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Loss of Transient Receptor Potential Ankyrin 1 Channel Deregulates Emotion, Learning and Memory, Cognition, and Social Behavior in Mice

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Abstract The transient receptor potential ankyrin 1 (TRPA1) channel is a non-selective cation channel that helps regulate inflammatory pain sensation and nociception and the development of inflammatory diseases. However, the potential role of the TRPA1 channel and the underlying mechanism in brain functions are not fully resolved. In this study, we demonstrated that genetic deletion of the TRPA1 channel in mice or pharmacological inhibition of its activity increased neurite outgrowth. In vivo study in mice provided evidence of the TRPA1 channel as a negative regulator in hippocampal functions; functional ablation of the TRPA1 channel in mice enhanced hippocampal functions, as evidenced by less anxiety-like behavior, and enhanced fear-related or spatial learning and memory, and novel location recognition as well as social interactions. However, the TRPA1 channel appears to be a prerequisite for motor function; functional loss of the TRPA1 channel in mice led to axonal bundle fragmentation, downregulation of myelin basic protein, and decreased mature oligodendrocyte population in the brain, for impaired motor function. The TRPA1 channel may play a crucial role in neuronal development and oligodendrocyte maturation and be a potential regulator in emotion, cognition, learning and memory, and social behavior.

Keywords Transient receptor potential ankyrin 1 channel · Anxiolytic-like behavior · Learning and memory · Cognition · Social preference · Neuron differentiation · Myelination

Introduction

The transient receptor potential ankyrin 1 (TRPA1) channel is a type of nonselective transmembrane cation channel [1, 2] characterized by a large number of N-terminal ankyrin repeats and mainly permeable to calcium (Ca^{2+}), which suggests its importance in the Ca^{2+} signaling pathway [2–5]. TRPA1 channels are abundant in the brain, Aδ fiber, C fiber, and dorsal root ganglia and are key players in acute inflammatory pain and nociception with a wide variety of stimuli such as cold temperature, reactive oxygen species, and allyl isothiocyanate (AITC) [1, 4–7]. During the development of sensory neurons, the TRPA1 channel participates in regulation of sensory neuron differentiation [8–12]. Besides being expressed on neurons, the TRPA1 channel is expressed on astrocytes and ependymal cells located at ventricles [13–16]. However, little is known about how this specific distribution contributes to various brain functions. Further investigation delineating the role and molecular mechanisms of the TRPA1 channel in brain functions is warranted.

In the central nervous system (CNS), neuron–glial communication plays a regulatory role in the physiological functions of the brain, including memory and cognitive skill formation, learning capacity, and capability for emotional responses and social interactions [17–19]. The protoplasmic protrusions of neurons, axons, and dendrites are essential for signal transmission between neurons and their target cells [20, 21]. In

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addition, the level of myelination on axons by oligodendrocytes (one kind of glia cell) is a critical determining factor in the regulation of motor functions [20–26]. Ca^{2+} signaling is implicated in the regulation of various stages of brain development, including neuron differentiation and myelination [27–31]. However, whether and how TRPA1-mediated Ca^{2+} signaling contributes to cerebral development and functions remain elusive.

Given the importance of the TRPA1 channel in regulating pathophysiological functions in the brain, we addressed the role of the channel in brain function. We examined the postnatal expression profile of the TRPA1 channel in mouse brains, and then assessed its role in development of emotional responses, learning and memory, cognition, and social preference in a TRPA1-channel loss-of-function mouse model and the underlying mechanism.

Materials and Methods

Reagents

Goat anti-rabbit FITC-conjugated antibody and mouse antibodies for glial fibrillary acidic protein (GFAP) and β3-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-mouse rhodamine-conjugated antibody, mouse antibody for α-tubulin and Flag, crystal violet, potassium dichromate, silver nitrate, retinoic acid (RA), bovine serum albumin (BSA), phosphatase inhibitor cocktails 1 and 2, TRPA1 antagonist HC030031, and agonist AITC were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit antibody for microtubule-associated protein 2 (MAP-2) and mouse antibodies for NeuN and myelin basic protein (MBP) were from Millipore (Billerica, MA, USA). Retrieval buffer was from Biocare Medical (Concord, CA, USA). Rabbit antibody for TRPA1 was from Novus (Littleton, CO, USA). The mounting medium with DAPI was from Vector Laboratories (Burlingame, CA, USA). Mouse antibody for O4 was from R&D Systems (Minneapolis, MN, USA). Scramble siRNA and TRPA1 small interfering RNA (siRNA) were obtained from Thermo Scientific Dharmacon (Lafayette, CO, USA).

Mice

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, eighth edition, 2011), and all animal experiments were approved by the Animal Care and Utilization Committee of the National Yang-Ming University. Eight-week-old male B6129PF2/J wild-type (WT) and B6;129P-Trpa1$^{tm1Kykw/J}$ (TRPA1$^{-/-}$) mice on a B6129PF2/J background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). TRPA1$^{-/-}$ mice were backcrossed to B6129PF2/J for at least ten generations. Mice were housed in barrier facilities on a 12-h/12-h dark cycle and fed a regular chow diet (Newco Distributors, Redwood, CA). At the end of the experiment, mice were euthanized with CO_2, and then brains were harvested for histological analysis and stored at −80 °C.

Western Blot Analysis

Frozen brains were homogenized. Cells and tissues were lysed in immunoprecipitation lysis buffer (50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 300 mmol/L NaCl, 1 % Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Aliquots (50 μg) of cell lysates were separated on SDS-PAGE, and then transferred to membranes and immunoblotted with primary antibodies, then horseradish peroxidase-conjugated secondary antibodies. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (PerkimElmer, Waltham, MA), and density was quantified by use of Imagequant 5.2 (Healthcare Bio-Sciences, PA).

Open Field Activity

The locomotor activity of mice was assessed in a cage (length × width × height 28.5 × 28.5 × 30 cm). Mice were placed in the center of the cage and allowed to explore the open field for 5 min. The behavior was recorded by video, and the movement distance, percentage of resting time in the zone, and trajectory were calculated for each mouse by use of Smart v3.0 software with the Panlab Harvard apparatus (Cornellà, Barcelona, Spain). The floor and internal walls were cleaned with ethanol between each trial.

Elevated Plus Maze

The elevated plus maze (EPM) was used for investigating the anxiety-like behavior of mice. The maze consisted of two open arms and two enclosed arms (30 × 6 cm with 20 cm high walls in black acrylic). The maze was elevated 50 cm from the floor. The mouse was placed in the center square facing an open arm and allowed to explore the maze for 10 min. The time spent among open arms, closed arms, or the central area was recorded by use of Smart v3.0. The floor and internal walls were cleaned with ethanol between each trial.

Social Preference Test

The protocol of the social interaction test was as described [32] with modification. Social interactions between the isolated mouse and a visitor were recorded. The social box area was 60 × 30 × 30 cm. The social preference assay consisted of two phases. In the habituation phase, the mouse was placed in the cage and freely explored the box for 5 min. In the social

preference phase, a strange or familiar mouse was placed into a small chamber and the original mouse was allowed to sniff. The time spent exploring the mouse area and the empty area was immediately recorded for 10 min. The individual path length, trajectory, and time in different zones were analyzed by use of Smart v3.0. The proportion of time in the target zone was calculated by the time in the zone with a strange or familiar mice/time in the two zones × 100.

Hippocampus-Dependent Object Cognition

The cognition testing area was 28.5 × 28.5 × 30 cm. The object cognition test consisted of two phases. In the habituation phase, the mouse was placed in the cage and freely explored two different identical objects placed at two diagonal corners for 10 min. Two different visual cues providing contextual markers were attached to the wall of the cage. After the habituation, mouse was returned to the home cage for 3 h. In the second phase, one object was relocated to a different corner, and the other object remained in the same area. The time spent exploring each object was recorded for 5 min for each trial. Contact of each object was manually recorded. To analyze cognitive performance, the two discrimination indexes were calculated according to the following formula: (time spent on novel object / time spent on both two objects × 100) and (contact number on novel object / total contact number on both objects × 100). The floor and internal walls were cleaned with ethanol between each trial.

Classical Fear Conditioning

The experiments were performed by using a computerized fear-conditioning system from Coulbourn Instruments (San Diego, CA, USA). The system consisted of a shock and a tone generator. Training took place in an apparatus consisting of a box (22 × 22 × 30 cm) with a simple gray interior and a 12-V light attached to the ceiling. At the beginning of the experiments, each mouse was exposed to the conditioning chamber for 2 min, hard upon; then mice were exposed to the conditioning stimuli (CS): a tone (2000 Hz, 80 dB) for 20 s and a footshock (FS) at 1.5 mA received in the last 3 s. This conditioning training was repeated three times with an intertrial delay of 30 s. Finally, the freezing behavior while exposed to the tone (CS) was analyzed to determine the learning activity. The freezing response was defined as the absolute lack of movement (excluding respiratory movements), monitored by an ultra-red ray detector and analyzed by use of a computer. On days 2 and 7, the conditioned mice were reexposed to the CS training for three times, each with duration of 20 s, and the percentage freeze response was scored.

Rotarod Test

The motor function of the mice was analyzed by rotarod as described [21]. The speed of the rotarod was fixed to 10 rpm. Each mouse was placed on a rotarod and the retention time on the wheel was recorded. The limit of retention time was 1 min. Each mouse was tested seven times in continuous practice with intervals of 2 min. Finally, the seven retention times for each mouse were averaged.

Morris Water Maze

The Morris water maze was used to evaluate hippocampus-dependent spatial learning and memory of mice. A large circular tank (0.8-m diameter, 0.4-m depth) was filled with water (25 ± 1 °C, 20-cm depth), and the escape platform (8 × 4 cm) was submerged 1 cm below the surface. Each section was monitored by a video system. The escape latency and the trajectory of swimming were recorded for each mouse. The hidden platform was located in the center of one of the four quadrants in the tank. The location of the platform was fixed throughout testing. Mice had to navigate using extra maze cues that were placed on the walls of the maze. From days 1 to 4, mice went through three trials, with an intertrial interval of 5 min. The mice were placed into the tank facing the side wall randomly at one of the four start locations and allowed to swim until they found the platform or for a maximum of 120 s. Mice that failed to find the platform within 120 s were guided to the platform. The animals then remained on the platform for 20 s before being removed from the pool. The day after the hidden platform training, a probe trial was conducted to determine whether the mice used a spatial strategy to find the platform. At day 5, the platform was removed from the pool and the mice were allowed to swim freely for 120 s. The amount of time spent in each quadrant of the pool and the number of times the mice crossed the former position of the hidden platform were recorded.

Histology and Staining

Mouse brain tissue was fixed with 4 % paraformaldehyde, embedded in paraffin and serially sectioned at 15 μm. Brain sections underwent Nissl staining by incubation with 0.1 % crystal violet in phosphate-buffered saline for 30 min. For Golgi staining, brain samples were placed into 3 % potassium dichromate in 4 % paraformaldehyde for 2 days to avoid light, then 2 % silver nitrate in distilled deionized water for an additional 2 days. After staining, brain samples were embedded in paraffin and serially sectioned at 20 μm.

Immunohistochemistry Staining

Brain sections were fixed in 4 % paraformaldehyde and 15-μm cross sections were prepared. Sections were incubated with retrieval buffer for 10 min, blocked with 2 % BSA for 60 min, and incubated with primary antibody overnight at 4 °C, and then FITC- or rhodamine-conjugated secondary antibody for 1 h at 37 °C. Antigenic sites were visualized under a Nikon TE2000-U microscope (Tokyo) with QCapture Pro 6.0 software (QImaging, BC, Canada).

Cell Culture

Neuro-2a cells from the Bioresource Collection and Research Center (Hsinchu, Taiwan) were cultured in Dulbecco's modified Eagle medium with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 μg/ mL streptomycin (HyClone, Logan, UT) at 37 C in a humidified cell culture incubator with 95 % air and 5 % CO_2. Neuro-2a cells were treated with RA 20 μM in 2 % fetal bovine serum for 48 h for differentiation. Measurement of neurite outgrowth was as described [33, 34]. In short, the definition of a neurite was by length greater than twofold the cell. We randomly selected five regions to determine the differential cells under a TE2000-U fluorescence microscope (Nikon, Japan).

Plasmid Construction and Transient Transfection

The coding region for the human TRPA1 DNA fragment was cloned into a pCMV5 N-Flag vector with MluI and HindIII restriction sites. The sequence of isolated DNA fragments was confirmed by sequence analysis. Lipofectamine 2000 (Invirogen, MA, USA) was used for transient transfection experiments according to the manufacturer's instructions. Briefly, 1 μg of vector or Flag-tag TRPA1 plasmid was transfected into Neuro-2a cells. Transfected cells were used in further experiments. Flag expression was examined by anti-Flag antibody overnight at 4 °C, and then FITC-conjugated secondary antibody for 1 h at 37 °C. Antigenic sites were visualized under a Nikon TE2000-U microscope (Tokyo) with QCapture Pro 6.0 software (QImaging, BC, Canada).

SiRNA Transfection

Neuro2a cells were transfected with scramble or TRPA1 siRNA (50 nmole/L) with use of Lipofectamine 2000 for 24 h for the indicated experiments.

Statistical Analysis

Data are presented as mean ± SEM. Mann–Whitney *U* test was used to compare two independent groups. Kruskal-Wallis followed by Bonferroni post hoc analyses was used to account for multiple testing. SPSS v20.0 (SPSS Inc, Chicago, IL) was used for analysis. Differences were considered statistically significant at $P < 0.05$.

Results

Genetic Ablation of TRPA1 Channel Decreases Anxiety-Like Behaviors in Mice

First, we found that the protein expression of TRPA1 channels in brain was increased in a postnatal development-dependent manner (Fig. 1a). Loss of function of TRPA1 channel in mice did not affect locomotor activity in the open field test (Fig. 1b–d). Compared with WT mice, $TRPA1^{-/-}$ mice showed relatively less anxiety-like behaviors, as evidenced by spending decreased times in the closed arm but increased time in open and central arms, with a slight decrease in locomotion in the EPM test (Fig. 1e–g). These results suggest that the TRPA1 channel may play an important role in anxiolytic behaviors.

Genetic Ablation of TRPA1 Channel Promotes Both Hippocampus-Dependent Fear-Related Learning and Amygdala-Dependent Fear-Related Memory

The model of fear condition test we used is schematized in Fig. 2a. Starting from trial 2 on Day 1, $TRPA1^{-/-}$ mice showed significantly higher freezing rate than WT mice did, thereby indicating better learning trajectory, which is hippocampus-dependent (Fig. 2b). On Day 2, the freezing rate after cue delivery was significantly higher for $TRPA1^{-/-}$ than for WT mice with all three cues (Fig. 2c). On Day 7, WT mice did not react to the cues until the third cue; however, $TRPA1^{-/-}$ mice reacted to all three cues with a significantly high freezing rate (Fig. 2d). Thus, the TRPA1 channel may be crucial in regulating hippocampus-dependent fear-related learning and amygdala-dependent fear-related memory.

Genetic Ablation of TRPA1 Channel Promotes Hippocampus-Dependent Learning and Memory and Cognition

On the Morris water maze (MWM) test, the time to find the hidden platform was greater for $TRPA1^{-/-}$ than for WT mice during all 4 days (Fig. 3a, b). However, the results from the probe trial on Day 5 revealed an increased number of times crossing the former position of the hidden platform for $TRPA1^{-/-}$ mice, which suggests that $TRPA1^{-/-}$ mice had better retention of hippocampus-dependent spatial memory than WT mice did (Fig. 3c). Additionally, $TRPA1^{-/-}$ mice spent longer time arriving at the visually cued platform compared with WT mice (Fig. 3d), so ablation of the TRPA1 channel might impair the motor ability in the MWM test. In the novel

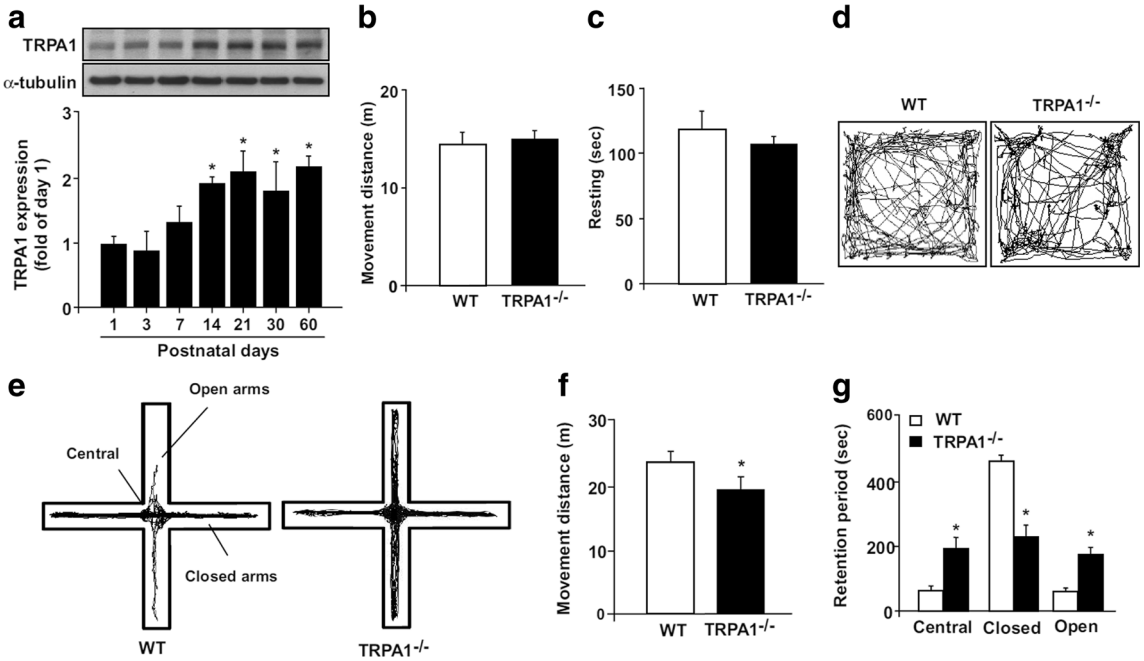

Fig. 1 Loss of function of TRPA1 channel decreases anxiety-like behavior in mice. **a** Brains were collected from wild-type (WT) mice ($n = 5$ in each group) at the indicated times. Western blot analysis of TRPA1 and α-tubulin. **b**, **c** The distance traveled and resting time for WT and TRPA1$^{-/-}$ male mice measured in the open field activity test ($n = 10$ in each group). **d** Representative video tracking analysis from male WT and TRPA1$^{-/-}$ mice. **e** Schematic diagram of elevated-plus maze (EPM) and representative running tracks for male WT and TRPA1$^{-/-}$ mice. **f**, **g** The distance traveled and time spent in the central, close and open arms for male WT and TRPA1$^{-/-}$ mice ($n = 10$ in each group). Data are mean ± SEM. In panel **a**, $^*p < 0.05$ vs. Day 1 group. In panels **f** and **g**, $^*p < 0.05$ vs. WT mice

location recognition test, TRPA1$^{-/-}$ mice spent more time and contacted the relocated object more than the other object compared with WT mice (Fig. 4). The preference for the relocated object implied that TRPA1$^{-/-}$ mice may have better spatial memory.

Genetic Ablation of TRPA1 Channel Enhances Social Recognition Behavior

In the social preference test, TRPA1$^{-/-}$ mice showed locomotor defects compared with WT mice in the presence of the strange or familiar mouse (Fig. 5a, b). Nevertheless, the time TRPA1$^{-/-}$ mice spent with strange and familiar mice was significantly greater than that for WT mice, which suggests enhanced social recognition capability of TRPA1$^{-/-}$ mice (Fig. 5c. d).

Loss of Function of TRPA1 Channel Increases Neurite Integrity In Vivo and In Vitro

WT and TRPA1$^{-/-}$ mice showed no difference in gross cerebral anatomy (Fig. 6a). However, brains of TRPA1$^{-/-}$ mice showed significant complex neurite circuitry in the cortex and hippocampus compared with WT mice (Fig. 6b). The protein level of MAP-2 in the brain was higher in TRPA1$^{-/-}$ than in WT mice (Fig. 6c, d).

We used RA-induced differentiation of Neuro-2a cells [35, 36] to examine neurite outgrowth in vitro. RA treatment increased the protein expression of the TRPA1 channel and induced Neuro-2a cell differentiation, as evidenced by the formation of neurites and the upregulation of MAP-2, β3-tubulin and NeuN (Fig. 7a–c). Pharmacological inhibition of TRPA1 channel function with HC030031 increased the mean neurite number (Fig. 7d–g), and activation of the TRPA1 channel function with AITC markedly decreased the number of neurite-bearing cells, mean neurite number, and mean neurite length (Fig. 7h–k). Moreover, inhibition of TRPA1 channel expression by siRNA increased the mean neurite number (Fig. 7l–p). In contrast, ectopic overexpression of TRPA1 channels significantly decreased the number of neurite-bearing cells, mean neurite number, and mean neurite length (Fig. 7q–u). Hence, TRPA1 channels may play a pivotal role in neuron differentiation.

Genetic Ablation of TRPA1 Channel Impairs Motor Function and Disrupts Axonal Bundle Organization and Oligodendrocyte Composition

We used the rotarod test to examine the vertical locomotion and vestibular function of mice. Compared to WT mice, TRPA1$^{-/-}$ mice showed impaired motor function, as

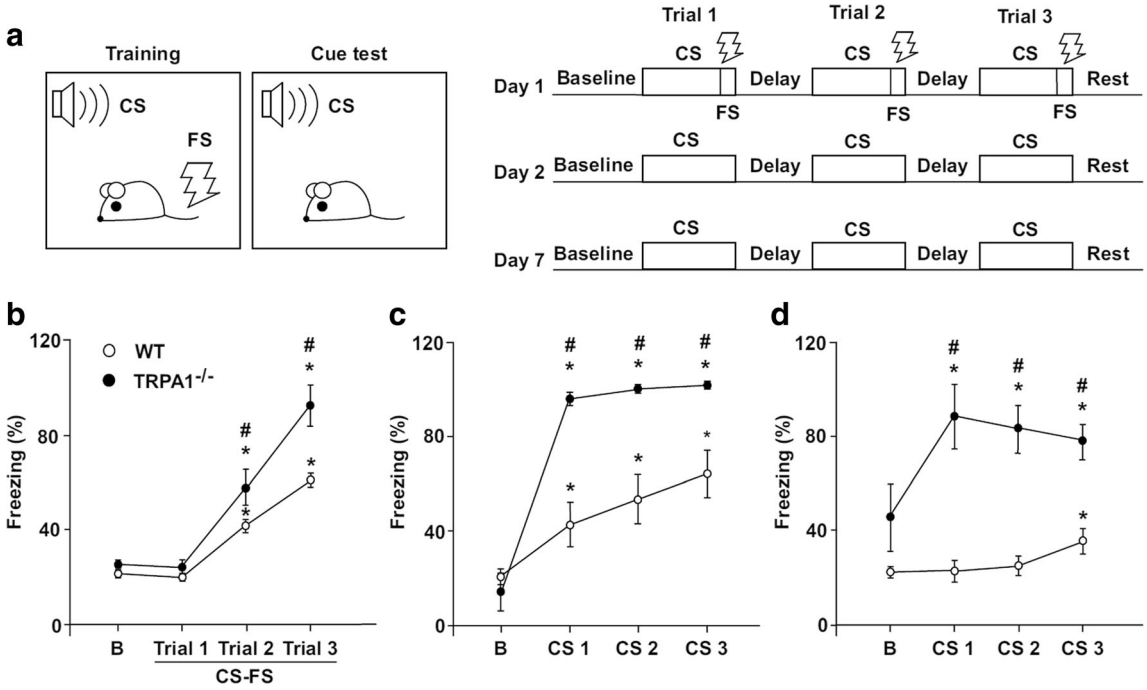

Fig. 2 Genetic deletion of TRPA1 channel function enhances the ability for hippocampus-dependent fear-related learning and amygdala-dependent fear-related memory in mice. **a** Schematic illustration of the experiment design for the fear conditioning test. **b** Freezing behavior after a foot shock (FS) was used to assess hippocampus-dependent learning on Day 1. **c**, **d** Freezing behavior after a condition stimuli (CS) on days 2 and 7, respectively, to assess amygdala-dependent memory. Data are mean ± SEM ($n = 10$ in each group). * $p < 0.05$ vs. baseline; #$p < 0.05$ vs. WT with corresponding Trial/Stimulus. *B* baseline

evidenced by decreased retention time on the rotarod (Fig. 8a). Golgi staining revealed loose and fragmented axonal bundles in the striatum in TRPA1$^{-/-}$ mice compared with WT mice (Fig. 8b). The protein expression of MBP, which plays an important role in myelination of nerves, was markedly decreased in TRPA1$^{-/-}$ mice compared with that in WT mice (Fig. 8c, d). Also, the composition of oligodendrocytes, the myelinating cells in the CNS, as evidenced by the O4 expression, was 50 % decreased in TRPA1$^{-/-}$ brains compared with those in WT mice (Fig. 8e).

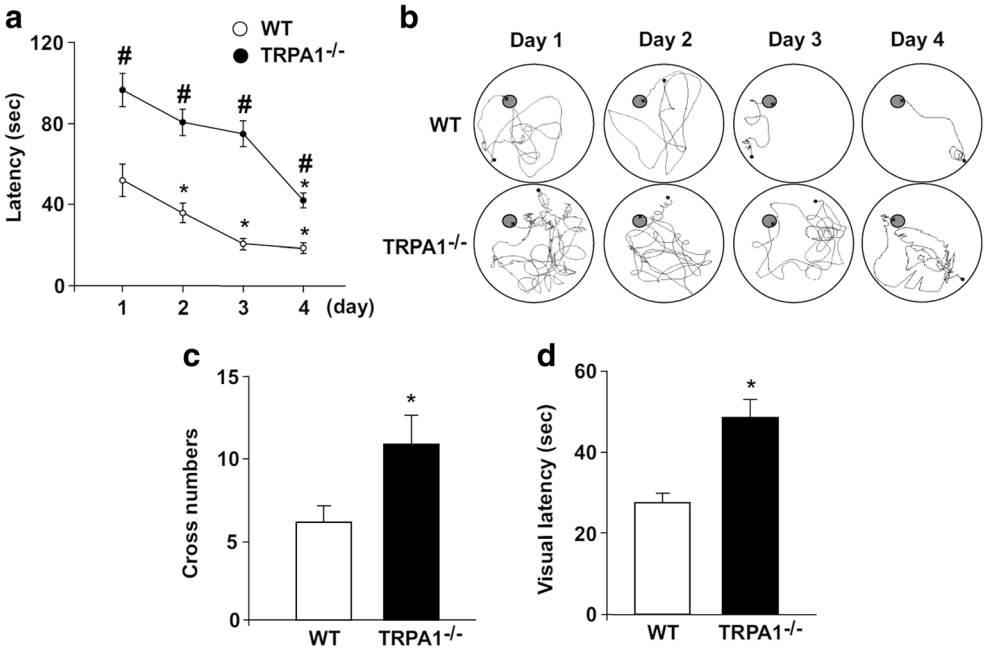

Fig. 3 TRPA1$^{-/-}$ mice require more time for learning but exhibit significant hippocampus-dependent spatial memory retention. **a** Latency of finding the platform in the Morris water maze test from days 1 to 4. **b** Representative trajectories for WT and TRPA1$^{-/-}$ mice in the hidden-platform trials from days 1 to 4. **c** The number of times crossing the former position of platform on Day 5. **d** Visual latency was combined with trajectories on Day 5 to assess differences in locomotion. Data are mean ± SEM ($n = 10$ in each group). In panel **a**, *$p < 0.05$ vs. Day 1, #$p < 0.05$ vs. WT mice. In panels **c** and **d**, *$p < 0.05$ vs. WT mice

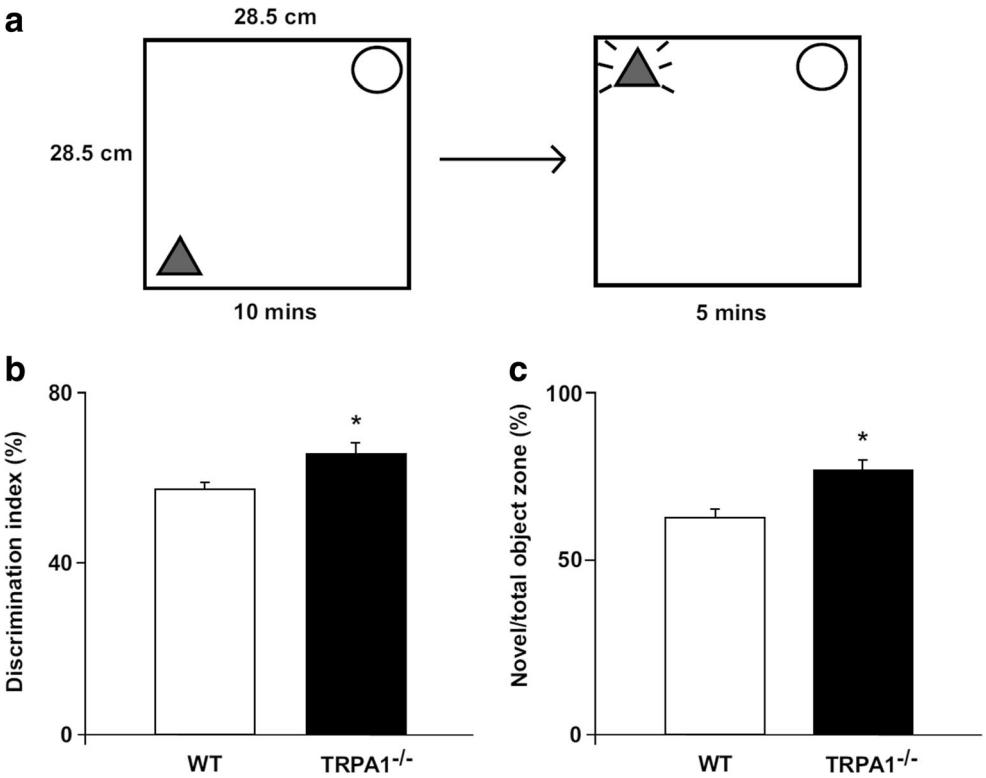

Fig. 4 TRPA1$^{-/-}$ mice show better performance in the hippocampus-dependent novel location recognition. **a** Schematic illustration of the experimental procedure of the novel location recognition test. **b** Time spent on the relocated object to the total time spent on both objects presented as a percentage. **c** The number of contacts with the relocated object to the total number of contacts with both objects presented as a percentage. Data are mean ± SEM ($n = 10$ in each group). *$p < 0.05$ vs. WT mice

Discussion

In this study, we characterized the novel role of the TRPA1 channel in regulating emotion, learning and memory, objective cognition, social interactions, and locomotion by using various behavioral mouse models. The protein expression of the TRPA1 channel was increased in the WT mouse brain during postnatal development. However, compared with WT mice, TRPA1$^{-/-}$ mice showed less anxiety-related behavior and better hippocampus-dependent brain functions, as evidenced by enhanced ability in fear-related or spatial learning and memory, and novel location recognition as well as social interactions. In contrast, TRPA1$^{-/-}$ mice showed impaired motor function by use of Morris water maze test and rotarod analysis. The TRPA1 channel-mediated regulation of the above behaviors was gender-independent (Supplementary

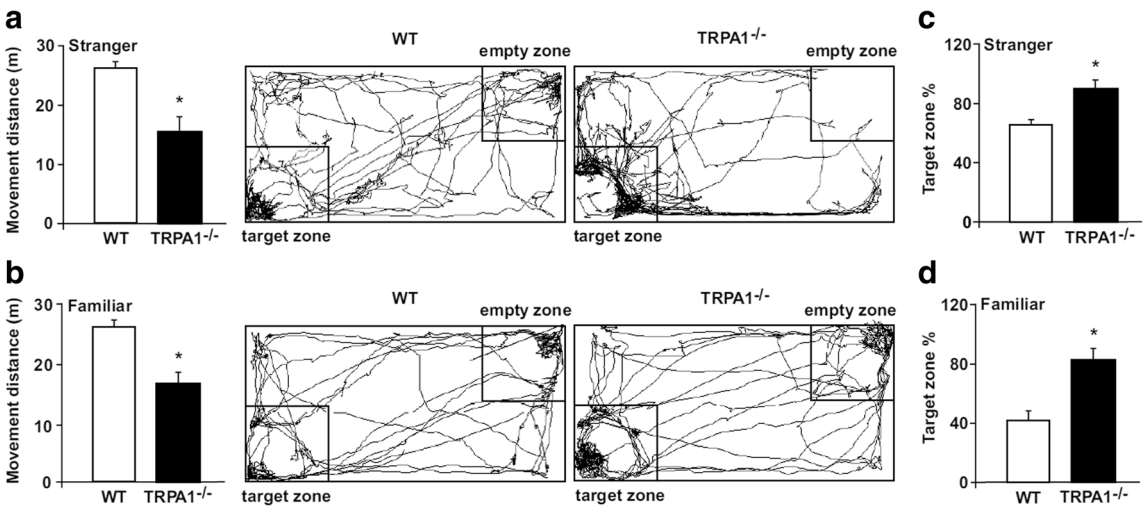

Fig. 5 TRPA1$^{-/-}$ mice exhibit enhanced social discrimination between strange and familiar mice as interactive partners. The ambulatory distance and representative trajectories of WT and TRPA1$^{-/-}$ mice with **a** a strange mouse in the target zone and **b** a familiar mouse in the target zone. **c**, **d** The percentage time spent in the target zone during the interactions of WT and TRPA1$^{-/-}$ mice. Data are mean ± SEM ($n = 10$ in each group). *$p < 0.05$ vs. WT mice

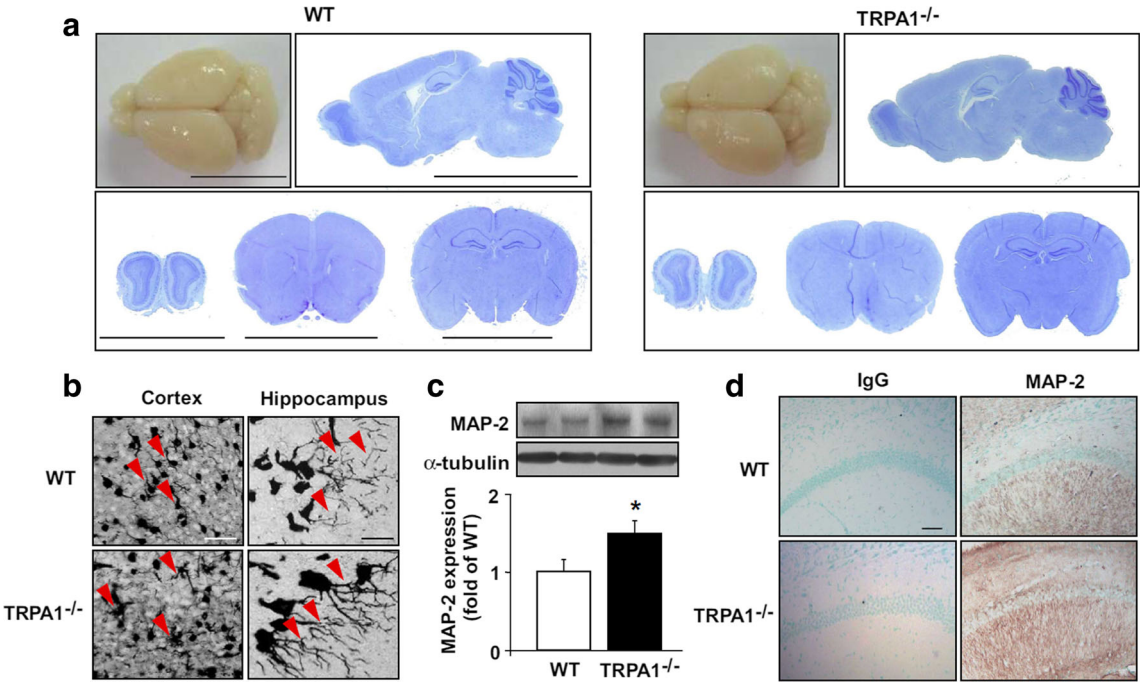

Fig. 6 TRPA1$^{-/-}$ mice show altered neurite structure of neurons in cortex and hippocampus with increased microtubule-associated protein 2 (MAP-2) protein expression. **a** Brain morphology of WT and TRPA1$^{-/-}$ mice. Scale bar = 1 cm. **b** Golgi staining of neurons in cortices and hippocampi of WT and TRPA1$^{-/-}$ mice. Scale bar = 25 μm. **c** Western blot analysis of MAP-2 and α-tubulin in brain lysates of WT and TRPA1$^{-/-}$ mice. **d** Immunohistochemistry of MAP-2 in hippocampus of WT and TRPA1$^{-/-}$ mice. *$p < 0.05$ vs. WT mice

information Figs. 1–4). At the cellular level, deletion of the TRPA1 channel in mice altered the neurite structure of neurons in the cortex and hippocampus. To study the role of TRPA1 channels at certain differentiated stage of neurons in vitro. We chose to use RA-induced differentiation of Neuro-2a neuroblastoma cells as our cell model. Neuro-2a neuroblastoma cells have been extensively used as a cell model of neurogenesis [35, 36]. They have the advantage of responding quickly to environmental stimuli such as serum deprivation and RA treatment by expressing crucial regulatory molecules including MAP-2, β3-tubulin, and NeuN that regulate the key events of the early stages of neuronal differentiation and neurite outgrowth [35, 36]. This could avoid the disadvantage of primary neurons that have individual difference in certain developmental stages when they were isolated from fetus or neonatal. Our results demonstrated that pharmacological inhibition of TRPA1 channel activity increased the number of neurites in neurons, and activation of TRPA1 channel activity blunted neuron differentiation. Ablation of TRPA1 channel function resulted in locomotive deficits, which may be attributed to fragmented axonal bundles, downregulation of MBP, and decreased number of oligodendrocytes in the brain. Collectively, our study provides both behavioral and cellular evidence supporting the pivotal role of the TRPA1 channel in maintaining normal brain functions.

The biological significance of the TRPA1 channel in the physiological functions of the peripheral nervous system has been well documented [37–41]. Most research focused on how the TRPA1 channel contributes to pain transduction in cold hypersensitivity via Aδ and C sensory fibers [37–41]. Nevertheless, little is known about the role of the TRPA1 channel in the CNS. Here, our results demonstrate that pharmacological modulation of TRPA1 channel activity affected neuron differentiation, which is consistent with previous findings that the TRPA1 channel participates in regulation of sensory neuron differentiation [8–12]. Our findings suggest that targeting the TRPA1 channel may have potential therapeutic value in treating anxiety and mood disorders, which agree with findings by de Moura et al., who found that inhibiting TRPA1 channel activity had antidepressant- and anxiolytic-like actions in experimental rodents [42]. Importantly, we also found that TRPA1$^{-/-}$ mice exhibited better hippocampus-dependent learning and memory, cognition, and social interactions, accompanied by a reformed neurite structure of neurons in the cortex and hippocampus. The nervous system function depends on the complex architecture of neuronal networks [17–19]. Thus, in terms of branching complexity and length of neurites, functional inhibition of the TRPA1 channel could modify the circuitry and be the cellular basis for the altered capacity in learning and memory and the social preferences we observed in TRPA1$^{-/-}$ mice. Accordingly, these findings suggest that the TRPA1 channel is an important player in regulating the physiological functions of the CNS.

We found that disrupting the TRPA1 channel function resulted in locomotor deficits observed in rotarod, EPM, MWM, and social preference tests. In parallel, our cellular and

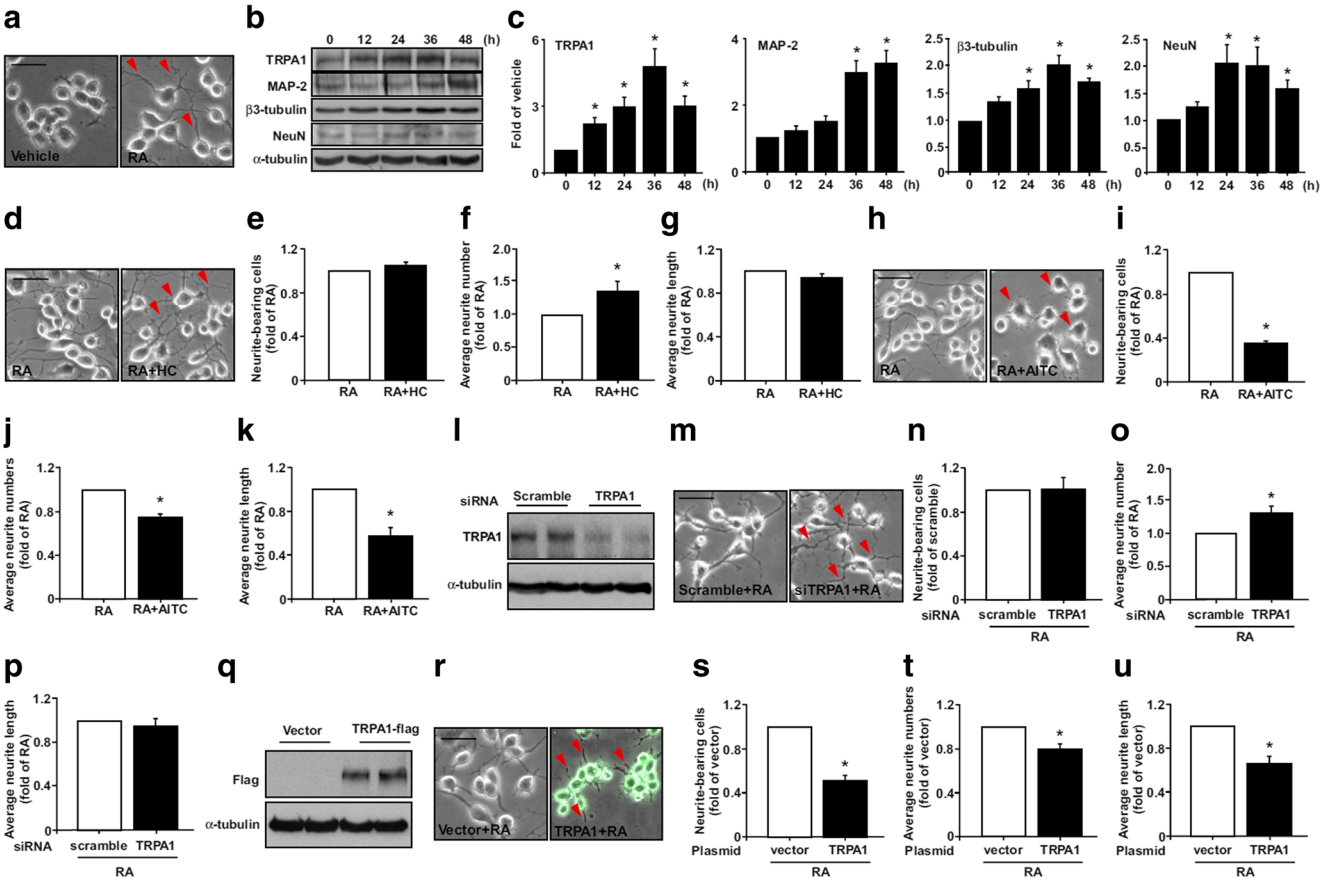

Fig. 7 TRPA1 plays a crucial role in retinoic acid (RA)-induced differentiation of Neuro-2a cells. The differentiation of Neuro-2a cells into neuron-like cells was induced by treatment with RA (20 μM) for 48 h. **a** Representative images of undifferentiated and differentiated Neuro-2a cells. Scale bar = 25 μm. **b**, **c** Western blot analysis of TRPA1, MAP-2, β3-tubulin, NeuN and α-tubulin in Neuro-2a cells. Cotreatment with (**d**–**g**) TRPA1 antagonist HC030031 (10 μM), **h**–**k** TRPA1 agonist AITC (10 μM), **l**–**p** knockdown of TRPA1 gene expression by siRNA or **q**–**u** overexpression of Flag-tag TRPA1 channels with 48-h RA-induced differentiation for evaluation of degree of neurite-bearing cells, neurite number, and neurite length. Scale bar = 25 μm. The neurites were indicated by *red arrows*. Green florescent signal-positive cells indicated Flag-tag TRPA1 channel-transfected cells. Data are mean ± SEM from five independent experiments. In panel **c**–**f**, $*p < 0.05$ vs. vehicle-treated group. In panel **g**–**s**, $*p < 0.05$ vs. RA-treated group

molecular evidence indicated that the TRPA1$^{-/-}$ brain showed fragmented axonal bundles and downregulated protein expression of MBP and O4 (oligodendrocyte marker). MBP plays a crucial role in maintaining the accurate structure of the myelin sheath of oligodendrocytes. Oligodendrocytes are the myelinating cells in the CNS, and their main functions are to provide support and insulation to axons [20–26]. Ample evidence indicates that antibodies to MBP or genetic deletion of MBP indeed results in the development of multiple sclerosis with the progressive loss of motor function [43]. Oligodendrocyte damage induced by many pathological conditions such as multiple sclerosis and ischemia is also highly associated with loss of motor function in humans or experimental animals [43]. Conceivably, our findings of a decrease in both MBP expression and mature oligodendrocyte population in the TRPA1$^{-/-}$ mouse brain may account for the locomotor deficits. However, how the TRPA1 channel regulates the MBP expression and oligodendrocyte biology and orchestrates behaviors remains for further investigation. Nevertheless, these findings strongly suggest that the TRPA1 channel may play an important role in regulating the motor function of the brain.

Intracellular Ca^{2+} dynamics via the integration of various Ca^{2+}-permeable channels is the key event in neuronal differentiation by regulating neurotransmitter specification, dendritic morphology, and axon growth and guidance [26]. For instance, $Ca_v1.2$, the most abundant neuronal L-type Ca^{2+} channel, combines with the TRPC channel to regulate Ca^{2+} influx during axonal guidance and outgrowth [44]. In terms of Ca^{2+} signaling, the alterations we observed in hippocampus-dependent behaviors including anxiety, learning and memory, cognition, and social preference in TRPA1$^{-/-}$ mice is likely due to the deregulation of Ca^{2+} mobilization by the TRPA1 channel in hippocampal neurons. This notion was also supported by our in vitro observations that pharmacological inhibition of the TRPA1 channel increased the complexity of neurite structure. In contrast, pharmacological activation of the TRPA1 channel had opposite effects. Therefore, this

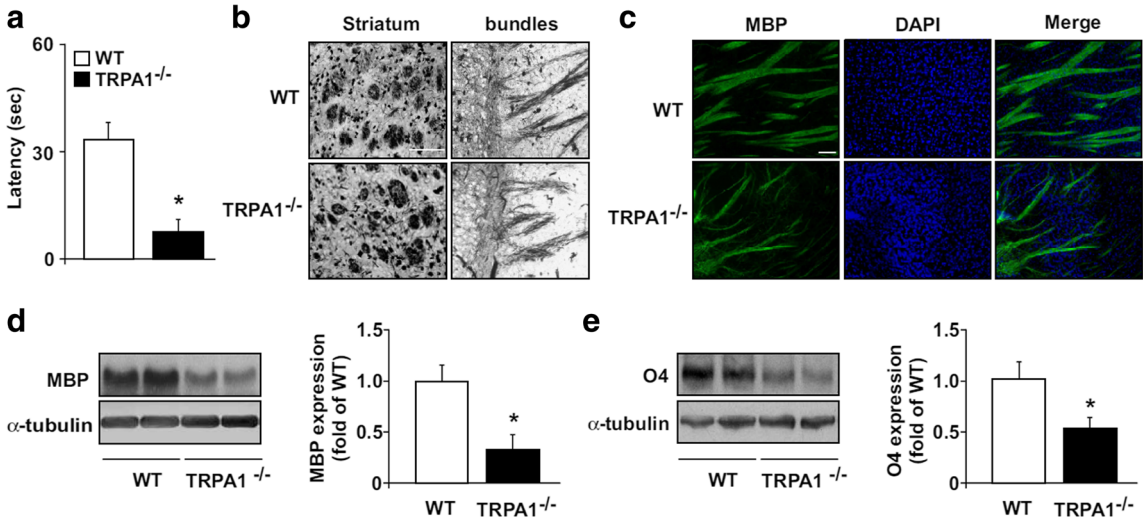

Fig. 8 TRPA1$^{-/-}$ mice show impaired motor function and possess fragmented axonal bundles and altered myelination profile in the brain. **a** The retention times on a rotarod was used to assess vertical locomotion and vestibular function. **b** Golgi staining of axonal bundles in striatum of brain sections from WT and TRPA1$^{-/-}$ mice. **c** Double immunofluorescence staining for myelin basic protein (MBP = *green*) and DAPI = *blue*. Scale bar = 100 μm. **d**, **e** Western blot analysis of MBP, O4 (an oligodendrocytic marker) and α-tubulin in brain lysates from WT and TRPA1$^{-/-}$ mice. Data are mean ± SEM ($n = 5$ in each group). *$p < 0.05$ vs. WT mice

enhanced neurite complexity resulting from functional inhibition of the TRPA1 channel may modify the circuitry and ultimately alter behavioral outputs.

However, many studies reported that Ca^{2+} signaling is involved in neuron differentiation and also in oligodendrocyte maturation and myelination [27–29]. Two routes of Ca^{2+} surge in oligodendrocytes: Ca^{2+} influx via voltage-gated Ca^{2+} channels and discharge of intracellular Ca^{2+} reservoir induced by extracellular stimuli such as cannabidiol [27–30]. Ca^{2+}-sensing receptors readily detect in immature oligodendrocytes and transduce these Ca^{2+} surges into signals for maturation and functional regulation [27, 31]. In line with these findings, our results showed decreased amount of mature oligodendrocytes and friable structure of axonal bundles, which might be attributed to impaired myelination regulated by oligodendrocytes in TRPA1$^{-/-}$ mice. These potential roles of TRPA1-Ca^{2+} signaling in oligodendrocytes provide evidence at a molecular level for our findings and also highlight the fundamental role of the TRPA1 channel in oligodendrocytes. Very recently, Hamilton et al. reported that TRPA1-mediated Ca^{2+} influx into oligodendrocytes plays a central role in ischemia-induced myelin injury [45]. Collectively, these findings discover a link between TRPA1 channels and oligodendrocyte biology, which broadens the implications of oligodendrocyte TRPA1 activation in pathogenesis of neurological diseases.

Astrocytes are specialized glial cells, essential for preserving CNS integrity and consequently the functions of CNS [46]. In addition to providing physical support, astrocytes protect neurons against toxic compounds, regulate synaptic transmission and plasticity, and offer metabolic support to ensure optimal neuronal functions [47]. Moreover, astrocytes are involved in neuroprotective mechanisms in numerous pathological states, including Alzheimer's disease, Parkinson's disease, and ischemia [46, 47]. Ca^{2+} signaling plays a regulatory role in astrocytic development and proliferation [48–51]. The potential increase in astrocytic population may provide additional support for enhanced hippocampal functions and again highlights the differential roles of TRPA1 in multiple cellular populations within the CNS. Whether the TRPA1 channel is involved in the communication of neurons, astrocytes, and oligodendrocytes remains in need for further investigation.

In addition to the TRPA1 channel, other TRP channel isoforms have been found in the CNS and are implicated in the regulation of brain functions [52]. For instance, TRPC4 was recently reported to be expressed in the amygdala, hippocampus, auditory cortex, and auditory thalamus and implicated in regulating fear-related behaviors [53]. Moreover, TRPV1-deficient mice exhibited less anxiety, conditioned fear, and hippocampal long-term potentiation [54]. However, whether other TRP channel isoforms participate in the less anxiolytic-like behavior in TRPA1$^{-/-}$ mice warrant further investigations. Notably, TRP channels form homoetramers or heterotetramers on multiple activation stimuli, which lead to elevated intracellular Ca^{2+} level and subsequent activation of various TRP channels or Ca^{2+}-permeable channels [55, 56]. Our previous research indicated that TRPA1 channels and TRPC channels are involved in a TRPV1-mediated intracellular Ca^{2+} signaling cascade elicited by simvastatin in endothelial cells [57]. Nonetheless, the detailed molecular mechanisms underlying the crosstalk between the TRPA1 and TRPV1 channels in regulating anxiolytic behavior are still unclear. Our finding here of less anxiolytic-like behavior in genetic loss of TRPA1 channel function in mice is in

agreement with Marsch et al. [54] who found that the pharmacological inhibition of TRPA1 channel activity decreased the anxiolytic-like behavior in mice. In spite of the unique role of TRPA1 channels discovered in these studies, the information about the relationship between TRPA1 channel and other TRP isoforms is limited. Because these TRP channels are considered to be heteromeric, i.e., composed of a mixture of different isomers from the same family and they are functionally complementary under certain circumstances. Nevertheless, we could not exactly identify which TRP isoform or Ca^{2+}-permeable channel associates with the TRPA1 channel to regulate Ca^{2+} signaling-mediated brain functions in this study.

In summary, we provide experimental evidence to support the critical role of TRPA1-Ca^{2+} signaling in regulating neuron differentiation and myelination to enable complex information processing and behavioral outputs in mice. Our findings provide new information for better understanding the biological functions of the TRPA1 channel in the mouse brain. The molecular mechanisms we established may offer new insights into the therapeutic value of the TRPA1 channel for treating neurological diseases.

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Compliance with Ethical Standards

Competing Financial Interests The authors declare that they have no competing interests.

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