Ethanol facilitates socially evoked memory recall in mice by recruiting pain-sensitive anterior cingulate cortical neurons

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Alcohol is a traditional social-bonding reinforcer; however, the neural mechanism underlying ethanol-driven social behaviors remains elusive. Here, we report that ethanol facilitates observational fear response. Observer mice exhibited stronger defensive immobility while observing cagemates that received repetitive foot shocks if the observer mice had experienced a brief priming foot shock. This enhancement was associated with an observation-induced recruitment of subsets of anterior cingulate cortex (ACC) neurons in the observer mouse that were responsive to its own pain. The vicariously activated ACC neurons projected their axons preferentially to the basolateral amygdala. Ethanol shifted the ACC neuronal balance toward inhibition, facilitated the preferential ACC neuronal recruitment during observation, and enhanced observational fear response, independent of an oxytocin signaling pathway. Furthermore, ethanol enhanced socially evoked fear response in autism model mice.
Alcohol has been enjoyed throughout the history of humankind, as it promotes sociality. Acute alcohol consumption reduces social stress, increases generosity, and improves recognition of facial expressions. Recent studies have demonstrated that it enhances social bonding and promotes smile contagion, suggesting that alcohol enhances affective empathy (or emotional contagion), which underlies altruistic motivation and prosocial behaviors. However, the neural mechanism of alcohol-driven sociality remains to be identified.

Sociality is enhanced by prior similar experiences. For example, prior similar experiences of the events in stories increase empathy for the persons in the stories. Freis et al. reported that prior pain exposures facilitate subsequent state empathy for pain. Human brain imaging studies suggest that vicarious experience of others’ pain involves shared representation of experienced and observed pain. This notion raises a possibility that this overlapping neural representation may underlie the experience-dependent facilitation of empathy. However, voxel-level analyses using functional magnetic resonance imaging do not necessarily support the shared representation hypothesis at the single-neuron level.

Empathy is a high-level affective process that is often expressed by humans, but rodents also manifest some aspects of empathy-like behaviors. Affective empathy is modeled using the fear observational system, in which an animal (observer) exhibits behavioral defensive immobility when it observes the distress of a conspecific (demonstrator) receiving electrical shocks. In this fear transmission system, the anterior cingulate cortex (ACC), a pain-relevant brain region, is critical for experiencing vicarious pain, consistent with human imaging studies. However, fundamental questions remain unsolved, including (i) whether firsthand and vicarious pain activate the same ACC neurons at the single-cell level and (ii) if so, whether ethanol modulates the overlapping ACC neuronal representation and observational fear.

Like ethanol, the neuropeptide oxytocin is also involved in social functioning, including consolation behavior, maternal care, and social recognition. Furthermore, intranasal oxytocin improves behavioral and neural deficits in autism. Regarding empathy, intranasal oxytocin enhances affective empathy in response to both positive- and negative-valence stimuli, and facilitates the perceptions of harm for victims. However, the social effect of oxytocin depends on context. For example, oxytocin does not modulate empathic responses to painful pictures but facilitates them when participants are asked to take the perspective of others. Moreover, the social effect of oxytocin differs between in-group and out-group members. In this work, we also examined the involvement of endogenous oxytocin signaling in observational fear transmission and compared it to the effect of exogenous ethanol. As a result, we found that mice treated with ethanol-increased defensive immobility while observing the distress of demonstrators. The effect of ethanol emerged independently of the oxytocin signaling.

Results
Ethanol enhances socially evoked fear memory recall. We first examined the effect of a prior shock experience on subsequent fear observation in mice. A total of 80 pairs of cagemates cohoosed for 1–2 weeks were randomly divided into four groups, no priming shock/fear observation (no-PS/FO), FO-only, PS-only, and PS + FO (Fig. 1a), and each pair consisted of a randomly chosen observer and demonstrator. In the PS + FO group, the observers were briefly placed in a shock chamber and given a single priming foot shock. Two hours later, the observers were placed in a fear observational chamber in which demonstrators in the neighboring compartment received repetitive foot shocks (every 12 s for 4 min). In the FO-only group, observers were placed in the same shock chamber but did not receive a priming shock and were then tested for fear observation. In the PS-only group, observers underwent a priming shock, but the demonstrators did not receive repetitive shocks. During the fear observation period, the defensive immobility of the observers in the PS + FO group was significantly higher than that in the three other groups (Fig. 1b), suggesting that the prior shock experience facilitated observational fear response. When the observers in the PS + FO group were re-exposed to the same context 24 h after the observation, they exhibited defensive immobility (Supplementary Fig. 1), indicating that they learned fear-associated contexts by observing their cagemates’ behavior. The observers in the PS-only group showed no apparent defensive immobility, and thus, the priming shock alone was insufficient to induce fear response (Fig. 1b and Supplementary Fig. 1). The facilitatory effect of the priming shock lasted for at least 4 w (Supplementary Fig. 2) and was abolished by intraperitoneal injection of 0.1 mg/kg MK801, an N-methyl-D-aspartate (NMDA) receptor inhibitor, 30 min before the priming shock (Supplementary Fig. 3a, b), but not 30 min before fear observation (Supplementary Fig. 3c). The priming shock could not be replaced with other aversive experiences, such as tail pinches or forced swimming (Supplementary Fig. 4), suggesting that observational fear response is facilitated by common experiences shared between demonstrators and observers.

A recent study has reported that acute intranasal treatment with oxytocin enhances observational fear in mice. Therefore, we examined the involvement of endogenous oxytocin in observational fear by preventing the oxytocin signaling in the FO-only group. Intrapерitoneal injection of 5 mg/kg L-368,899, an oxytocin receptor antagonist, 30 min before the fear observation reduced the duration of defensive immobility (Supplementary Fig. 5). A similar effect of L-368,899 was produced when observer mice had experienced a priming shock (Fig. 1c).

We investigated the effect of ethanol on observational fear response. We adopted a dose of 1.5 g/kg, based on the blood ethanol concentration observed with daily alcohol consumption in humans. Observers that had received intraperitoneal injection of ethanol 30 min before the fear observation (i.e., 90 min after the priming shock) exhibited more defensive immobility than saline-treated controls (Fig. 1c), an effect that was not reduced by L-368,899 (Fig. 1c). Thus, ethanol enhances observational fear response in an oxytocin-independent manner. The facilitatory effect of ethanol on observational fear cannot be explained by modulations of locomotor, anxiety, or pain-related behavior because, consistent with previous reports, this dose of ethanol facilitated locomotor activity (Supplementary Fig. 6a, b) and tended to decrease anxiety-like behaviors (Supplementary Fig. 6c–f), but did not alter pain sensitivity (Supplementary Fig. 6g, h). Moreover, the effect of ethanol was not likely due to enhanced consolidation or retrieval of a simple context-dependent priming shock memory, given that ethanol prevented contextual fear memory recall (Supplementary Fig. 7), and also because observational fear response was enhanced 24 h after the priming shock when ethanol was administered 30 min before the fear observation but not when ethanol was administered 90 min after the priming shock (Supplementary Fig. 8a, b). Importantly, ethanol failed to enhance fear transmission in the FO-only group (Supplementary Fig. 8c). Thus, we conclude that ethanol facilitates socially evoked recall of observer’s own fear memory.

Firsthand and vicarious pain share neuronal representations. To examine the neuronal representation underlying observational fear at a cellular resolution, we conducted cellular compartment...
analyses of temporal activity by fluorescent in situ hybridization (catFISH)\textsuperscript{31}, which can discriminate neurons that are activated during two time windows, the firsthand pain (primary shock) and the vicarious pain (fear observation), by taking advantage of the intracellular localization of the immediate early gene Arc in response to neuronal activity, Arc mRNA appears in the nucleus within 5 min and moves to the cytoplasm 20–45 min later. Therefore, in the experimental schedule depicted in Fig. 2a, nuclear and cytoplasmic Arc-positive (Nuc\textsuperscript{+} and Cyto\textsuperscript{+}, respectively) cells are expected to represent neurons that were activated by the fear observation and the priming shock, respectively. We first analyzed the Arc expression in ACC neurons (Fig. 2b). The percentages of Nuc\textsuperscript{+} cells were consistent with those reported in a previous study\textsuperscript{32}, and did not differ between the PS + FO and FO-only groups (Fig. 2c), suggesting that the facilitatory effect of the priming shock was not mediated simply by an increase in ACC neuronal activity. We then computed the overlap score for nuclear/cytoplasmic double-positive (Double\textsuperscript{+}) cells (Fig. 2d), which represents the extent to which neurons were commonly activated by both the priming shock and the fear observation. The PS + FO group had a significantly higher overlap score than the FO-only control group (PS + FO: 57.9 ± 3.5, FO-only: 16.0 ± 1.4, \(t_{10} = 11.2, P = 5.70 \times 10^{-2}, n = 6\) mice per group). Consistent with the behavioral data, the scores of the PS + FO group were decreased and increased by intraperitoneal L-368,899 and ethanol, respectively (Fig. 2e), and L-368,899 did not affect ethanol-induced increases in scores (Fig. 2e).

We also analyzed Arc mRNA in the anterior insular cortex, the basal/lateral amygdala (BLA), and the dorsal hippocampal CA1 region (Supplementary Fig. 9), but unlike in the ACC, we failed to find significant overlap scores (Fig. 2f), though these brain regions exhibited significant percentages of Double\textsuperscript{+} cells; note that the overlap score was defined such that it excluded the possible effects of population sizes\textsuperscript{33}. Consistent with that result, local injections of 4.1 µg/side L-368,899 and 7.0 µg/side ethanol into the ACC were sufficient to reduce and enhance observational fear response, respectively (Fig. 3a). Moreover, individual-animal analyses demonstrated that the duration of defensive immobility during observation was positively correlated with the overlap scores of ACC neurons, but not with the percentages of Cyto\textsuperscript{+} or Nuc\textsuperscript{+} cells (Fig. 3b–d).

Although the overlap scores in the BLA did not differ between the PS-only and PS + FO groups (Fig. 2f), the BLA receives anatomically direct afferents from the ACC, and this neural pathway is a part of the neuronal substrate for observational fear learning\textsuperscript{13,34}. Thus, the overlapping ACC activity may be transmitted to the BLA. We retrogradely labeled the neurons projecting to the BLA by injecting Alexa 594-conjugated cholera toxin subunit B (CTB, 0.5 µg/side) into the BLA and conducted Arc catFISH for ACC neurons (Fig. 4a–d). CTB-positive cells were more frequently observed in Cyto\textsuperscript{+} cells than in nuclear-only Arc-positive cells or double-negative cells (Fig. 4e, \(n = 4\) mice). Thus, ACC neurons that projected to the BLA were preferentially recruited by a priming shock, potentially indicating
that the increased overlap of ACC neuron ensembles reflects increased functional connectivity between the ACC and BLA.

**Ethanol shifts the E/I-balance in ACC neurons.** To examine how ethanol modulates ACC neuronal activity, we recorded miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively) from layer II/III ACC pyramidal neurons in acute brain slices and bath-applied 50 mM ethanol (Fig. 5a), which corresponds to the normal intrabrain concentrations after systemic injection of ethanol. Ethanol decreased the amplitudes and frequencies of mEPSCs and increased the frequencies of mIPSCs, whereas it did not alter the amplitudes of mIPSCs (Supplementary Fig. 10). Overall, ethanol decreased the mEPSC conductances (Fig. 5b, left) and increased the mIPSC conductances (Fig. 5b, middle), thereby reducing the net excitatory to inhibitory (E/I) ratio (Fig. 5b, right). To examine how the E/I-balance shift affects socially evoked fear memory recall, we injected 79 ng/side clonazepam, a traditional benzodiazepine, or 0.3 µg/side picrotoxin, a γ-aminobutyric acid (GABA)γ-receptor inhibitor, into the ACC. Note that clonazepam is reported to reduce the synaptic E/I ratios and thereby ameliorate social behavioral deficits. As we expected, clonazepam increased observational fear response in control mice, whereas picrotoxin reduced it (Fig. 5c). Picrotoxin also abolished ethanol-enhanced observational fear response (Fig. 5c).

**Ethanol rescues social impairment of poly(I:C) mice.** Finally, we examined observational fear in a mouse model of maternal immune activation. We treated pregnant mice with poly-inosine:cytosine (poly(I:C)), a synthetic analogue of double-stranded RNA that mimics a molecular pattern of viral infection, and tested the offspring in the fear observation test. The priming shock failed to enhance observational fear response in these poly(I:C) mice (Fig. 6a). However, intraperitoneal ethanol administration in poly(I:C) mice restored observational fear response to levels comparable to those in control mice (Fig. 6b). Poly(I:C) mice exhibited normal defensive immobility during their own contextual fear conditioning and higher defensive immobility during subsequent memory tests (Supplementary Fig. 11). Thus, neither fear expression nor fear learning ability was impaired in poly(I:C) mice. Arc catFISH revealed that poly(I:C) mice exhibited a lower ACC overlap score than control mice (Fig. 6c), whereas there were no effects on the percentages of either Cyto+ or Nuc+ cells per se (Supplementary Fig. 12). The overlap score of poly(I:C) mice was increased by intraperitoneal ethanol (Fig. 6d).
consistent with a report showing that the strength of fear memory is not coded by the overall size of memory trace in the lateral amygdala. Thus, overlapping populations of ACC neurons may integrate firsthand and vicarious pain information and facilitate socially evoked fear memory recall.

Our findings suggest that overlapping neuronal representations are shaped by neuronal E/I balance, consistent with other studies about learning and memory. The E/I balance in individual neurons is a fundamental determinant of memory allocation. The E/I balance regulates the neuronal overlap and thereby determines whether two memories are integrated or segregated. A computational study has also shown that balanced E/I ratios are required for noise-robust neuronal selection. Moreover, imbalanced E/I ratios are one of the major biological characteristics of human autism spectrum disorders, in which the number of parvalbumin-expressing interneurons and the GABA concentration in the cerebral cortex are reduced. GABAergic signaling is also disrupted in mouse models of autism. In normal mice, an artificial elevation of the E/I balance leads to social deficits. However, the mechanisms linking the E/I balance and social impairments remain unclear. Based on our results, we suggest that ethanol reduced the E/I ratio, increased the shared neuronal representations for experienced and observed pain, and enhanced observational fear in both normal and autism model mice, thus possibly providing a cell-level framework for both sociality and social impairments in developmental disorders. However, our data must be interpreted with caution. We performed only pharmacological manipulations on the excitability of neuronal circuits of the ACC and did not provide a direct link between the E/I balance and social behavior. Moreover, we found that injection of picrotoxin alone abolished the observational fear response. Therefore, it is possible that picrotoxin caused dysfunction of the ACC neuronal circuits, rather than a modulation of the E/I balance, and impaired social behavior.

We rule out the possibility that the facilitatory effect of ethanol on defensive immobility during fear observation resulted merely from ethanol-induced sedation because ethanol-increased locomotor activity and had no effect on immobility time in the open field test at the dose (1.5 g/kg) used in our study. Consistent with our observations, ethanol is known to increase locomotor frequency but does not alter immobility in the open field test at similar doses. Another concern of using ethanol was the acute effect on memory. In humans, ethanol administered after training is reported to facilitate the ability to learn various tasks; the results from animal studies are contradictory. Posttraining ethanol can promote learning or impair learning. Thus, in our study, it is possible that ethanol-enhanced consolidation of the priming shock memory facilitated observational fear. However, we demonstrated that injection of ethanol prior to the fear observation, but not after the priming shock, promoted observational fear, suggesting that the enhanced memory consolidation cannot account for the facilitatory effect of ethanol. The defensive immobility in the PS + FO group contains an aspect of memory recall. Therefore, ethanol may increase defensive immobility by enhancing the recall of observer’s own fear memory. We confirmed that ethanol did not promote fear memory recall in a classical fear-conditioning paradigm. Previous reports have also demonstrated that ethanol impairs, rather than facilitates, memory retrieval in the Morris water maze task and the passive avoidance task. Therefore, we believe that ethanol-enhanced associative memory recall in a classical fear-conditioning paradigm. Previous reports have also demonstrated that ethanol impairs, rather than facilitates, memory retrieval in the Morris water maze task and the passive avoidance task. Therefore, we believe that ethanol-enhanced associative memory recall in a classical fear-conditioning paradigm. Previous reports have also demonstrated that ethanol impairs, rather than facilitates, memory retrieval in the Morris water maze task and the passive avoidance task. Therefore, we believe that ethanol-enhanced associative memory recall in a classical fear-conditioning paradigm.
that is evaluated by tail-decubitus assays. The specificity of the systemic injection of ethanol induces analgesia and reduces the intensity of electric shock needed to induce pain-related responses, suggesting that ethanol did not apparently affect pain sensitivity. Under our experimental conditions, therefore, ethanol-enhanced analgesia may be simply reflect an increase or decrease in the minimal electric shock intensity needed to induce pain-related responses, including tail-squeeze and paw-flinch responses. This discrepancy may be due to differences in the types or intensities of pain stimuli, in the experimental protocols, or in the behavioral endpoints measured. Further studies are needed to clarify the mechanisms underlying ethanol's effects on pain sensitivity.

Interestingly, alcohol-dependent patients are known to exhibit a lower level of empathy. This fact is apparently contradictory to our findings, but chronic ethanol treatment induces neurochemical changes in both excitatory and inhibitory synaptic transmissions in the cerebral cortex, and those changes are different from the acute effect of ethanol. Chronic ethanol increases the mRNA level of NMDA receptors and decreases the mRNA level of GABA receptors, potentially shifting the E/I balance toward excitation. In fact, cortical inhibition is reduced in alcoholic patients. By contrast, we found that the acute application of ethanol shifted the E/I balance toward inhibition. Therefore, ethanol seems to exert opposite effects in acute or chronic treatment.

There are also conflicting reports regarding the effects of ethanol on the perception of one's own pain. Several studies have demonstrated that systemic injection of ethanol induces analgesia that is evaluated by tail-deflection or tail-flick assays, whereas other reports have shown that similar doses of ethanol have no effect on motor responses to various intensities of electrical shocks. This discrepancy may be due to differences in the types or intensities of nocuous stimuli delivered to animals. In our study, intraperitoneal injection of 1.5 g/kg ethanol did not alter the minimal electrical shock intensity needed to induce pain-related responses or the frequency of acetic acid-induced writhing responses, suggesting that ethanol did not apparently affect pain sensitivity. Under our experimental conditions, therefore, ethanol-enhanced observational fear is unlikely attributable to a sensitized pain system.

In contrast to our findings, some studies have demonstrated that ethanol enhances aggression in mice. These studies assessed aggressive behaviors using resident–intruder tests, in which a resident mouse attacks an unfamiliar mouse that intrudes the territory. This paradigm differs from the situation in our fear observation test in which an observer and a demonstrator are cagemates and do not exhibit offensive behaviors against one another. In general, familiarity between two animals is crucial for establishing empathy-related behaviors, such as fear transmission, prosocial behavior, and social modulation of pain. In humans, oxytocin augments not only in-group favoritism but also out-group derogation. Ethanol may work in a similar way and facilitate socially evoked fear memory recall only to in-group members.

Also in contrast to our study showing that oxytocin increases observational fear expression, several reports have demonstrated that oxytocin attenuates fear per se through modulating the activity of the central amygdala. These studies showed that artificial augmentation of oxytocin signaling, such as microinjection of oxytocin receptor agonist and optogenetically evoked axonal oxytocin release, in the central amygdala causes fear reduction, but they did not mention whether naturally released endogenous oxytocin affects defensive immobility. Indeed, systemic administration of L-368,899 (5 or 10 mg/kg) did not alter defensive immobility in Pavlovian conditioning. A meta-analysis of human studies also found that intranasal oxytocin did not significantly influence the expression of negative emotions, including fear. Therefore, it is unclear whether naturally released oxytocin in the amygdala is truly involved in fear, even though artificially elevated oxytocin signaling can attenuate fear.

On the other hand, we showed that intra-ACC injection of L-368,899 is sufficient to reduce observational fear response, suggesting that oxytocin differently affects Pavlovian-conditioned fear and socially evoked fear. This difference may simply reflect
the difference between the brain regions, and it is also possible that oxytocin-reduced fear is surpassed by oxytocin-induced facilitation of socially evoked fear.

In summary, we demonstrated that ACC neuronal representation of self-experienced pain and vicarious pain overlaps at the single-cell level and that this overlapping representation may underlie shared affective states between individuals. Moreover, our study revealed that the overlapping representation was increased by acute administration of ethanol. Our data were obtained exclusively from a fear observational system in mice, and extrapolation to other empathetic states requires care; however, our findings provide insights into the role of ethanol in sociality.

### Methods

#### Animals

Male 4- to 12-week-old C57BL/6J mice (SLC, Shizuoka, Japan) were housed under conditions of controlled temperature and humidity (23 ± 1°C, 55 ± 5%) on a 12:12-h light/dark cycle with the lights on from 7:00 a.m. to 7:00 p.m., and had access to food and water ad libitum. Animal experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval numbers: P24-70 and P29-11) and according to the University of Tokyo guidelines for the care and use of laboratory animals. These experimental protocols were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alllevation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006) and the Guidelines on the Method of Animal Disposal (Prime Minister’s Office, Notice No. 40 of 1995).

#### Drugs

The doses of ethanol (1.5 g/kg for systemic injection, 7.0 g/kg for intra-ACC injection, Wako) were determined based on the blood ethanol concentrations observed with daily alcohol consumption in humans and on previous studies of rodent behavior. The concentration of ethanol (50 mM) in in vitro experiments using brain slices was based on the normal intrabrain concentration after systemic injection of ethanol. The doses of other drugs were also chosen with reference to previous reports of rodent behavior (MK801: 0.1 mg/kg, Tocris Bioscience; L-368,899: 5 mg/kg, 4.1 g/kg, Tocris Bioscience; picrotoxin: 0.3 g/kg, Nacarai Tesque; clonazepam: 79 ng/g, Wako). For behavioral experiments, all drugs except for clonazepam were diluted in 0.9% saline. Clonazepam...
Acetic acid-induced writhing test. Pain sensitivity was evaluated by measuring the acetic acid-induced writhing responses. Acetic acid (0.9%) was intraperitoneally injected at a volume of 10 ml/kg into mice that were placed in a plastic cage (18 cm in width, 11 cm in depth, and 11 cm in height) 5 min before the experiment. A blinded observer judged whether the writhing behaviors were present or absent in these 5-s video clips. The writhing frequency was expressed as the percentage of video clips with writhing responses.

Sample preparation and fluorescence in situ hybridization. Mice were sacrificed 5 min after the fear observation test was finished, and their brains were removed and frozen quickly. In situ hybridization was performed as follows: coronal brain sections (20 μm) were hybridized with digoxigenin-labeled riboprobe (1.5 μg/ml). The signals were detected with an anti-digoxigenin-alkaline phosphatase antibody (1:500; Roche, Cat# 1120773910, RRID: AB_514500) and then with biotinylated tyramide signal amplification (40× objective lens (Yokogawa). The majority of cells exhibited whole, large nuclei stained diffusely with the Hoechst dye. Only these putative neurons were included in the analysis. The designation "nuclear positive" or "Nuc+" indicates neurons that contained one or two of the intensely fluorescent intranuclear bodies. The designation "cytoplasmic positive" or "Cyto+" indicates neurons that contained perinuclear/cytoplasmic labeling across multiple optical sections. An overlap score between neuronal populations that were active during the priming shock and the fear observation test was identified as (D+−C)/N (D: chance level), in which (C) represents the total number of Cyto+ cells, (D) represents the total number of cells in which Cyto+ signals were observed both in the cytoplasm and nucleus; the chance level was defined as C×N/100.

Surgery for microinjection. Under intraperitoneal xylazine (10 mg/kg) and pentobarbital (25 mg/kg) anesthesia, mice were bilaterally implanted with 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA, United States) into the ACC (A/P 1.0 mm, L/M±0.2 mm, D/V 2.0 mm) or BLA (A/P 1.3 mm, L/M±3.3 mm, D/V 4.8 mm). These cannulae were fixed to the skull using a mixture of acrylic and dental cement, and 33-gauge dummy cannulae were then inserted into the guide cannulae to prevent clogging. Mice were given at least seven days of postoperative recovery time. For microinjection, an infusion cannula was carefully inserted into each guide cannula. Drug solution or an equivalent volume of saline was bilaterally infused at a rate of 0.25 μl/min under free-moving conditions in a plastic cage (18 cm in width, 11 cm in depth, and 11 cm in height). The infusion cannulae were left in place for 2 min to allow backward diffusion of the solution into the guide cannulae.

In vitro electrophysiology. Acute neocortical slices containing the ACC area were prepared from postnatal day 3–4 mice. Mice were anesthetized with isoflurane and decapitated, and the brain was coronally sliced (400 μm thick) in an ice-cold oxygenated cutting solution consisting of (in mM) 222.1 sucrose, 27 NaHCO3, 1.4 NaH2PO4, 2.5 KCl, 1 CaCl2, 7 MgSO4, and 0.5 ascorbic acid using a vibratome. Slices were allowed to recover for at least 1 h while submerged in an oxygenated artificial cerebrospinal fluid (aCSF) at room temperature. aCSF consisted of (in mM) 27 NaCl, 26 NaHCO3, 1.6 KCl, 1.24 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, and 10 glucose. Recordings were performed in a submerged chamber perfused at 3 ml/min with oxygenated aCSF at 34–37 °C. Whole-cell patch-clamp current-clamped recordings were obtained, before and during bath application of 50 mM ethanol or 500 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), from layer II/III ACC pyramidal cells, which were visually identified under an infrared differential interference contrast microscope (DAGE-MTI IR-1000). Patch pipettes (3–6 MΩ) were filled with a Cs-based solution consisting of (in mM) 130 CsMeSO4, 10 CsCl, 10 HEPES, 1 MgCl2, 1.3 Na2ATP, and 10 QX-314. Fast-spiking interneurons were isolated based on their short time constants (≤0.2 ms) and nonadaptive spiking patterns. The synaptic conductance was calculated by dividing the charge (the time integral of excitatory/inhibitory postsynaptic currents) by the driving force (clamped voltage) [V]. This measure is believed to reflect general synaptic efficacy, including the amplitudes, durations, and frequencies of synaptic events.
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Author contributions

T.S. and Y.I. designed the study and wrote the manuscript. T.S. conducted the behavioral experiments and data analyses. S.I. conducted the immunohistochemistry. M.O. and K.O. conducted the electrophysiological experiments. K.O. prepared the MIA mouse model. All authors discussed the results and commented on the manuscript.

Additional information

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