with a post-hoc Dunnnett's test; non-significant differences [$p = 0.0820, F_{\text{genotypes}}(1, 7) = 4.117, p = 0.7705$; $F_{\text{concentration} \times \text{genotype}}(2, 7) = 0.2707$] **(b)** or two-tailed t-test; $p < 0.05, *$ **(c–e)**. **(f)** The development of aversion-resistant ethanol seeking is impaired in *seb-3lf* animals. A two-way ANOVA comparison [$F_{\text{Genotype}}(1, 17) = 24.64, p < 0.0001$; $F_{\text{Concentration}}(3, 30) = 18.35, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 40) = 6.005, p = 0.0018$]. Significant post hoc differences (Dunnnett's test) between no barrier versus 2 mM, 5 mM, or 10 mM in each genotype ($p < 0.001, ***$; $p < 0.0001, ****$). Comparison between genotype is also shown ($p < 0.05, *$; $p < 0.0001, ****$).

microarray data with qRT-PCR (Fig. 7c). For the functional evaluation of *tkr-1* in association with the ethanol preference and compulsive ethanol seeking, we tested the KO mutant of *tkr-1* (*ok2886*) in the assay for aversion-resistant ethanol seeking. The *C. elegans* genome includes 3 genes predicted to encode Neurokinin receptor family

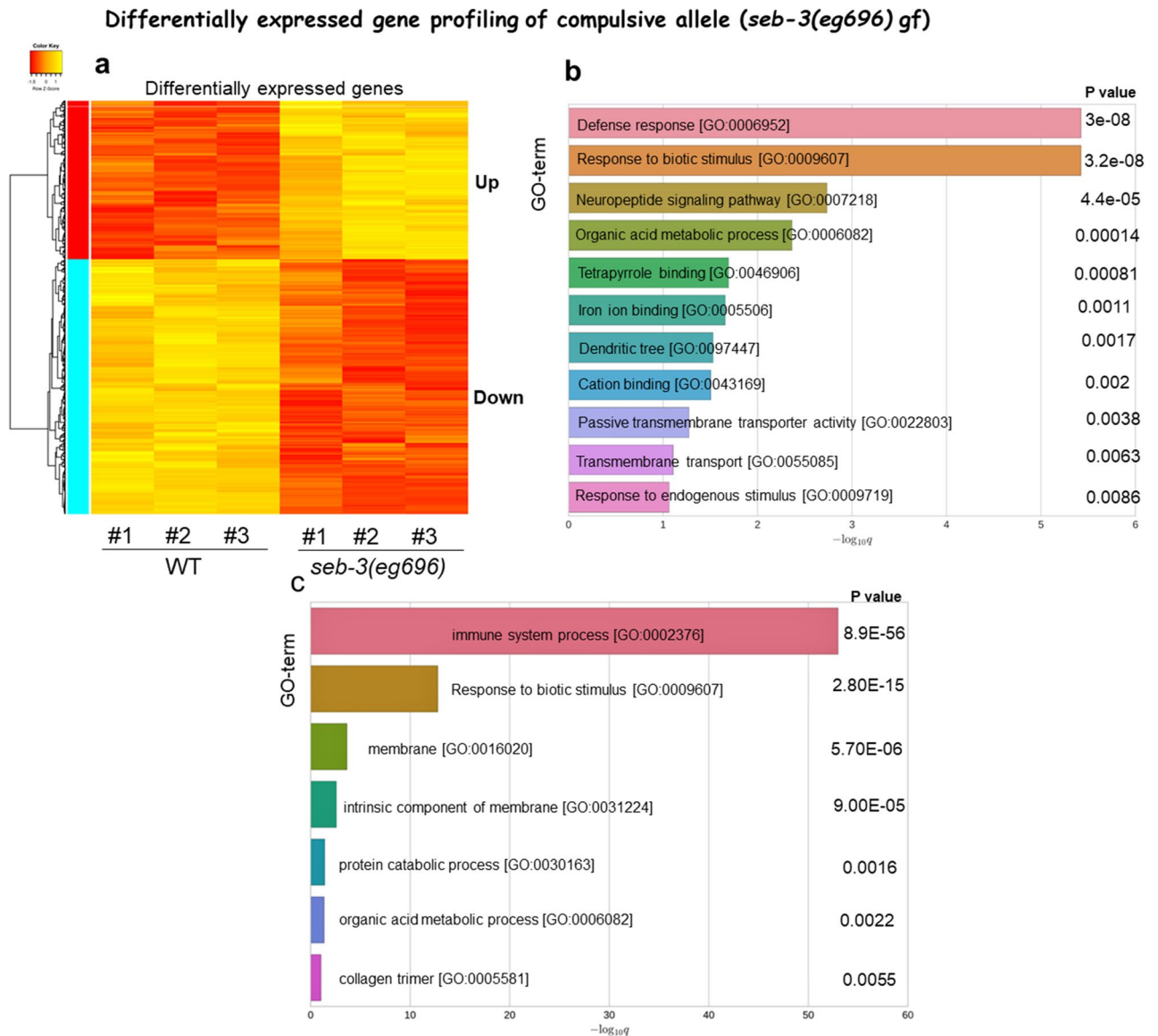


Figure 6. Differentially expressed gene profiling of genetic variant vulnerable to compulsive seeking behavior. (a) Altered expression of transcripts in *seb-3gf*. Gene Ontology (GO) enrichment analysis revealed 11 GO terms that upregulated genes are enriched (b) and 7 GO terms of downregulated genes (c).

proteins, *tkr-1*, *tkr-2*, and *tkr-3* as shown in the phylogenetic analysis of the Tachykinin/Neurokinin Receptor family (Fig. 7b). The phylogenetic tree was constructed by COBALT (constraint-based alignment tool for multiple protein sequences) using minimum evolution method²⁵. We also tested KO mutant animals of *tkr-2* (*ok1620*) and *tkr-3* (*ok381*) in the neurokinin receptor family. The tested are putative KO strains due to the deletion of genomic loci (Fig. 7d–f). The evident phenotype was observed in *tkr-1* (*ok2886*) animals. A Fig. 7d showed significantly low SI values of *tkr-1* KO compared to WT animals in ethanol seeking assay against the Cu²⁺ barrier in overall concentrations. Remarkably, *tkr-1* KO animals did not cross even low concentration of Cu²⁺ barrier after ethanol pretreatment like *seb-3* KO animals. Interestingly, the KO animals of *tkr-2*, predicted as a NK3R orthologue¹⁸, also showed slightly reduced development of aversion-resistant ethanol seeking induced by ethanol pretreatment whereas *tkr-3* KO animals displayed ethanol preference and aversion-resistant ethanol seeking as much as wild type (Fig. 7e,f). Furthermore, epistatic analysis demonstrated that compulsive ethanol seeking in *seb-3(eg696) gf* animals was suppressed by KO in the *tkr* family, *tkr-1* and *tkr-2* (Fig. 7g,h). It suggests that TKR-1 and TKR-2 function downstream of SEB-3 with redundancy in the family (Fig. 7g,h, and Sup. 5). TKR-1 is expressed in the glial cells such as AMsh and sensory/interneurons such as URA (or CEP), ASJ, and AIY confirming the expression in the consortium data (Fig. 8a and⁷⁹). This promoter and the genomic *tkr-1* could rescue the *tkr-1* KO phenotype (Fig. 8b). Neural processes in worms are surrounded by glial cells such as AMsh⁸⁰. Growing evidence shows *C. elegans* glial-neuron interactions are involved in the regulation of animal behavior such as olfaction, gustation, and thermosensation⁸¹. Indeed, NK1R is expressed in human glia cells and functionally respond to its ligand⁸². AMsh, in which neurons such as AWC, which are expected to be involved in ethanol seeking, and nociceptive neurons, such as ASH and ADL, are simultaneously connected through adhesive junctions. Along

with downstream interneurons, functions of TKR-1 in AMsh will be further investigated for the conduits of maladaptation that induce compulsivity in multisensory integration. Our results, impaired development of aversion-resistant ethanol seeking behavior in the absence of TKR-1, was evident and reflect the importance of the involvement of TKR-1 function in the progress of compulsive ethanol seeking. Combined, these data suggest that SEB-3 transcriptionally regulates the TKR-1 to enhance and progress the compulsive ethanol seeking behavior in ethanol experienced animals.

Discussion

Compulsivity is defined as repetitive attempts despite facing adverse consequences and compel individuals to perform to be relieved from stress or anxiety⁸³. The repetitive or obsessive aspects and compulsive drinking scale has been adapted and help to evaluate the severity of AUD in human genetic studies for reliable assessing alcohol craving^{6,26,27}, that has been described as a compelling urge to intake alcohol and considered crucial for the maintenance of AUD⁵. The compulsive alcohol seeking is characterized by an imbalance between superior drive to alcohol and disruption in control of alcohol use^{12,13}. To model the development of compulsive engagement of alcohol seeking in *C. elegans*, we showed following: (1) enhanced preference of ethanol, (2) repetitive attempts to seek, and (3) enhanced aversion-resistant ethanol seeking.

Despite baseline aversive response to ethanol in acute exposure, *C. elegans* exhibit state-dependent ethanol seeking behavior, which is a significantly potentiated preference for ethanol after the prolonged experience of ethanol²¹. In addition, *C. elegans* also recapitulates the behavioral traits of alcohol-dependent animals, known as studies in mammals. Ethanol pretreated worms showed distinct orientation while seeking for ethanol and a pronounced tendency to stay in the limited region where ethanol is, even though their locomotion and exploratory behavior were not damaged by the pretreatment of ethanol (Figs. 1 and 2). The increase in exploratory behavior during ethanol withdrawal is consistent with the evident demonstration of ethanol-withdrawal symptom in *C. elegans*, as seen in our previous study²², and nicotine, classified as a psychostimulant like ethanol, can also be compared to promoting withdrawal-induced motor stimulation in *C. elegans*⁸⁴. The locomotion trajectories are depending on the transition between distinct motor states, which is the modulation of random exploration behavior^{85–88}. Since the long-term behavioral state of exploration in *C. elegans* is modulated by neuropeptide signals including *seb-3*^{22,89,90}, the change in exploration behavior of ethanol pretreated animals can be considered in association with the dependence modulation by neuropeptides signaling.

Ethanol pretreated animals also represented repetitive attempts and endurance to cross the chemical barrier to move to the ethanol area and consequently, crossed the barrier (Fig. 3). Chemotaxis behaviors are regulated primarily by the chemosensory neurons and modulated by integration of signaling with interneurons^{91,92}. Recent studies on multisensory integration of the worms have provided sophisticated experimental manipulation with complex behavioral decision paradigms in vivo animals where information from distinct sensory networks is concurrently processed to demonstrate the comprehensible representation of the environment^{93–95}. We introduced nociceptive stimuli, of which perception and mediation are well defined^{43,96–98}, as an obstacle to interfere with the animals' ethanol seeking behavior. Since ethanol pretreatment does not change the worm's sensitivity to the aversive stimulus (Fig. 3b), ethanol seeking against the aversive chemical barrier promise as an endophenotype for compulsive ethanol seeking. Together with persistent drug seeking despite aversive consequences, aversion-resistant alcohol intake is increasingly recognized as a pivotal characteristic of addiction and is being investigated as a model of compulsive behavior^{99–102}. The progress of compulsivity is hypothesized to be imbalanced between enhanced ethanol seeking and loss of controlling avoidance program. *C. elegans* detect the aversive stimuli in the environment and avoid noxious chemicals to cope with unfavorable situation. After repeated trial, crossing over aversive stimuli for ethanol seeking is depending on the concentration of aversive barrier suggesting the strength of an animal's innate state that modulate ethanol preference results in compulsive seeking. We introduced various nociceptive chemicals, of which avoidance is mediated by polymodal sensory neurons for nociception^{43,44}, to rule out the possibility that overcoming aversive barrier for ethanol seeking was specific to certain aversive stimuli. For example, in those cases where only the receptors for the copper sensation are downregulated during pretreatment instead of modulation of the overall circuit of attraction or avoidance program depending on the state. Cu^{2+} and denatonium benzoate effectively interfere with ethanol seeking behavior in concentration dependent manner. Quinine also works in a similar way, but only a low concentration was applicable due to the solubility in ethanol as a solvent (data not shown). Together with increased ethanol preference, we demonstrate the ethanol dependent behaviors of *C. elegans* that parallels compulsive alcohol seeking behavior in mammals.

Neuromodulation via neuropeptides signaling has been shown to have a pivotal roles in the development of AUD from worms to human^{20,22,33,103–106}. We demonstrate that SEB-3, a CRF (corticotropin-releasing factor) receptor-like GPCR in *C. elegans*, positively regulates ethanol preference and also has a role in the progress of compulsive ethanol seeking over adverse stimuli after prolonged preexposure to ethanol. Neuropeptidergic signaling of CRF has been studied as a key element that lead to AUD and comorbidities in a way to compensate for the distress associated with substance withdrawal and discontinuation. Neuropeptides signaling provides powerful mechanisms for rapid physiological adjustment to kaleidoscopic environmental changes and maintain systemic function by precise integration of signal flow. A maladaptation in multisensory integration has been reported in individuals diagnosed with compulsive behaviors^{107,108}. Previously, we have shown insight into how SEB-3 facilitates the progress of compulsive behavior by demonstrating that SEB-3 enhances motivational state and leads animals to engage obsessively in sexual drive, superseding the avoidance program of locomotion under negative stimuli³⁸. The animal has to consider its physiological statuses such as hunger and sexual drive and must monitor its environment to determine whether stressful conditions warrant the expression of their innate urges. The CRF (corticotropin-releasing factor) system plays a pivotal role in mediating stress responses in the brain from amphibians to primates^{109–112}. A CRF (corticotropin-releasing factor) receptor has been implicated

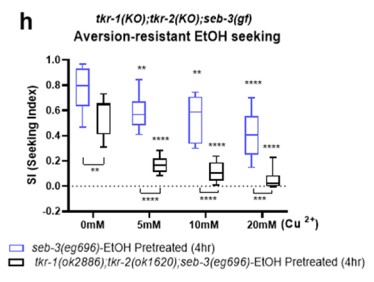
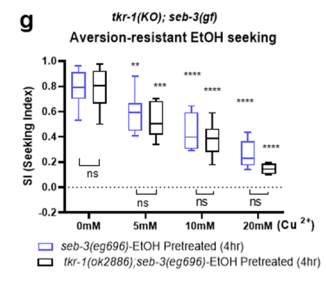
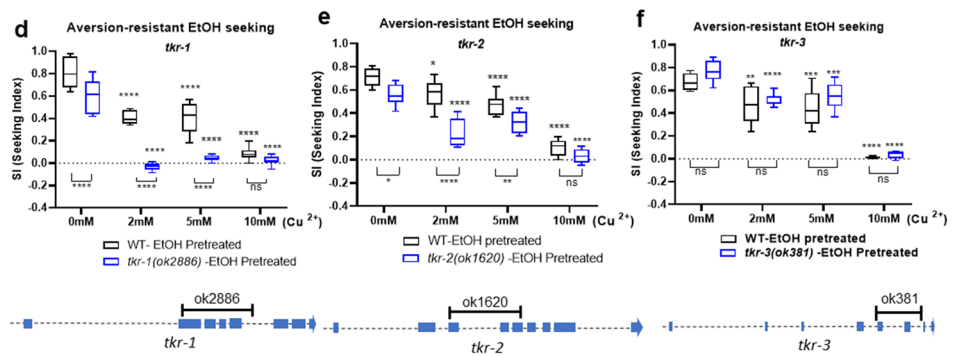
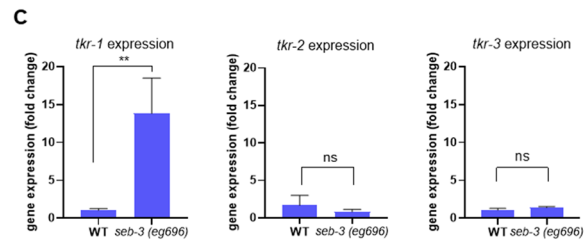
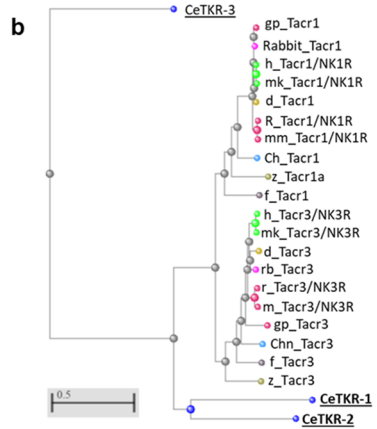
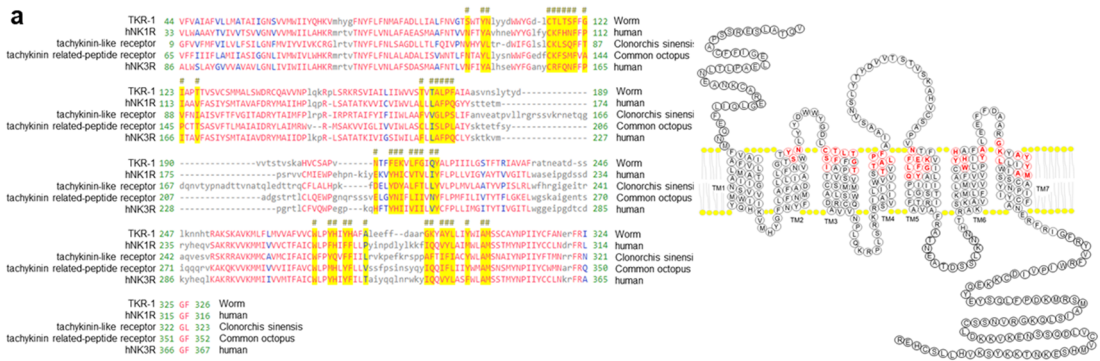


Figure 7. Neurokinin receptors in *C. elegans*. (a) Sequence alignment of TKR-1 (NP_499064.2), humanTACR1/NK1R (2KS9_A), humanTACR3/NK3R (P29371), and tachykinin-like receptors of liver fluke (GAA51416) and octopus (BAD93354.1) revealed the consensus sequences of putative ligand binding pocket of TKR-1 (Yellow highlights). Red indicates highly conserved amino acids and blue indicates lower conservation. The diagram of full-length TKR-1 topology represents transmembrane helix domains and the consensus sequences of putative peptide ligand binding pocket (Red amino acids). (b) Phylogenetic analysis of the Tachykinin/Neurokinin Receptor family (minimum evolution method). (c) Upregulation of *tkr-1* in *seb-3(eg696)* determined by qRT-PCR (n = 3 biological replication). RNA was extracted from 10 young adult worms in each biological replication. A paired-t test show significance ($p < 0.01$, **). (d–h) Aversion-resistant ethanol seeking assay in *tkr-1(ok2886)* (d), *tkr-2(ok1620)* (e), *tkr-3(ok381)* (f), *tkr-1(ok2886); seb-3(eg696)* double (g), and *tkr-1(ok2886); tkr-2(ok1620)* triple (h). A two-way ANOVA comparison shows the impaired development in *tkr-1(ok2886)* [$F_{\text{Genotype}}(1, 58) = 106.6, p < 0.0001$; $F_{\text{Concentration}}(3, 58) = 140.7, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 58) = 9.495, p < 0.0001$]. Significant post hoc differences (Dunnett's test) between no barrier versus 2 mM, 5 mM, or 10 mM in each genotype ($p < 0.0001$, ****); *tkr-2(ok1620)* [$F_{\text{Genotype}}(1, 16) = 38.37, p < 0.0001$; $F_{\text{Concentration}}(3, 34) = 76.82, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 34) = 6.030, p = 0.0021$], *tkr-1(ok2886); seb-3(eg696)* [$F_{\text{Genotype}}(1, 18) = 2.031, p = 0.1712$; $F_{\text{Concentration}}(3, 36) = 64.04, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 36) = 0.5236, p = 0.6688$], and *tkr-1(ok2886); tkr-2(ok1620); seb-3(eg696)* [$F_{\text{Genotype}}(1, 18) = 63.25, p < 0.0001$; $F_{\text{Concentration}}(3, 35) = 33.21, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(3, 35) = 2.358, p = 0.0084$]. Significant post hoc differences (Dunnett's test) between no barrier versus 2 mM, 5 mM, or 10 mM in each genotype ($p < 0.05$, *; $p < 0.0001$, ****). Comparison between genotype is also shown ($p < 0.001$, ***; $p < 0.0001$, ****).

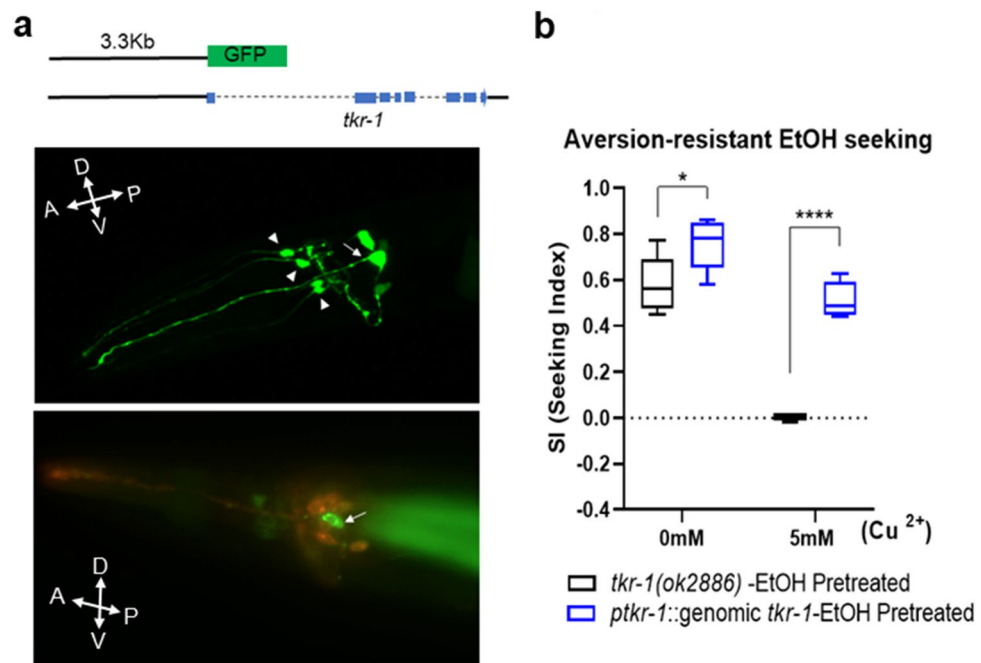


Figure 8. TKR-1 is required for compulsive ethanol seeking. (a) *tkr-1* is expressed in the amphid neurons such as URA or CEP (arrowhead) and Amphid sheath glial cells (AMsh, arrow). Green fluorescent protein (GFP) is translationally fused with one amino acid of TKR-1 driven by 3.3 kb of 5' promoter region. DiI lipophilic dye visualized the head sensory neurons (ASK, ADL, ASI, AWB, ASH, and ASJ). Using this as a marker, the amphid sheath cell was identified through its shape and location. Distinct expression in the amphid neurons such as URA or CEP (arrowhead) and Amphid sheath glial cells (AMsh, arrow) was observed. Faint expression is also observed in AIY-like and ASJ-like neurons. The double-headed arrows indicate A/P, D/V polarities. (b) The genomic DNA of *tkr-1* containing 3.4 kb of promoter region (a) rescue the impaired development of compulsive ethanol seeking of *tkr-1(ok2886)* animals. A two-way ANOVA comparison shows [$F_{\text{Genotype}}(1, 8) = 63.44, p < 0.0001$; $F_{\text{Concentration}}(1, 8) = 107.4, p < 0.0001$; $F_{\text{Genotype} \times \text{Concentration}}(1, 8) = 17.20, p = 0.0032$]. Significant post hoc differences (Dunnett's test) between strains are represented ($p = 0.0143$, *; $p < 0.0001$, ****).

in the pathophysiology of compulsive behavior such as anxiety and AUD^{113–115}. Stress pathways have been studied as key fundamentals of neural systems that drive alcohol and drug dependence. The brain stress system is activated and sensitized during repeated withdrawal and lead to a negative emotional state that promotes dependence¹¹⁶. Indeed, the *seb-3(eg696)* gf animals, originally isolated as a genetic variant showing enhanced

arousal and represented altered susceptibility to AUD such as enhanced acute functional tolerance to ethanol and withdrawal behavior²².

Like CRF, Tachykinin/Neurokinin is one of the strongest conserved neuropeptide systems across bilaterians, of which both peptide and receptor orthologues are represented in *C. elegans*⁶⁹. The *tkr-1*, *tkr-2*, and *tkr-3* have been categorized into tachykinin/neurokinin receptor-like group^{117–119} and RNAi functional screens revealed *tkr-1* affects fat metabolism and deposition¹²⁰. We report *tkr-1* and *tkr-2*, close to NK1R and NK3R, have a prominent role in the progress of compulsive ethanol seeking. NK1R preferentially mediates the signal of Substance P (SP), belongs to the tachykinin/neurokinin family of neuropeptides^{121,122}. SP and NK1R are widespread in the nervous system in mammals. NK1R has been studied in correlation with the stress response related to anxiety and depression^{73–75}. Recently after the failure of CRFR1 antagonist in Clinical trials^{123,124}, the increasing evidence of neurokinin receptors related to alcohol and drug dependence has been more highlighted. An activation of both NK1R and NK3R facilitate dopamine release in the NAc¹²⁵ and NK1R antagonist was reported to reduce cocaine-induced DA release¹²⁶. Antagonism of NK1R and KO mice reduces alcohol consumption¹²⁷ and Antagonism of NK1R decreases alcohol self-administration in alcohol-preferring rats¹²⁸. Furthermore, adeno-associated virus-mediated overexpression of NK1R in the central amygdala increased alcohol self-administration¹²⁹, which is consistent with our finding. A Family-based association study in human genetics have also reported NK3R is associated with alcohol and cocaine dependence¹³⁰.

We report that SEB-3 facilitates animal drive to seek ethanol over noxious stimuli representing enhanced compulsive seeking. Our functional genomics study revealed that TKR-1 is upregulated in *seb-3* gf strain, which is defined as compulsive ethanol seeking animals, and functions in the development of compulsive ethanol seeking. Interestingly, *sodh-1*, which showed a significant alcohol intoxication phenotype in the orthogonal test for functional validation of ADH (alcohol dehydrogenase) as a human GWAS candidate related to heavy alcohol consumption²⁵, is also upregulated in *seb-3* gf animals (Sup. 2). These results also demonstrate the potential of our investigation as a scalable model to accelerate the functional validation of alcohol dependence associated with networks of epistatic interactions. Despite strong evidence of the crucial role of the CRF system in the development of alcohol dependence, there are concerns about the importance of the CRF system for drug target based on recent failures of CRF1 antagonist clinical trials for alcohol dependence. Here, the conservation of ethanol phenotypes across species suggests that a gradual increase of dependence-induced compulsivity is progressed by the interplay between CRF and Neurokinin signaling. Further investigation of interactions will provide significance to understand the neurobiological mechanism to progress AUD for advanced clinical benefits.

Received: 18 June 2021; Accepted: 7 January 2022

Published online: 02 February 2022

References

- Kaplan, H. L., Sellers, E. M., Hamilton, C., Naranjo, C. A. & Dorian, P. Is there acute tolerance to alcohol at steady state? *J. Stud. Alcohol* **46**, 253–256 (1985).
- Korpi, E. R. *et al.* Mechanisms of action and persistent neuroplasticity by drugs of abuse. *Pharmacol. Rev.* **67**, 872–1004. <https://doi.org/10.1124/pr.115.010967> (2015).
- Wallace, M. J. *et al.* Acute functional tolerance to ethanol mediated by protein kinase Cepsilon. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **32**, 127–136. <https://doi.org/10.1038/sj.npp.1301059> (2007).
- Rohsenow, D. J. & Monti, P. M. Does urge to drink predict relapse after treatment? *Alcohol Res. Health* **23**, 225–232 (1999).
- Koob, G. F. Animal models of craving for ethanol. *Addiction* **95**(Suppl 2), S73–81. <https://doi.org/10.1080/09652140050111663> (2000).
- Flannery, B. A. *et al.* The role of craving in alcohol use, dependence, and treatment. *Alcohol. Clin. Exp. Res.* **25**, 299–308 (2001).
- Koob, G. F. & Le Moal, M. Drug abuse: Hedonic homeostatic dysregulation. *Science* **278**, 52–58. <https://doi.org/10.1126/science.278.5335.52> (1997).
- Funk, C. K., O'Dell, L. E., Crawford, E. F. & Koob, G. F. Corticotropin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. *J. Neurosci. Off. J. Soc. Neurosci.* **26**, 11324–11332. <https://doi.org/10.1523/JNEUROSCI.3096-06.2006> (2006).
- Spanagel, R. & Kiefer, F. Drugs for relapse prevention of alcoholism: Ten years of progress. *Trends Pharmacol. Sci.* **29**, 109–115. <https://doi.org/10.1016/j.tips.2007.12.005> (2008).
- Wise, R. A. & Koob, G. F. The development and maintenance of drug addiction. *Neuropsychopharmacology* **39**, 254–262. <https://doi.org/10.1038/npp.2013.261> (2014).
- Kranzler, H. R. *et al.* Genome-wide association study of alcohol consumption and use disorder in 274,424 individuals from multiple populations. *Nat. Commun.* **10**, 1499. <https://doi.org/10.1038/s41467-019-09480-8> (2019).
- Baler, R. D. & Volkow, N. D. Drug addiction: The neurobiology of disrupted self-control. *Trends Mol. Med.* **12**, 559–566. <https://doi.org/10.1016/j.molmed.2006.10.005> (2006).
- Koob, G. F. & Volkow, N. D. Neurobiology of addiction: A neurocircuitry analysis. *Lancet Psychiatry* **3**, 760–773. [https://doi.org/10.1016/S2215-0366\(16\)00104-8](https://doi.org/10.1016/S2215-0366(16)00104-8) (2016).
- White, J. G., Southgate, E., Thomson, J. N. & Brenner, S. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **314**, 1–340. <https://doi.org/10.1098/rstb.1986.0056> (1986).
- Varshney, L. R., Chen, B. L., Paniagua, E., Hall, D. H. & Chklovskii, D. B. Structural properties of the *Caenorhabditis elegans* neuronal network. *PLoS Comput. Biol.* <https://doi.org/10.1371/journal.pcbi.1001066> (2011).
- Jarrell, T. A. *et al.* The connectome of a decision-making neural network. *Science (New York, N.Y.)* **337**, 437–444. <https://doi.org/10.1126/science.1221762> (2012).
- Lai, C. H., Chou, C. Y., Chang, L. Y., Liu, C. S. & Lin, W. Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. *Genome Res.* **10**, 703–713. <https://doi.org/10.1101/gr.10.5.703> (2000).
- Kim, W., Underwood, R. S., Greenwald, I. & Shaye, D. D. OrthoList 2: A new comparative genomic analysis of human and *Caenorhabditis elegans* genes. *Genetics* **210**, 445–461. <https://doi.org/10.1534/genetics.118.301307> (2018).
- Davies, A. G. *et al.* A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* **115**, 655–666. [https://doi.org/10.1016/S0092-8674\(03\)00979-6](https://doi.org/10.1016/S0092-8674(03)00979-6) (2003).

20. Davies, A. G., Bettinger, J. C., Thiele, T. R., Judy, M. E. & McIntire, S. L. Natural variation in the npr-1 gene modifies ethanol responses of wild strains of *C. elegans*. *Neuron* **42**, 731–743. <https://doi.org/10.1016/j.neuron.2004.05.004> (2004).
21. Lee, J., Jee, C. & McIntire, S. L. Ethanol preference in *C. elegans*. *Genes Brain Behav.* **8**, 578–585. <https://doi.org/10.1111/j.1601-183X.2009.00513.x> (2009).
22. Jee, C. *et al.* SEB-3, a CRF receptor-like GPCR, regulates locomotor activity states, stress responses and ethanol tolerance in *Caenorhabditis elegans*. *Genes Brain Behav.* **12**, 250–262. <https://doi.org/10.1111/j.1601-183X.2012.00829.x> (2013).
23. Barclay, J. W. *et al.* Presynaptic targets for acute ethanol sensitivity. *Biochem. Soc. Trans.* **38**, 172–176. <https://doi.org/10.1042/bst0380172> (2010).
24. Bettinger, J. C. & Davies, A. G. The role of the BK channel in ethanol response behaviors: Evidence from model organism and human studies. *Front. Physiol.* **5**, 346. <https://doi.org/10.3389/fphys.2014.00346> (2014).
25. Thompson, A. *et al.* Functional validity, role, and implications of heavy alcohol consumption genetic loci. *Sci. Adv.* **6**, eaay5034. <https://doi.org/10.1126/sciadv.aay5034> (2020).
26. Anton, R. F., Moak, D. H. & Latham, P. K. The obsessive compulsive drinking scale: A new method of assessing outcome in alcoholism treatment studies. *Arch. Gen. Psychiatry* **53**, 225–231. <https://doi.org/10.1001/archpsyc.1996.01830030047008> (1996).
27. Modell, J. G., Glaser, F. B., Cyr, L. & Mountz, J. M. Obsessive and compulsive characteristics of craving for alcohol in alcohol abuse and dependence. *Alcohol. Clin. Exp. Res.* **16**, 272–274. <https://doi.org/10.1111/j.1530-0277.1992.tb01375.x> (1992).
28. Valdez, G. R., Sabino, V. & Koob, G. F. Increased anxiety-like behavior and ethanol self-administration in dependent rats: Reversal via corticotropin-releasing factor-2 receptor activation. *Alcohol. Clin. Exp. Res.* **28**, 865–872 (2004).
29. Nie, Z. *et al.* Ethanol augments GABAergic transmission in the central amygdala via CRF1 receptors. *Science* **303**, 1512–1514. <https://doi.org/10.1126/science.1092550> (2004).
30. Hansson, A. C. *et al.* Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 15236–15241. <https://doi.org/10.1073/pnas.0604419103> (2006).
31. Gehlert, D. R. *et al.* 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine: A novel brain-penetrant, orally available corticotropin-releasing factor receptor 1 antagonist with efficacy in animal models of alcoholism. *J. Neurosci. Off. J. Soc. Neurosci.* **27**, 2718–2726. <https://doi.org/10.1523/JNEUROSCI.4985-06.2007> (2007).
32. Weiss, F. *et al.* Compulsive drug-seeking behavior and relapse. Neuroadaptation, stress, and conditioning factors. *Ann. N. Y. Acad. Sci.* **937**, 1–26. <https://doi.org/10.1111/j.1749-6632.2001.tb03556.x> (2001).
33. Gelernter, J. *et al.* Genome-wide association study of maximum habitual alcohol intake in >140,000 U.S. European and African American veterans yields novel risk loci. *Biol. Psychiatry* **86**, 365–376. <https://doi.org/10.1016/j.biopsych.2019.03.984> (2019).
34. Spierling, S. R. & Zorrilla, E. P. Don't stress about CRF: Assessing the translational failures of CRF(1) antagonists. *Psychopharmacology* **234**, 1467–1481. <https://doi.org/10.1007/s00213-017-4556-2> (2017).
35. Pomrenze, M. B., Fetterly, T. L., Winder, D. G. & Messing, R. O. The corticotropin releasing factor receptor 1 in alcohol use disorder: Still a valid drug target?. *Alcohol. Clin. Exp. Res.* **41**, 1986–1999. <https://doi.org/10.1111/acer.13507> (2017).
36. Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94 (1974).
37. Jansen, G., Hazendonk, E., Thijssen, K. L. & Plasterk, R. H. Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. *Nat. Genet.* **17**, 119–121. <https://doi.org/10.1038/ng0997-119> (1997).
38. Jee, C., Goncalves, J. F., LeBoeuf, B. & Garcia, L. R. CRF-like receptor SEB-3 in sex-common interneurons potentiates stress handling and reproductive drive in *C. elegans*. *Nat. Commun.* **7**, 11957. <https://doi.org/10.1038/ncomms11957> (2016).
39. Reiner, D. J. *et al.* Behavioral genetics of *Caenorhabditis elegans* unc-103-encoded erg-like K(+) channel. *J. Neurogenet.* **20**, 41–66. <https://doi.org/10.1080/01677060600788826> (2006).
40. Sulston, J. E. & Brenner, S. The DNA of *Caenorhabditis elegans*. *Genetics* **77**, 95–104 (1974).
41. Ward, S. Chemotaxis by the nematode *Caenorhabditis elegans*: Identification of attractants and analysis of the response by use of mutants. *Proc. Natl. Acad. Sci. U.S.A.* **70**, 817–821. <https://doi.org/10.1073/pnas.70.3.817> (1973).
42. Bargmann, C. I., Hartwig, E. & Horvitz, H. R. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* **74**, 515–527. [https://doi.org/10.1016/0092-8674\(93\)80053-h](https://doi.org/10.1016/0092-8674(93)80053-h) (1993).
43. Hilliard, M. A., Bergamasco, C., Arbucci, S., Plasterk, R. H. & Bazzicalupo, P. Worms taste bitter: ASH neurons, QUI-1, GPA-3 and ODR-3 mediate quinine avoidance in *Caenorhabditis elegans*. *EMBO J.* **23**, 1101–1111. <https://doi.org/10.1038/sj.emboj.7600107> (2004).
44. Hilliard, M. A., Bargmann, C. I. & Bazzicalupo, P. *C. elegans* responds to chemical repellents by integrating sensory inputs from the head and the tail. *Curr. Biol. CB* **12**, 730–734. [https://doi.org/10.1016/s0960-9822\(02\)00813-8](https://doi.org/10.1016/s0960-9822(02)00813-8) (2002).
45. Gish, W. & States, D. J. Identification of protein coding regions by database similarity search. *Nat. Genet.* **3**, 266–272. <https://doi.org/10.1038/ng0393-266> (1993).
46. Papadopoulos, J. S. & Agarwala, R. COBALT: Constraint-based alignment tool for multiple protein sequences. *Bioinformatics* **23**, 1073–1079. <https://doi.org/10.1093/bioinformatics/btm076> (2007).
47. Lu, S. *et al.* CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Res.* **48**, D265–d268. <https://doi.org/10.1093/nar/gkz991> (2020).
48. Anderson, E. N. *et al.* *C. elegans* lifespan extension by osmotic stress requires FUDr, base excision repair, FOXO, and sirtuins. *Mech. Ageing Dev.* **154**, 30–42. <https://doi.org/10.1016/j.mad.2016.01.004> (2016).
49. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B (Methodol.)* **57**, 289–300 (1995).
50. Ashburner, M. *et al.* Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nat. Genet.* **25**, 25–29. <https://doi.org/10.1038/75556> (2000).
51. Mi, H., Muruganujan, A., Casagrande, J. T. & Thomas, P. D. Large-scale gene function analysis with the PANTHER classification system. *Nat. Protoc.* **8**, 1551–1566. <https://doi.org/10.1038/nprot.2013.092> (2013).
52. The gene ontology resource: 20 years and still GOing strong. *Nucleic Acids Res.* **47**, D330–d338. <https://doi.org/10.1093/nar/gky1055> (2019).
53. Angeles-Albores, D., Raymond, N. L., Chan, J. & Sternberg, P. W. Tissue enrichment analysis for *C. elegans* genomics. *BMC Bioinform.* **17**, 366. <https://doi.org/10.1186/s12859-016-1229-9> (2016).
54. Grove, C. *et al.* Using WormBase: A genome biology resource for *Caenorhabditis elegans* and related nematodes. *Methods Mol. Biol.* **1757**, 399–470. https://doi.org/10.1007/978-1-4939-7737-6_14 (2018).
55. Ly, K., Reid, S. J. & Snell, R. G. Rapid RNA analysis of individual *Caenorhabditis elegans*. *MethodsX* **2**, 59–63. <https://doi.org/10.1016/j.mex.2015.02.002> (2015).
56. Heimbucher, T. *et al.* The deubiquitylase MATH-33 controls DAF-16 stability and function in metabolism and longevity. *Cell Metab.* **22**, 151–163. <https://doi.org/10.1016/j.cmet.2015.06.002> (2015).
57. Rao, X., Huang, X., Zhou, Z. & Lin, X. An improvement of the 2^{-ΔΔCT} method for quantitative real-time polymerase chain reaction data analysis. *Biostat. Bioinform. Biomath.* **3**, 71–85 (2013).
58. Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J. & Vanfleteren, J. R. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*. *BMC Mol. Biol.* **9**, 9. <https://doi.org/10.1186/1471-2199-9-9> (2008).
59. Heimbucher, T., Hog, J., Gupta, P. & Murphy, C. T. PQM-1 controls hypoxic survival via regulation of lipid metabolism. *Nat. Commun.* **11**, 4627. <https://doi.org/10.1038/s41467-020-18369-w> (2020).

60. Alaimo, J. T. *et al.* Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*. *Alcohol. Clin. Exp. Res.* **36**, 1840–1850. <https://doi.org/10.1111/j.1530-0277.2012.01799.x> (2012).
61. Ahlenius, S. & Engel, J. Behavioral stimulation induced by ethanol withdrawal. *Pharmacol. Biochem. Behav.* **2**, 847–850. [https://doi.org/10.1016/0091-3057\(74\)90121-x](https://doi.org/10.1016/0091-3057(74)90121-x) (1974).
62. Burchi, E., Makris, N., Lee, M. R., Pallanti, S. & Hollander, E. Compulsivity in alcohol use disorder and obsessive compulsive disorder: Implications for neuromodulation. *Front. Behav. Neurosci.* <https://doi.org/10.3389/fnbeh.2019.00070> (2019).
63. Hyman, S. E. & Malenka, R. C. Addiction and the brain: The neurobiology of compulsion and its persistence. *Nat. Rev. Neurosci.* **2**, 695–703. <https://doi.org/10.1038/35094560> (2001).
64. Tiffany, S. T. & Carter, B. L. Is craving the source of compulsive drug use? *J. Psychopharmacol.* **12**, 23–30. <https://doi.org/10.1177/026988119801200104> (1998).
65. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. & Lefkowitz, R. J. Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J. Biol. Chem.* **267**, 1430–1433 (1992).
66. Nishihara, E. *et al.* A novel thyrotropin receptor germline mutation (Asp617Tyr) causing hereditary hyperthyroidism. *Endocr. J.* **54**, 927–934. <https://doi.org/10.1507/endocrj.k07-088> (2007).
67. Abadji, V., Lucas-Lenard, J. M., Chin, C. & Kendall, D. A. Involvement of the carboxyl terminus of the third intracellular loop of the cannabinoid CB1 receptor in constitutive activation of Gs. *J. Neurochem.* **72**, 2032–2038. <https://doi.org/10.1046/j.1471-4159.1999.0722032.x> (1999).
68. Nassel, D. R., Zandawala, M., Kawada, T. & Satake, H. Tachykinins: Neuropeptides that are ancient, diverse, widespread and functionally pleiotropic. *Front. Neurosci.* **13**, 1262. <https://doi.org/10.3389/fnins.2019.01262> (2019).
69. Mirabeau, O. & Joly, J. S. Molecular evolution of peptidergic signaling systems in bilaterians. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E2028–2037. <https://doi.org/10.1073/pnas.1219956110> (2013).
70. Steinhoff, M. S., von Mentzer, B., Geppetti, P., Pothoulakis, C. & Bunnett, N. W. Tachykinins and their receptors: Contributions to physiological control and the mechanisms of disease. *Physiol. Rev.* **94**, 265–301. <https://doi.org/10.1152/physrev.00031.2013> (2014).
71. Gayen, A., Goswami, S. K. & Mukhopadhyay, C. NMR evidence of GM1-induced conformational change of substance P using isotropic bicelles. *Biochim. Biophys. Acta* **127**–139, 2011. <https://doi.org/10.1016/j.bbamm.2010.09.023> (1808).
72. Schöppe, J. *et al.* Crystal structures of the human neurokinin 1 receptor in complex with clinically used antagonists. *Nat. Commun.* **10**, 17. <https://doi.org/10.1038/s41467-018-07939-8> (2019).
73. Ebner, K., Rupniak, N. M., Saria, A. & Singewald, N. Substance P in the medial amygdala: Emotional stress-sensitive release and modulation of anxiety-related behavior in rats. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 4280–4285. <https://doi.org/10.1073/pnas.0400794101> (2004).
74. Ebner, K., Singewald, G. M., Whittle, N., Ferraguti, F. & Singewald, N. Neurokinin 1 receptor antagonism promotes active stress coping via enhanced septal 5-HT transmission. *Neuropsychopharmacology* **33**, 1929–1941. <https://doi.org/10.1038/sj.npp.1301594> (2008).
75. Hoppe, J. M. *et al.* Association between amygdala neurokinin-1 receptor availability and anxiety-related personality traits. *Transl. Psychiatry* **8**, 168. <https://doi.org/10.1038/s41398-018-0163-1> (2018).
76. Otsuka, M. & Yoshioka, K. Neurotransmitter functions of mammalian tachykinins. *Physiol. Rev.* **73**, 229–308. <https://doi.org/10.1152/physrev.1993.73.2.229> (1993).
77. De Felipe, C. *et al.* Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* **392**, 394–397. <https://doi.org/10.1038/32904> (1998).
78. Hökfelt, T., Kellerth, J. O., Nilsson, G. & Pernow, B. Substance p: Localization in the central nervous system and in some primary sensory neurons. *Science* **190**, 889–890. <https://doi.org/10.1126/science.242075> (1975).
79. Hammarlund, M., Hobert, O., Miller, D. M. 3rd. & Sestan, N. The CeNGEN project: The complete gene expression map of an entire nervous system. *Neuron* **99**, 430–433. <https://doi.org/10.1016/j.neuron.2018.07.042> (2018).
80. Sulston, J. E., Albertson, D. G. & Thomson, J. N. The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. *Dev. Biol.* **78**, 542–576 (1980).
81. Singhvi, A. & Shaham, S. Glia-neuron interactions in *Caenorhabditis elegans*. *Annu. Rev. Neurosci.* **42**, 149–168. <https://doi.org/10.1146/annurev-neuro-070918-050314> (2019).
82. Burmeister, A. R. *et al.* Human microglia and astrocytes constitutively express the neurokinin-1 receptor and functionally respond to substance P. *J. Neuroinflamm.* **14**, 245. <https://doi.org/10.1186/s12974-017-1012-5> (2017).
83. Berlin, G. S. & Hollander, E. Compulsivity, impulsivity, and the DSM-5 process. *CNS Spectr.* **19**, 62–68. <https://doi.org/10.1017/S1092852913000722> (2014).
84. Feng, Z. *et al.* A *C. elegans* model of nicotine-dependent behavior: Regulation by TRP-family channels. *Cell* **127**, 621–633. <https://doi.org/10.1016/j.cell.2006.09.035> (2006).
85. Pierce-Shimomura, J. T., Morse, T. M. & Lockery, S. R. The fundamental role of pirouettes in *Caenorhabditis elegans* chemotaxis. *J. Neurosci.* **19**, 9557–9569. <https://doi.org/10.1523/jneurosci.19-21-09557.1999> (1999).
86. Pierce-Shimomura, J. T. *et al.* Genetic analysis of crawling and swimming locomotory patterns in *C. elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 20982–20987. <https://doi.org/10.1073/pnas.0810359105> (2008).
87. Wakabayashi, T., Kitagawa, I. & Shingai, R. Neurons regulating the duration of forward locomotion in *Caenorhabditis elegans*. *Neurosci. Res.* **50**, 103–111. <https://doi.org/10.1016/j.neures.2004.06.005> (2004).
88. Klein, M. *et al.* Exploratory search during directed navigation in *C. elegans* and *Drosophila* larva. *Elife* <https://doi.org/10.7554/eLife.30503> (2017).
89. Flavell, S. W. *et al.* Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in *C. elegans*. *Cell* **154**, 1023–1035. <https://doi.org/10.1016/j.cell.2013.08.001> (2013).
90. Campbell, J. C., Polan-Couillard, L. F., Chin-Sang, I. D. & Bendena, W. G. NPR-9, a GALANIN-LIKE G-protein coupled receptor, and GLR-1 Regulate interneuronal circuitry underlying multisensory integration of environmental cues in *Caenorhabditis elegans*. *PLoS Genet.* <https://doi.org/10.1371/journal.pgen.1006050> (2016).
91. Wes, P. D. & Bargmann, C. I. *C. elegans* odour discrimination requires asymmetric diversity in olfactory neurons. *Nature* **410**, 698–701. <https://doi.org/10.1038/35070581> (2001).
92. Hukema, R. K., Rademakers, S. & Jansen, G. Gustatory plasticity in *C. elegans* involves integration of negative cues and NaCl taste mediated by serotonin, dopamine, and glutamate. *Learn. Mem. (Cold Spring Harbor, N.Y.)* **15**, 829–836. <https://doi.org/10.1101/lm.994408> (2008).
93. Ghosh, D. D. *et al.* Neural architecture of hunger-dependent multisensory decision making in *C. elegans*. *Neuron* **92**, 1049–1062. <https://doi.org/10.1016/j.neuron.2016.10.030> (2016).
94. Ishihara, T. *et al.* HEN-1, a secretory protein with an LDL receptor motif, regulates sensory integration and learning in *Caenorhabditis elegans*. *Cell* **109**, 639–649. [https://doi.org/10.1016/S0092-8674\(02\)00748-1](https://doi.org/10.1016/S0092-8674(02)00748-1) (2002).
95. Shinkai, Y. *et al.* Behavioral choice between conflicting alternatives is regulated by a receptor guanylyl cyclase, GCY-28, and a receptor tyrosine kinase, SCD-2, in AIA interneurons of *Caenorhabditis elegans*. *J. Neurosci.* **31**, 3007–3015. <https://doi.org/10.1523/jneurosci.4691-10.2011> (2011).

96. Hilliard, M. A. *et al.* In vivo imaging of *C. elegans* ASH neurons: Cellular response and adaptation to chemical repellents. *Embo J.* **24**, 63–72. <https://doi.org/10.1038/sj.emboj.7600493> (2005).
97. Guo, M. *et al.* Reciprocal inhibition between sensory ASH and ASI neurons modulates nociception and avoidance in *Caenorhabditis elegans*. *Nat. Commun.* **6**, 5655. <https://doi.org/10.1038/ncomms6655> (2015).
98. Marques, F. *et al.* Identification of avoidance genes through neural pathway-specific forward optogenetics. *PLoS Genet.* **15**, e1008509. <https://doi.org/10.1371/journal.pgen.1008509> (2019).
99. Deroche-Gamonet, V., Belin, D. & Piazza, P. V. Evidence for addiction-like behavior in the rat. *Science* **305**, 1014–1017. <https://doi.org/10.1126/science.1099020> (2004).
100. Vengeliene, V., Celerier, E., Chaskiel, L., Penzo, F. & Spanagel, R. Compulsive alcohol drinking in rodents. *Addict. Biol.* **14**, 384–396. <https://doi.org/10.1111/j.1369-1600.2009.00177.x> (2009).
101. Seif, T. *et al.* Cortical activation of accumbens hyperpolarization-active NMDARs mediates aversion-resistant alcohol intake. *Nat. Neurosci.* **16**, 1094–1100. <https://doi.org/10.1038/nn.3445> (2013).
102. Barbier, E. *et al.* Dependence-induced increase of alcohol self-administration and compulsive drinking mediated by the histone methyltransferase PRDM2. *Mol. Psychiatry* **22**, 1746–1758. <https://doi.org/10.1038/mp.2016.131> (2017).
103. Egli, M. Advancing pharmacotherapy development from preclinical animal studies. *Handb. Exp. Pharmacol.* **248**, 537–578. https://doi.org/10.1007/164_2017_85 (2018).
104. Pastor, R. *et al.* Ethanol concentration-dependent effects and the role of stress on ethanol drinking in corticotropin-releasing factor type 1 and double type 1 and 2 receptor knockout mice. *Psychopharmacology* **218**, 169–177. <https://doi.org/10.1007/s00213-011-2284-6> (2011).
105. Ciccocioppo, R. *et al.* Stress-related neuropeptides and alcoholism: CRH, NPY, and beyond. *Alcohol* **43**, 491–498. <https://doi.org/10.1016/j.alcohol.2009.08.003> (2009).
106. Schank, J. R., Ryabinin, A. E., Giardino, W. J., Ciccocioppo, R. & Heilig, M. Stress-related neuropeptides and addictive behaviors: Beyond the usual suspects. *Neuron* **76**, 192–208. <https://doi.org/10.1016/j.neuron.2012.09.026> (2012).
107. Huey, E. D. *et al.* A psychological and neuroanatomical model of obsessive–compulsive disorder. *J. Neuropsychiatry Clin. Neurosci.* **20**, 390–408. <https://doi.org/10.1176/jnp.2008.20.4.390> (2008).
108. Ansari, Z. & Fadardi, J. S. Enhanced visual performance in obsessive compulsive personality disorder. *Scand. J. Psychol.* **57**, 542–546. <https://doi.org/10.1111/sjop.12312> (2016).
109. Clements, S., Schreck, C. B., Larsen, D. A. & Dickhoff, W. W. Central administration of corticotropin-releasing hormone stimulates locomotor activity in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol.* **125**, 319–327. <https://doi.org/10.1006/gcen.2001.7707> (2002).
110. Johnson, R. W., von Borell, E. H., Anderson, L. L., Kojic, L. D. & Cunnick, J. E. Intracerebroventricular injection of corticotropin-releasing hormone in the pig: Acute effects on behavior, adrenocorticotropin secretion, and immune suppression. *Endocrinology* **135**, 642–648. <https://doi.org/10.1210/endo.135.2.8033811> (1994).
111. Lowry, C. A. & Moore, F. L. Corticotropin-releasing factor (CRF) antagonist suppresses stress-induced locomotor activity in an amphibian. *Horm. Behav.* **25**, 84–96 (1991).
112. Winslow, J. T., Newman, J. D. & Insel, T. R. CRH and alpha-helical-CRH modulate behavioral measures of arousal in monkeys. *Pharmacol. Biochem. Behav.* **32**, 919–926 (1989).
113. Lee, E. H. & Tsai, M. J. The hippocampus and amygdala mediate the locomotor stimulating effects of corticotropin-releasing factor in mice. *Behav. Neural Biol.* **51**, 412–423 (1989).
114. Goeders, N. E. & Guerin, G. F. Non-contingent electric footshock facilitates the acquisition of intravenous cocaine self-administration in rats. *Psychopharmacology* **114**, 63–70 (1994).
115. Piazza, P. V. & Le Moal, M. The role of stress in drug self-administration. *Trends Pharmacol. Sci.* **19**, 67–74 (1998).
116. Koob, G. F. A role for brain stress systems in addiction. *Neuron* **59**, 11–34 (2008).
117. Froominckx, L. *et al.* Neuropeptide GPCRs in *C. elegans*. *Front. Endocrinol.* **3**, 167. <https://doi.org/10.3389/fendo.2012.00167> (2012).
118. Cardoso, J. C., Félix, R. C., Fonseca, V. G. & Power, D. M. Feeding and the rhodopsin family g-protein coupled receptors in nematodes and arthropods. *Front. Endocrinol. (Lausanne)* **3**, 157. <https://doi.org/10.3389/fendo.2012.00157> (2012).
119. Hobert, O. The neuronal genome of *Caenorhabditis elegans*. *WormBook* <https://doi.org/10.1895/wormbook.1.161.1> (2013).
120. Ashrafi, K. *et al.* Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421**, 268–272. <https://doi.org/10.1038/nature01279> (2003).
121. Regoli, D., Drapeau, G., Dion, S. & D'Orléans-Juste, P. Pharmacological receptors for substance P and neurokinins. *Life Sci.* **40**, 109–117. [https://doi.org/10.1016/0024-3205\(87\)90349-3](https://doi.org/10.1016/0024-3205(87)90349-3) (1987).
122. Hökfelt, T., Pernow, B. & Wahren, J. Substance P: A pioneer amongst neuropeptides. *J. Intern. Med.* **249**, 27–40. <https://doi.org/10.1046/j.0954-6820.2000.00773.x> (2001).
123. Kwako, L. E. *et al.* The corticotropin releasing hormone-1 (CRH1) receptor antagonist pexacerfont in alcohol dependence: A randomized controlled experimental medicine study. *Neuropsychopharmacology* **40**, 1053–1063. <https://doi.org/10.1038/npp.2014.306> (2015).
124. Schwandt, M. L. *et al.* The CRF1 antagonist verucerfont in anxious alcohol-dependent women: Translation of neuroendocrine, but not of anti-craving effects. *Neuropsychopharmacology* **41**, 2818–2829. <https://doi.org/10.1038/npp.2016.61> (2016).
125. Elliott, P. J., Mason, G. S., Stephens-Smith, M. & Hagan, R. M. Behavioural and biochemical responses following activation of midbrain dopamine pathways by receptor selective neurokinin agonists. *Neuropeptides* **19**, 119–126. [https://doi.org/10.1016/0143-4179\(91\)90141-5](https://doi.org/10.1016/0143-4179(91)90141-5) (1991).
126. Noailles, P. A. & Angulo, J. A. Neurokinin receptors modulate the neurochemical actions of cocaine. *Ann. N. Y. Acad. Sci.* **965**, 267–273. <https://doi.org/10.1111/j.1749-6632.2002.tb04168.x> (2002).
127. Thorsell, A., Schank, J. R., Singley, E., Hunt, S. P. & Heilig, M. Neurokinin-1 receptors (NK1R:s), alcohol consumption, and alcohol reward in mice. *Psychopharmacology* **209**, 103–111. <https://doi.org/10.1007/s00213-010-1775-1> (2010).
128. Schank, J. R. *et al.* Tacr1 gene variation and neurokinin 1 receptor expression is associated with antagonist efficacy in genetically selected alcohol-preferring rats. *Biol. Psychiatry* **73**, 774–781. <https://doi.org/10.1016/j.biopsych.2012.12.027> (2013).
129. Nelson, B. S. *et al.* Escalated alcohol self-administration and sensitivity to yohimbine-induced reinstatement in alcohol preferring rats: Potential role of neurokinin-1 receptors in the amygdala. *Neuroscience* **413**, 77–85. <https://doi.org/10.1016/j.neuroscience.2019.06.023> (2019).
130. Foroud, T. *et al.* The tachykinin receptor 3 is associated with alcohol and cocaine dependence. *Alcohol. Clin. Exp. Res.* **32**, 1023–1030. <https://doi.org/10.1111/j.1530-0277.2008.00663.x> (2008).

Acknowledgements

This work was supported by College of Medicine, University of Tennessee Health Science Center (UTHSC). We thank the *C. elegans* Genetics Center (CGC) for providing strains, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Authors declare no conflict of interests.

Author contributions

C.S. participated in generating Figs. 4, 5, 6, and 7 and writing. A.K.K. generated and analyzed Figs. 2, 6, and 7. A.K.K. also participated in writing. E.B. participated in generating Figs. 7 and 8. E.C.P. analyzed the data for Fig. 1. C.J. Conducted the works for Fig. 1, 3, 5, 7, and 8. C.J. designed the all experiments and direct the overall project including analysis and writing.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-022-05256-1>.

Correspondence and requests for materials should be addressed to C.J.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2022