ORIGINAL ARTICLE

Altered functional properties of a missense variant in the TRESK K⁺ channel (*KCNK18*) associated with migraine and intellectual disability

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Abstract

Mutations in the *KCNK18* gene that encodes the TRESK K2P potassium channel have previously been linked with typical familial migraine with aura. Recently, an atypical clinical case has been reported in which a male individual carrying the p.Trp101Arg (W101R) missense mutation in the *KCNK18* gene was diagnosed with intellectual disability and migraine with brainstem aura. Here we report the functional characterization of this new missense variant. This mutation is located in a highly conserved residue close to the selectivity filter, and our results show although these mutant channels retain their K⁺ selectivity and calcineurin-dependent regulation, the variant causes an overall dramatic loss of TRESK channel function as well as an initial dominant-negative effect when co-expressed with wild-type channels in *Xenopus laevis* oocytes. The dramatic functional consequences of this mutation thereby support a potentially pathogenic role for this variant and provide further insight into the relationship between the structure and function of this ion channel.

Keywords TRESK K2P · *KCNK18* gene missense mutation · *Xenopus* oocytes · Two-electrode-voltage-clamp · Calcineurin-dependent regulation

Introduction

The *KCNK18* gene codes for the TWIK-related spinal cord K⁺ channel (TRESK). The relevant protein (K2P18.1) belongs to

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This article is part of the special issue on Channelopathies: from mutation to diseases in Pflügers Archiv—European Journal of Physiology

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the two-pore domain (K2P) family of K⁺ channels involved in the control of cellular electrical excitability [7, 8, 24]. The regulation of TRESK activity by the calcium-dependent phosphatase calcineurin [3, 4], as well as its expression in dorsal root [27] and trigeminal ganglia [2] led to a proposed role for this channel in a variety of pain pathways [26]. In particular, a frameshift mutation (F139Wfsx24) in TRESK was identified in a multigenerational pedigree where it co-segregated with typical migraine with aura [18]. Furthermore, functional analysis revealed that this mutation caused a dominant-negative loss of TRESK function and that the truncated subunit was also capable of down regulating wild-type channel function. This therefore highlighted a potential role for *KCNK18* in the pathogenesis of familial migraine with visual aura [18]. Interestingly, a very recent study [23] has shed further light on the unusual molecular mechanism by which this particular frameshift mutation works by introducing an alternative translation initiation site to produce a truncated protein that not only down regulates wild-type TRESK activity, but also that of several unrelated K2P channels in trigeminal ganglion neurons. However, the relationship of other missense variants in

TRESK to migraine and/or any other disease states remains unclear.

Recently, an atypical clinical case has been reported in which a male individual was diagnosed with intellectual disability (IQ 65) at the age of 10. It was reported that the patient presented with an acute confused state, agitation, speech impairment, disorientation, and loss of sense of place and time at the age of 12. It was also reported that approximately 1 h before this change occurred, he had experienced severe head pain and dizziness accompanied by vomiting and nausea, aggravated by movement. There was a familial history of migraine, with both his mother and elder sister having a history of migraine attacks with visual impairment and nausea, and mild intellectual disability. The formulated diagnosis for the proband was intellectual disability associated with migraine with brainstem aura. Targeted next generation sequencing and Sanger sequencing revealed that the proband harbored a heterozygous missense mutation (c.301 T > C; p.Trp101Arg; W101R) in *KCNK18* that was maternally inherited [11]. However, at the time, the potential effects of this mutation on TRESK channel function were not assessed and its pathogenic relevance not corroborated. In this study, we have investigated the functional effect of the W101R variant to probe the potential association between TRESK channel dysfunction, migraine with brainstem aura and intellectual disability. Our results demonstrate a significant functional impairment of TRESK channel activity associated with this mutation.

Materials and methods

Molecular biology

Human TRESK was subcloned between the 5′ and 3′ UTR of the *Xenopus* β-globin gene in the oocyte expression vector, pFAW. The W101R mutant was introduced by site directed mutagenesis and confirmed by automated sequencing. mRNA for wild-type and mutant channels was synthesized using the T7 mMESSAGE mMACHINE kit (Ambion, Life Technologies, Carlsbad, CA, USA) and mRNA concentrations were quantified by spectrophotometric analysis prior to injection.

Electrophysiological recordings

Unless otherwise stated, equal quantities of either wild-type or mutant mRNAs were microinjected into defolliculated *Xenopus laevis* oocytes according to standard protocols and in accordance with international standards of animal care, the Maltese Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Whole-cell currents were recorded 1 to 6 days after injection, at room temperature (20–22 °C), using the two-electrode voltage clamp method

(Axoclamp-2B, Axon DIGIDATA 1550B, Axon Instruments, Foster City, CA, Axon). Oocytes were held in a small recording chamber and were continuously perfused with control low K^+ extracellular solution, (in mM 95.4 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 HEPES pH 7.5 with NaOH). Recording electrodes were back-filled with 3 M KCl and their resistances varied from 0.3 to 1 MΩ. Currents were filtered at 100 Hz and digitized at 1 kHz for analysis. Oocytes were held at −80 mV, voltage commands were applied, and currents were recorded using Clampex 10.7 software (Axon Instruments, Foster City, CA, USA). TRESK currents were usually measured in low K^+ solution, at the end of 1 s long voltage steps from a holding potential of − 80 mV delivered in 20 mV increments from − 120 mV to + 60 mV. For the experiments with ionomycin, TRESK currents were measured in high K^+ solution (in mM 17.4 NaCl, 80 KCl, 1.8 $CaCl_2$, 5 HEPES, pH 7.5 with NaOH) at the end of 300 ms-long voltage steps from 0 mV to −100 mV. Ionomycin (free acid form), was made as a stock solution of 1 mM in DMSO and diluted in the high K^+ solution to the appropriate test concentration (0.5 μM) on the day of the experiment. Data were analyzed with Clampfit 10.7 (Axon instruments, Foster City, CA, USA), Igor and Kaleida Graph programs.

Statistics

Data are given as mean values ± standard error of the mean (SEM), where *n* represents the number of oocytes. Results were reproducible in at least 2–3 different batches of oocytes. Statistical significance was determined using a student's t test. When error bars are not shown they are smaller than the size of the symbol.

Results

Location of the missense variant in the TRESK channel

Using a previously generated homology model of human TRESK [1], the predicted location of the W101R missense variant within the TRESK channel is shown in Fig. 1c. This tryptophan (W101) is located within the first extracellular loop, upstream of the selectivity filter and within the first pore domain (P1) (Fig. 1a, b). Interestingly, this residue also resides within a very highly conserved region and is found at this position in all 15 members of the human K2P family (Fig. 1a). Furthermore, the structure reveals W101 to be in close proximity to the first pore-helix and adjacent to the selectivity filter which has been implicated in the control of K2P channel gating [22]. The highly conserved nature of this residue and its predicted location therefore suggest that it may play a critical role in the structure and/or function of the TRESK channel.
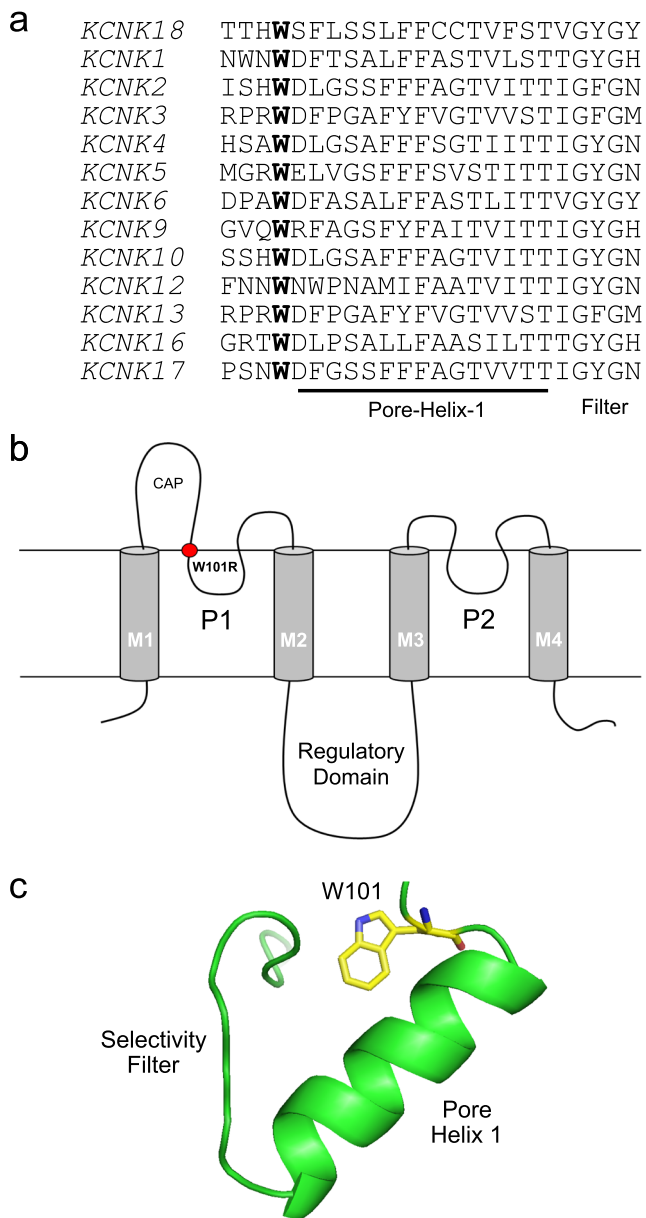
a

KCNK18	TTH**W**SFLSSLFFCCTVFSTVGYGY
KCNK1	NWN**W**DFTSALFFASTVLSTTGYGH
KCNK2	ISH**W**DLGSSFFFAGTVITTIGFGN
KCNK3	RPR**W**DFPGAFYFVGTVVSTIGFGM
KCNK4	HSA**W**DLGSAFFFSGTIITTIGYGN
KCNK5	MGR**W**ELVGSFFFSVSTITTIGYGN
KCNK6	DPA**W**DFASALFFASTLITTVGYGY
KCNK9	GVQ**W**RFAGSFYFAITVITTIGYGH
KCNK10	SSH**W**DLGSAFFFAGTVITTIGYGN
KCNK12	FNN**W**NWPNAMIFAATVITTIGYGN
KCNK13	RPR**W**DFPGAFYFVGTVVSTIGFGM
KCNK16	GRT**W**DLPSALLFAASILTTTGYGH
KCNK17	PSN**W**DFGSSFFFAGTVVTTIGYGN

Pore-Helix-1   Filter

b

c

Fig. 1 Localization of the W101 residue. **a** Alignment of human K2P channel sequences in the region of the W101R missense variant. This indicates that this highly conserved residue (in bold) is located adjacent to Pore-Helix 1 and the selectivity filter of the first pore-domain (P1). **b** Schematic topology of the human K_2P TRESK subunit showing the position of the W101R variant. **c** Homology model of the TRESK channel in this region shows the predicted location of this highly-conserved tryptophan, just before the first pore-helix

Loss of TRESK function in the W101R variant

We next examined whether the W101R variant affects the functional properties of human TRESK channels. Figure 2a shows representative whole-cell basal currents recorded from *Xenopus* oocytes injected with mRNA encoding either wild-type (WT) or mutant W101R TRESK channel. While the WT TRESK channels exhibited large outwardly-rectifying K^+ currents similar to those previously reported [17, 24], the W101R mutant expressed markedly reduced current amplitudes (Fig. 2a, b). Although the W101R currents were very small compared to WT, residual current could still be detected for this variant (Fig. 2) and when greater quantities of mRNA (5 ng) were injected, we found that larger whole-cell currents could be recorded indicating that the channel is not completely non-functional.

TRESK channels typically produce a leak K^+ current that stabilizes the resting membrane potential and we observed that the expression of WT TRESK shifted the cell resting membrane potential of the oocytes towards the K^+ equilibrium potential (E_K) (Fig. 2c). Assuming an intracellular K^+ concentration of ~ 140 mM in the oocyte, the predicted value for E_K is −107 mV. This hyperpolarizing effect was observed at both 24 and 48 h after injection of WT RNA. On the other hand, the resting membrane potential of oocytes expressing the mutant channel were only marginally more negative than uninjected oocytes at both time periods (Fig. 2c).

Given the location of this mutation close to the selectivity filter which forms an important part of the gating mechanism in K2P channels, we next examined whether the residual W101R mutant currents were still capable of responding to calcineurin activation. In WT TRESK channels expressed in *Xenopus* oocytes, an increase of intracellular Ca^{2+} produced by the calcium ionophore ionomycin, produces a calcineurin-dependent activation of TRESK [3, 4]. We therefore determined the effect of ionomycin on this TRESK variant. As expected, 0.5 μM ionomycin induced a large, robust and reversible activation of WT TRESK (Fig. 3a). However, we also found that ionomycin activated the W101R variant, although the absolute values of Ca^{2+}-activated whole-cell currents were significantly smaller than that for the WT channel (Fig. 3a–d). The response of un-injected control oocytes to ionomycin was insignificant as previously reported [28] (Fig. 3).

Interestingly, we found that extent of activation (I_{Iono}/I_{Basal} ratios) for both WT and W101R TRESK appeared similar when a lower amount of mRNA (0.13 ng) was injected (Fig. 3e). But, when larger amounts of mRNA (1 ng) were injected the I_{Iono}/I_{Basal} ratios for W101R were far more variable and possibly greater than that observed for WT TRESK (Fig. 3e). However, due to the relatively small size of the unstimulated I_{Basal} currents for the W101R mutant these values may be overestimated. Nevertheless, these results suggest that the W101R mutation reduces the basal activity of the TRESK channels, but does not markedly impair their ability to respond to calcineurin activation.

The location of the W101R mutation near the selectivity filter of the channel (Fig. 1) might also alter the K^+ selectivity of the channel. To address this, the extracellular K^+ concentration was gradually increased by replacing Na^+ with K^+ while measuring the reversal potential of the currents. We found no difference between the WT and W101R reversal
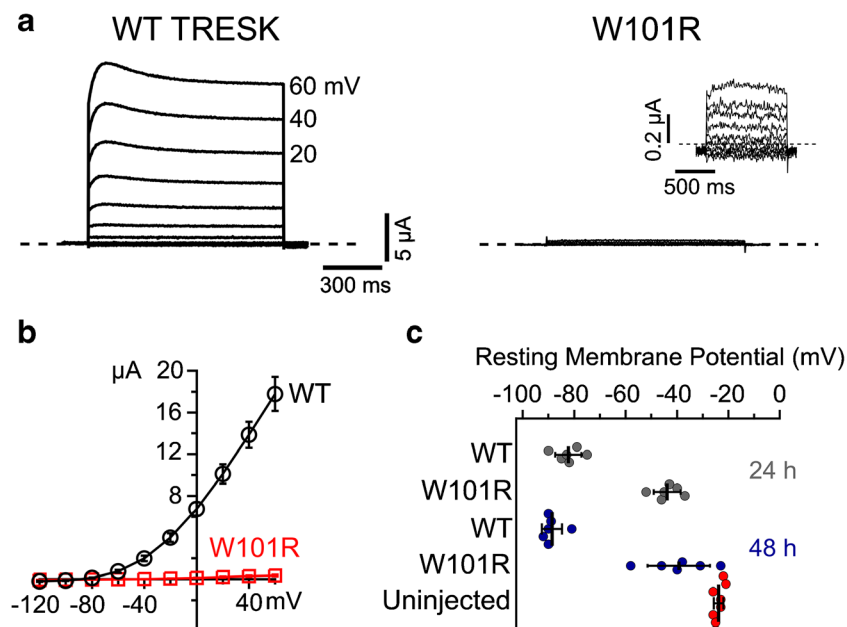
Fig. 2 W101R mutation reduces the basal current and abolishes the ability of TRESK channels to control the cell resting potential. **a** Representative families of current traces for the WT (left) and W101R (right) channels. Note that unlike the WT channel, W101R produces little current. The inset on the right shows an enlargement of the W101R currents. Recordings were done in K^+ 2 mM. Cells membrane potential was held at -80 mV. Each family of currents was evoked by 1 s long voltage steps from -120 mV to 60 mV, with 20 mV increments. The recordings were performed 48 h after the injection of 1 ng of mRNA for each channel type. **b** Average of plateau current as a function of voltage (IV relationships) calculated from experiments as in A for WT (circles) and W101R (squares). The data points are mean ± standard error

($n = 12$). The averaged current amplitudes for W101R channels are statistically different from those of WT from -40 to $+60$ mV ($p < 0.001$). **c** Resting membrane potentials from individual oocytes uninjected (red dots), injected with 1 ng of WT or W101R mRNA and recorded 24 (gray dots) or 48 (blue dots) hours after injection. Note that while the expression of the WT channels shifts the resting potential towards K^+ reversal potential, the W101R has little effect even at 48 h post-injection of the relevant mRNA. The dots in **c** represent single cell recordings. The data are mean ± standard deviation. At 24 and 48 h the statistical differences are as follow: WT vs W101R, $p < 0.001$; WT vs uninjected, $p < 0.001$; W101R vs uninjected, n.s.

potential plots, indicating the mutant channel retains normal K^+ selectivity (Fig. 4) and oocytes expressing these mutant W101R channels remained viable for many days, indicating a lack of cellular toxicity that is sometimes associated with altered ionic selectivity [25].

K2P channels assemble as dimers and the reported proband was heterozygous for the W101R variant. Previous studies that examined *loss-of-function* mutations in the TRESK channel also report a reduction in WT channel activity when mutant and WT TRESK are co-expressed in a 1:1 ratio, i.e. a situation that mimics the heterozygous state [18]. We therefore examined whether the W101R variant had a similar effect. Co-injection of WT and W101R mutant mRNAs in a 1:1 ratio reduced the whole-cell currents nearly 70% at day 1 post mRNA injection (Fig. 5a). Similar reductions were also observed when the WT:W101R mRNA ratio was increased to 1:5 and the time course of whole cell current expression was also altered (Fig. 5b). Overall, our results suggest that the W101R variant has the potential to produce a dominant negative effect by co-assembly with WT TRESK channels.

Discussion

The majority of intellectual disability in patients remain undiagnosed, and this can have considerable adverse effects on the proband and family members, such as failure to identify proper management, as well as failure to provide anticipatory support and neurological prognosis. In this study, we have functionally characterized a newly identified missense variant in the *KCNK18* gene which may provide important insights into the proposed association between this gene and the reported phenotype of the described patient. This W101R variant was identified in a male proband first diagnosed with a disability characterized by limited intellectual function associated with increasing neurological dysfunction including migraine with brainstem aura. The mutation was maternally inherited, and the mother also presented with mild intellectual disability as well as migraine attacks with visual impairment and nausea. The results presented here, therefore expand the phenotypic spectrum associated with TRESK channel dysfunction, a phenomenon not uncommon in K^+ channelopathies (e.g., Episodic Ataxia Type 1, EA1) where in addition to the core
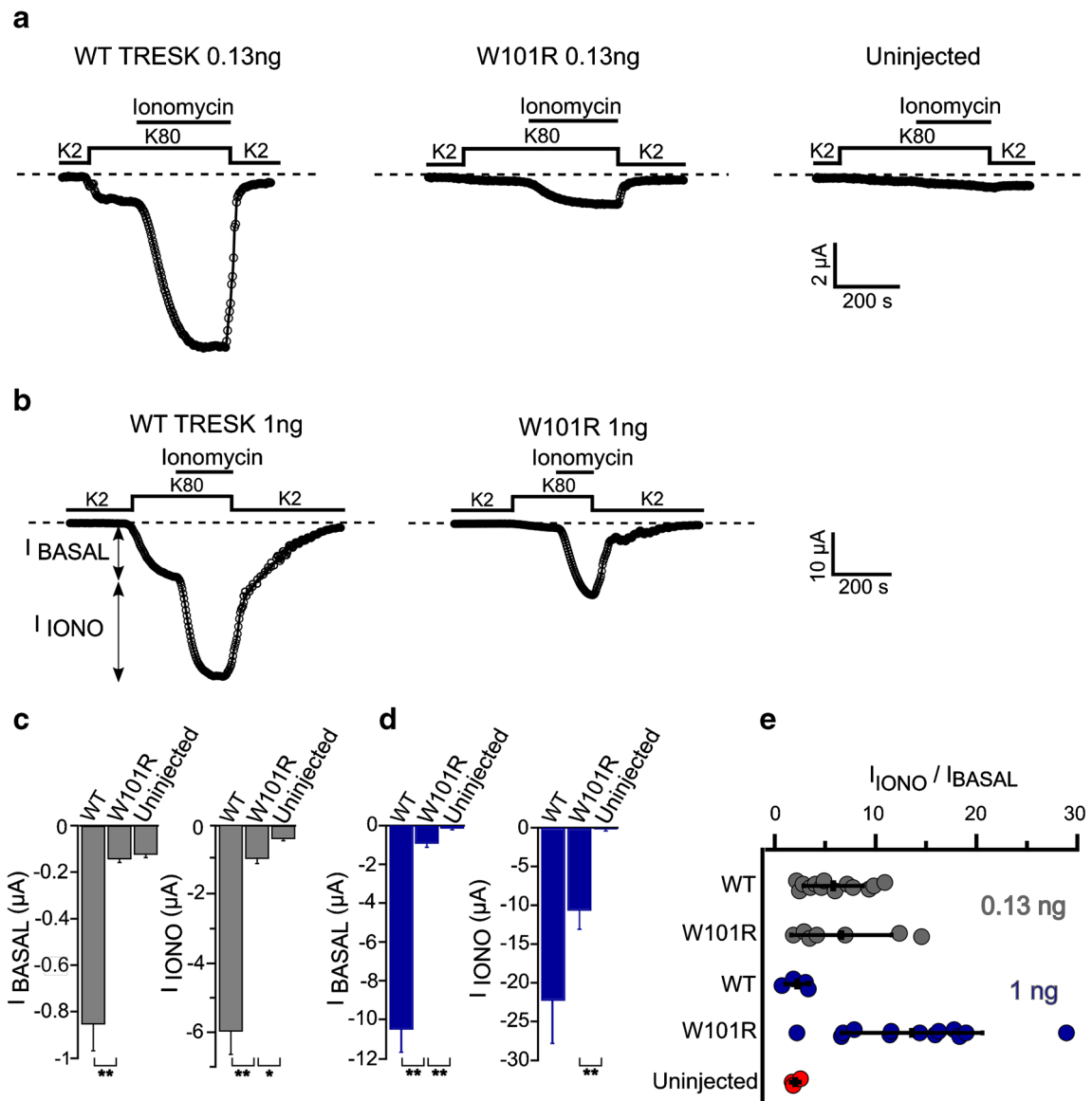
Fig. 3 Ionomycin activates both WT and W101R TRESK channels. Representative data points showing ionomycin activation of TRESK WT and W101R currents as well as endogenous currents from uninjected oocytes (**a**, **b**). Currents were evoked from oocytes injected with 0.13 or 1 ng of mRNA of the corresponding and indicated channel type and by 300 ms long voltage steps from 0 mV to -100 mV. The sampled data represent the average of 50 ms long period of the steady-state currents recorded at -100 mV. The steady-state basal current amplitude (I_{Basal}) and the ionomycin activated current amplitude (I_{Iono}) is indicated in panel **b**. Ionomycin activates both WT and W101R channels but have little effect on uninjected oocytes. Note that the current decay upon reapplication of a solution containing 2 mM K^+ in the recording chamber is due to the reduced K^+ concentration rather than ionomycin washout. Bar graphs (**c**, **d**) showing the mean steady-state basal currents (I_{Basal}) and the ionomycin activated currents (I_{Iono}) both recorded in K^+ 80 mM. The data are mean ± standard error ($*p < 0.05$; $**p < 0.01$). Normalized ionomycin activation (I_{Iono}/I_{Basal}) for the WT, W101R TRESK (injected with 0.13 ng or 1 ng) and uninjected oocytes (**e**). Each data point represents the steady state ionomycin activated current divided by the steady-state basal current (I_{Iono}/I_{Basal}) recorded in the presence of 80 mM extracellular K^+. The statistical differences are as follow: WT vs W101R (0.13 ng of mRNA) n.s.; WT vs W101R (1 ng of mRNA) $p < 0.001$ (mean ± sd)

symptoms of a disease, unexpected atypical symptoms and/or co-morbidities can be present [5, 6].

The reduction in whole cell current associated with this W101R variant is consistent with the location of this mutation adjacent to the selectivity filter where substitution of a large highly conserved aromatic side-chain with a positively charged arginine is presumably disruptive to channel structure and/or function, especially since the selectivity filter has been implicated in the control of K2P channel gating [19]. However, the mutated channels retained their K^+ selectivity indicating that the structure of the filter is not dramatically compromised. Interestingly, despite its low basal currents,
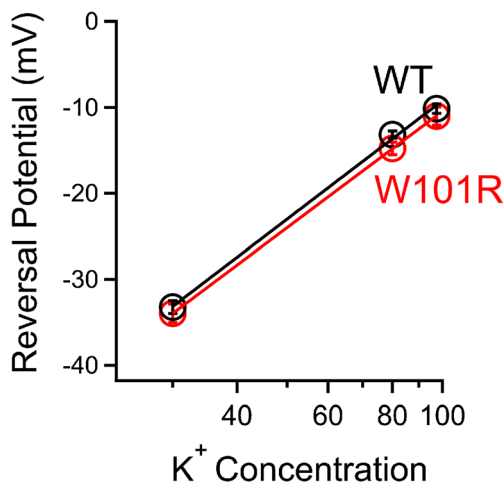
Fig. 4 The W101R mutation does not alter the ion selectivity of TRESK channel. The reversal potentials for the WT and W101R TRESK channels are plotted vs the corresponding extracellular K^+ concentration and fitted with the Nernst's equation. The slope of the fit is 49 and 48 for WT and W101R TRESK channels, respectively (each data point for WT and W101R represents the mean ± SEM and are not statistically different; $n = 8$)

the W101R mutation still retained its ability to respond to calcineurin-dependent activation, and this may be significant in differentiating variants that have a complete *loss-of-function* with those that retain not only some activity, but also the ability to respond to Ca^{2+}-dependent signaling pathways. Whether the mutation leads to an intrinsic loss of function and/ or problems with channel structure/assembly that impair its trafficking to the cell surface remains to be determined, though such questions are best be addressed in more native cell types. Nevertheless, our results clearly indicate compromised TRESK channel activity associated with this variant that reduces the channel's ability to perform its physiological role, especially in the control of the resting membrane potential.

The ability of the W101R variant to functionally interact with, and reduce the activity of, the co-expressed WT TRESK, as would be observed in the heterozygous state, could also further reduce overall TRESK channel activity through heteromeric co-assembly. Often in K^+ channel diseases the mutant subunits further reduce channel activity through 'dominant-negative' co-assembly. For tetrameric K^+ channels this can sometimes result in a > 90% suppression of channel activity [12–15]. For dimeric K2P channels, however, this effect is not predicted to be as severe because at least 25% homomeric WT channels exist. The W101R variant initially exerts a strong dominant negative effect on the WT subunit, although this effect appears to gradually reduce over time. The reasons for this are unclear and further investigation of the underlying cell biology and trafficking of this mutant channel in a more relevant line will be required to fully understand this effect. Nevertheless, our results show that the mutant subunit clearly has the potential to exert a dominant negative effect when initially coexpressed with WT subunits. The phenotypic
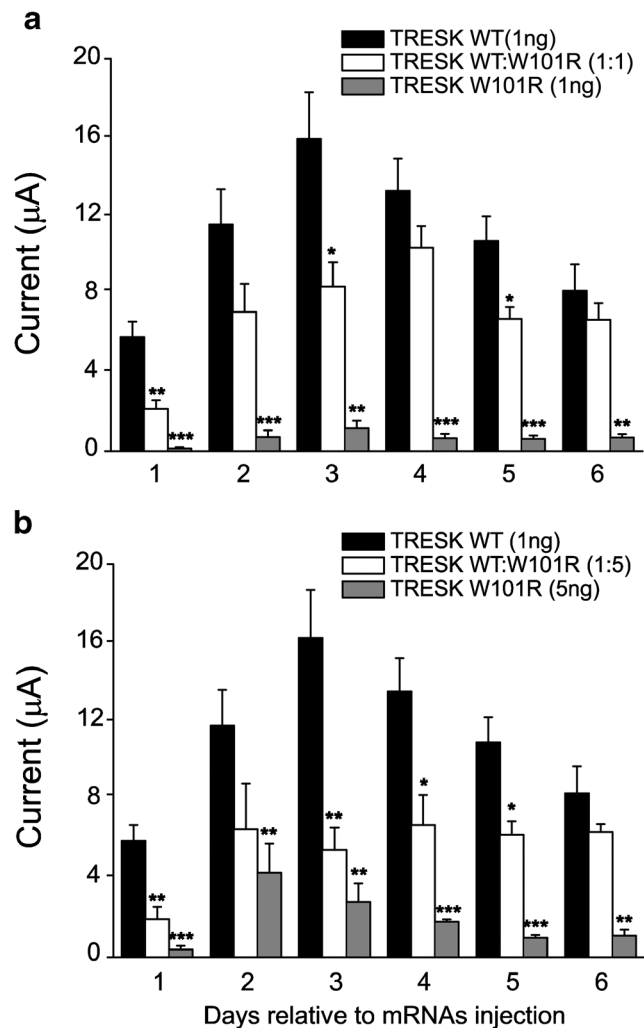

Fig. 5 Expression time course for WT TRESK, W101R and WT:W101R channels in Xenopus oocytes. Steady-state whole-cell current amplitudes were recorded at +60 mV and plotted as a function of days after the injections of the indicated mRNAs at 1:1 (**a**) and 1:5 (**b**) ratios into oocytes drawn from the same frog (data points are mean ± SEM; $n = 10$, significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs WT values)

effects of a dominant negative mutation are also predicted to be different to that of a complete gene deletion which would be unable to co-assemble. Notably, complete loss of TRESK function by means of genetic ablation of *Kcnk18* in mice causes mechanical and thermal hyperalgesia or exaggerated nocifensive behaviors during an inflammatory headache model [10, 21].

TRESK channels are expressed in a number of neuronal populations in different regions of the central and peripheral nervous systems in addition to the spinal cord and dorsal root ganglia [16, 17, 19] and can be regulated by $G\alpha q$-signaling pathways [4, 8, 20]. This strongly suggests that TRESK may contribute broadly to the maintenance and regulation of the resting membrane potential in these cell types; channel dysfunction during development of the CNS may therefore also need to be considered because K^+-dependent alterations of

neuronal membrane potential and excitability are known to cause serious neurodevelopmental disorders with intellectual disability [9]. Establishing a causative role of this TRESK channel variant in neuronal dysfunction clearly requires further investigation, nevertheless the severely perturbed functional properties associated with this variant provides important information towards this goal.

Compliance with ethical standards

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