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Loss of the zinc receptor ZnR/GPR39 in mice enhances anxiety-related behavior and motor deficits, and modulates KCC2 expression in the amygdala

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Abstract

Background Mood disorders, particularly depression and anxiety, are associated with zinc dyshomeostasis and aberrant GABAergic signaling. Activation of ZnR/GPR39 by synaptic zinc in the hippocampus triggers phosphorylation of extracellular regulated kinase (ERK1/2), which regulates the K^+/Cl^- cotransporter (KCC2) and thereby GABAergic inhibitory neurotransmission and seizure activity. Therefore, we studied whether impaired ZnR/GPR39 signaling is linked to anxiety-related behavior in male or female mice.

Results Using the acoustic startle response, elevated plus maze, and open field test, we found increased anxiety-related behavior in ZnR/GPR39 knockout (KO) mice. Despite a well-established sex difference, where females are typically more prone to anxiety, both male and female ZnR/GPR39 KO mice exhibited increased anxiety-related behavior compared to wildtype (WT) mice. Additionally, ZnR/GPR39 KO mice displayed impaired motor coordination in the pole and rotarod tests but did not show reduced muscle strength, as indicated by a grip test. Finally, we found intrinsic alterations in the expression level of KCC2, a major Cl^- transporter regulating GABAergic signaling, in the amygdala of naïve ZnR/GPR39 KO mice compared to controls.

Conclusions Our findings indicate that loss of ZnR/GPR39 enhances anxiety-related behavior in both male and female mice. Moreover, ZnR/GPR39 KO mice exhibit impaired motor coordination, which may be associated with increased anxiety. Finally, we demonstrate that loss of ZnR/GPR39 modulates the expression of KCC2 in the amygdala. Thus, we propose that ZnR/GPR39 can serve as a target for regulating GABAergic signaling in anxiety treatment.

Keywords Zinc, ZnR/GPR39, Anxiety-related, KCC2, Motor coordination, Zinc signaling

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Background

Zinc is selectively sequestered into synaptic vesicles of the neocortex, amygdala and hippocampus during early postnatal development by the zinc transporter, ZnT3 [1, 2]. This pool of Zn^{2+} ions is released during neuronal activity to modulate the function of several postsynaptic targets, affecting cognitive function [3–5]. In the amygdala, synaptic Zn^{2+} induces long-term potentiation in pyramidal neurons and is associated with fear conditioning [6–8]. In addition, restriction of dietary zinc facilitated fear extinction, and this was related to increased expression of the immediate-early genes c-Fos and Zif268 in cortico-amygdala regions [9].

A distinct target for Zn^{2+} is a Gq-coupled zinc sensing receptor ZnR/GPR39, which is activated by the synaptically released Zn^{2+} [10, 11]. This previously orphan receptor was initially suggested to act as a receptor for obestatin [12]; however, these results were not reproduced [13], and Zn^{2+} was shown to act as the ligand for this receptor [14–16]. The ZnR/GPR39 mRNA was largely localized to the hippocampus and amygdala, and was also found in the auditory cortex [17], but not in the hypothalamus where obestatin was expected to play a role. Thus far, Zn^{2+} remains the only known physiological ligand [10, 18].

Activation of synaptic release from Zn^{2+}-containing fibers triggers ZnR/GPR39-dependent Ca^{2+} rise and ERK1/2 activation. However, a post-synaptic response is absent in ZnR/GPR39 knockout (KO) mice [19, 20], further suggesting that Zn^{2+} is the distinct ligand of this receptor. Activation of ZnR/GPR39 signaling enhances transport rates of the K^+/Cl^- cotransporter, KCC2 [11, 19]. Neuronal KCC2 is the major Cl^- extruder that controls transmembrane Cl^- gradients, thus regulating $GABA_A$-dependent Cl^- influx and the inhibitory drive [21, 22]. Increase in the expression of this transporter plays a major role in the developmental shift that drives GABA from excitatory to inhibitory neurotransmission during early development [23, 24]. As such, activation of ZnR/GPR39 signaling that upregulates KCC2 activity in the hippocampus enhances inhibitory tone and protects from kainate-induced epileptic seizures [25]. Dramatically lower severity scores and shorter duration of seizures were monitored in wildtype (WT) compared to ZnR/GPR39 KO mice [19, 26]. Moreover, changes in ZnR/GPR39 expression were associated with morbid developmental seizures, an effect that was rescued by dietary zinc supplementation [27]. Recent studies using the GABA channel blocker, pentylenetetrazole, to induce seizures showed that a putative ZnR/GPR39 agonist enhances epileptogenesis [28, 29]. However, the canonical pathway of ZnR/GPR39-activation of KCC2, which is mediated by the GABA currents, is blocked by pentylenetetrazole and cannot underlie the effects of the putative agonist. Additionally, ZnR/GPR39 modulates cannabinoid signaling in the dorsal cochlear nucleus region in the auditory brainstem [20]. Thus, ZnR/GPR39 regulates GABAergic inhibitory signaling, by modulating the neuronal Cl^- gradients maintained by KCC2.

In humans, epilepsy is associated with mood disorders, specifically anxiety, via a mechanism that is not fully understood [30, 31]. It is well-established, that excitatory/inhibitory imbalance is implicated in mood disorders, including anxiety and depression, and specifically regulation of the GABAergic pathway is crucial in emotional dysfunction [32, 33]. The inhibitory pathway in the hippocampus and amygdala plays a major role in the etiology of anxiety [34]. Consistent with these findings, a mutant mouse that expresses approximately 30% of normal KCC2, a major regulator of GABAergic signaling, shows increased susceptibility to seizures and anxiety-related behavior [35]. Dietary zinc deficiency was suggested to reduce KCC2 expression in the prefrontal cortex but not hippocampus of male mice [36]. The general role of ZnR/GPR39 regulation of GABAergic responses suggests that this receptor may be involved in anxiety-related behavior. Yet, whether ZnR/GPR39 regulation of KCC2 is the mechanism linking zinc to mood disorders remains not well understood.

Zinc deficiency is considered a risk factor in mood disorders, and is associated with anxiety and depression [37, 38]. In concordance with this, rats fed a zinc-deficient diet exhibit increased anxiety-related behavior [39]. Importantly, anxiety is associated with synaptic Zn^{2+} deficiency, as ZnT3 KO mice, lacking synaptic Zn^{2+}, exhibit slower travel speed in the open field test and a lower score in a social preference test [40]. The synaptic Zn^{2+} activates ZnR/GPR39, and this receptor was suggested to play a role in depression- and anxiety-like behavior in mice [41]. Indeed, ZnR/GPR39 KO mice showed increased immobility time in forced swim and tail suspension tests, and fewer entries into a lit compartment in light/dark box test [42–44], which were correlated with higher c-fos activation in the medial amygdala [45]. While several Zn^{2+} transporters have been associated with diseases [46], the signaling pathways linking ZnR/GPR39 to mood disorders are not well understood.

Previous behavioral studies of ZnR/GPR39 KO animals did not perform sex-dependent analysis, although the prevalence of anxiety disorders is higher in females compared to males [47, 48] and female animals present an important and reproducible model [49, 50]. Moreover, mice deficient in synaptic Zn^{2+} (ZnT3 KO) show sex-dependent behavioral deficits, with female mice exhibiting impaired skilled learning and a motor deficit in the pole test, while male mice show autistic-like behavior [40, 51, 52]. Therefore, we asked if ZnR/GPR39 has a direct role in anxiety-related behavior, specifically in male or

female ZnR/GPR39 KO mice. We found that loss of ZnR/GPR39 enhances anxiety-related behavior and is linked to motor coordination deficits, but not strength, in both male and female mice. Interestingly, we monitored changes in the expression of the Cl^- transporters that regulate GABAergic signaling in the amygdala and hippocampus of ZnR/GPR39 KO mice. Thus, we suggest a role for ZnR/GPR39 in modulating inhibitory signaling that underlies anxiety-related behavior.

Materials and methods

Animals

Transgenic mice lacking the gene that encodes for ZnR/GPR39 [53] were used in this study and compared to littermate WT mice as controls (30±4 g males, 23±2 g females, no significant weight difference between genotypes, $F(1,82)=1.32$, $p=.25$). Genotypes were verified by polymerase chain reaction (PCR), using DNA fragments that were isolated from tail biopsy samples. Specific primers for WT (ZnR/GPR39$^{+/+}$) allele (forward: 5'AACA GCGTCACCATCAGGGTT, reverse: 5'TGCGAGAGAG GTTGCAGTTGA) and KO allele (forward: 5'GGAACT CTCACTCGACCTGGG; reverse: 5'GCAGCGCATCGC CTTCTATC) were used to amplify an exclusive sequence for each genotype. Mice were housed in individually ventilated cages (97sqcm floorX13cm height) with woodchip bedding and environmental enrichment using cardboard tubes and shredded paper for hiding and nesting. Mice had unrestricted access to food and tap water. The cages were kept in a temperature-controlled room (22°C) under a 12:12 h light/dark cycle (lights on during the day). Adult WT (GPR39$^{+/+}$) and KO (GPR39$^{-/-}$) C57BL6 mice of both sexes were tested and housed in groups of 2–5 mice of the same sex and genotype per cage. Mice were 3–5 months old at the time of the testing (4±0.7 months). All mice were inspected before any behavioral tests were conducted and any that displayed injuries or health problems were removed. Naïve mice were used for the experimental paradigms, to make sure that previous experiments did not affect stress levels and thereby affect the anxiety results. All tests were performed at ambient temperature and low background noise. Note that behavioral measures may be affected by the strain, however we compared WT and ZnR/GPR39 KO mice of the same strain, which is considered highly active [54]. The sample size for each experiment is reported in each respective figure. All experimental procedures and animal handling were performed in accordance with a protocol approved by the Committee for the Ethical Care and Use of Animals in Experiments at the Faculty of Health Sciences at Ben-Gurion University (AAALAC approved facility).

Behavioral testing: acoustic startle response (ASR) and prepulse inhibition (PPI)

ASR experiments were used to determine anxiety-related behavior. In this paradigm, mice were presented with 30 startle trials in which a loud sound (110 dB SPL white noise, 40 ms in duration) was presented, with random time intervals (5 to 25 s) between the pulses. The first two pulses were not used for the analysis. In the PPI experiment, a modified ASR protocol was used to study sensorimotor gating. We presented prepulse stimuli (80 dB SPL, 40 ms duration), which were completed 90 ms before the onset of the startle pulse. In addition, 20 startle-only trials were performed and the prepulse trials were randomly ordered between them, separated by a quiet inter-trial interval ranging randomly from 8 to 24 s. The ASR averaged within each prepulse frequency condition was then normalized to the average ASR in the startle-only trials. Since previous studies suggested that loss of synaptic Zn^{2+} affects frequency discrimination [55], we asked if a similar effect on frequency discrimination is also found in ZnR/GPR39 KO mice. We therefore used different frequencies (7.2, 8, or 9 kHz; seven times each) for the prepulse stimulus and tested if the PPI varies at different frequencies. Experiments were performed using the LE0823G Startle and Fear Combined system (Panlab, Harvard Apparatus), and test protocols were programmed with Packwin V2.0.06 software. The system contained two sound-attenuated startle chambers, and animals were tested in pairs, with chambers counterbalanced across the different experimental groups. On the load cell platform, mice were placed in a holder (LE117M) small enough to restrict movement but big enough to enable turning around. Voltage waveforms that were converted from the movements of the mice were digitized and stored on a computer. The Panlab calibration unit was used routinely to ensure consistent stabilimeter sensitivity between test chambers and over time. Sound levels within each test chamber were measured routinely with the software calibration tests and by a sound level meter (TFA-Dostmann SOUND BEE) to ensure consistent sound presentation. All experiments were performed during the light cycle. Each session started with five minutes of acclimation without background noise.

Behavioral testing: elevated plus maze (EPM) and open field test (OFT)

The EPM test was used to evaluate the anxiety-related behavior of the mice. Experiments were done between 5pm-8pm, just before the dark cycle, in a dimly lit room 150–170 lx across the maze. The EPM apparatus consisted of two opposing transparent closed arms (47 cm length × 5 cm width × 20 cm height) and two opposing open arms (47 cm length × 5 cm width, with a 3 cm ledge

to prevent falling) in the form of a plus, elevated 75 cm above the floor in a room illuminated by dim light. The mice were placed in the center of the maze facing an open arm, and behavior was recorded for 5 min on the maze. The maze was cleaned with a dilute solution of ethanol (70%) in water after each mouse. The digital recordings were analyzed with EthoVision 3.1 software [56] to measure entries and duration of time in the open and closed arms and the center region. During 300 s, we monitored the duration in open arms, the time spent in the center zone between the arms of the maze, the number of entries into open arms, total arm entries (the number of entries into open and closed arms, as a relative pure index of locomotor activity) and total distance traveled. All measures referred to the body center point of the mice. To create a single dependent variable, the Anxiety Index (AI) for each EPM exposure was calculated as follows [57, 58]:

$$\mathrm{AI} = 1 - \left[\frac{\left(\frac{\text{Duration in open arms}}{\text{Duration in open and closed arms}}\right) + \left(\frac{\text{Open arm entries}}{\text{Total arm entries}}\right)}{2}\right]$$

The open field apparatus consisted of a square area (1×1 m) divided into 4 compartments (50×50×50 cm) with dark walls. Four mice of the same genotype and sex were tested simultaneously, with one mouse in each compartment. and their behavior was recorded [59]. Experiments were done just after the light cycle begins at 9am-12pm, in a dimly lit room 140–150 lx across the apparatus. Initially, the mouse was placed in a peripheral corner, facing the wall. It was allowed to move freely around the arena and explore the environment for a single session of 10 min. The digital recordings were analyzed using EthoVision 3.1 software [56]. Each compartment was divided into central (~20% of total area) and peripheral parts [60]. The frequency and duration of entries of the mouse (body center point) to the central part of the arena were assessed. In addition, we monitored the total time of no movement (freezing time), total rotations (clockwise and counterclockwise), and the total distance traveled on the apparatus. The apparatus was cleaned with 70% ethanol solution after every trial to eliminate any olfactory clues.

Behavioral testing for strength and coordination: Pole test, rotarod and grip test

These tests that do not directly assess anxiety were performed during the light hours, 2pm-6pm, in a lit room. The pole test was performed to assess motor dysfunction and coordination. Mice were placed with their head upward on the top of a 50 cm vertical pole with a diameter of 1 cm that was wrapped with porous cotton fabric tape to avoid slippage of the mice. The time to turn and the total time to descend to the base of the pole were recorded, starting from when the animal began the turning movement. This was conducted three times for each animal on separate days; the first trial was a training trial, and the times to descend in the second and third trials were averaged. The time to descend was normalized compared to the WT male mice on that same day and the time for each animal is presented in the graph. The pole was cleaned with 70% ethanol solution after each animal completed the test.

The Rotarod experiments, to further test motor coordination, were performed on the accelerating Ugo Basile Model 7650 Rota-rod apparatus (Ugo Basile, Camerio, Italy). A mouse was placed on the cylinder, which increased rotation speed from 5 to 40 rpm over a 300 s period. Mice were first given one trial to become acquainted with the Rotarod apparatus before the test. The results presented are the latency to fall averaged over 2 consecutive trials with a 30 min rest period between them, similar to the expected time previously measured for this strain and age [61, 62]. For detection, a group of 5 mice was placed in individual compartments on the rotating rod before starting the acceleration program. The time each mouse remained on the rod was registered automatically. If the mouse remained on the rod for 300 s (top speed of the rod) the test was completed and scored as 300 s.

Grip strength experiments were performed to directly assess the strength of the ZnR/GPR39 KO mice compared to controls. In this test, mice forelimb grip strength and four-paws grip strength were monitored using a mouse-specific strength gauge (Chatillon® DFE II Series device, Ametek). The average grip force (recorded in gram-force [gF]) of three trials was used for statistical analysis. In addition, we performed an inverted screen test as previously described [63]. Briefly, untrained animals were placed in the middle and on top of a 43×43 cm, 1 mm thick wire mesh with 12×12 mm squares. The mesh was rotated by 180° with the head of the animal descending first so that the animal was inverted, above soft bedding, while holding the mesh with all four limbs. The time before the animal fell was monitored and animals were allowed to stay up to 12 min. The test was repeated three times and the average latency to fall for each animal was used for statistical analysis.

Western blot analyses of protein levels in the amygdala and hippocampus

Mice were euthanized according to approved protocol and the amygdala was dissected from brains harvested from naïve WT and GPR39 KO animals aged 12–15 weeks. Following the isoflurane euthanasia and decapitation, brains were rapidly removed and placed in ice-cold phosphate buffer saline. The cerebellum and ventral third

of the cerebrum were removed and brains were mounted on the vibratome holder, submerged in ice-cold PBS (frontal side facing up and oriented towards the blade). Slices were discarded until the hippocampal formation was visible, after which, 4 consecutive, 300 µm slices were collected (corresponding to brain region between Sects. 66–78 in the Mouse Brain Atlas (https://atlas.brain-map.org/atlas)) and transferred into a new dish containing fresh ice-cold PBS and dissected on ice. The amygdala was collected bilaterally from all 4 slices and combined into a lysis buffer containing vessel. The same sections were used to bilaterally dissect hippocampal tissue samples. The tissue was lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% IGEPAL CA-630; 0.5% sodium deoxycholate; 0.1% SDS) freshly supplemented with protease and phosphatase inhibitor cocktails. The proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Following blocking with 5% bovine serum albumin in tris-buffered saline (supplemented with 0.1% Tween20), membranes were incubated with primary antibodies overnight at 4 °C (KCC2, Cell Signaling; 94725, 1:1000; NKCC1, Abcam, 303518, 1:1000; tERK1/2, Cell Signaling; 4696, 1:1000, and tCaMKII, Santa Cruz Biotechnology sc13141, 1:1000), and subsequently with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. β-actin or β-tubulin served as a loading control. The immunoblotting bands were quantified by densitometry using ImageJ software and normalized to the loading control. For KCC2 quantification, oligomer fraction was normalized to KCC2 monomer level.

Statistical analysis

To analyze the effects of genotype and sex on behavioral measures, two-way analyses of variance (ANOVA) were performed. PPI was analyzed with a mixed design repeated measures ANOVA, with prepulse frequency as a within-subject independent variable. All Statistical tests were conducted using JASP (JASP Team 2022, https://jasp-stats.org/).

Results

Anxiety-like: acoustic startle response and elevated plus maze

We initially asked if ZnR/GPR39 KO female and male mice exhibit similar anxiety-related behavior by measuring the well-established acoustic startle response (ASR). The mean startle amplitude was significantly higher in both male and female ZnR/GPR39 KO mice compared to WT mice (F (1,21) = 25.5, $p < .001$), without significant interaction between genotype and sex (Fig. 1a). In addition, we found that startle habituation (the proportion of the mean ASR amplitude in the last 5 trials compared to

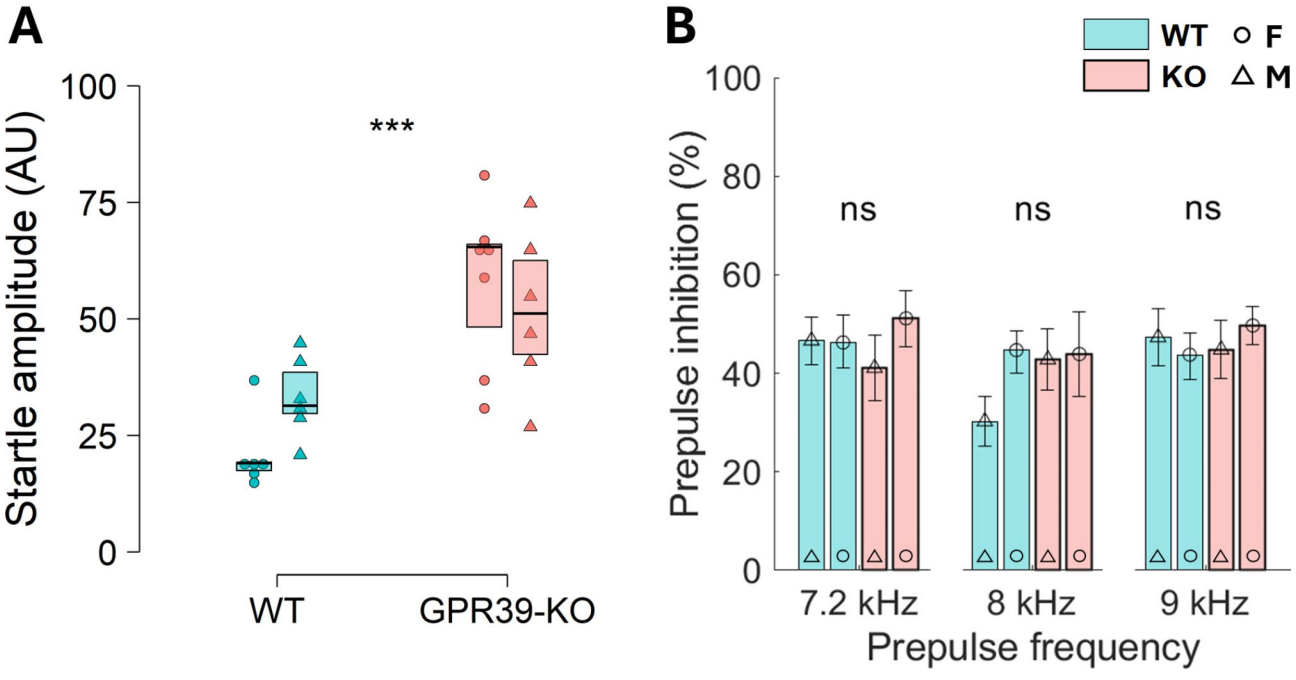

Fig. 1 Acoustic Startle Response (ASR) and Prepulse Inhibition (PPI). (**A**) ASR amplitudes. Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 6–7$ / group. (**B**) PPI across different prepulse frequencies. Error bars represent the means ± 1 standard error, $n = 8$ / group. Asterisks denote the effect of genotype, ***$p < .001$, ns non-significant, AU arbitrary units

the first 5 trials in each animal) was not significantly different between WT and KO mice (F (1,21)=0.01, p=.92). To assess sensorimotor gating, we used the prepulse inhibition paradigm (PPI) paradigm. A modified version of this protocol was previously used to show that loss of synaptic Zn^{2+} affects frequency discrimination [55]. We therefore also used different frequencies for the prepulse stimuli (7.2, 8, or 9 kHz; seven times each) and studied if ZnR/GPR39 may be associated with frequency discrimination. If auditory acuity at these frequencies is impaired, then the degree of inhibition should be smaller following the prepulse. Notably, our results indicated that regardless of the prepulse frequency, the startle responses were attenuated to a similar extent (~40–50%) in both WT and KO mice of both sexes (Fig. 1b) (non-significant effects of genotype or interaction prepulse frequency*genotype; F (1,28)=0.32, p=.58; F (2,56)=0.46, p=.64; respectively). This indicates that sensorimotor gating and frequency discrimination are not modulated by ZnR/GPR39 signaling and are not likely to explain differences in ASRs. Having ruled out weight differences (see Methods, Sect. 2.1) and sensory differences, we tested whether the higher startle response in both male and female ZnR/GPR39 KO mice may be linked to higher anxiety levels.

As an additional test for anxiety-related behavior, we conducted the elevated plus maze (EPM) and monitored the time spent in open arms or in the center of the apparatus, and the number of entries into the open arms (Fig. 2a-c). Both male and female ZnR/GPR39 KO mice spent less time in the open arms and entered them less frequently (F (1,39)=7.5, p=.009; F (1,39)=14.0, p<.001), providing further evidence of anxiety-related behavior in ZnR/GPR39 KO mice. Note that time spent in the center of the apparatus was similar across groups (Fig. 2b), likely reflecting basic anxiolytic behavior of mice [54]. To account for potential differences in locomotor abilities, we analyzed the total number of arm entries (Fig. 2d) and the total distance traveled in the apparatus (Fig. 2f). ZnR/GPR39 KO mice traveled a smaller total distance (F (1,39)=8.8, p=.005), consistent with fewer total arm entries (F (1,39)=4.8, p=.035). Despite the reduced locomotion, the proportional entry rate into the open arms was lower in ZnR/GPR39 KO mice, suggesting higher anxiety (Fig. 2c). To integrate all parameters, we calculated a general Anxiety Index [64] (Fig. 2e), which showed a significant effect of genotype (F (1,39)=11.0, p=.002), without a significant interaction with sex (F (1,39)=0.6, p=.44). The results presented in this set of experiments suggest that ZnR/GPR39 KO mice exhibit anxiety-related behavior compared to WT mice, and that this effect is consistent across both sexes.

Locomotion: open field test

Because ZnR/GPR39 KO mice exhibited hypo-locomotion patterns in the EPM, we examined the differences in motor activity and strength between the genotypes. We first used the open field test (OFT), which assesses both anxiety and mobility. To determine if increased anxiety-related behavior can be monitored, we analyzed the frequency of entries to the central zone (Fig. 3a). ZnR/GPR39 KO animals showed a significantly lower frequency of entries to the center zone compared to WTs, supporting increased anxiety-related behavior of the ZnR/GPR39 KO mice of both sexes (F (1,21)=5.3, p=.031). Nevertheless, the time spent in the center zone of the arena (Fig. 3b) was not significantly different between WT and ZnR/GPR39 KO mice, which could be possibly related to anxiety due to a non-significant increase in the freezing time (Fig. 3c) of ZnR/GPR39 KO compared to WT mice (F (1,21)=3.9, p=.06). Regarding mobility effects, the number of rotations was not different between the genotypes (Fig. 3d, F (1,21)=1.2, p=.28), and similar total distance traveled in the apparatus was monitored for both genotypes (Fig. 3e, F (1,21)=2.1, p=.16). Altogether, this test also indicates that ZnR/GPR39 KO mice present increased anxiety-related behavior.

Coordination and strength: Pole, rotarod and grip tests

To assess whether motor coordination or strength could influence locomotion in the EPM or ASRs, we conducted two complex motor tests that involve both strength and coordination requirements. In the pole test (Fig. 4a-b), ZnR/GPR39 KO mice were significantly slower to turn (F (1,41)=5.5, p=.024) and descend the pole (F (1,41)=5.9, p=.02) compared to WT mice. Similarly, in the accelerating rotarod test (Fig. 4c), ZnR/GPR39 KO mice stayed on the rod for a significantly shorter time than WT mice, corresponding to much slower rates of turning (F (1,51)=23.1, p<.001). In both tests, there were no significant effects of sex or interaction between genotype and sex (Pole: F (1,41)=0.05, p=.83; F (1,41)=0.2, p=.64; Rotarod: F (1,51)=0.02, p=.92; F (1,51)=0.009, p=.92). This suggests that motor coordination abilities or strength of ZnR/GPR39 KO mice may be impaired in both males and females.

To assess potential differences in strength between genotypes that could affect the performance in the pole and rotarod tests, we directly measured the muscle strength of ZnR/GPR39 KO mice using the grip test. Both female and male ZnR/GPR39 KO mice exhibited significantly greater strength of their forelimbs (Fig. 5a) or all four limbs (Fig. 5b) compared to WT mice (F (1,37)=6.1, p=.018; F (1,37)=10.8, p=.002; respectively). However, in the horizontal grid test, ZnR/GPR39 KO mice showed similar latencies to fall off the grid as WT mice (F (1,37)=0.03, p=.86) (Fig. 5c). There was a significant

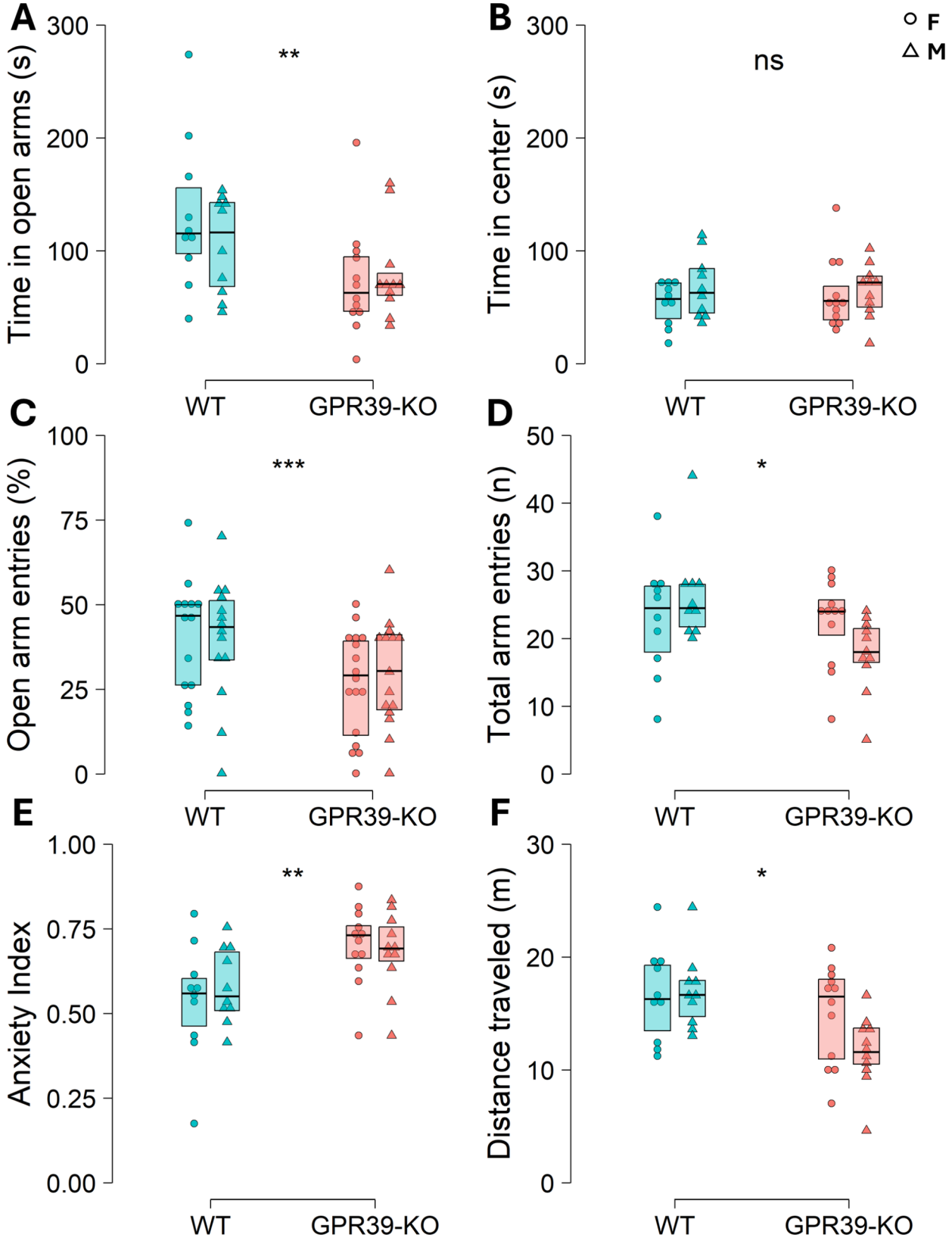

Fig. 2 Elevated Plus Maze (EPM). (**A**) Time spent in the open arms (**B**) Time spent in the center (**C**) Number of entries into the open arms relative to total number of entries into the open and closed arms (**D**) Total number of entries into open and closed arms (**E**) Anxiety index (see Methods) (**F**) Total distance traveled. Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 10–12$ / group, asterisks denote the effect of genotype $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, ns non-significant

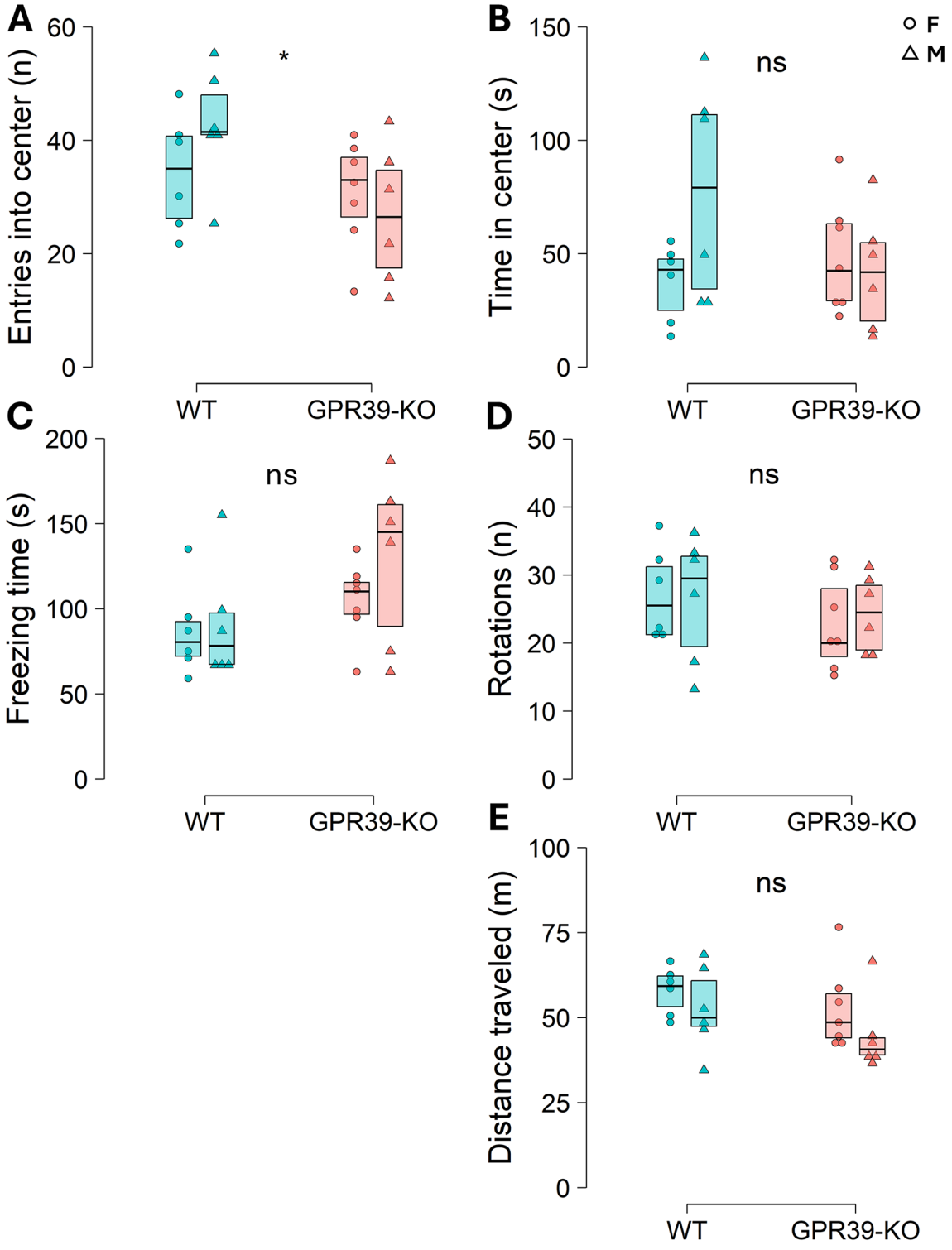

Fig. 3 Open Field Test (OFT). (**A**) Total number of entries into the center (**B**) Time spent in the center (**C**) Total time of freezing (**D**) Number of rotations (**E**) Total distance traveled. Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 6$ / group, effect of genotype $^*p < .05$, ns non-significant

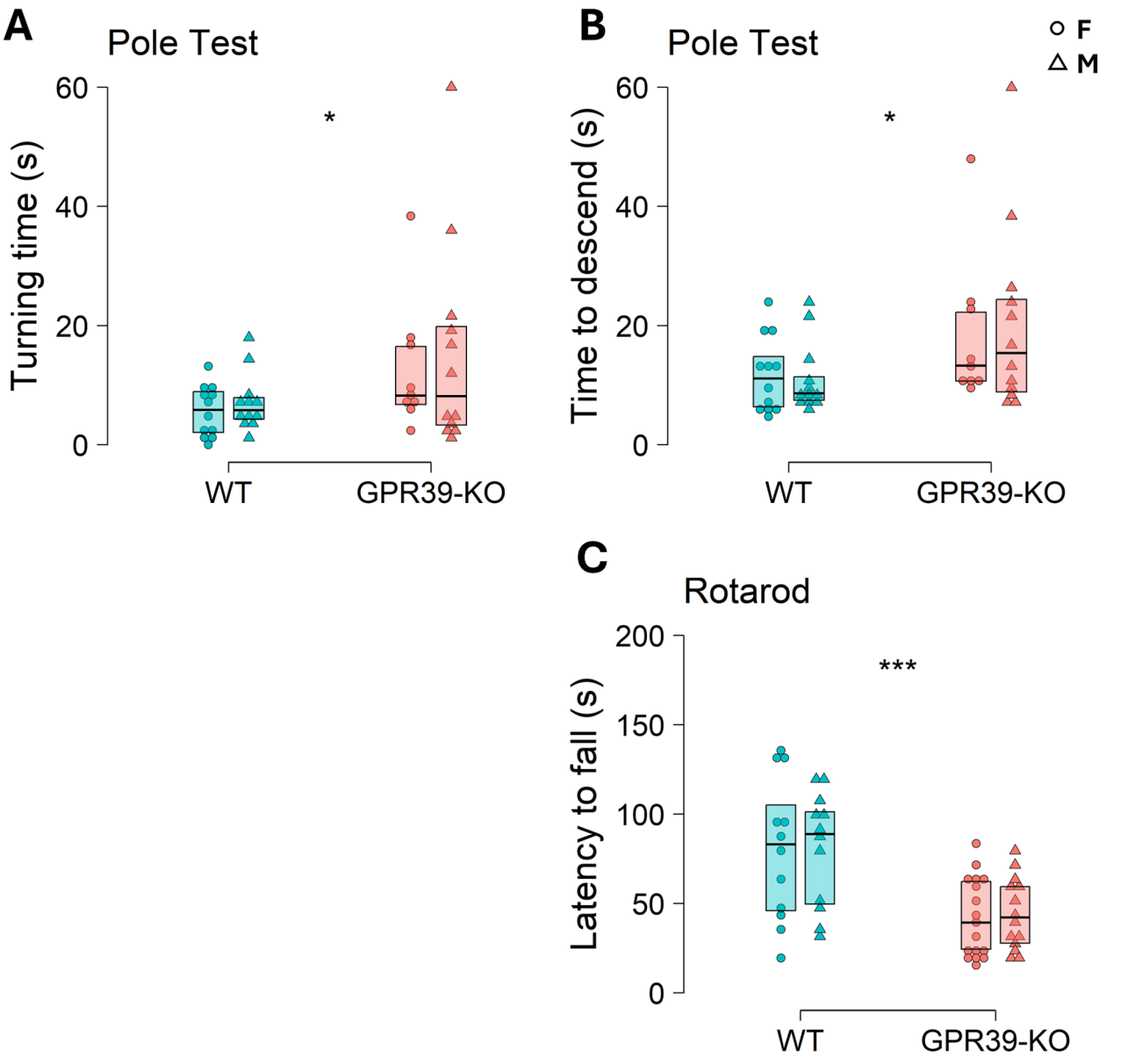

Fig. 4 Motor Coordination Tests. (**A**) Time taken to turn and (**B**) Total time to descend the vertical Pole Test, $n = 9–12$ / group (**C**) Latency to fall off the Rotarod. Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 12–17$ / group, asterisks denote the effect of genotype $^{*}p < .05$, $^{***}p < .001$

effect of sex in the horizontal grid, indicating that males exhibited increased strength (F (1,37) = 12.8, $p < .001$). However, there were no significant interaction effects between sex and genotype in these measures (Grip: F (1,37) < 0.001, $p = .97$; F (1,37) = 0.1, $p = .71$; Grid: F (1,37) = 0.2, $p = .62$). Overall, this suggests that both male and female ZnR/GPR39 KO mice do not exhibit muscle weakness compared to WT mice.

Signaling pathway underlying ZnR/GPR39 regulation of anxiety

Previous studies linked anxiety-related behavior to the GABAergic pathway, and specifically to changes in the K^+/Cl^- cotransporter, KCC2 [35, 65] that is regulated by the ZnR/GPR39 [19, 66]. To further explore the intrinsic mechanism that underlies the increased anxiety-related behavior observed in ZnR/GPR39 KO mice, we measured levels of KCC2 in the amygdala and hippocampus of naïve mice. We compared the level of KCC2 oligomers, the functional form of this transporter [67], normalized to KCC2 monomer levels, between WT and

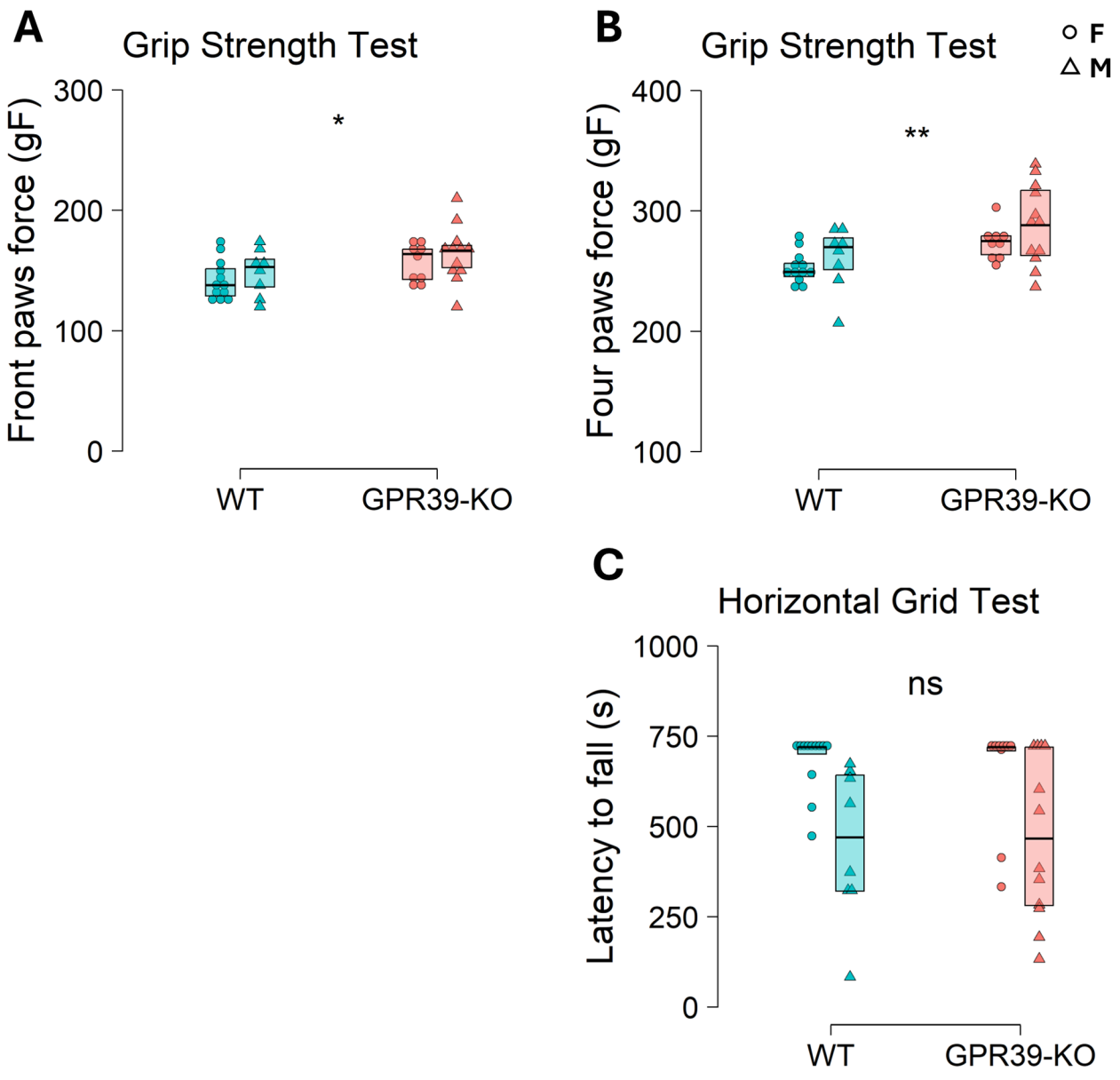

Fig. 5 Strength Tests. (**A**-**B**) Muscle strength mean values measured for each mouse using its forelimbs (**A**) or all four limbs (**B**). (**C**) Latency to fall off the inverted wire screen. Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 8–12$ / group. Asterisks denote the effect of genotype $^{*}p < .05$, $^{**}p < .01$, ns non-significant, gF gram-force

ZnR/GPR39 KO mice (Fig. 6a-b). We found an increase in KCC2 expression in the amygdala of ZnR/GPR39 KO mice (F (1,19) = 7.3, $p = .014$) but not in the hippocampus. We further asked if the Na^+-dependent K^+/Cl^- cotransporter NKCC1 that is driving Cl^- influx, opposing KCC2, is affected by loss of ZnR/GPR39 (Fig. 6c-d). We found that NKCC1 levels in WT and ZnR/GPR39 KO mice are similar in the amygdala and hippocampus of ZnR/GPR39 KO mice compared to WT (F (1,7) = 6.3, $p = .037$). Activation of ZnR/GPR39 in the hippocampus also enhances calcium calmodulin kinase (CaMKII) phosphorylation [11]. The phosphorylation of these pathways is triggered by synaptic transmission, since we are measuring differences in baseline conditions, we measured tCaMKII level in the amygdala and hippocampus of WT and ZnR/GPR39 KO tissue (Fig. 6e-f). We found that under baseline conditions, tCaMKII is not different in ZnR/GPR39 KO mice compared to WT. Although there were no significant effects of genotype, we did see significant effects of sex and significant interaction between genotype and sex in both the amygdala (F (1,8) = 11.1, $p = .003$) and the hippocampus (F (1,8) = 9.3, $p = .016$). Simple effects analyses indicated that ZnR/GPR39 KO females showed higher tCaMKII protein levels in the amygdala ($p = .005$),

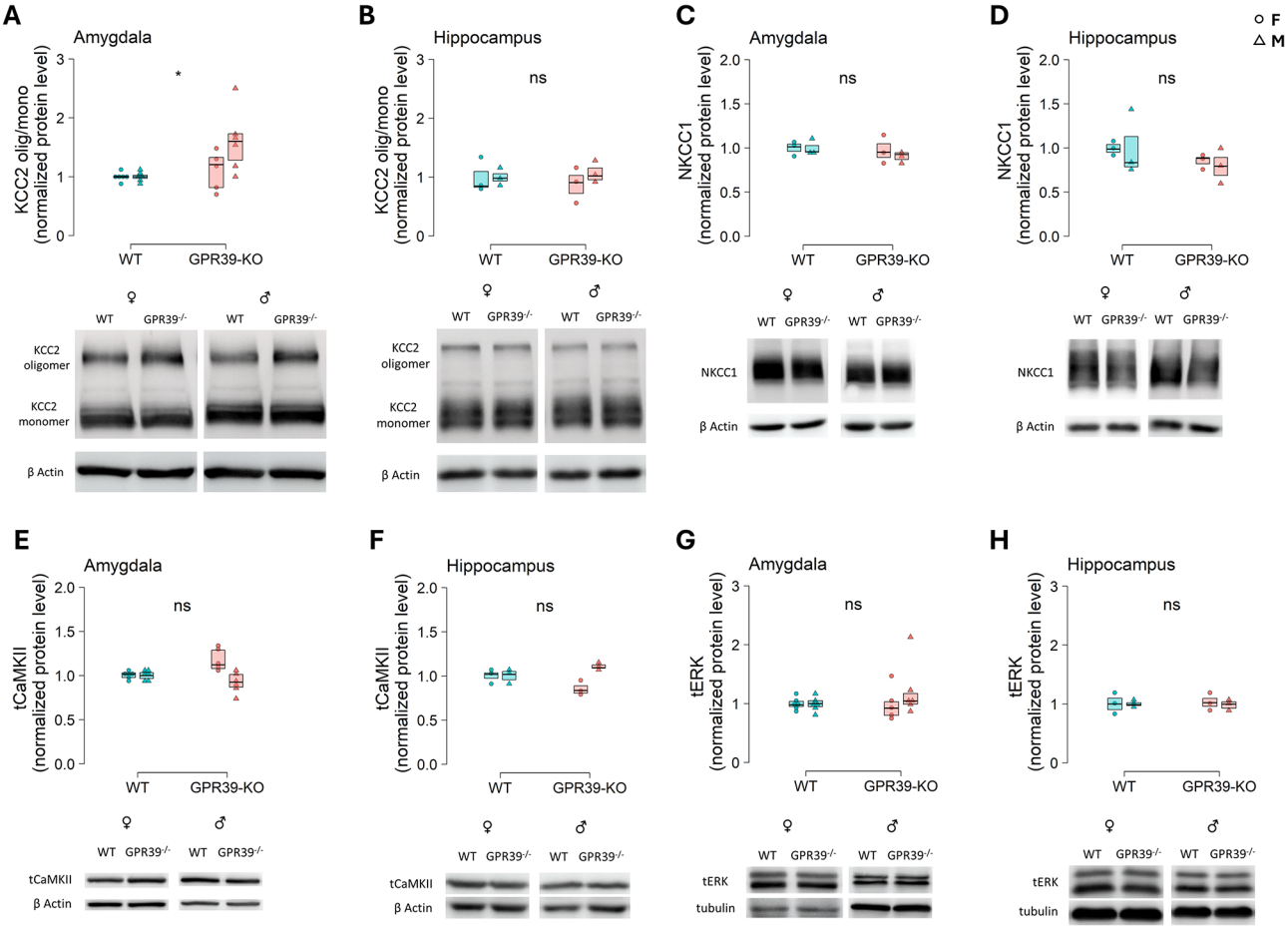

Fig. 6 Protein expression levels in the amygdala and hippocampus. (**A**-**B**) Level of K^+/Cl^- cotransporter (KCC2) oligomers normalized to KCC2 monomer (**C**-**D**) Level of Na^+-dependent K^+/Cl^- cotransporter (NKCC1) (**E**-**F**) Level of total calcium calmodulin kinase (tCaMKII) (**G**-**H**) Level of total extracellular regulated kinase (tERK). Box central marks indicate the median, bottom and top edges indicate the 25th and 75th percentiles, $n = 3$–6 / group, asterisks denote the effect of genotype, $^*p < .05$

and lower protein levels in the hippocampus ($p = .039$). Finally, we asked if extracellular regulated kinase (ERK1/2) that mediates ZnR/GPR39 activation of KCC2 is altered in the ZnR/GPR39 KO mice (Fig. 6g-h). We did not see a significant effect of genotype or sex in total levels of ERK1/2 in the amygdala or hippocampus, likely due to the fact that their activity depends on phosphorylation that is very short [68].

Discussion

Our current analysis aimed to study the behavioral aspects of ZnR/GPR39 using both male and female mice, based on established sex-dependent effects on anxiety disorders. While anxiety is typically more prominent in females [47, 48], we observed that increased anxiety-related behavior in ZnR/GPR39 KO mice does not show sex-dependent effects. Previous studies observed an anxiety-like phenotype in another strain of GPR39 KO male mice [42]. While that study did not directly investigate the relationship between anxiety and ZnR/GPR39 regulation of GABAergic signaling, we find an important link via changes in KCC2 expression that suggests that modulation of the GABAergic pathway in ZnR/GPR39 KO mice may underlie the anxiety-like phenotype. In addition, hippocampal-dependent memory deficits were observed in these male ZnR/GPR39 KO mice, which were not found in female mice [69]. Importantly, while our study uses a mouse model, ZnR/GPR39 mRNA expression in the human brain is similar to that observed in the mouse brain, and found in the hippocampus and amygdala, both regions closely associated with anxiety (https://www.proteinatlas.org/ENSG00000183840-GPR39/brain/amygdala).

Zinc deficiency itself is strongly linked to mood disorders and specifically anxiety [70]. Animal studies have shown that a 2 week exposure to a diet that is low in zinc, reduced neuronal zinc levels and led to anxiety-like behavior [39]. This link is also relevant in human patients, as a systematic review suggested that lower serum zinc levels are found in anxiety patients and zinc intake can

reduce symptoms of anxiety [71]. A mechanism that links zinc to anxiety was not clearly shown, but zinc regulation of GABA signaling and its effects on excitatory/inhibitory balance is suggested to be involved in this function of zinc. The GABAergic system plays an important role in anxiety-like behavior [72, 73]. Moreover, parental stress affects GABAergic system development, and leads to anxiety-like behavior in offspring [65]. Thus, ZnR/GPR39 that modulates GABAergic activity, is well-positioned to control anxiety levels. The role of the receptor, and the effects seen on KCC2 expression in our study, provide a distinct handle to explain the link between zinc, GABA responses and anxiety.

Loss of ZnR/GPR39 results in a lack of activation of the major Cl^- cotransporter, KCC2, in the hippocampus [19]. Interestingly, a decrease in KCC2 expression and function was associated with anxiety-related behavior [65], with a dramatic effect on open arm entry in the EPM test [35]. Moreover, KCC2 regulation of Cl^- gradients directly affects $GABA_A$ receptors activity and the inhibitory tone, which are associated with anxiety disorders [72]. Impaired regulation of KCC2 activity, by its phosphorylation, has also been linked to abnormal vocalization and deficits in social behavior [74]. Here, we found elevated expression of the oligomeric, functional KCC2 [22, 75], in the amygdala of both male and female ZnR/GPR39 KO mice. In contrast, hippocampal KCC2 expression was not different between WT and ZnR/GPR39 KO mice, in agreement with previous studies [69]. Surprisingly, the increased anxiety-related behavior in ZnR/GPR39 KO mice was associated with increased expression of KCC2 in the amygdala, while previous studies associated anxiety with its decrease. This suggests a potential compensatory mechanism, of increased KCC2 expression in the amygdala that nevertheless is not sufficient to confer protection against anxiety in the ZnR/GPR39 KO mice. Moreover, our examination of adult mice revealed that the expression of NKCC1, which typically decreases during development concomitantly with KCC2 increase, was not affected by the loss of Zn/GPR39. Aberrant GABA inhibitory signaling in the hippocampus, basolateral amygdala, and interconnected amygdaloid nuclei, including the central and lateral nuclei, plays a critical role in anxiety disorders [34, 76, 77]. The role of KCC2 and NKCC1 in modulating GABA inhibitory signaling suggests that the general elevated expression of KCC2 in the amygdaloid complex of ZnR/GPR39 KO mice may link between inhibitory signaling in the amygdaloid complex circuitry and ZnR/GPR39. However, specific roles of individual amygdala subregions in ZnR/GPR39-dependent GABAergic signaling and anxiety behavior require further investigation in future studies. Nevertheless, the identification of ZnR/GPR39 as an upstream target for the effects of $GABA_A$ and KCC2 on anxiety may suggest a more subtle handle for the regulation of this pathway and thereby of anxiety.

In the amygdala, CaMKII phosphorylation has been shown to increase in corticosterone-treated mice that developed anxiety-related behavior [78, 79]. Under baseline conditions, we did not detect differences in this pathway in the ZnR/GPR39 KO mice in general. However, female mice showed significant variations in the levels of CaMKII in both the amygdala and hippocampus. This gender-specific phenotype may suggest that developmental changes in the global ZnR/GPR39 KO mouse model used in this study could potentially mask behavioral differences between sexes. As such, we cannot exclude the possibility of developmental compensatory effects or the involvement of other systems in the behavioral phenotype. For example, immune system function and microbiota are major players in anxiety disorders [80]. Of note, loss of ZnR/GPR39 did not modulate immune responses in the digestive system of KO mice [81]. Similarly, no interaction was found between zinc deficiency and immune response effects on behavior [82].

In addition to increased anxiety, our data show that ZnR/GPR39 KO mice also have impaired motor coordination. In contrast to KCC2 knockdown animals [35], ZnR/GPR39 KO mice show a pronounced effect in the rotarod test with a much shorter time to fall off the rod. While this could be attributed to loss of strength, direct measure of muscle strength indicated that these mice are not weaker than the WT mice. This suggests that the faster fall of the ZnR/GPR39 KO mice from the rotarod is likely associated with coordination deficits and/or anxiety itself. The pole test, also utilized for assessing motor coordination, revealed that the descent of ZnR/GPR39 KO mice was considerably slower. This could be explained by the enhanced anxiety of the mice [83], together with their increased strength, enabling them to hold onto the pole and descend at a slower pace. Considering that ZnR/GPR39 KO mice show even more muscle strength in the grip test, their impaired performance in both pole and rotarod tests suggests a coordination deficit rather than direct motor impairment. Finally, ZnR/GPR39 KO mice displayed a slightly reduced distance traveled in the EPM test, which could be interpreted as a locomotor impairment. However, the results of the grip tests do not support a direct effect of reduced muscle strength in the ZnR/GPR39 KO mice. Therefore, we propose that the observed hypo-locomotion pattern may be attributed to the increased anxiety levels and impaired coordination in these mice. A link between loss of coordination and anxiety has been identified in humans [84–86]. A similar association is well-established in mouse models. For example, the treatment with memantine, a drug inhibiting excitatory NMDA

function, resulted in enhanced anxiety and impaired motor coordination in mice, as assessed by the rotarod paradigm [87]. Moreover, increased anxiety in the EPM and OFT was reported in the mutant Headbanger (Hdb) model, which is characterized by coordination and balance deficit due to vestibular malformations. Using this model they further showed that balance training alleviate the Hdb mutants symptoms of anxiety [88]. Furthermore, chronic stress induces a combination of anxiety-related behavior and motor deficits in mice, accompanied by alterations in neuronal populations. The causality in these reports is not clear - whether anxiety and stress affect motor coordination or if the loss of motor coordination leads to anxiety. It will require further study to determine whether loss of ZnR/GPR39 and the observed increase in strength result in impaired coordination and thereby enhanced anxiety.

In conclusion, our results indicate that loss of ZnR/GPR39 is associated with impaired KCC2 regulation in the amygdala and increased anxiety in both male and female mice. Additionally, we found that motor coordination, rather than muscle strength, is impaired in ZnR/GPR39 KO mice, potentially enhancing anxiety-related behavior. These results suggest that ZnR/GPR39 provides an important mechanistic handle to the previously established link between zinc deficiency and anxiety [89]. Moreover, ZnR/GPR39 may serve as a significant target for regulating the inhibitory-excitatory balance in the amygdala.

Supplementary Information

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Supplementary Material 1

Author contributions

R.S planned and performed the experiments, analyzed and interpreted the data and wrote and edited the manuscript. M.C, M.B, planned and performed the experiments, analyzed the data and wrote the manuscript. H.A, I.S, O.K and H. C. designed the experiments, interpreted the data and edited the manuscript. M.H conceived and designed the experiments, analyzed and interpreted the data, wrote and edited the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

All experimental procedures involving animals were performed according to approved protocols and in accordance with the committee for Ethical Care and Use of Animal in Experiments at the Faculty of Health Sciences at Ben-Gurion University, and according to ARRIVE guidelines. Animals were housed on a 12/12 h light/dark cycle, at a temperature of 20–24 °C and 30–70% relative humidity and were provided with mice chow and water ad libitum.

Competing interests

The authors declare no competing interests.

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