Functional characterisation of missense variations in the Kir4.1 potassium channel (KCNJ10) associated with seizure susceptibility

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Abstract

Recent genetic linkage studies have identified an association between missense variations in the gene encoding the Kir4.1 potassium channel (KCNJ10) and seizure susceptibility phenotypes in both humans and mice. The results of this study demonstrate that these variations (T262S and R271C) do not produce any observable changes in channel function or in predicted channel structure. It is therefore unlikely that the seizure susceptibility phenotypes associated with these missense variations are caused by changes in the intrinsic functional properties of Kir4.1.

1. Article

Inwardly-rectifying (Kir) potassium channels are expressed in a wide variety of excitable and non-excitable tissues throughout the body where they play a key role in the maintenance of the resting membrane potential and thereby the control of cellular excitability. Many Kir channels also play an important role in K\(^{+}\) homeostasis by contributing to a wide range of different K\(^{+}\) transport pathways [2,18].

The Kir4.1 potassium channel was first cloned from the brain where it is expressed predominantly, but not exclusively, in glial cells including both oligodendrocytes and astrocytes [3,17,20]. In these cells, Kir4.1 is thought to play an important role in the ‘spatial buffering’ of extracellular K\(^{+}\) concentrations ([K\(^{+}\)]\textsubscript{o}) which is essential for the maintenance of neuronal activity; this prevents the accumulation of excess [K\(^{+}\)]\textsubscript{o} which would otherwise interfere with neuronal excitability [16,20]. These ‘K\(^{+}\) transport’ properties of Kir4.1 are also highlighted by its role in the inner ear where its expression in the stria vascularis is important for the maintenance of the endocochlear potential [9].

In addition to forming homomeric channels, Kir4.1 also heteromultimerises with Kir5.1 [15]. Heteromeric Kir4.1/Kir5.1 channels are abundantly expressed in the brainstem, in particular the locus coeruleus where their extreme sensitivity to changes in pH\(_{i}\) suggest that they may contribute to the chemosensitivity of this brain region [24]. Kir4.1/Kir5.1 channels are also present in the basolateral membrane of renal tubular epithelia where they appear to be involved in K\(^{+}\) recycling across these membranes [21,22].

The functional importance of Kir4.1 has been demonstrated by targeted ablation of the Kir4.1 gene (Kcnj10) in mice. Homozygous Kir4.1 knockout mice die within 2–3
weeks of birth demonstrating severe motor impairment caused by dysmyelination and axonal degeneration [10]. The severity of this phenotype suggests that complete ‘loss of function’ mutations are unlikely to be prevalent in the human population. Nevertheless, subtle changes in the functional activity of Kir4.1 could give rise to a defined, but viable, phenotype. In humans, the KCNJ10 gene is located on chromosome 1q22–23. This region has been linked to Type 2 diabetes in the Pima Indian population, although missense variations in the KCNJ10 gene have now been discounted as the cause of this linkage [7]. However, two recent genetic linkage studies have indicated a linkage between missense variations in Kir4.1 and seizure susceptibility [5,8]. The DBA/2 mouse strain exhibits a susceptibility to induced seizures compared to the C57BL/6 strain. Previous QTL mapping identified the seizure susceptibility locus (Ssz1) on the distal region of mouse chromosome 1 and further fine mapping studies suggested that a missense variation (Thr262Ser) in Kcnj10 was the likely candidate for this linkage [8]. In a second linkage study, a variation in the human KCNJ10 gene (Arg271Cys) was associated with seizure resistance in groups of patients with either focal or generalised epilepsy [5].

Subtle changes in Kir4.1 function could result in defective regulation of \([K^+]_o\), in the brain and presents an attractive mechanistic hypothesis for association between the reported genetic variations and a seizure susceptibility (or resistance) phenotype. We therefore determined to examine whether these variations cause any measurable changes in the functional properties of Kir4.1, or heteromeric Kir4.1–Kir5.1 channels. Using standard methods of site-directed mutagenesis (QuikChange II, Stratagene), we introduced these mutations (T262S, R271C) into the rat Kir4.1 gene previously cloned into the pBF expression vector. The protein sequence of the rat Kir4.1 exhibits >99% identity with both the human and murine Kir4.1 sequences and exhibits no functional differences (data not shown). The same mutations were also introduced into a tandemly linked Kir4.1–Kir5.1 sequence (in the pBF vector) [15]. This dimeric construct is a well-established method for expression of heteromeric Kir4.1–Kir5.1 channels as it controls both subunit composition and orientation without affecting the functional properties of the channels [15,16,25]. All mutations were verified by automated sequencing.

In order to accurately compare any differences in intrinsic channel properties and expression levels caused by these mutations, we chose the Xenopus oocyte expression system. Messenger RNA encoding both wild-type and mutant Kir4.1 (or heteromeric Kir4.1–Kir5.1) was then in vitro transcribed using the SP6 mMessageMachine system (Ambion). mRNA concentrations were quantified and standardised by spectrophotometric analysis, and mRNA quality assessed by electrophoresis and ethidium bromide staining. Equal quantities of either wild-type or mutant mRNAs were then microinjected into Xenopus oocytes (0.25 ng/oocyte) according to standard protocols. After approximately 18–24 h, macroscopic whole cell currents were recorded by two-electrode voltage clamp analysis [15,16,25].

Fig. 1A demonstrates that no differences were observed in the average whole-cell currents for oocytes expressing either the Kir4.1(T262S) or Kir4.1(R271C) variant compared to wild-type channels. Similarly, no difference in average current levels was observed for either variant when expressed as a heteromeric Kir4.1–Kir5.1 channel (Fig. 1B). Homomeric Kir4.1 exhibits a relatively low sensitivity to inhibition by intracellular pH (pK_a 6.0). However, coassembly with Kir5.1 enhances the intrinsic sensitivity of Kir4.1 [16,21,22]. We therefore examined the effect of these variants on the pH-sensitivity of heteromeric Kir4.1–Kir5.1. We have previously shown that inhibition of Kir4.1–Kir5.1 currents expressed in Xenopus oocytes can be achieved using a well-established K-acetate buffering system [16,21,22]. Fig. 2A shows that, like wild-type, both variants exhibit reversible inhibition when exposed to intracellular acidification. Quantitation of this inhibitory effect reveals no apparent difference in the inhibition of either variant by a reduction in intracellular pH (Fig. 2B). WT Kir4.1–Kir5.1 exhibited 91.9 ± 1.1% inhibition (n = 8), compared to 93.5 ± 0.9% (n = 8) for Kir4.1(T262S)–Kir5.1 and 92.4 ± 2.1% (n = 7) for Kir4.1(R271C)–Kir5.1.

Examination of the current traces shown in Fig. 2A also demonstrates that neither variant produces any apparent difference in either the rectification at depolarised potentials, or the highly characteristic slow activation seen at hyperpolarising potentials [15]. The slow activation at −120 mV was quantified by fitting a single exponential function. For WT Kir4.1–Kir5.1 τ = 1.45 s ± 0.07 (n = 6), compared to Kir4.1(T262S)–Kir5.1 τ = 1.51 s ± 0.03 (n = 6) and Kir4.1(R271C)–Kir5.1 τ = 1.42 s ± 0.05 (n = 6).

![Fig. 1](image_url) Average macroscopic whole-cell currents from Xenopus oocytes expressing either (A) wild-type or mutant homomeric Kir4.1, or (B) wild-type or mutant heteromeric Kir4.1–Kir5.1. Oocytes were injected with equal quantities of mRNA for WT or mutant channels (typically 0.25 ng/oocyte). Steady state currents were recorded by two-electrode voltage clamp at −100 mV in a standard bath solution containing 90 mM KCl [14,22]. Average currents represent mean ± SEM.
Heteromeric Kir4.1/Kir5.1 channels are sensitive to inhibition by extracellular barium [19]. We therefore tested the effect of 50 μM extracellular BaCl₂ on both wild-type and mutant Kir4.1–Kir5.1 channels. Fig. 3 shows that neither variant exhibits any major difference in sensitivity to extracellular barium. WT Kir4.1–Kir5.1 exhibited 92.5 ± 1.1% inhibition (n = 6), compared to 92.1 ± 1.2% (n = 6) for Kir4.1(T262S)–Kir5.1 and 93.6 ± 0.5% (n = 6) for Kir4.1(R271C)–Kir5.1.

As a final attempt to detect any functional differences caused by these missense variations, we used cell-attached single channel patch clamp recordings to examine the properties of both wild-type and mutant Kir4.1–Kir5.1 channels. Single channel currents were recorded over a range of different voltages between +40 and −120 mV. No obvious difference in either single channel current amplitude or open probability was observed. This is illustrated in Fig. 4 which shows single channel currents recorded from wild-type and mutant Kir4.1–Kir5.1 channels recorded at −100 mV. Current voltage relationships were constructed from the measured single channel amplitudes and the slope conductance determined between −60 and −120 mV; these were 42.3 ± 0.6 pS (n = 3) for wild-type Kir4.1–Kir5.1, compared to 43.8 ± 2.3 pS (n = 3) for Kir4.1(R271C)–Kir5.1 and 44.7 ± 1.1 pS (n = 3) for Kir4.1(T262S)–Kir5.1. Similarly, no change in single channel conductance values was observed for either wild-type homomeric Kir4.1, Kir4.1–T262S or Kir4.1–R271C (data not shown).

Given the apparent lack of functional differences observed for these two missense variations, we next determined whether they were likely to have any effect on channel structure. The recent determination of X-ray crystal structures for the KirBac1.1 channel [11] and the intracellular...
domains of Kir3.1 [14] combined with the high degree of sequence conservation between different family members enables 3D homology modelling studies to be performed. Using both the KirBac1.1 and Kir3.1 structures as templates, we generated a 3D homology model of a heteromeric Kir4.1/Kir5.1 channel (Fig. 5) [1]. The position of these missense variations within the structure reveals that neither of them is at an intersubunit interface and so they are unlikely to affect interactions with either a neighbouring Kir4.1 subunit in a homomeric channel, or Kir5.1 subunit in a heteromeric channel. Threonine 262 is found at the bottom end of the inner vestibule exposed to the cytoplasm and the T262S variant simply removes a methyl group from this side chain. Arginine 271 is also located at the bottom end of the channel and exposed to the cytoplasm, but in this case, the side chain is on the outside of the channel. Neither variant is likely to produce any major structural disturbance. However, to more accurately assess this, we performed a Ligplot analysis of interactions in close proximity to residues T262 and R271 [23]. Fig. 6 shows that only one interaction occurs near T262; between D260 and the backbone of T262.

However, this interaction is not altered by the T262S variation. Similar analysis of the area surrounding R271 reveals no apparent interaction of R271 with any neighbouring residues and that substitution of a cysteine into this position does not introduce any novel interactions (Fig. 6).

The principal conclusion of this study is that neither the T262S, nor the R271C variant affects the intrinsic properties of Kir4.1 or heteromeric Kir4.1–Kir5.1 channels. The variants appear to have no effect on the macroscopic current levels and basic channel properties, or on the single channel properties of Kir4.1 or Kir4.1–Kir5.1 channels. Both wild-type and mutant channels also exhibit identical block by extracellular barium which would highlight any changes within the pore of the channels. These results indicate that the intrinsic biophysical properties and basic subunit trafficking/processing properties are not affected by these missense variations. The regulation by intracellular pH also appears to be intact with both wild-type and mutant channels exhibiting similar degrees of inhibition by intracellular acidification. In support of our functional studies, the 3D molecular modelling studies also predict that no major changes in channel structure are produced by these variants.

Sequence alignments of the Kir channel family show that although the regions around T262 and R271 are highly conserved between Kir4.1 from different species, these regions are not highly conserved between different Kir channels (alignments not shown). We have also recently identified a Kir4.1 homolog from *Xenopus tropicalis* which exhibits >85% sequence identity with the mouse Kir4.1.

Fig. 4. Cell-attached patch recordings from oocytes expressing either wild-type or mutant Kir4.1–Kir5.1 channels. Currents were recorded at −100 mV using the standard cell-attached recording configuration. The patch pipette contained 120 mM KCl [15,16]. In each case, the dotted line indicates the zero current level. No differences in either the amplitude of the current or open probability are visible. Recordings are also shown on an expanded timescale (marked by asterisk) below each trace. The expanded traces show that both wild-type and mutant channels also exhibit the characteristic sub-conductance states previously reported [15,25].

Fig. 5. 3D-homology model of heteromeric Kir4.1–Kir5.1 indicating the position of residues T262 and R271. The right hand panel shows a model of the heteromeric channel. The Kir4.1 subunit is coloured pink and the Kir5.1 subunit is blue. The expanded figure in the left hand panel shows the spatial arrangement of T262 and R271 in the Kir4.1 subunit. Only one Kir4.1 subunit is expanded for clarity. The T262 and R271 residues are rendered as van der Waal’s spheres and coloured cpk. T262 faces the inner vestibule whilst R271 faces the outside of the channel. Models were generated as previously described [1] and chosen based on their low energy criteria. The best model was then energy minimised using Gromacs v3.1.4. The mean RMSD of the selected model was 1.2 Å calculated over C-alpha residues. Mutant models were generated using Insight II (www.accelrys.com). Mutant residues were superpositioned over the wild-type, thereby maintaining the spatial orientation within the model. The quality and stereochemical properties of the original and mutant models were evaluated using ProCheck [13].
sequence at the amino acid level. The *X. tropicalis* sequence contains a serine at position 262 and functional expression of this clone also reveals no major differences in channel function (data not shown). Furthermore, a recent immunohistochemical study has demonstrated that there is no difference in either the patterns, or levels of Kir4.1 expression between the brains of C57BL/6 and the seizure-sensitive DBA/2 mice which contain the T262S variant[14].

In our view, it is therefore highly unlikely that these missense variations produce changes in the intrinsic properties of Kir4.1 (or Kir4.1/Kir5.1) which underlie the reported association of these variants with seizure susceptibility. However, our study is unable to comprehensively disprove this association and alterations in Kir4.1 channel activity remain an attractive mechanistic hypothesis. It is also unclear why these mutations would only result in a neurological phenotype; Kir4.1 exhibits a widespread pattern of expression throughout the body[3,9] and if these mutations affected cell trafficking or interaction with other proteins then additional tissue phenotypes would be likely. Indeed, in Kir4.1 knockout animals, a multisystem pathology is observed[10].

It is possible that these mutations cause differences in the interaction with other proteins; Kir4.1 contains a PDZ-binding motif at the distal C-terminus which is known to mediate interaction with a variety of PDZ-proteins including cell scaffolding proteins[6,12]. However, neither mutation affects this PDZ-binding motif. It is also possible that other expression systems may reveal differences in cell processing/trafficking of these mutant channels. However, despite these qualifications, we suggest that future investigations of the association between these variants and seizure susceptibility phenotypes should either examine how these variants could produce subtle changes in their interaction with (as yet unknown) cell-specific trafficking or regulatory proteins, or perhaps, more realistically, focus on other candidate genes within this associated chromosomal region.

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**References**


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*Fig. 6. Ligplot analysis of interactions affected by the T262S and R271C variants. Interactions involving (A) T262 and (C) R271 in the wild type channel. Only one interaction between D260 and the backbone of T262, indicated by the dotted green line, is visible in the wild-type channel. R271 does not contribute to any visible interactions. In panels (B) and (D), these residues were mutated and similar interactions plotted to identify any changes. No observable difference in interactions between the wild type and the mutated residues is visible.*
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