Neuropeptidergic regulation of compulsive ethanol seeking in C. elegans

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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 consumption1–3. Subsequently, during the withdrawal or abstinence, alcohol craving and seeking are reinstated4–6. Since these three phases model has provided an 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 disorders8–10. 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 use12,13. 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. elegans14–16.

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 orthologues17,18, 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 AUD19–25. 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 dependence6,20,27. Subsequently, we discovered that seb-3 (Secretin family Class B GPCR), a corticotropin-releasing factor (CRF) receptor-like GPCR in C. elegans,
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\textsuperscript{30-33}. Additionally, its function has been implicated in compulsive drug self-administration and stress-induced reinstatement of drug seeking in mammalian studies\textsuperscript{32}. Furthermore, a recent GWAS identified CRFR1 loci is associated with habitual alcohol intake\textsuperscript{34}. 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\textsuperscript{35,36}. 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 °C\textsuperscript{36} 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)gf previously isolated from a genetic screening and the seb-3(tm1848)gf strain was obtained from S. Mitani (Tokyo Women’s Medical University, Tokyo, Japan) and backcrossed twice with N2\textsuperscript{37}. Double and triple mutant strains were generated by PCR, taking advantage of the feature that tkr-1(ok2886) and tkr-2(ok1620) are deletion alleles\textsuperscript{32}; tkr-1(ok2886) III; seb-3(eg696) X, tkr-2(ok1620) IV; seb-3(eg696)X, tkr-1(ok2886) III; seb-3(eg696) X, tkr-2(ok1620) IV; seb-3(eg696)X.

Transgenic strains include the following: tkr-1(ok2886) chjEx101 [pCJ201 pkr-1:tkr-1(1WT); pofm-1:gfp, and pha-1(e2123) chjEx102 [pCJ210 pkr-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 pCJ201. About 800 bp of Flanking sequences are sequenced by M13 Forward and backward primers. pCJ201 plasmid was injected into the germline of wild-type C. elegans with pofm-1:gfp construct as an injection marker. The concentration of the final injection mixtures was 150 ng μl\textsuperscript{-1}. Pha-1 (+), was co-injected at 50 ng μl\textsuperscript{-1} into pha-1(e2123) as an injection marker. Using pha-1 (+) as an injection marker as described\textsuperscript{38}. The pCJ210 was injected at 50 ng μl\textsuperscript{-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 pDG15\textsuperscript{39}, 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\textsuperscript{-1}. A Pofm-1:gfp plasmid was co-injected at 50 ng μl\textsuperscript{-1}. The pCJ201 was injected at 5–50 ng μl\textsuperscript{-1}. Multiple lines of extrachromosomal arrays were maintained, and L4 animals expressing 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 cm\textsuperscript{2}) of the culture dish and recorded locomotion of animals were analyzed. NGM was added to each well of 4 well plate of the culture dish and recorded locomotion of animals were analyzed. NGM was added to the mid-region of 4-well plate by gentle Trajectory analysis of the animals were allowed to move freely on the assay plate, which contains ethanol in only one of the 4 well (1.9 cm\textsuperscript{2}) of the culture dish and recorded locomotion of animals were analyzed. NGM was added to each well of 4 well plate of the culture dish and recorded locomotion of animals were analyzed. NGM was added to the mid-region of 4-well plate by gentle movement 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)),\textsuperscript{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\textsuperscript{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\textsuperscript{15}) 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 CuSO\(_4\) 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 CuSO\(_4\) (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 CuSO\(_4\) solution, the filter paper was gently removed using forceps. Meanwhile, 1 µl of 1 M NaNO\(_2\) 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 \([\text{(number of animals in A + number of animals in B)/Total number of animals} \times \text{Seeking index SI = (A – B)/Total(A + B + C + D)}]\). To measure the copper sensitivity, 20 naïve or Ethanol-pretreated animals were placed on the aversion-resistant assay plate with 5 mM CuSO\(_4\) 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 CuSO\(_4\)) was conducted as shown in previous study\(^{43,44}\), to measure the sensitivity to nociceptive stimuli of WT and seh-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 search\(^{45}\) and the multiple protein sequences were simultaneously aligned using the COBALT, a constraint based alignment tool\(^{46}\). 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_03340.3). 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 seh-3(eg696) were 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\(^\circ\). 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, seh-3(eg696), FUDR-treated WT, and FUDR-treated seh-3(eg696), respectively. Since FUDR affects some physiological function such as osmotic stress related life span extension\(^{48}\), 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 Conndole. 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. Benjamin Hochberg false discovery rate method was applied in order to obtain the adjusted \( p \) value for each gene\(^2\). 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 significant\(^3\). 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 in\(^55\), 10 worms of FUdR-treated WT, and FUdR-treated seb-3ge(e6966), respectively, were transferred to a drop of ~20 µl of S-basal buffer to remove bacteria. Washed worms were transferred into 2 µl 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 previously\(^56\). 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)) Method\(^57\) 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 µg 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 \( krt-1 \) 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 experience\(^60\). 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 mM\(^60\). 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 chronically\(^21\). 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 area 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 speed\(^19\). 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 systems\(^20\). 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 systems\(^61\). 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
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 Cu²⁺ 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 Cu²⁺ barrier in time and Seeking Index (SI) was obtained as shown in (Fig. 3a). We tested what range of Cu²⁺ 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 Cu²⁺ response in a concentration-dependent...

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.
manner (Fig. 3c). The ethanol preference developed by pretreatment enabled WT animals to cross the Cu²⁺ 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 Cu²⁺ barrier. 4 h pretreatment of ethanol was not enough to enable WT animals to cross the aversive Cu²⁺ 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 consequences⁶²–⁶⁴, 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 mice⁵². 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 symptom⁵². 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 Cu²⁺ 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 proteins⁵²–⁵⁷. First, ethanol preference of seb-3(eg696) animals and seb-3(tm1848) Knockout animals due to its deletion 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) Ethanol pretreated animals demonstrates more animals cross over the Cu barrier for ethanol (aversion-resistant seeking), in low concentration (2 and 5 mM), than does Naïve animals [F_{EtOH pretreated}(1, 18) = 46.64, p < 0.0001; F_{Concentration(4,72)} = 99.01, p < 0.0001; F_{pretreated×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 naïve 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 ± SEM. N = 10 in each conc. (d) Ethanol pretreated animals demonstrates more animals cross over the Denatonium barrier for ethanol (aversion-resistant seeking), in low concentration, than does Naïve animals [F_{EtOH pretreated}(1, 6) = 598.8, p < 0.0001; F_{Concentration(1,712)} = 119, p < 0.0001; F_{pretreated×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 naïve 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 ± SEM. N = 4 in each conc.
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 Cu²⁺ (Fig. 5a). Nevertheless, sensory perception of aversive Cu²⁺ 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 Cu²⁺ 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 identity 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 (≥ 1.5×, 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 receptor. Tachykinins/Neurokinins and their receptors are neuropeptides signaling conserved from invertebrates to mammals. 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). 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 core. 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. We confirmed the upregulation of tkr-1 observed in the

Figure 4. SEB-3 facilitates the development of ethanol preference. (a) Diagrammatic representation of ethanol preference assay. Naive or ethanol pretreated animals remain free to explore on the quadrants plate before counting. (b) Naive 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 × 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–3gf) at naive, 2 h, and 4 h are shown (p < 0.05,* p < 0.01,** p < 0.001,****).
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 (Dunnett’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 CuSO4). 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 Dunnett’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 (Dunnett’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
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 method. 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 orthologue, 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 and 79). 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

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).
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 anxiety. 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, that has been described as a compelling urge to intake alcohol and considered crucial for the maintenance of AUD. The compulsive alcohol seeking is characterized by an imbalance between superior drive to alcohol and disruption in control of alcohol use. 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 ethanol. 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 study, and nicotine, classified as a psychostimulant like ethanol, can also be compared to promoting withdrawal-induced motor stimulation in *C. elegans*. The locomotion trajectories are depending on the transition between distinct motor states, which is the modulation of random exploration behavior. Since the long-term behavioral state of exploration in *C. elegans* is modulated by neuropeptide signals including seb-3, 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. 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. We introduced noxious stimuli, of which perception and mediation are well defined, 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. The progress of compulsivity is hypothesized to be balanced 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 noxious chemicals, of which avoidance is mediated by polymodal sensory neurons for nociception 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* 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. 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. 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 stimuli. 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. 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), human TACR1/NK1R (2K99_A), human TACR3/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); seb-3(eg696)* triple (h). A two-way ANOVA comparison shows the impaired development in *tkr-1(ok2886)* [*F*<sub>Genotype</sub>(1, 58) = 106.6, *p* < 0.0001; *F*<sub>Concentration</sub>(3, 58) = 140.7, *p* < 0.0001; *F*<sub>Genotype×Concentration</sub>(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, ****). Comparison between genotype is also shown (*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. Dil 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*<sub>Genotype</sub>(1, 8) = 63.34, *p* < 0.0001; *F*<sub>Concentration</sub>(3, 8) = 107.4, *p* < 0.0001; *F*<sub>Genotype×Concentration</sub>(3, 8) = 17.20, *p* = 0.0032]. 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, ****).
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. elegans23. The tkr-1, tkr-2, and tkr-3 have been categorized into tachykinin/neurokinin receptor-like group17-19 and RNAi functional screens revealed tkr-1 affects fat metabolism and deposition20. 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 neuropeptides21,22. 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 depression73–75. Recently after the failure of CRF1 antagonist in Clinical trials123,124, 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 NA125 and NK1R antagonist was reported to reduce cocaine-induced DA release26. Antagonism of NK1R and KO mice reduces alcohol consumption27 and Antagonism of NK1R decreases alcohol self-administration in alcohol-prefering rats128. Furthermore, adeno-associated virus-mediated overexpression of NK1R in the central amygdala increased alcohol self-administration28, 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 sed-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 sed-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.

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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.

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