• Basic Research •

Elevated expression of TREK-TRAAK K_{2P} channels in the retina of adult rd1 mice

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Abstract

• AIM: To examine the expression of Twik-related K^+ channel 1 (TREK-1), Twik-related K^+ channel 2 (TREK-2), and Twik-related arachidonic acid-stimulated K^+ channel (TRAAK) in the retina of adult rd1 mice and to detect the protective roles of TREK-TRAAK two-pore-domain K^+ (K_{2P}) channels against retinal degeneration.

• METHODS: Twenty-eight-day-old C57BL/6J mice and 28-day-old rd1 mice were used in this study. Retinal protein, retinal RNA, and embedded eyeballs were prepared from these two groups of mice. Real-time quantitative polymerase chain reaction and Western blot analyses were used to assess the gene transcription and protein levels, respectively. Retinal structures were observed using hematoxylin and eosin (H&E) staining. Immunohistochemistry was utilized to observe the retinal localization of TREK-TRAAK channels. Current changes in retinal ganglion cells (RGCs) after activation of TREK-TRAAK channels were examined using a patch-clamp technique.

• RESULTS: Compared with C57BL/6J mice, rd1 mice exhibited significantly higher retinal mRNA and protein expression levels of TREK-1, TREK-2, and TRAAK channels. In both groups, immunohistochemistry showed expression of TREK-TRAAK channels in retinal layers. After addition of the TREK-TRAAK channel agonist arachidonic acid (AA), whole-cell voltage step evoked currents were significantly higher in RGCs from rd1 mice than in RGCs from control C57BL/6J mice, suggesting that TREK-TRAAK channels were opened in RGCs from rd1 mice.

• CONCLUSION: TREK-TRAAK K_{2P} channels' expression is increased in adult rd1 mice. AA induced the opening of TREK-TRAAK K_{2P} channels in adult rd1 mice and may thus counterbalance depolarization of RGCs and protect the retina from excitotoxicity. TREK-TRAAK channels may play a protective role against retinal degeneration.

• KEYWORDS: TREK-TRAAK channels; arachidonic acid; retinal ganglion cells; retinal degeneration

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INTRODUCTION

Retinitis pigmentosa (RP) is an inherited retinal degenerative disease involving the degeneration of rod photoreceptors followed by cone cells, ultimately leading to blindness[1-2]. Several interventions can either delay photoreceptor degeneration or replace lost photoreceptors[3-7]. However, the success of RP treatment largely relies on retinal ganglion cells (RGCs), whose axons transmit visual information to the central nervous system[8-9]. Thus, it is essential to understand the changes in these cells that accompany the degenerative loss of photoreceptors.

Twik-related K^+ channel 1 (TREK-1), Twik-related K^+ channel 2 (TREK-2), and Twik-related arachidonic acid-stimulated K^+ channel (TRAAK) are two-pore-domain K^+ (K_{2P}) channels that feature 4TMS/2P structures. TREK-TRAAK potassium channels can be strongly activated by arachidonic acid (AA)[10]. In addition to the neuronal functions of K_{2P} channels[11], their roles in the rd1 mouse retina remain unknown. A previous study reported that K_{2P} channels are expressed in the mouse retina[12]. However, the roles of these channels in rd1 mice, which are characterized by rapid photoreceptor degeneration, have not been clarified. The rd1 mouse is an RP model and carries a loss-of-function mutation in the rod-specific Pde6β gene that leaves a single layer of cone photoreceptors in the outer nuclear layer (ONL) by the time the mouse reaches 4wk of age[13]. The rd1 model is widely used for studying retinal degeneration[13]. We sought to examine the role of TREK-TRAAK K_{2P} channels in rd1 mice, with a particular focus on RGCs after photoreceptor degeneration. In this study, real-time quantitative polymerase chain reaction (RT-qPCR), Western blot, hematoxylin and eosin (H&E) staining, immunohistochemistry and patch-clamp recording were used to analyze TREK-

TRAAK channels in the rd1 mouse retina. Our aim was to reveal correlations between changes in TREK-TRAAK potassium channels expression and retinal degeneration.

MATERIALS AND METHODS

Ethical Approval Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethical Committee of Zhongshan Ophthalmic Center. For this study, rd1 (C3H/HeJ) mice were obtained from Nanjing University, and C57BL/6J mice were provided by Zhongshan Ophthalmic Center.

Animal Use and Welfare At postnatal day 28 (P28), rd1 and C57BL/6J mice were sacrificed to detect TREK-TRAAK expression in the retina. C57BL/6J mice of the same age were used as the controls.

RT-qPCR C57BL/6J mice and rd1 mice were euthanized at P28, and the eyes were enucleated. Total RNA was extracted from the retina with TRIzol (Takara, Japan) and converted into cDNA using PrimeScript RT Master Mix (Takara). The primer sequences were as follows: TREK-1 forward, 5'-CTGACGACCATTGGATTTGGAG-3'; TREK-1 reverse, 5'-AAGTAGCCAGCCAACGAG-3'; TREK-2 forward, 5'-TGGAATTGGAGACCACTTGGAA-3'; TREK-2 reverse, 5'-GATGCAGCCAGCCAAGATGA-3'; TRAAK forward, 5'-CCATCCCTGTGTGAGCCAGA-3'; TRAAK reverse, 5'-CTGCTATTGGTCCAGCTGGTTTC-3'; GAPDH forward, 5'-TGTGTCCGTCGTGGATCTGA-3'; and GAPDH reverse, 5'- TTGCTGTTGAAGTCGCAGGAG-3'.

Western Blotting Retina were removed from rd1 and C57BL mice at P28. Protein was extracted with RIPA lysis buffer containing protease inhibitors. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skim milk at 25℃ for 1h, and incubated overnight with primary antibodies at 4℃. Subsequently, the membranes were incubated with secondary antibodies for 1h at 25℃. Detection was then performed using a chemiluminescent HRP substrate (Millipore, USA). The primary and secondary antibodies included anti-TREK-1 (1:1000 dilution, Alomone Labs, Israel), anti-TREK-2 (1:1000 dilution, Alomone Labs), anti-TRAAK (1:1000 dilution, Alomone Labs), anti-GAPDH (1:1000 dilution, Cell Signaling Technology, USA) and HRP-conjugated IgG (1:5000 dilution, Cell Signaling Technology).

Hematoxylin and Eosin Staining H&E staining was performed as described previously[3]. Eyeballs were removed from normal C57BL/6J mice and rd1 mice at 28d and processed to create paraffin sections. The sections were then stained with H&E using standard methods. After H&E staining, the slides were dehydrated.

Immunohistochemistry Eyes were fixed in 4% paraformaldehyde, embedded in paraffin wax, and deparaffinized according to standard procedures. The sections were incubated with 0.3% H_2O_2 at room temperature for 1h and blocked with bovine serum albumin for 30min. They were then incubated with primary antibody at 4℃ overnight, and incubated with secondary antibody at room temperature for 30min. Negative control sections were incubated with 0.01 mol/L PBS instead of primary antibody. The primary antibodies used included anti-TREK-1 (1:100 dilution, Alomone Labs), anti-TREK-2 (1:100 dilution, Alomone Labs) and anti-TRAAK (1:100 dilution, Alomone Labs).

Patch-clamp Recordings The retina were carefully dissected from the pigment epithelium in artificial cerebrospinal fluid (ACSF). Whole-cell currents in response to voltage step stimuli were recorded in voltage-clamp mode with real-time P/N leak subtraction. To activate the TREK-TRAAK channels, 10 μmol/L AA was added to the perfusing ACSF and directly applied to the retina. The pipette solution contained 120 mmol/L potassium gluconate (with 120 mmol/L potassium chloride used instead to measure spontaneous synaptic current), 5 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L HEPES, 2 mmol/L ATP, and 0.5 mmol/L GTP and was adjusted to pH 7.2 with 1 mol/L KOH.

The currents evoked by voltage steps from -80 mV to +60 mV were measured under control and AA-treated conditions. For each cell, the current under the AA condition was normalized to the corresponding current under the control condition.

Statistical Analysis The data are presented as the means±SEM with $n \geq 3$ and were analyzed by GraphPad Prism (version 7.0, USA). Between-group differences were compared using Student's *t*-test.

RESULTS

Expression of TREK-TRAAK in the Retina of P28 C57BL/6J and rd1 Mice Detected by RT-qPCR and Western Blotting In comparisons between C57BL/6J and rd1 mice, the latter mice exhibited significantly greater gene transcription of TREK-1 (1.89±0.15 *vs* 3.29±0.30, n=4, P<0.01), TREK-2 (0.19±0.05 *vs* 0.40±0.03, n=3, P<0.05), and TRAAK (0.78±0.13 *vs* 1.87±0.07, n=4, P<0.001; Figure 1). At the protein level, greater quantities of these channels were also consistently observed in rd1 mice than in C57BL/6J mice (Figure 2, n=3). The relative protein levels (rd1/C57BL) for TREK-1, TREK-2, and TRAAK were 1.55±0.04 (P<0.001), 2.31±0.18 (P<0.01), and 1.64±0.15 (P<0.05), respectively. In retinal tissues, the detected levels of TREK-TRAAK K_{2P} channel proteins were broadly consistent with our mRNA results.

Retinal Layers of C57BL/6J and rd1 Mice at P28 Observed by H&E Staining H&E staining showed the retinal structures

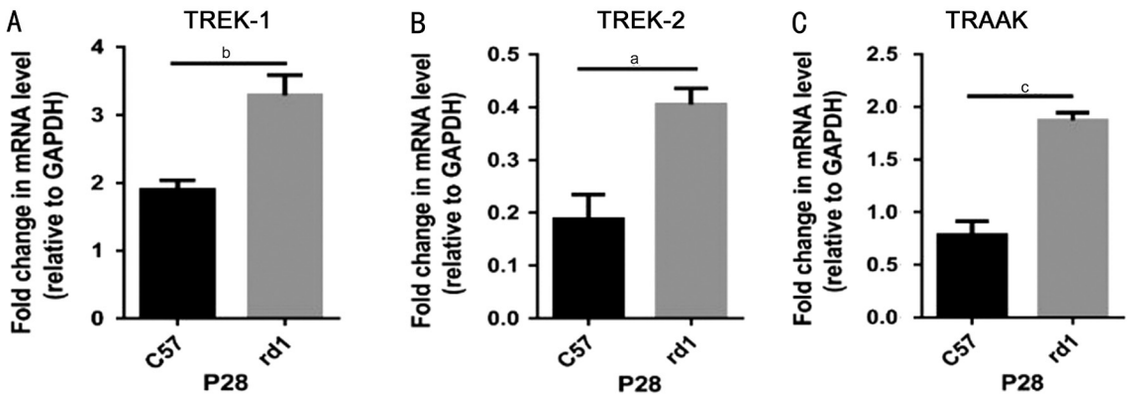

Figure 1 Relative mRNA expression of TREK-TRAAK in the retinae of P28 C57BL/6J (C57) and rd1 mice as determined by RT-qPCR. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

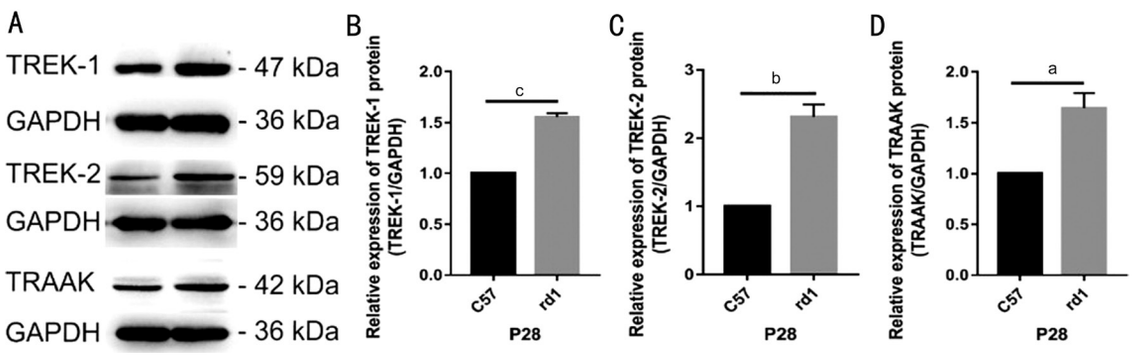

Figure 2 Western blot quantification of TREK-TRAAK protein expression in the retinae of C57BL/6J (C57) and rd1 mice at P28 $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

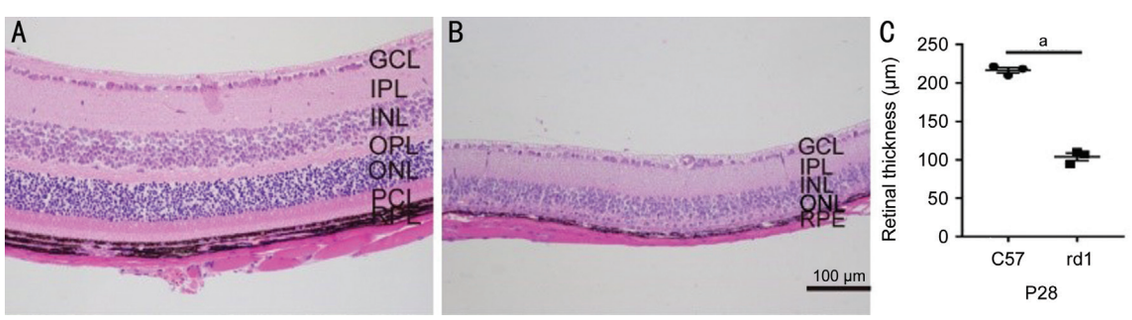

Figure 3 H&E-stained retinal layers of C57BL/6J (C57) and rd1 mice at P28 A: Retinal layers of C57 mice; B: Retinal layers of rd1 mice; C: The total thickness of the retina was lower in rd1 mice than in C57 control mice; $^{a}P<0.0001$.

of rd1 and C57BL mice (Figure 3). In C57BL mice, at P28, the observed retinal layers were the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), ONL, photoreceptor cell layer (PCL), and retinal pigment epithelium (RPE). In rd1 mice, at P28, the photoreceptors had completely degenerated, and the observed retinal layers were the GCL, IPL, INL, ONL and RPE. Compared to that in the C57BL/6J mice, the total retinal thickness was lower in rd1 mice (216.7±3.50 μm *vs* 103.8±4.95 μm, $P<0.0001$).

Expression of TREK-TRAAK in the Retina of P28 C57BL/6J and rd1 Mice Detected by Immunohistochemistry and Patch-clamp Recordings We detected strong expression of TREK-TRAAK in the retina of both rd1 and C57BL/6J mice (Figure 4). TREK-TRAAK expression was visible in the GCL, IPL, INL, OPL, ONL, PCL, and RPE in C57BL/6J mouse retina and in the GCL, IPL, INL, ONL and RPE in rd1 mouse retina. Interestingly, we found that TREK-TRAAK channels were all expressed by RGCs in both C57BL/6J and rd1 mouse retina, with no discernible differences in expression. To verify whether these TREK-TRAAK channels were functional, electrophysiological experiments were performed. First, we tested whether retinal RGCs expressed functional TREK-TRAAK channels by assessing whether AA could activate TREK-TRAAK-mediated currents in RGCs. For wild type C57BL/6J mice, after cells had been bathed in ACSF containing 10 μmol/L AA, we failed to detect a significant increase in current responses to voltage step stimuli (Figure 5).

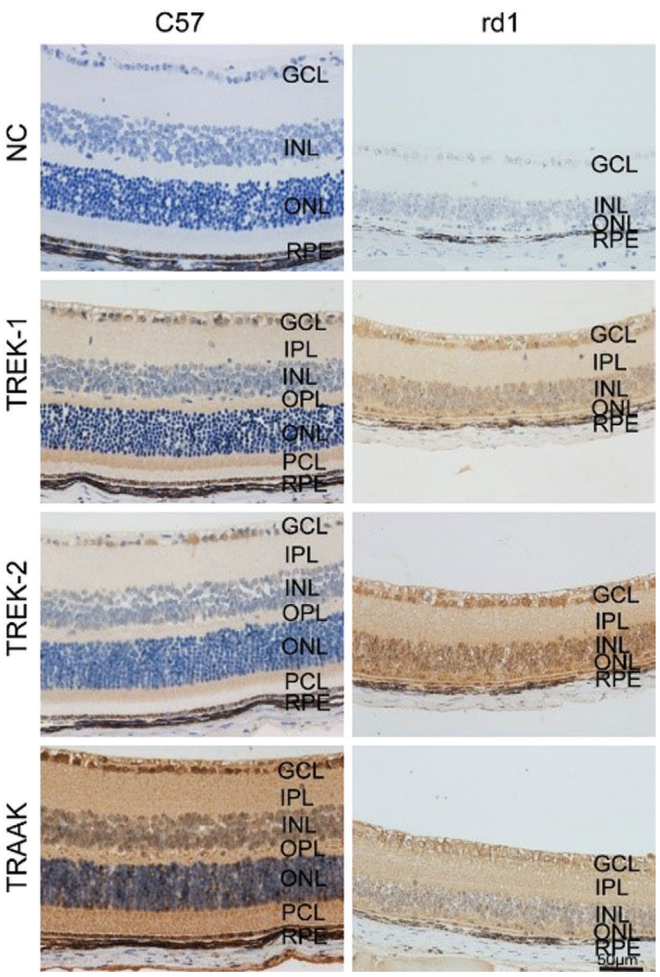

Figure 4 Expression of TREK-TRAAK channels in the retina of C57BL/6J (C57) and rd1 mice NC: Negative control.

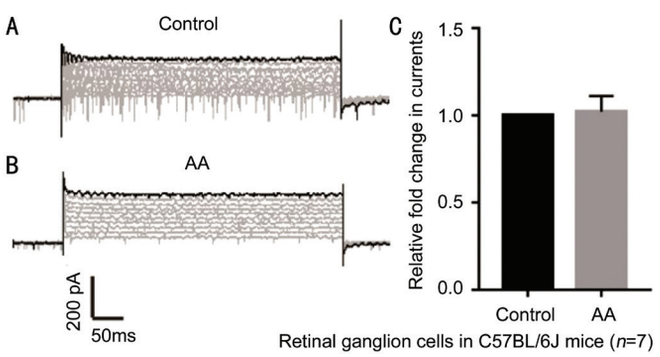

Figure 5 Current response of C57BL/6J (C57) mouse RGCs to voltage step stimuli A: Whole-cell currents under the control condition; B: Whole-cell currents under the AA-treated condition; C: Difference in current between cells under the control and AA-treated conditions. AA: Arachidonic acid. P=0.813.

This result suggested that the TREK-TRAAK expression revealed by immunostaining may not reflect actual expression patterns or that TREK-TRAAK channels expressed by RGCs are not functional. For rd1 mice, for which rod loss begins at P8 and nearly all rods have died by P21[14], we observed that AA can amplify the current response of RGCs to voltage step stimuli 48% (P<0.001; Figure 6), indicating that TREK-TRAAK was activated by AA in these cells.

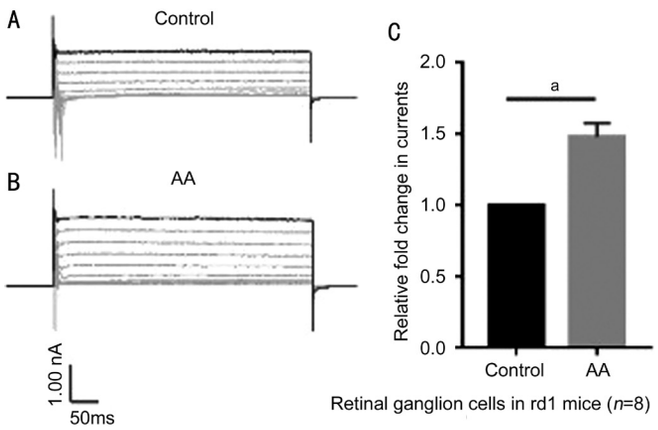

Figure 6 Current response of rd1 mouse RGCs to voltage step stimuli A: Whole-cell currents under the control condition; B: Whole-cell currents under the AA-treated condition; C: Difference in current between cells under the control and AA-treated conditions. AA: Arachidonic acid. [a]P<0.001.

DISCUSSION

Given the variety of biological effects of K_{2P} potassium channels, great interest has developed in identifying the protective roles of these channels against diseases[15-17].

Previous research studied the localization of K_{2P} channels in the adult C57BL/6J mouse retina[12], while this research assessed TREK-TRAAK ion channels in the retina of rd1 mice. Both the mRNA and protein expression levels of TREK-TRAAK were higher in rd1 mice than in C57BL/6J mice. TREK-TRAAK channels are associated with resting potential and cellular excitability[18]. In retinal degeneration, TREK-TRAAK K_{2P} channels may be meaningful targets for suppressing pathological hyperactivity in RGCs[9,12,19]. Upregulation of TREK-TRAAK channels may produce K^+ currents and hyperpolarize the resting membrane potential, leading to decreased cellular excitability in rd1 mice. Thus, in such mice, upregulation of TREK-TRAAK channels in RGCs might suppress the excitability of these cells and play a protective role. In a previous study, increased expression of TREK-1 was found to be a protective feedback mechanism under pathological conditions. TREK-1 has also previously been observed to be upregulated in the dorsal root ganglion (DRG) of rats with detrusor overactivity; this upregulation might suppress the excitability of DRG neurons and protect the bladder from overactivity[20]. In the current study, we found that TREK-TRAAK levels were upregulated in retinal tissue from rd1 mice compared to that from control mice. In RP, a family of blinding diseases that result in photoreceptor degeneration, approximately 20% of RGCs were reduced in rd mutant mice[21]. In vision, RGCs ultimately project light information to retinorecipient areas of the brain. Given the critical role of RGCs in the visual pathway, it is necessary to delay the functional decay of RGCs, a process that can be studied in rd1 mice[8].

A previous study has reported the effects of TREK-TRAAK channels in human RPE cells under oxidative stress[22], whereas our research concentrated on RGCs in retinal degeneration. The previous study did not show patch-clamp recording-based evidence of protective effects. In patch-clamp experiments, the TREK-TRAAK agonist AA was chosen to explore the functional expression of TREK-TRAAK channels in RGCs in retinal degeneration. In RGCs from rd1 mice, addition of the TREK-TRAAK channel agonist AA significantly increased whole-cell voltage step evoked currents, suggesting that TREK-TRAAK channels were opened. Thus, AA induced the opening of K2P channels in adult rd1 mice and may therefore limit RGC depolarization and protect the retina from excitotoxicity. Our results show that TREK-TRAAK channels may protect the retina from degeneration.

Our study had certain limitations. The ways in which overexpression of TREK-TRAAK channels regulates the excitability of RGCs remain unknown and warrant further investigation. Moreover, we did not detect whether upregulation of potassium ion channels increased the action potential threshold of RGCs. To better elucidate the function of TREK-TRAAK channels in retinal degeneration, future studies should include the use of specific inhibitors of these channels.

In summary, this study showed marked upregulation of TREK-TRAAK K^+ channels in the retina of rd1 mice after photoreceptor degeneration; this upregulation might suppress the excitability of RGCs and play a protective role against RP. Upregulation of TREK-TRAAK potassium channels in the retina may be a form of protective feedback in response to retinal degeneration in rd1 mice. Therefore, it is likely that increased expression of TREK-TRAAK K^+ channels plays a protective role against retinal degeneration in rd1 mice. Whether TREK-TRAAK channels can be a potential interventional target in the treatment of RP needs additional investigation.

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Conflicts of Interest: Zhang XT, None; **Xu Z**, None; **Shi KP,** None; **Guo DL,** None; **Li H,** None; **Wang L,** None; **Zhu XB,** None.

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