

# Memory Impairment Related to NLRP3 Inflammasome Activation and TRPC5 Decrease in Hippocampal Excitatory Synapses in Microglia Knockout IL-10 Mice

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## Research Article

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# Abstract

Members of the transient receptor potential canonical (TRPC) protein family are widely distributed in the hippocampus of mammals and exert respective and cooperative influences on the functions of neurons. The relationship between specific TRPC subtypes and neuroinflammation is receiving increasing attention. Here, using Cx3cr1<sup>CreER</sup> IL-10<sup>-/-</sup> transgenic mice and their littermates, we demonstrated that Cx3cr1<sup>CreER</sup> IL-10<sup>-/-</sup> mice displayed spatial memory deficits in object location recognition (OLR) and Morris water maze (MWM) tasks. The decreased levels of TRPC4 and TRPC5 in the hippocampal regions were verified via reverse transcription polymerase chain reaction, western blotting, and immunofluorescence tests. The expression of postsynaptic density protein 95 (PSD95) and synaptophysin in the hippocampus decreased with an imbalance in the local inflammatory environment in the hippocampus. The number of cells positive for ionized calcium binding adaptor molecule 1 (Iba1), a glial fibrillary acidic protein (GFAP), increased with the high expression of interleukin 6 (IL-6) in Cx3cr1<sup>CreER</sup> IL-10<sup>-/-</sup> mice. The nod-like receptor protein 3 (NLRP3) inflammasome was also involved in this process, and the cytokines IL-1 $\beta$  and IL-18 activated by NLRP3 were also elevated by western blotting. The colocalization of TRPC5 and calmodulin-dependent protein kinase IIa (CaMKIIa) significantly decreased TRPC5 expression in excitatory neurons. AAV9-CaMKIIa-TRPC5 was used to upregulate TRPC5 in excitatory neurons in the hippocampus. The results showed that the upregulation of TRPC5 improved the memory performance of Cx3cr1<sup>CreER</sup> IL-10<sup>-/-</sup> mice by inhibiting NLRP3 inflammasome-associated inflammatory activity.

## Introduction

The TRPC protein family has seven members (TRPC1 to TRPC7) that form homo- and/or heteromeric tetramers. TRPC is a non-selective cationic membrane channel with Ca<sup>2+</sup> permeability. According to sequence homology, the family can be divided into three subfamilies: TRPC1, C4, and C5; TRPC3, C6, and C7; and TRPC2, which is a pseudogene in humans [1].

The physiological and pathological functions of TRPC, particularly in the central nervous system, are of increasing concern due to the extensive localization of TRPC, especially in the hippocampus. Evidence is converging to show the involvement of TRPC channels in cognitive functions, since multiple TRPC subtypes are highly expressed in the hippocampus.

TRPC1 is indispensable for environmental enrichment-induced spatial memory enhancement, which is related to long-term potentiation (LTP) induction and hippocampal neurogenesis[2]. The function of the TRPC1/4/5 subfamily plays a key role in spatial working memory formation[3], since TRPC1/4/5<sup>-/-</sup> mice exhibited deficiencies in adapting to a new challenge in a relearning task.

Neuroinflammation is a complex response to brain injury and a major contributor to progressive neuronal damage. IL-10 is a major anti-inflammatory cytokine that maintains the balance of the immune response and is an important molecule in the modulation of neuronal homeostasis and cell survival. At the level of

the hippocampus, it has been shown that IL-10 plays a key role in improving the learning and memory ability of animals under physiological and pathological conditions[4]. IL-10 helped to improve spatial memory performance in Sprague–Dawley rats treated with *Escherichia coli*[5]. Increased IL-10 levels played a role in the process of enriched environment alleviated LPS-induced spatial learning and memory impairment[6]. IL-10 is an important molecule in the modulation of learning and memory dysfunction, as shown by the IL-10<sup>tm1/tm1</sup> mice that exhibited behavioral deficits in the MWM test [7].

Microglial cells are the most investigated innate immune cells in the brain and are the main cytokine producers, including IL-10. Many studies have confirmed that IL-10 secreted by microglial cells plays a key role in the pathological process of neuroinflammation [8, 9].

In our study, we focused on the effects of IL-10 induced from microglial cells on animal cognitive and behavioral abilities to explore the role of TRPC in the hippocampus during this process.

## Materials And Methods

### Animals.

All experiments were performed on male mice that were 8–10 weeks old and weighed 22–26 g. All animals were on a C57BL/6 background and were maintained in a reversed 12-h light-dark cycle with free access to food and water. Tamoxifen (Sigma-Aldrich, MO, USA) was administered as a solution in corn oil (20 mg/mL) by intraperitoneal injection (80  $\mu$ L per mouse). All protocols were approved by the Ethics Committee of Nankai University. B6.129-Il10<sup>tm1.1</sup>(Flox) Smoc mice and Cx3cr1<sup>CreER</sup> transgenic mice were purchased from Shanghai Model Organisms Center, Inc.

### Novel Object Recognition (NOR) task and OLR task

Each mouse was gently handled for 3 min every day for three consecutive days before the behavioral test. The test was conducted in a bare square box (48 cm long, 48 cm wide, 36 cm high) made of compressed wood. Briefly, the NOR and OLR tasks consisted of two sessions: the training phase and the test phase. In the NOR task, during the test period, the mice were placed in the empty box and allowed to explore freely for 5 min to adapt to the environment. Two identical objects (plastic boxes, 4–5 cm high) were arranged in a straight line along one side of the wall, 8 cm from the sides. The experimental mice were placed in the box facing the opposite wall and were free to explore and adapt for 5 min. After a 2-hour interval, the animals were reintroduced into the experimental box for free exploration during the experimental period. At this point, one of the two objects used during training was replaced by an object of similar size. If any of the mice pointed or touched the new object with its nose within 1 cm, it was considered exploratory. The objects were thoroughly cleaned between trials to avoid olfactory cues. The mice were tracked using a charge-coupled device camera connected to a personal computer (Ethovision 2.0, Noldus, Wageningen, Netherlands).

In the OLR task, the experimental procedure was similar to the NOR task in the training stage. The difference was that in the test stage, the two unchanged objects were placed diagonally, as shown in Figure 1.

During the training phase in both the NOR and OLR tasks, if the mouse total exploration time for the two objects was less than 10 s in 5 min, the data was eliminated in a later study. The discrimination index (DI) and object duration were used as the NOR and OLR task evaluation index. The DI is the percentage of time each mouse spent exploring new objects and positions.

## **MWM task and RMWM task.**

The mice swam freely in the pool without platform 90s on 1d before the test, so that they could get familiar with the maze environment. In the training stage, each mouse was placed into the water with their heads facing the wall, and randomly selected one of the four starting positions of east, west, south and north. Record the time it takes the animal to find the underwater platform (escape latency). if the escape latency exceeded 60s, the mouse was guided to the platform and stayed on the platform for 10s.

Training was conducted once a day at each of the four entry points, and the results of the day were statistically analyzed using the mean value of the four escape latencies. The training lasted for four days.

The concealed platform was removed 24 h after the test. After the mice were placed in the water, their swimming trails for 60 s were recorded, and the residence time of the mice in the original platform quadrant and the times of platform crossover were statistically analyzed. The reversal phase started after the test, and the platform was moved to the opposite quadrant of the tank. The platform remained in this northwest quadrant location for all training trials on days 1, 2, and 3, but not for day 4 of the test trial (Figs. 2 and S2). The swimming activity of each mouse was automatically recorded using a video tracking system (Ethovision 2.0, Noldus, Wagenigen, Netherlands).

## **Quantitative PCR.**

Total RNA was extracted from hippocampal tissues with Trizol (Biosharp, China). The Total RNA was reverse transcribed into complementary DNA (cDNA) by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The primers used to measure gene expression are the following TRPC1 Forward-CGTGCGACAAGGGTGACTATTAT, Reverse- TGCATCTGCGGACTGACAAC; TRPC3 Forward-ACCCTGCTTTTACCACGGTT, Reverse- GCATGTTGAGCAGAACGACC; TRPC4 Forward-AAACCCATCGGAAGTACC, Reverse- GCTAGTCCATCATCTCCGCA; TRPC5 Forward-TTTGCCAACGGACTGAACCA, Reverse- GAAGGGTTTCAAAGAGCGTGG; TRPC6 Forward-AAGTGAACGAAGGGGAGCTG, Reverse- ACAGTCTCTCCCAAGCTTTC; Forward-TCCCTTTAACCTGGTGCCGAGTC, Reverse- TTCAGCATGCCCATTTCCAGG.

## Western blotting.

Protein sample concentrations were measured using the Pierce BCA Protein Assay Kit (Biosharp, Hefei, China). Equal amounts of proteins (10 µg per lane) were run on 10% or 12% SDS-PAGE and then transferred to PVDF membranes by electroblotting (Bio-Rad, Hercules, CA, USA). The PVDF membranes were blocked with 5% skim milk powder (BD-Difco, USA) diluted with Tween/0.1M PBS (TBST) for one h at room temperature before incubation with the primary antibody.

The membranes were washed with TBST three times for 10 min after the primary antibodies were incubated at 4 °C overnight. The membranes were then incubated with secondary antibodies for 1 h at room temperature, washed three times for 10 min in TPBS, and reacted with chemiluminescent substrate (Biosharp, Hefei, China). The bands were obtained using an ECL luminescence imaging system (Tanon 5200, China). The densities of the target protein bands were measured using Image J and normalized to corresponding β-actin bands.

## Immunostaining.

Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> ( $n=3$ ) and littermate ( $n=3$ ) male mice were sacrificed and perfused with PBS (pH=7.4), followed by 4% paraformaldehyde in PBS. The samples were immersed in a fixed solution overnight and then dehydrated in a gradient solution of 10%, 20%, and 30% sucrose in PBS. Each brain was embedded in OCT and coronal sections of 20µm thickness were prepared using a freezing microtome (Leica CM 1860, Germany). The slices were washed three times with PBS for 5min before staining. The tissue sections were permeabilized in 0.3% Triton X 100 for 30min and then blocked in 10% normal goat serum. The blocked slices were incubated with the corresponding primary antibodies overnight at 4 °C. The samples were washed in PBS three times for 5 min, and then incubated with fluorescent secondary antibodies for 1 h at room temperature. After being washed in PBS three times for 5min, the slices were incubated with DAPI (Beyotime, China, 1:5000). Representative images were obtained using a fluorescence microscope (Olympus, BX53, Japan) or confocal microscopy (Olympus, FV1000, Japan).

## AAV injection.

The hippocampal region was injected with AAV vectors that included AAV9-CaMKIIa promoter-TRPC5 2928 ( $9.7 \times 10^{13}$  µg/mL) or AAV9-GFP. A total of 10 mice received AAV9-TRPC5, and another 10 mice received AAV9-GFP. All vectors were generated and tittered using Vigene Biosciences (Vigene Biosciences, Inc., China).

After the mice were anesthetized (10% chloral hydrate, 3.5 mL/kg), their brains were fixed to a stereotactic locator and the virus was injected into the bilateral middle regions of the hippocampus (0.5 µl/hemisphere) using a 2 µl microsyringe according to the following stereotaxic coordinates referenced in mm from the bregma (AP=-2 mm; ML=±1.4 mm; DV=-1.5 mm). Following injection, the microsyringe

was left in place for 5 min to prevent backflow of the solution. After surgery, the mice were single-housed for one week to recover well.

## Data analysis.

All data were expressed as mean  $\pm$ S.E.M. One-way or Two-way ANOVA were used for data analysis based on different experimental designs or data sets followed by multiple comparison test or by unpaired, two-tailed *t* test. Values of  $P < 0.05$  were considered statistically significant.

## Results

### Microglial cell knockout (KO) IL-10 impaired the learning and memory ability of mice.

Before the experiment, all mice were detected by PCR and classified (Fig. S1a). Colocalization of microglial markers Iba1 and IL-10 was also examined after tamoxifen injection (Fig. S1b). To test the learning and memory abilities of mice, we tested NOR and OLR in our study. Figure 1a shows the experimental procedure. There was no difference between the two groups during NOR detection in both the training and test stages, not only in the discrimination index, but also in object duration time history detection ( $P > 0.05$ ,  $n=8$ , *t* test, Fig 1.b-d).

During the OLR task,  $Cx3cr1^{CreER}IL-10^{-/-}$  mice showed reduced memory capacity. In the test stage, discrimination index ( $t=2.387$ ,  $P=0.031 < 0.05$ ,  $n=8$ , *t* test,) and object duration ( $t=2.357$ ,  $P=0.034 < 0.05$ ,  $n=8$ , *t* test) decreased in the  $Cx3cr1^{CreER}IL-10^{-/-}$  group (Fig. 1e-g).

There was no difference between the two groups in NOR, but there was a difference in OLR, indicating that the two groups had differential sensitivity in spatial location recognition [10]. We further detected the learning and memory abilities of the two groups using MWM. The results showed that both the escape latency to the platform ( $F(1, 14) = 10.96$ ,  $P < 0.01$ , Two-way ANOVA, Fig.2 b) and the average distance ( $F(1, 14) = 10.36$ ,  $P < 0.01$ , Two-way ANOVA, Fig.2 c) in the target quadrant increased in KO group during the learning stage. In the test stage, the mice in the KO group showed a decrease in both platform crossover times ( $P < 0.05$ ,  $n=8$ , *t* test,) and time spent in the target quadrant ( $P < 0.05$ ,  $n=8$ , *t* test, Fig.2.f). In the reversal MWM test, the KO mice showed a decreased ability in the learning stage, but not in the memory stage (Fig. S2).

### TRPC4 and TRPC5 decreased in hippocampi of $Cx3cr1^{CreER}IL-10^{-/-}$ mice.

Since spatial learning relies heavily on hippocampal activity, in addition to TRPC2, other TRPC subtypes were detected via RT-PCR since TRPC2 is not expressed in the hippocampus[11]. RT-PCR results showed that the mRNA levels of TRPC1,3,4 and 5 decreased in the hippocampus (Fig.3.a). Using western blot to detect the protein expression levels of these TRPC subtypes, also revealed that TRPC4 ( $t=3.130$ ,  $P=0.020<0.05$ ,  $n=4$ ,  $t$  test) and TRPC5 ( $t=5.910$ ,  $P=0.001<0.01$ ,  $n=4$ ,  $t$  test) were decreased in the hippocampus (Fig.3 b-f). Immunofluorescence results in the CA3 region further verified the results (Fig.3.g).

## Synaptic proteins decreased in hippocampi of **Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup>** mice.

Hippocampal synaptic proteins are one of the structural bases of spatial learning and memory. We tested the expression of glutamate receptors NR2A and NR2B, synaptic protein postsynaptic density protein 95 (PSD95), and synaptophysin in the hippocampus. In our results, NR2A and NR2B were not affected in the KO mice ( $P>0.05$ ,  $n=4$ ,  $t$  test, Fig4.a,b and c), but PSD95 ( $t=4.398$ ,  $P=0.005<0.01$ ,  $n=4$ ,  $t$  test) and synaptophysin ( $t=2.850$ ,  $P=0.029<0.05$ ,  $n=4$ ,  $t$  test) were decreased in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice compared to their littermates, as determined by western blot analyses (Fig.4.a d and e). The immunofluorescence results of PSD95 and synaptophysin in the CA3 region are shown in Figure 4f, which was consistent with that of western blotting. The fluorescence intensity was reduced in both PSD95 and synaptophysin in the KO group.

Synaptophysin is an integral membrane protein of small synaptic vesicles and has been identified as a useful marker for synaptic density[12]. PSD95 is a protein localized to the postsynaptic density of synapses[13] and plays a key role in synapse stabilization and plasticity [14], suggesting that microglia-derived IL-10 may play a role in synaptic protein synthesis through underlying mechanisms, thus affecting the spatial learning and memory ability of Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice.

## Hippocampal inflammatory activity enhanced in **Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup>** mice.

To determine whether changes in IL-10 levels in microglia affect inflammation in vivo, the levels of glial marker Iba1 (for microglia) and GFAP (for astrocytes) were tested. Our results showed increased protein expression of GFAP ( $t=4.583$ ,  $P=0.005<0.01$ ,  $n=4$ ,  $t$  test,) and Iba1( $t=2.698$ ,  $P=0.036<0.05$ ,  $n=4$ ,  $t$  test, Fig.5 a,b and c), which was consistent with enhanced immunofluorescence levels of Iba1 and GFAP-positive cells in the hippocampal CA3 region (Fig 5.i). The increased expressions of GFAP and Iba1 suggested that the behavioral changes in KO mice were involved in inflammation activation.

We also investigated whether the inflammasome nod-like receptor protein 3 (NLRP3) pathway was upregulated in the hippocampus. Protein expression levels of NLRP3 inflammasome components, NLRP3

( $t=3.976$ ,  $P=0.007<0.01$ ,  $n=4$ ,  $t$  test), IL-1 $\beta$  ( $t=5.784$ ,  $P=0.001<0.01$ ,  $n=4$ ,  $t$  test), and IL-18 ( $t=3.092$ ,  $P=0.02<0.05$ ,  $n=4$ ,  $t$  test), were significantly increased in the hippocampi of KO mice compared to their littermates (Fig. 5a, d–f). In addition, our results showed that IL-10 ( $t=2.922$ ,  $P=0.03<0.05$ ,  $n=4$ ,  $t$  test) decreased and IL-6 ( $t=4.887$ ,  $P=0.003<0.01$ ,  $n=4$ ,  $t$  test) increased in the hippocampi of Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice.

## **TRPC5 down-regulated in excitatory neurons in the hippocampi of Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice.**

The balance between the central inhibitory and facilitatory systems may serve as a principal mechanism of memory activation and regulation[15]. TRPC is expressed in both the inhibitory and excitatory neurons of the CNS [16]. We explored how downregulation of TRPC in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice affects memory by affecting network excitation and inhibitory balance. Our results showed that gene KO did not affect the content of GAD67 ( $t=1.430$ ,  $P=0.20>0.05$ ,  $n=4$ ,  $t$  test, Fig.6. a,b) but could enhance the content of Pv ( $t=13.53$ ,  $P<0.0001$ ,  $n=4$ ,  $t$  test, Fig.6. a,c), which is a specific subtype of gamma aminobutyric acid (GABA) interneurons that may subserve distinct behavioral functions and behavior-dependent network activities[17]. Inhibition of the GABAergic system has memory-facilitating effects, whereas stimulation produces memory impairment. These results suggest that the effect of TRPC4 or 5 downregulation on learning and memory in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice mainly acted on excitatory neurons. Immunofluorescence showed increased TRPC5 colocalization in the hippocampus with CaMKII $\alpha$  (an excitatory neuron marker) (Fig.6. d).

## **Upregulation of TRPC5 in excitatory neurons can improve memory impairment in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice.**

CaMKII $\alpha$  was used as the promoter of the AAV9 virus, specifically upregulating TRPC5 in excitatory neurons (Fig.7. b, c). After injection of the virus for four weeks, we performed MWM tests in all groups of mice. The results of MWM showed differences in escape latency to the platform ( $F(3, 16)=8.137$ ,  $P<0.01$ ) and average swimming distance ( $F(3, 16)=13.00$ ,  $P<0.001$ ) during the learning phase. The KO mice treated with AAV-9 TRPC5 injection showed an improved behavioral performance compared to KO-GFP group ( $P<0.05$ , Fig.7d, e). In the training stage, the KO mice treated with TRPC5 exhibited alleviated memory damage in both platform crossover times ( $F(3, 16)=7.053$ ,  $P<0.01$ , Fig.7. g) and in time spent in the target quadrant ( $F(3, 16)=12.81$ ,  $P<0.001$ , Fig.7 h).

## **The effects of TRPC5 on Cx3cr1<sup>CreER</sup>IL10<sup>-/-</sup> mice by suppressing NLRP3-associated neuroinflammation.**

The mechanism of TRPC5 upregulation in excitatory neurons contributes to the improvement behavioral performance in KO mice. We used immunofluorescence to detect GFAP, Iba1, and NLRP3 in the brain regions of the hippocampus. The results showed that increasing the expression of TRPC5 inhibited the number of GFAP ( $F(3,8)=28.01$ ,  $P<0.001$ , Fig.8. a, d), Iba1 ( $F(3,8)=8.011$ ,  $P<0.01$ , Fig.8. b, e) and NLRP3 ( $F(3,8)=49.59$ ,  $P<0.001$ , Fig.8. c, f) positive cells in the hippocampus, especially in the CA3 region.

## Discussion

IL-10 is a key cytokine that represses excessive inflammatory responses and is linked to anti-inflammatory and protective functions in the CNS[18]. In the CNS, IL-10 is mainly produced by astrocytes and microglia. Our results showed that  $Cx3cr1^{CreER}IL-10^{-/-}$  mice showed impaired spatial cognition, suggesting that microglia-targeted production of IL-10 plays an important role in hippocampal function. This result is similar to the results of other relevant studies, in which  $IL-10^{tm1/tm1}$  male mice with a low expression of IL-10 exhibited defective learning and memory behaviors in the MWM test[7]. A recent study showed that IL-10 produced from microglial cells in non-learned helpless mice is necessary to maintain learning and memory[19]. Intranasal administration of IL-10 increased dendritic spine density by 2.0- and 4.3-fold in the dentate gyrus of non-learned helpless and learned helpless mice[19].

We detected mRNA levels of TRPC subtypes in the hippocampus other than TRPC2, a pseudogene in humans. TRPC1, 3, 4, and 5 mRNA levels decreased. Further results confirmed that TRPC4 and TRPC5 protein levels were downregulated. This result could be linked to another related study, in which the TRPC1/4/5 channels were relevant to synaptic transmission for working memory formation and in relearning tasks in the hippocampus [3]. Controversially, TRPC1/4/5 KO did not affect mouse reference memory in that study. Based on the synaptic plasticity deficit, it could be difficult to explain how TRPC1/4/5 KO can specifically affect working memory without affecting the reference memory [3]. Notably, during the training phase in the MWM task,  $TRPC1/4/5^{-/-}$  mice showed a similar decrease in learning ability. This difference may also be related to the interaction of TRPC subtypes, since the expression of TRPC1 protein in our study did not change in mice.

The cognitive impairment caused by  $Cx3cr1^{CreER}IL-10^{-/-}$  mice is closely related to a decrease in synaptic transmission. Synaptic transmission involves the release of neurotransmitters from presynaptic neurons, which then bind to specific postsynaptic receptors. Synaptic proteins PSD95 and synaptophysin were tested in our study, and an obvious decrease in presynaptic synaptophysin and postsynaptic density protein PSD95 suggested that the memory deficits of  $Cx3cr1^{CreER}IL-10^{-/-}$  mice depend on structural changes in synaptic associated proteins, which is a dual mechanism involving presynaptic and postsynaptic processes. Notably, the glutamate receptors NR2A and NR2B were not involved in this process.

IL-10 is a key cytokine that has been shown to inhibit excessive inflammation associated with anti-inflammatory and protective functions in the CNS. IL-10 exerts anti-inflammatory effects by inhibiting monocyte/macrophage-derived cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8. It has been reported that

immune dysfunction is commonly associated with the progression of many CNS diseases, such as neuropsychiatric disorders and neurodegenerative disorders. [18, 23]. Moreover, the role of cytokines is of particular interest because they are involved in cognitive impairment in hippocampal-dependent memory[24, 25].

The increase in the number of GFAP-and Iba1 positive cells indicates an imbalance in local neuroinflammation in the hippocampus. Our results confirmed the upregulation of the inflammasome NLRP3 signaling pathway, accompanied by the upregulation of IL-6. These results suggest that KO IL-10 from microglia may affect the behavioral performance of animals by altering the balance of local pro-inflammatory and anti-inflammatory networks. The NLRP inflammasome has been identified as a multi-protein complex that plays a pathogenic role in nervous system diseases. Among these types of inflammasomes, NLRP3 has been implicated in several chronic inflammatory responses and is associated with many CNS diseases[27]. Neuroinflammation can trigger cognitive impairment, and the role of inflammasome NLRP3 processes involved in recognition impairment in a variety of nervous system diseases, has been supported by experimental evidence, especially in recent years. Several studies have shown that NLRP3 can be a target for improving memory impairment in diabetes[28–31], sepsis-associated encephalopathy[32], hypoxemia[33], epilepsy[34, 35], AD[36, 37], intracerebral hemorrhage[38], cerebral ischemia[39], and aged[40]. Baicalin increases the performance of APP/PS1 transgenic mice in MWM by suppressing NLRP3 inflammasomes to alleviate microglia-mediated neuroinflammation[41]. Activation of the NLRP3 inflammasome plays a role in the Gastrodin-induced amelioration of cognitive impairment in diabetic rats [28], and NLRP3 inflammation, a molecular marker involved in inflammatory response, is known to play a key role in the development of cognitive impairment.

NLRP3 activation can trigger IL-1 $\beta$  and IL-18 production and secretion[42], and enhanced IL-1 $\beta$  production in astrocytes is associated with the pathogenesis of major depressive disorder. Our results showed an increase in NLRP3, IL-1 $\beta$ , and IL-18 in microglial cells knocked out in IL-10 mice compared to their littermates, suggesting that the NLRP3 pathway could play an important role in memory impairment in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice.

TRPC channels are widely expressed in the brain and are related to a variety of neuronal functions [43]but in general, TRPC4 and TRPC5 are the predominant subtypes in the rodent brain[44]. In our study, we observed that both TRPC4 and 5 decreased in hippocampi of KO mice, which indicated that both channels could contribute to the spatial memory impairment of Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice, although it is difficult to judge which of these channels contributes to cognitive impairment. TRPC5 is highly expressed in the hippocampus [45, 46]. TRPC channels have been implicated in presynaptic and postsynaptic neuronal processes. To date, the physiological function of TRPC channels in the brain is unknown. In cultured neurons, TRPC5 insertion and TRPC5-mediated Ca<sup>2+</sup> influx are important determinants of hippocampal neurite growth rate and growth cone morphology[47, 48].

In our study, we used CaMKII $\alpha$  as a promoter to specifically express TRPC5 in pyramidal neurons. The results showed that high expression of TRPC5 could improve spatial cognitive impairment in IL-10 KO mice. Furthermore, the results suggested that the effect of TRPC5 on behavioral improvement in IL-10 KO mice might be related to its inhibition of neuroinflammation. Increasing the expression of TRPC5 in excitatory neurons can reduce the high expression of GFAP and Iba1 in Cx3cr1<sup>CreER</sup>IL-10<sup>-/-</sup> mice. Several other studies have also focused on the role of TRPC5 in inflammation. Growing evidence has linked the activation of TRPC5 complexes to inflammation. TRPC5<sup>-/-</sup> mice showed enhanced synovitis and local inflammation, and the TRPC4/5 antagonist ML204 increased the levels of TNF- $\alpha$  and IL-10 in synovial fluid. In TRPC5 KO and wild-type mice treated with TRPC4/5 antagonists, IL-10 secretion was found to be elevated to regulate a highly inflammatory response. The absence or antagonism of TRPC5 increases the local secretion of many key pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ [49]. TRPC5<sup>-/-</sup> mice pretreated with thioredoxin also showed that cytokines (TNF- $\alpha$  and IL-6) in the peritoneum were exacerbated in the systemic inflammatory response[50]. There have also been conflicting studies on the relationship between TRPC5 and inflammation. Expression of TRPC5 in nasal polyps was positively correlated with the number of eosinophils, IL-6 expression and inflammation[51], suggesting that these pathways may respond differently to different inflammatory responses. There are few studies on the relationship between TRPC and NLRP3. TRPC1 downregulation affects IL-1 $\beta$  release through the Caspase-11 pathway, a process associated with NLRP3 activation[52].

TRPC5 may also directly affect the learning and memory ability of animals by improving the efficiency of synaptic transmission. Some studies have shown that TRPC5 regulates synaptic plasticity by changing the presynaptic Ca<sup>2+</sup> homeostasis of hippocampal neurons [53] and both TRPC4 and 5 channels contribute to persistent firing in CA1 pyramidal cells[54]. In another study, TRPC5 channels, profoundly regulate synaptic plasticity and elevate the rate of spontaneous release, indicating a key role of TRPC5 in short-term plasticity. In addition, the specific activation of TRPC4/5 induced a significant increase in the mEPSC frequency in hippocampal neurons of wild-type mice [53]. TRPC5 is also an important determinant at neurite outgrowth rates, growth cone morphology[48] and plateau potentials of excitatory neurons in the hippocampus [55].

## Conclusions

Our results using Cx3cr1<sup>CreER</sup> IL-10<sup>-/-</sup> mice indicated the involvement of TRPC4 and 5 channels in recognition impairment. Specific high expression of the excitatory neuron TRPC5 can improve the behavioral performance of KO mice. However, a limitation in our present study does not preclude the role of TRPC4 in the process. Future research should focus on resolving the contradictory observations mentioned above and determining the molecular mechanisms between TRPC and the inflammasome NLRP3 system. In a specific environment, an attempt should be made to clarify the relationship between the cooperative compensation of various subtypes of TRPC functions and the behavioral representation of animals

## Abbreviations

TRPC: [Transient Receptor Potential Canonical](#); IL-10: Interleukin-10; GFAP: Glial fibrillary acidic protein; Iba1: ionized calcium binding adaptor molecule 1; PSD95: Postsynaptic density protein 95; NLRP3: nod-like receptor protein 3; CaMKII: calmodulin-dependent protein kinase II; LTP: Long-term potentiation; CNS: Central nervous system; MWM: Morris Water Maze; Nor: Novel object recognition; Olr: Object location recognition; DI :discrimination index; PVDF: polyvinylidene difluoride; Pv: Pavalbumin; DG: Dentate gyrus; ANOVA: Analysis of variance; NLRP: NOD-like receptor pyrin domain containing; AD: Alzheimer disease; KO: Knock out; mEPSC: miniature excitatory postsynaptic current

## Declarations

## Acknowledgments

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## Conflicts of interest

The authors declare no conflicts of interest.

## Availability of data and material

The datasets used during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

Shiji Huo, Jiling Ren, Ahsawle Ozathaley and Wenjian Yuan performed the experiments. Shiji Huo and Jiling Ren analyzed data and plotted. Zhaowei Liu designed the experiment and wrote the original draft. Yunqing Ma, Hong Ni and Dong Li provided reagents and technical support.

## Ethical Approval

All procedures performed in the study involving animals were in accordance with the ethical standards of the institution or practice. This article does not contain any studies with human participants performed by any of the authors.

## Consent to participate

Informed consent was obtained from all individual participants included in the study.

## Consent for Publication

All individual participants have consented to the submission of the manuscript to the journal.

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## Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

## Figures

Fig.1

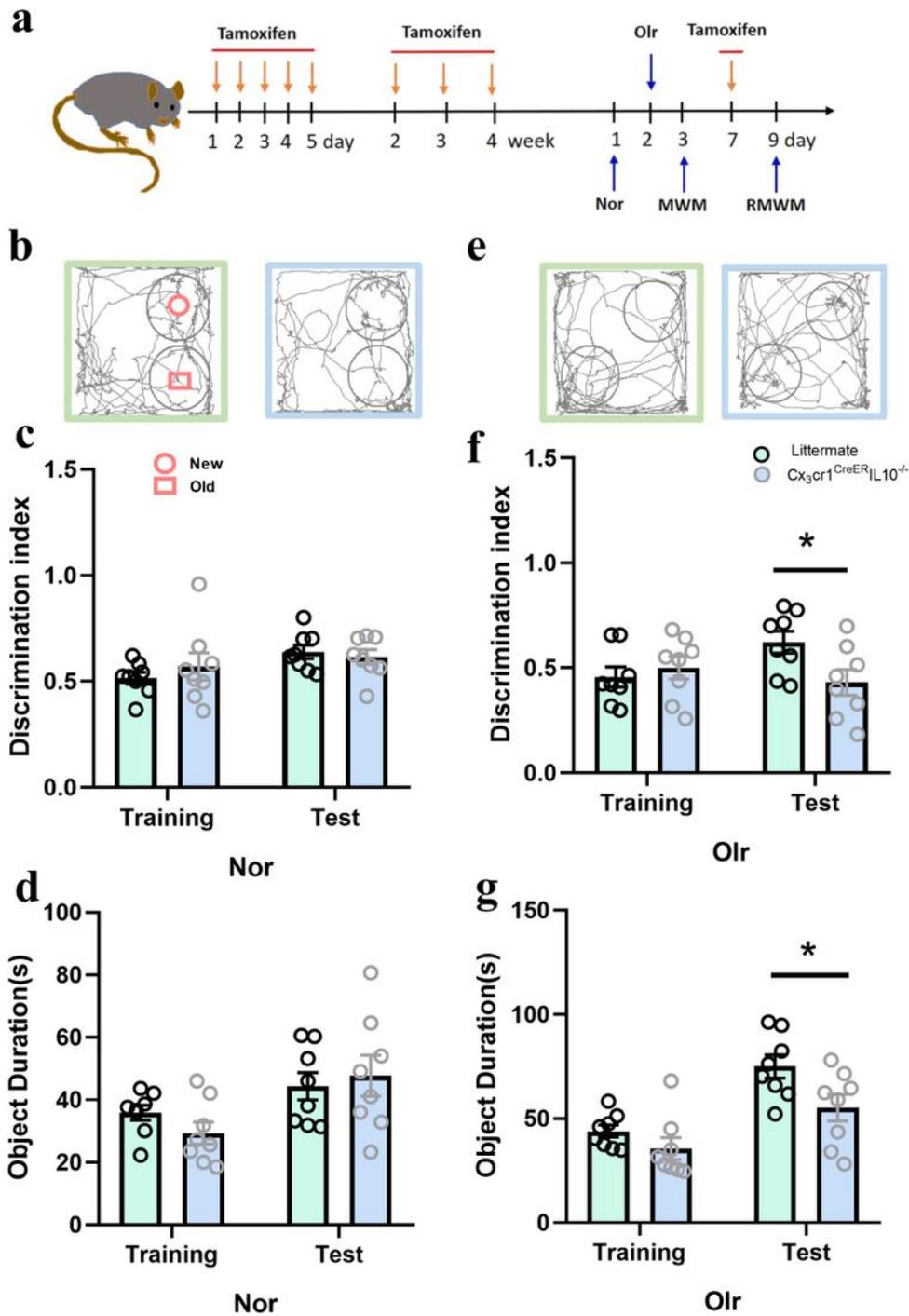


Figure 1

$Cx3cr1^{CreER}IL10^{-/-}$  mice showed a decrease recognition impairment in OLR task. a: Schematic of experimental design and schedule. The animal experimental protocol indicated the time course of various interventions utilized during the experiment. b: Representative movement traces from the two groups on the test stage of the NOR task. There were no significant differences between the two mice in the different groups. There was no significant difference between the two groups in the discrimination index (c) and

the new object duration (d). e: Representative movement traces from the two groups on the test stage of the OLR task. There was a significant decrease in the location of new objects in the Cx3cr1CreER IL-10<sup>-/-</sup> group. There was a significant decrease in the two groups in discrimination index (f) and new object duration (g) in the test stage in the Cx3cr1CreER IL-10<sup>-/-</sup> group. Each dot represents a mouse. Bars represent mean±SEM. n=8 in each group. Significant differences were established by t test, \*P<0.05.

Fig.2

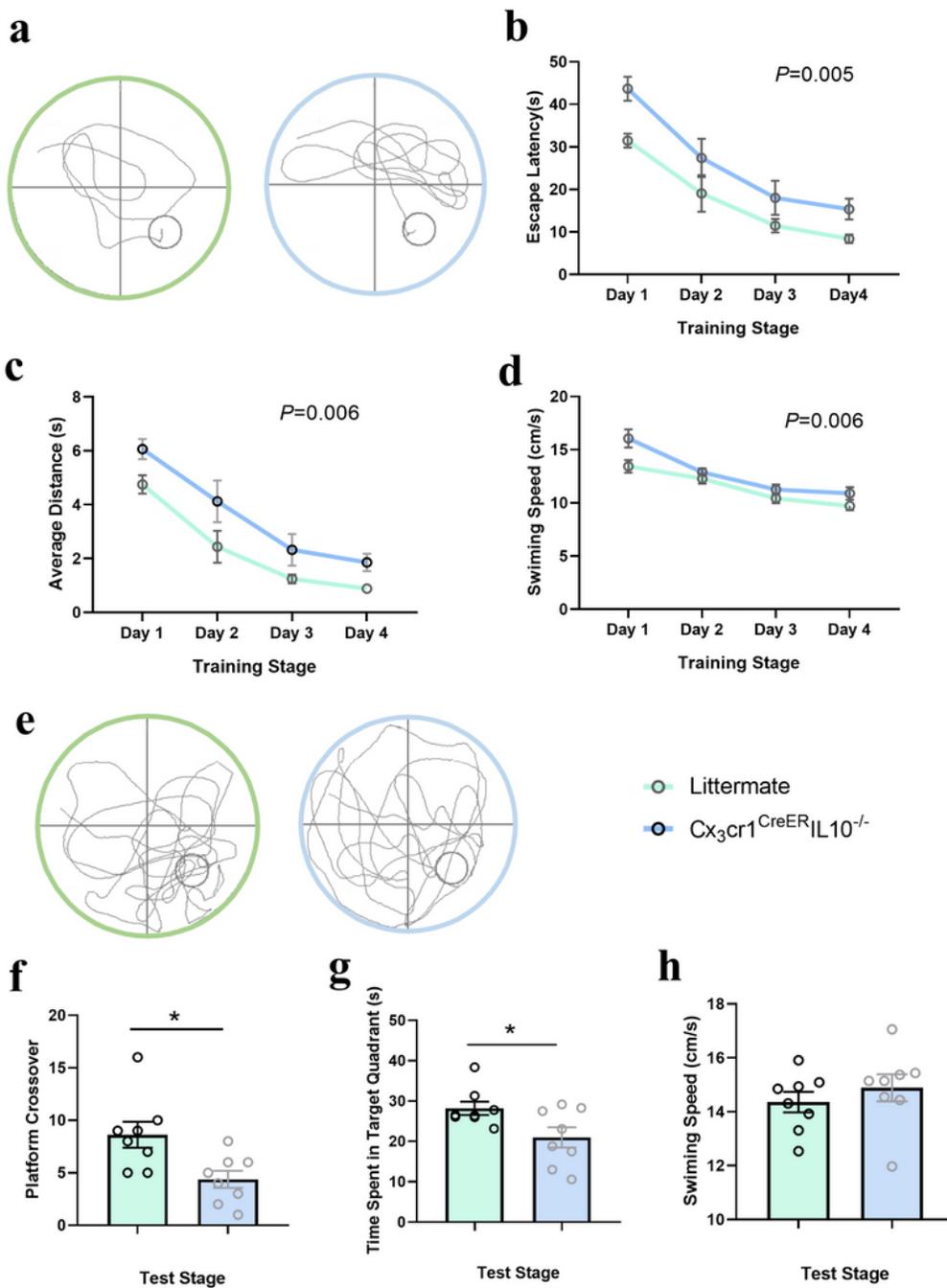


Figure 2

Cx3cr1CreER IL-10<sup>-/-</sup>-mice showed a decrease recognition impairment in MWM task. a: Representative movement traces from two groups during the training stage of the MWM task. Cx3cr1CreER IL-10<sup>-/-</sup>-mice had more dispersed paths in the training stage, suggesting impairments in learning ability. There was a significant increase in escape latency (b) and average distance (c) in Cx3cr1CreER IL-10<sup>-/-</sup> mice in the training stage, while Cx3cr1CreER IL-10<sup>-/-</sup> mice swam faster in the MWM task (d). e: Representative movement traces from the two groups on the test stage of the MWM task. Cx3cr1CreER IL-10<sup>-/-</sup>-mice had more dispersed paths in the test stage, suggesting memory impairments. There was a significant decrease in both the platform crossover times (f) and time spent in the target quadrant (g) in the Cx3cr1CreER IL-10<sup>-/-</sup> group in the test stage, while the swimming speed was similar between the two groups during the test stage (h). Each dot represents a mouse. Bars represent mean±SEM. n=8 in each group. Significant differences were established by two-way ANOVA (b-d) and t test in other bar graphs, \*P<0.05.

Fig.3

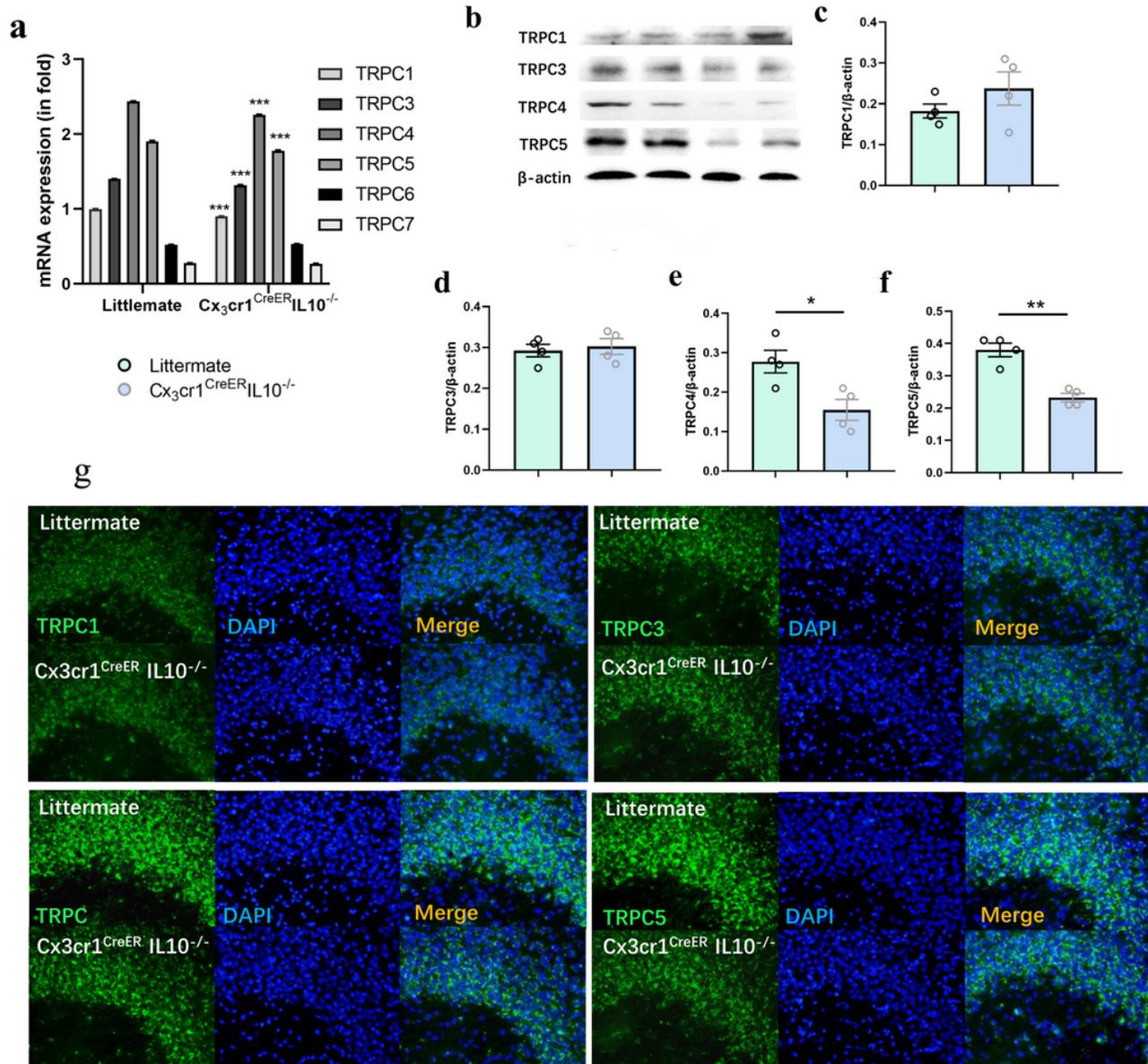


Figure 3

The decrease of both TRPC4 and TRPC5 in the hippocampi of Cx3cr1CreER IL-10<sup>-/-</sup> mice. a: RT-PCR results of TRPC isoforms in the hippocampus (n=3 in each group, t-test \*\*\*P<0.001). b: western blotting bands of TRPC1, TRPC3, TRPC4, and TRPC5 in littermates and in Cx3cr1CreER IL-10<sup>-/-</sup> mice. Bar graphs represent densitometric plots of protein expression in littermate and Cx3cr1CreER IL-10<sup>-/-</sup> mice in TRPC1 (c), TRPC3 (d), TRPC4 (e), and TRPC5 (f). Each dot represents a mouse. Bars represent mean $\pm$ SEM. n=4 in each group, Significant differences were established by t test. \*P<0.05, \*\*P<0.01. g: Expression of TRPC1, TRPC3, TRPC4, and TRPC5 in the CA3 region of mouse hippocampal slices. Immunofluorescence images were captured with a 20 $\times$  objective, green, immunoreactivity of TRPC1, TRPC3, TRPC4, and TRPC5; blue, nuclei stained with DAPI. Merged images of each TRPC isoform and DAPI staining

Fig.4

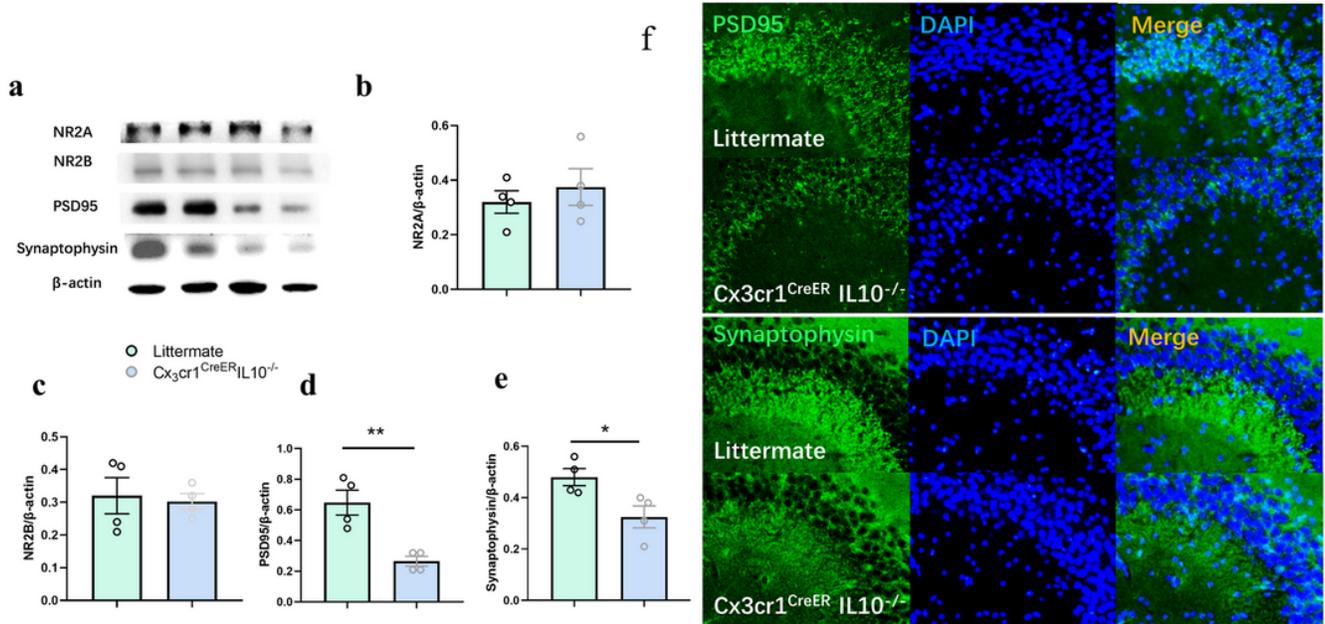


Figure 4

The expression of synaptic proteins in hippocampus of  $Cx3cr1^{CreER} IL-10^{-/-}$  mice. a: western blotting bands of NR2A, NR2B, PSD95, and synaptophysin in littermates and in  $Cx3cr1^{CreER} IL-10^{-/-}$  mice. Bar graphs represent densitometric plots of protein expression in littermate and  $Cx3cr1^{CreER} IL-10^{-/-}$  mice in NR2A (b), NR2B (c), PSD95 (d), and synaptophysin (e). Each dot represents a mouse. Bars represent mean  $\pm$  SEM. n=4 in each group. Significant differences were established by t test. \*P<0.05, \*\*P<0.01. g: Expression of PSD95 and synaptophysin in the CA3 region of mouse hippocampal slices. Immunofluorescence images were captured with a 20 $\times$  objective, green, immunoreactivity of TRPC1, TRPC3, TRPC4, and TRPC5; blue, nuclei stained with DAPI. The merged images of PSD95, synaptophysin, and DAPI staining

Fig.5

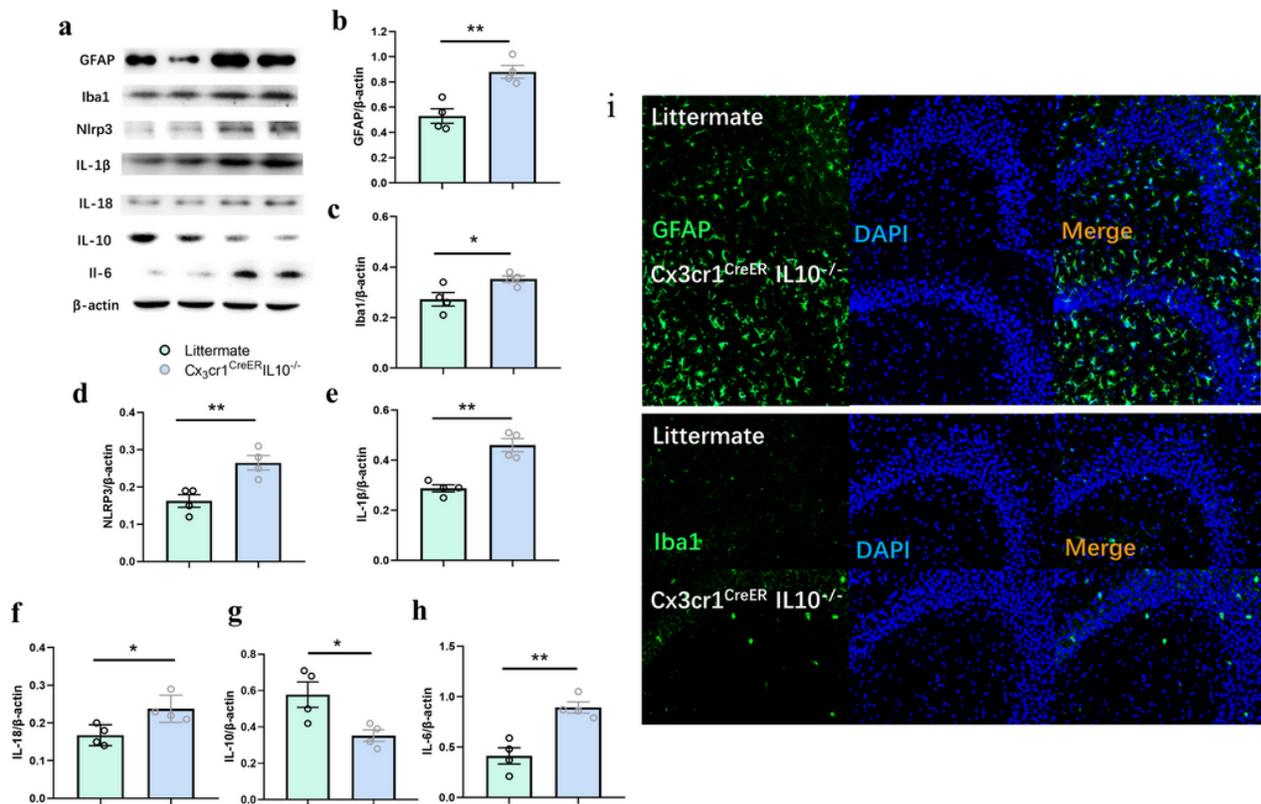


Figure 5

Neuroinflammatory activity enhanced in the hippocampus in Cx3cr1CreER IL-10<sup>-/-</sup>-mice a: western blotting bands of inflammation-related molecules in littermates and Cx3cr1CreER IL-10<sup>-/-</sup>-mice. Bar graphs represent densitometric plots of protein expression in littermate and Cx3cr1CreER IL-10<sup>-/-</sup>-mice in GFAP (b), Iba1 (c), NLRP3 (d), IL-1 $\beta$  (e), IL-18 (f), IL-10 (g), and IL-6 (h). Each dot represents a mouse. Bars represent mean $\pm$ SEM. n=4 in each group. Significant differences were established by t test, \*P<0.05, \*\*P<0.01. i: Expression of GFAP and Iba1 in CA3 region in mice hippocampal slices. Immunofluorescence images, captured with a 10 $\times$  objective, Green, immunoreactivity of GFAP and Iba1; blue, nuclei staining with DAPI. The merged images of GFAP, Iba1 and DAPI staining.

Fig.6

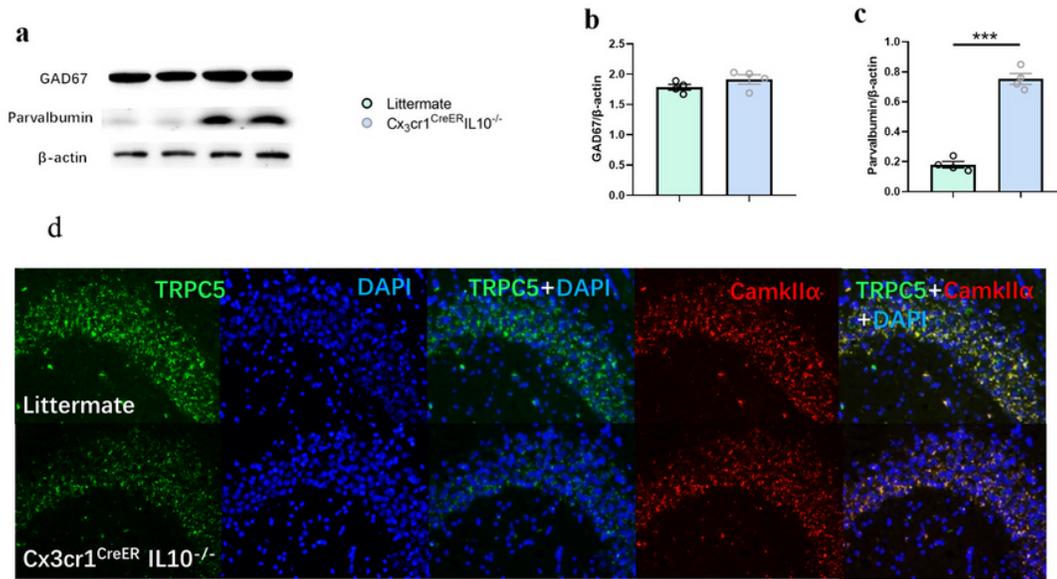
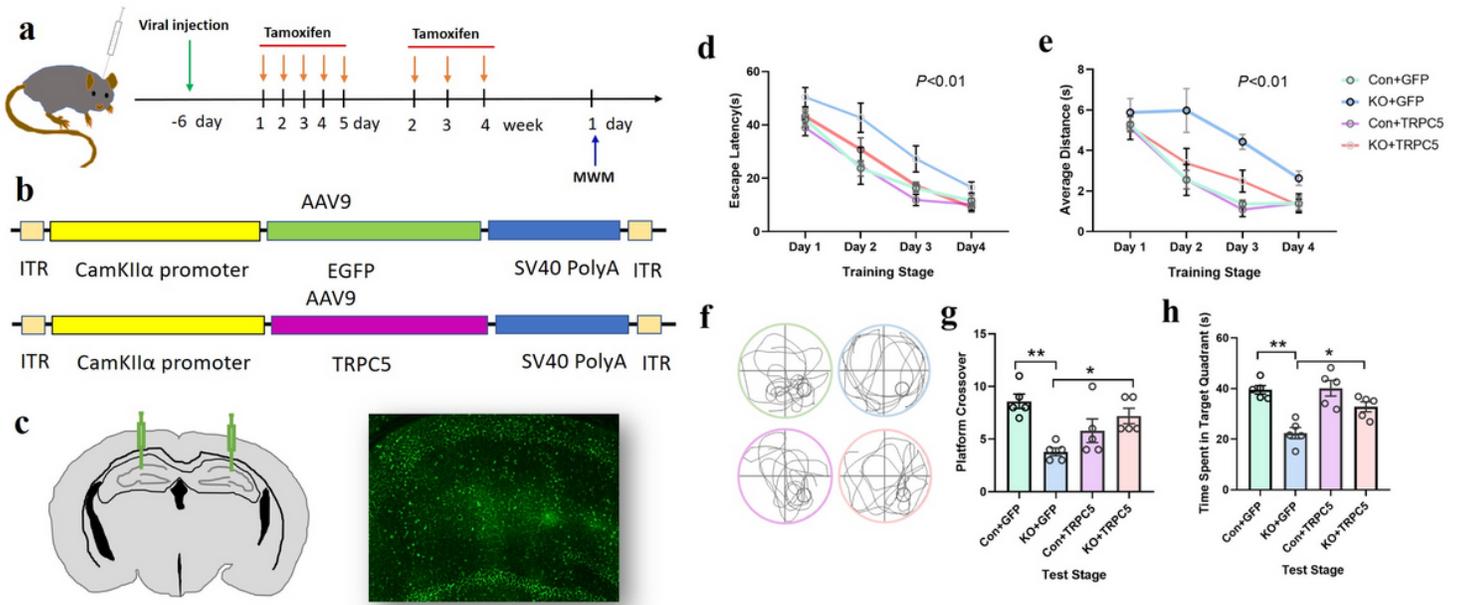


Figure 6

The effects of knocking out IL-10 from Microglia in interneuron and the increase of TRPC5 in excitatory neuron a: western blotting bands of GAD67 and parvalbumin in littermates and in Cx3cr1CreER IL-10<sup>-/-</sup> mice. Bar graphs represent densitometric plots of protein expression in littermate and Cx3cr1CreER IL-10<sup>-/-</sup> mice in GAD67 (b) and parvalbumin (c). Each dot represents a mouse. Bars represent mean ± SEM. n=4 in each group, Significant differences were established by t test, \*\*\*P<0.001. d: colocalization of TRPC5 and CaMKIIα in CA3 region in hippocampal slices. Immunofluorescence images, captured with a 20× objective, Green, immunoreactivity of TRPC5; red, immunoreactivity of CaMKIIα, a marker of excitatory neurons, blue, nuclei staining with DAPI. The merged images of TRPC5, CaMKIIα and DAPI staining.

**Fig.7**



**Figure 7**

Upregulation of TRPC5 in hippocampal excitatory neurons improving the learning and memory ability of Cx3cr1CreER IL-10<sup>-/-</sup> mice. **a**: Schematic of experimental design and schedule. The animal experimental protocol indicated the time course of various interventions utilized during the experiment. **b, c**: Schematics of AAV9-CamKIIα-TRPC5 (**b**) and stereotaxic injection in the hippocampus (**c**). There was a significant increase in both escape latency (**d**) and average distance (**e**) in KO+TRPC5 compared to the KO group in the training stage. **f**: Representative movement traces from the four groups on the test stage of the MWM task. The mice in the KO+TRPC5 group had more convergent paths in the test stage in the target quadrant, suggesting memory improvements. There was a significant increase in both the platform crossover times (**g**) and time spent in the target quadrant (**h**) in the KO+TRPC5 group in the test stage compared with the KO group (**h**). Each dot represents a mouse. Bars represent mean ± SEM. n=5 in each group, Significant differences were established by two-way ANOVA (**d,e**) and one-way ANOVA (**g,h**), \*P<0.05, \*\*P<0.05.

Fig.8

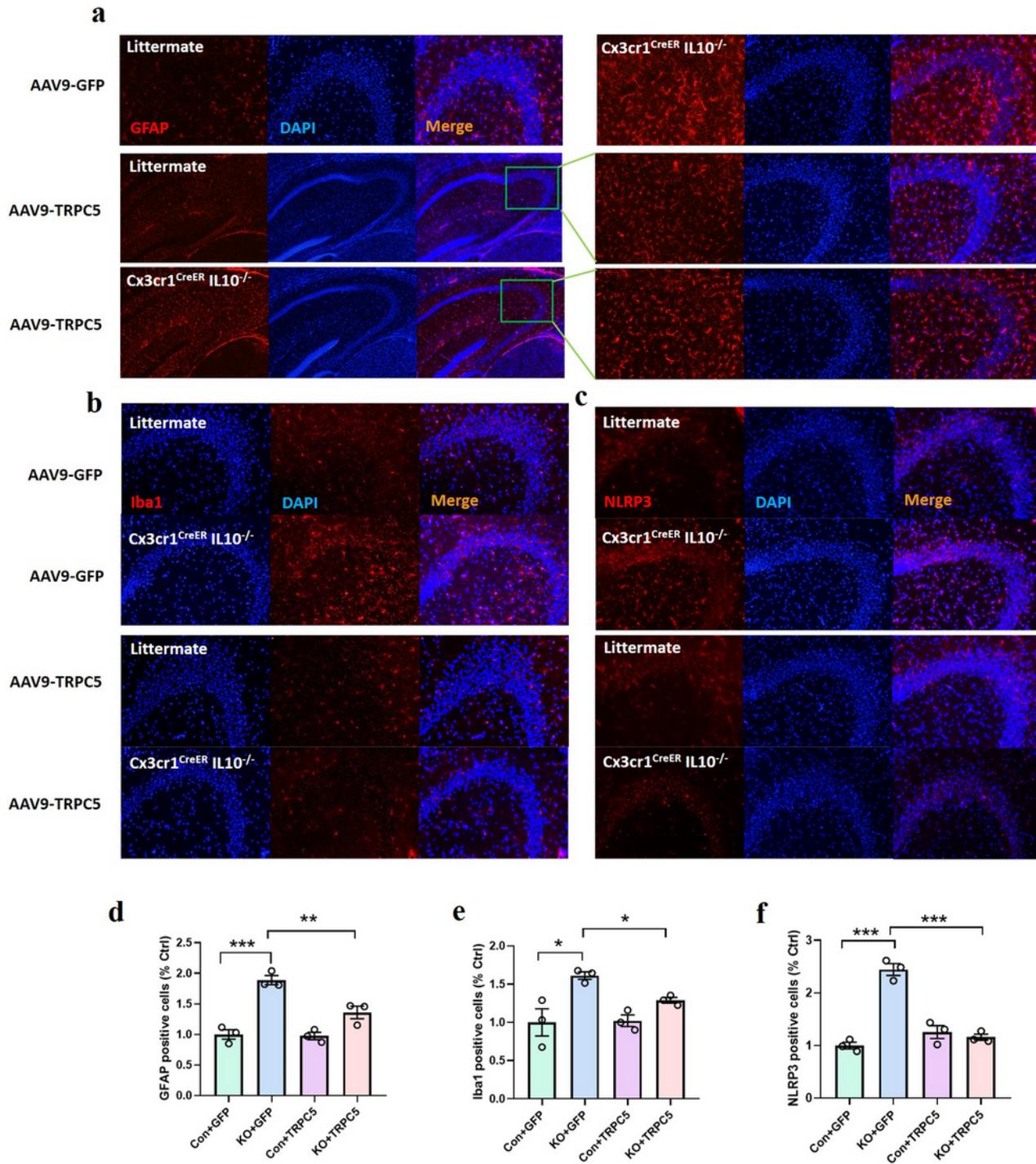


Figure 8

Upregulation of TRPC5 in hippocampal excitatory neurons inhibiting the neuroinflammation in hippocampus. a-c: Expression of GFAP (a), Iba1 (b), and NLRP3 (c) in the CA3 region of hippocampal slices. Immunofluorescence images were captured with a 5× or 10× objective, red, immunoreactivity of GFAP, Iba1, and NLRP3; blue, nuclei stained with DAPI. The merged images of GFAP, Iba1 and DAPI

staining. Bars represent positive cells in GFAP(d), Iba1(e) and NLRP3(f) at mean±SEM. n=3 in each group, Significant differences were established by one-way ANOVA (g,h) \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

## Supplementary Files

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