Cryo-EM structure of the human Kv3.1 channel reveals gating control by the cytoplasmic T1 domain

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Abstract

Kv3 channels have distinctive gating kinetics tailored for rapid repolarization in fast-spiking neurons. Malfunction of this process due to genetic variants in the *KCNC1* gene causes severe epileptic disorders, yet the structural determinants for the unusual gating properties remain elusive. Here, we present cryo-EM structures of the human Kv3.1a channel, revealing a unique arrangement of the cytoplasmic T1 domain which facilitates interactions with C-terminal axonal targeting motif and key components of the gating machinery. Additional interactions between S1/S2 linker and turret domain strengthen the VSD/PD interface. Supported by MD simulations and electrophysiological and mutational analyses, we identify close communication between $\alpha 6$ helix of T1 domain, S4/S5 linker and S6T helix as responsible for the ultra-fast activation/deactivation and open state stabilisation that are unique to Kv3 channels. These findings provide fundamentally new insights into gating control and disease mechanisms and guide strategies for the design of pharmaceutical drugs targeting Kv3 channels.

Introduction

High frequency firing of action potentials in the central nervous systems is essential for a multitude of physiological functions, ranging from early development and axonal path finding in neuronal progenitors, learning and memory in hippocampal neurons, sensory processing in auditory nuclei and specialized cells of the retina, to circadian rhythms in the suprachiasmatic nucleus of the hypothalamus. The precise interplay of ligand-gated ion channels and a diversity of voltage-gated sodium and potassium channels with different activation thresholds is required to tailor the frequency of action potential firing to each of these physiological processes. Voltage-gated K channels of the Kv3 family represent an important subfamily among the 70 different Kv channel genes in the human genome, with specialized biophysical properties to sustain high-frequency firing of action potentials [1, 2]. The combination of ultra-fast activation and deactivation kinetics and high activation potential of these channels are key for their ability to repolarize the membrane and terminate the action potential. Even slight changes in the biophysical properties of Kv3 channels can affect their ability to sustain high-frequency firing in different brain regions and can cause severe neurological disorders, including ataxias, epilepsies and schizophrenia [3-9].

Like other pore-forming Kv channels, Kv3 members are comprised of an N-terminal cytoplasmic T1 domain, which is involved but not required for Zn^{2+} -mediated tetramerization [10, 11], followed by the voltage-sensing domain with transmembrane helices S1-S4 which is connected via a helical linker (S4/S5L) to the pore forming domain of the channel. The latter contains the selectivity filter for potassium ions at the end of the pore helix (PH) and two transmembrane helices S5 and S6 with the lower gate of the channel located below a conserved PVP hinge region in S6.

Due to the overall similarity in architecture with other Kv channels, it has been unclear which structural determinants are responsible for the unique biophysical properties of Kv3 channels. Nevertheless, a number of functional studies have addressed this question by analysing chimeric constructs with

channels from different Kv subfamilies or other voltage dependent proteins. For example, a study by Labro *et al.* [12] showed that Kv3.1 channels exhibit ultrafast voltage-sensor relaxation, resulting in resurgent K⁺ currents during repolarization and this property was linked to the S3-S4 loop of the voltage sensor domain. Another study showed that the fast activation and deactivation kinetics could be transplanted onto the voltage-sensing phosphatase Ci-VSP if the S3/S4 portion of the enzyme was replaced by the respective Kv3.1 region [13]. This is not surprising considering that the length and composition of the S3/S4 linker has been identified as major factor shaping characteristics of the VSD in the Shaker K⁺ channel [14]. However, some of the largest sequence-differences to other members of the Kv1-4 families (Suppl. Figure S1) are within the cytoplasmic T1 domain, the C-terminal extension beyond S6, and the so-called turret domain, which is located between S5 and the PH helix. In the longer splice variant of Kv3.1 channels (Kv3.1b), an axonal targeting motif (ATM), which is located C-terminal of S6, was shown to interact with ankyrin G and promote enrichment of the channel in the axon [15]. The shorter Kv3.1a variant however is retained in somatodendritic membranes, resulting in lower frequency firing [16]. It was demonstrated that this is caused by a masking of the ATM motif in Kv3.1a through interactions with the N-terminal T1 domain [15]. Interactions between N- and C-terminal domains have been suggested for other Kv channels [17], but this region is unresolved in the currently available T1-containing Kv1 channel structures [18-21]. In contrast to the Kv1 subfamily where characterization of T1-deleted Shaker variants has ruled out an essential role of the T1 domain for normal gating and inactivation [22], multiple studies for other Kv subfamilies have provided experimental evidence suggesting that the cytoplasmic domains may play a major role in controlling channel gating [23-28], but structural data corroborating this hypothesis is currently missing.

Here, we present cryo-EM structures of the human Kv3.1a channel in an activated state, providing the first mechanistic insights into the modulation of Kv3 channels by the cytoplasmic T1 domain. The structure reveals multiple interactions of the most C-terminally located helix in T1 ($\alpha 6$) with the S4/S5 linker, as well as with residues within the S6 extension (S6T) containing the axonal targeting motif. The structure also shows the architecture of the turret domain in Kv3.1, featuring a unique interface with the extracellular S1/S2 linker of the VSD. Together with functional studies of aptly chosen mutants and molecular dynamics simulations, these findings illuminate the electromechanical coupling mechanism in Kv3.1 channels and provide a molecular explanation for the characteristic gating properties of Kv3 channels.

Results

Overall structure and cytoplasmic T1/ATM interaction

To elucidate the structural determinants underlying the biophysical properties of Kv3 channels, we purified the shorter α -isoform of Kv3.1 from mammalian cells and determined cryo-EM structures under different sample conditions (apo, Zn²⁺-containing vs. Zn²⁺-free, see Suppl. Table 1). We achieved 3D reconstructions between 3.1-3.5 Å for the different conditions (Suppl. Figure S2, S3 and S14), which show only minor differences in the overall structure, and no clear densities for additional Zn²⁺ sites other than

the known sites in the T1 domain [10] (see supplementary discussion). In the following structural analysis, we thus focus mostly on observations from the best resolved structure (Figure 1 A) which was obtained in digitonin and in the presence of 400 μM Zn^{2+} (representative densities for different regions of the channel are shown in Suppl. Figure S4). The locations of Kv3.1a residues affected by disease mutations in the *KCNC1* gene are illustrated in Figure EV1 D and in more detail in Suppl. Figure S5.

Kv3.1a exhibits a typical domain-swapped Kv channel architecture which brings the VSD from one subunit of the channel close to the S5/S6 helices of the pore domain from an adjacent subunit (Figure 1 A, Movie M 1). However, a striking difference to all previously published T1-containing full-length Kv structures [18, 19, 29] is the relative orientation between TMD and the T1 domain. When superposing the TM region of Kv3.1a with the respective region in the structure of the Kv1.2-Kv2.1 chimera (Figure 1 B), the T1 domain of Kv3.1a is rotated clockwise by approximately 48° with respect to the T1 domain of Kv1.2-2.1 (Figure 1 C). Superposition of the isolated T1 domains highlights the different orientation of the $\alpha 6$ helices of the two channels (Figure 1 D), whereas the rest of the domain exhibits a similar fold in both structures (RMSD=0.7). Small conformational differences to Kv1.2-2.1 and Kv1.3 are also present in $\alpha 3$ of T1, and the different conformation seen in Kv3.1a would result in a clash with the β -subunits present in the Kv1.2-2.1 and Kv1.3 complex structures (Suppl. Figure S6 A). As a result of these differences, the $\alpha 6$ helix in the context of the full Kv3.1a channel is rotated by an almost 90° angle compared to Kv1.2-2.1, bringing it in much closer proximity to the S4/S5 linker (Figure 1 B).

Due to this rotation of T1 in Kv3.1a, the extension beyond S6 that contains the ATM motif is located near $\alpha 1$, $\alpha 4$ and the $\alpha 5/\alpha 6$ linker of T1 from a neighbouring subunit (Figure 1 A, E-F, Suppl. Figure S7). A cluster of acidic charged residues (D97, E98, D100, E102) in the $\alpha 5/\alpha 6$ linker of T1 creates a negatively charged patch on T1 which electrostatically attracts the positive charges from a series of five lysines in the ATM (K454-K458). K458 forms a salt-bridge with D100 and further C-terminal the ATM/T1 interaction is stabilized by a polar contact between R462 and S24 in $\alpha 1$ (Figure 1 F). In a subclass of particles, we were able to tentatively trace more of the C-terminus for two diagonally related chains of the tetramer (Suppl. Figure S4 H, I), showing that this extension is stabilized by a series of intrasubunit interactions with residues in $\alpha 2$ and $\alpha 3$ of the T1 domain (Suppl. Figure S7). Despite the overall close prediction of the Kv3.1a structure by AlphaFold2 [30], the confidence of the model is low for the ATM region, and the interactions with T1 seen in our structure were not faithfully modelled (Suppl. Figure S8 A, B). This region is also unresolved by the EM maps of Kv3.1a reported in a recent preprint [31].

A frameshifting variant in the *KCNC1* gene associated with progressive myoclonic epilepsy 7 (EPM7, ClinVar ID: 692088) causes premature termination at K457 and therefore deletes many of the residues involved in this T1/ C-terminal interaction. The phenotype of this variant suggests that either the full extension is required for proper axonal targeting or to maintain normal gating properties of Kv3.1 channels, but the mutation has not yet been functionally characterized. Moreover, the interface between ATM and T1 also harbors a range of single point mutations identified in patients with EPM7; with S44N, H45Y and F46L in $\alpha 3$ helix of T1 domain and G467R, N470S and S474Y in ATM reported to date (Suppl.

Figure S5). Therefore, our structure warrants exploration of the functional significance of ATM beyond intracellular targeting.

Of note, T1/C-terminal interactions have been suggested for other Kv channels, including Kv2.1 [32] and Kv4.1 [17] and a superposition with structures of the isolated T1 domains for Kv4.2 and Kv4.3 show that the $\alpha 6$ conformation in these domains is more similar to Kv3.1 than the $\alpha 6$ conformation in the full-length structures from Kv family members (Suppl. Figure S6 B-D). Furthermore, the T1 domains of Kv4.2 and Kv4.3 share similar electrostatic properties with Kv3.1 for the central vestibule flanked by $\alpha 4$ helices in T1 (Suppl. Figures S9, S10). Whilst in Kv3/Kv4 T1 structures, acidic residues in the $\alpha 4/\alpha 5$ loop contribute to the overall negative surface potential (Suppl. Figure S10 A, D-E), the same region in Kv1 structures is dominated by positive surface charges (Suppl. Figure S10 B, C). In contrast to T1 domains from Kv1 and Kv4 channels which have characteristic salt bridges to stabilize $\alpha 4$ helices from neighboring subunits against each other (Suppl. Figure S10 B-E), this interaction is missing in Kv3 channels due to substitutions by uncharged residues (Suppl. Figure S10 A inset).

MD simulations of K^+ ions accessing the TM pore region from the cytoplasm via T1 fenestrations show that a ring of D81 residues (Figure EV1 C) plays a critical role for maintaining an abundance of K^+ ions in the lower vestibule (Suppl. Figure S11 D). Introducing a charge-inverting mutation (D81K) in the MD simulations causes a significant drop of the K^+ occupancy at the interstitial fenestrations (Suppl. Figure S11 E). We suspect that the negative electrostatic potential arising from the presence of D81 in the Kv3.1 vestibule may play a role in preventing local K^+ depletion after repetitive firing of fast-spiking action potentials [33] and could help to maintain the large conductance characteristic for Kv3 channels [34].

Voltage sensor domain conformation and pore arrangement in Kv3.1a

Consistent with the high activation potential of Kv3.1, the S4 helix of the VSD is in a less activated conformation compared to Kv1.2/Kv2.1, with only three of the six voltage-sensing charges located above the conserved F256 (in S2), which is part the hydrophobic charge transfer center (Figure 2A, B). In Kv3.1, the positively charged side chain R4 (R320) is engaged in a cation- π interaction with the aromatic ring of F256 and also forms an electrostatic interaction with D282 (located in S2, see Figure EV2 D). This gating charge residue is affected by mutation R320H, a dominant negative loss-of-function variant linked to EPM7 [4, 5]. The corresponding R4 side chain (R303) in Kv1.2/2.1 is located above the conserved Phe and interacts with E226 (in S1, Figure EV2 B). A similar interaction is observed for Kv3.1a between residues E249 and R3 (R317). As a result, the S4 sensor in Kv3.1 is shifted downwards by one 3_{10} helical turn compared to the more activated Kv1.2-2.1 channel structure (Figure EV2 C, F). Another notable difference is that a second glutamate (E213 in S2) in Kv3.1 neutralizes the positive gating charges in S4, substituting T184 in the Kv1.2/Kv2.1 structure (Figure EV2 A, B). Interestingly, both S2/S3 and S3/S4 linkers in Kv3.1 are shorter and exhibit a more structured and compact conformation compared to Kv1.2/2.1, in particular S2/S3 which is mostly helical, similar to the conformation in the Na_vAb channel [35]. It is conceivable that the more compact linkers could act like springs on both ends of S4 and hence speed up movement in either direction, hence accelerating the kinetics of VSD activation and

deactivation, respectively. These areas also show substantial sequence differences and therefore explain how their transplantation on Kv channels of other families can confer gating properties similar to Kv3.1a. Another interesting difference in our Kv3.1a structures is that the backbone carbonyl oxygen of gating charge R6 (R326) interacts with the positively charged side chain of R332 in the S4/S5 linker. This interaction is absent in Kv1.2-2.1 because the respective S4/S5 linker sequence lacks an arginine in this position and the nearby K308 side chain is too far away for a similar interaction (Figure 2 A-B, D). In the Kv4 subfamily, however, an analogous stabilizing interaction could exist because the R332 residue in the S4/S5 linker is conserved (Figure EV 3 D).

Despite the less activated S4 position in the VSDs, the lower S6 gate of Kv3.1 is captured in a dilated conformation, exhibiting a pore radius near the PVP hinge region large enough to accommodate a hydrated K^+ ion (Figure 2 C, E, F). MD simulations confirm that the pore is found in a stable conductive state, displaying a sustained hydration of the inner gate over MD simulation timescales (Suppl. Figure S11 A-C). Our maps also show densities in the selectivity filter which likely correspond to K^+ ions from the purification buffer (Suppl. Figure S10 A). Opening of the lower S6 gate however is unexpected, because of the high activation potential of Kv3.1a ($V_{0.5} = +10$ mV, see Suppl. Figure S12), and consequently only a minor fraction of the channels (<20%) should be activated at 0 mV. Since we observe distinct densities below the selectivity filter, it is possible that the lower gate of Kv3.1a is propped open by an unidentified molecule trapped within the pore (See Suppl. Figure S10 A and supplementary discussion). Similar densities are also present in the open pore of the Kv1.2/2.1 structure determined by cryo-EM [19].

Interestingly, two disease mutations are located between SF and the PVP hinge (A421V [6] and V425M [9], see Figure EV1 D). V425M is a gain-of-function mutation with leftward-shifted activation potential (by -30 mV) and higher sensitivity towards inhibition by the open pore blocker fluoxetine [9]. Since the side chain of V425 is one of the key pore lining residues (Figure 2 E), a replacement by the larger methionine could introduce steric clashes when the lower S6 gate is closed, hence reducing the threshold for opening and explaining the gain-of-function phenotype of the mutation, as well as the higher potency of fluoxetine in blocking the mutant channels.

S4/S5 linker and lower S6 gate interact with $\alpha 6$ of the T1 domain

The unique T1 arrangement in Kv3.1a brings $\alpha 6$ in close proximity to two areas of the TM domain with known importance for gating and electromechanical coupling, i.e., the C-terminal end of S6 (S6T) and the S4/S5 linker. In contrast to Kv1.2/2.1 where $\alpha 6$ of the T1 domain is located distant from these regions (Figure 3 A), the $\alpha 6$ helices in Kv3.1a create a ring-like structure which runs right below the cuff formed by the S4/S5 linker helices (Figure 3 B, D). In addition to the Zinc finger interactions of C104/C105 in $\alpha 6$, two acidic residues (E116 and D120) at the C-terminal end of $\alpha 6$ are forming salt bridges to K449 in S6T (Figure 3 C, E). The side chains of W106 and M107 located at the other end of $\alpha 6$ are engaged in hydrophobic interactions with A448 in S6T of a neighboring subunit (Figure 3 E). Due to this arrangement, the interactions mediated by either end of the four $\alpha 6$ helices in T1 may stabilize the lower S6 gate in the open state.

Sequence alignments with other Kv channels reveal that three methionines in S6 are unique and conserved within the Kv3 subfamily (M430, M441, M447 in Kv3.1a, see Figure EV3 A-D). M430 near the PVP hinge has been identified as a major factor for pore stability in Kv3.1a, because mutation to leucine caused flicker of the currents [36]. Our structure may provide a molecular explanation for this phenotype, because the partial charge on the side chain sulfur in M430 forms a sulfo-aromatic interaction with the edge of the aromatic ring from F345 in S5 (Figure EV3 C). This interaction exists only in Kv3 channels, because both residues are leucines in other Kv channels and it may strengthen the interlock between S5, S6 and S4/S5 linker. Whilst M430 is a known factor for open pore stability, the roles of methionines M441 and M447 in S6T is less clear and merits further investigation in future studies, in particular because M441L is a known variant associated with EPM7 but currently of unknown functional significance (ClinVar ID 848761).

Notably, the three-way interaction between $\alpha 6$, S6T and S4/S5 linker is only possible due to the close proximity of these helices, which in turn is facilitated by an abundance of alanine residues within this region: A446 and A448 in S6T, A115 and A118 in $\alpha 6$, and A340 in the S4/S5 linker (Figure 3 E and Figure EV3). The compact side chains in these hydrophobic residues allow a tight packing of $\alpha 6$ against Y442 in S6T. The two alanines in $\alpha 6$ and S6T are unique in Kv3 channels (Figure EV3 D and alignment in Suppl. Figure 1). Interestingly, the C-terminal end of $\alpha 6$ is also rich in acidic residues creating a negatively charged surface area which interacts with the polar side of the amphipathic S4/S5 linker. The latter carries excess positive charges from the side chains of R332 and R339 and H336 (Figure 3 C and Suppl. Figure S9 A). Furthermore, exposed backbone carbonyls of L119 and D120 interact with the imidazole ring of H336 in the centre of the S4/S5 linker (Figure 3 C). The exposure of these backbone carbonyls is a result of the partial unwinding at the C-terminal end of $\alpha 6$. In our maps, the density beyond D120 is weak, indicating structural flexibility. The recent AlphaFold2 model predicts a slightly longer helical region, albeit with low confidence (Suppl. Fig S8 B, D). This matches well with our MD simulations showing that this region can be helical as well as without secondary structure, with the latter enabling backbone interactions with S4/S5L and S6T.

Role of inter-domain interactions for open state stabilization and fast activation/deactivation

To test the functional significance of these interdomain interactions for open pore stability and gating kinetics, we characterized a series of alanine substitutions by TEVC in *Xenopus* oocytes (Suppl. Figure S12 and Figure 4). In terms of voltage dependence, the most profound effects are caused by replacements of R332 in the S4/S5 linker and K449 in S6T. The clear shift of the respective G/V curves of these mutants to more positive potentials (Figure 4 A, B) suggests that the interaction between R332 and the backbone of S4 voltage sensor R6 (contact A in Figure EV4 B), as well as the S6T/ $\alpha 6$ contact mediated by K449 (contact B in Figure EV4 B) could be important for efficient VSD coupling or open-pore stability of Kv3.1a. To investigate the significance of the latter contact further, we analyzed MD simulations at depolarizing potentials and monitored changes in the Z-position of gating charges R1-R4 relative to the CTC (Figure EV4 A). Upon a 4 μ s application of depolarizing voltages, we observe upward movement of R1-R4 in two out of four subunits of Kv3.1a (Figure EV4 A and Movie M2). The same

subunits also exhibited a significantly higher propensity of the E116/K449 salt bridge (Figure EV4 C), indicating that the contact gets stronger with S4 activation. This is in agreement with the functional data suggesting that the interaction stabilizes Kv3.1a channels in the open state.

Furthermore, we examined mutations in the respective $\alpha 6$ residues involved in contact B and observed small changes in $V_{0.5}$ for E116A, D120A and the E116A/E120A double mutant (Figure 4 C, D). Whilst the individual mutations E116A and D120A have only mild effects (Suppl. Figure S12 B, C), the E116A/E120A double mutant shows a substantial change in the activation and deactivation kinetics across the whole voltage range (Figure 4 E), indicating that the combined charge-neutralization at these $\alpha 6$ residues affects the rapid gating kinetics of Kv3.1a. Notably, we observed a similar “sluggish” gating phenotype for mutant H336A located at the centre of the S4/S5 linker (Figure 4 F). The mutation is situated at a mechanistically important position and interrupts polar interactions with several backbone carbonyl oxygens in the $\alpha 6$ helix (Figure 3 C). In the AlphaFold2 model, H336 interacts with the side chain and the backbone carbonyl of S121 at the C-terminal end of the slightly elongated $\alpha 6$ helix predicted by the algorithm (Suppl. Figure 8 A, D). This might be a consequence of the different S4/S5 linker conformation in the AlphaFold2 model, which captures S4 in a more activated state (Suppl. Figure S8 C). Hence, H336 may change its interaction partners in $\alpha 6$ to accommodate structural rearrangements in S4 and the attached S4/S5 linker which occur in response to a change in the membrane potential.

Our alanine scanning approach also identified a charge-neutralizing mutant in $\alpha 6$ (D114A) which causes a substantial hyperpolarizing shift in the activation potential (Figure 4 A, B). D114 is located within a cluster of other negatively charged residues in $\alpha 6$, but unlike E117, E116 and D120, the negative side chain is not neutralized by electrostatic interactions to nearby arginines or lysines (Figure 3 C and Figure EV3 B). Instead, D114 is sandwiched between M107 and M441 in S6T and introduces further negative repulsion with the negative partial charges on the methionine side chains. The D114A phenotype is thus consistent with a relative stabilization of the open state due to reduced electrostatic repulsion with adjacent residues.

Turret and S1/S2 linker create a second VSD/PD interface for efficient electromechanical coupling

A recently described disease mutation of Kv3.1 [6] is located at the C-terminal end of S1, close to the S1-S2 loop C208Y (Figure 5 D and Figure EV 1D). This cysteine is conserved in other Kv channels and was suggested to be part of a second, co-evolved interface between VSD and pore domain, with a major role in electromechanical coupling [37]. In Kv3.1, C208 has been identified as a key residue involved in inhibition by extracellular Zn^{2+} [38]. In our EM structure, C208 is indeed located at interacting distance to a conserved proline located of the pore helix (Figure 5 A, D). Kv3 channels have a conserved histidine (H212 in Kv3.1a) and glutamate (E213 in Kv3.1a) in this region, which are contributing additional polar interactions to strengthen the VSD-PD interface further (Figure 5D). Interestingly, mutant H212A results in a dramatically increased activation constant and reduced current amplitude [38], thus hinting at a prominent role of this interface for ultra-fast activation of Kv3 channels. Another set of Kv3-specific histidines is located nearby, in the S5-PH linker region (H381 and H383, see Figure 5 B). Again, mutation

of these histidines to alanines was shown to affect activation kinetics and the H381A/H383A double mutant had the same dramatic phenotype as the H212A mutant [38]. The side chains of H381 and H383 are interacting with Q372 and Q409, respectively, and H383 also forms a H-bond with Y407, which in turn interacts with Y403 near the K selectivity filter (Figure 5 A, B).

This region is also known as the turret domain of Kv channels and only members of the Kv3 subfamily contain an extra stretch of amino acids which is absent in other Kv families (Figure 5 C and Figure EV5 A). A serine uniquely present in Kv3.1 (S377) and Kv3.2 interacts with E238 in the S1/S2 β -hairpin (Figure 5 A). It must be noted that the S1/S2 loop is flexible and therefore resolved at lower resolution than most of the channel (see local resolution maps in Suppl. 2-3). 3D variability analysis was utilized to identify a small subset of particles with a 20° rotated S1/S2 linker region compared to the rest of the particles. In this alternative conformation, S377 forms a hydrogen bond with the backbone carbonyl of R237 (Figure EV5 B). None of the other published Kv channel structures show the same S1/S2 β -hairpin structure, indicating that the more structured conformation of this loop in Kv3 channels could be of functional importance for the turret interactions and may explain why this is a hot spot for a range of disease variants (Suppl. Figure S5).

Lipid interactions and potential drug binding sites in Kv3.1a

In all our Kv3.1 EM maps we observe clear densities for a native lipid in the pocket between PH and VSD (Site II in Figures EV1 A-D, Suppl. Figure S4 A). The polar head group of the lipid is stabilized by interactions with the imidazole group of H212 in the S1/S2 linker and backbone interactions with Q409 and M414 in S6. The non-polar side chains of W411 in S6 and W392, P388 and F391 in PH are interacting with the hydrophobic tails of the lipid (Figure EV5 C). Interestingly, the site is analogous to the anionic lipid interaction capable of modulating the prokaryotic analogue KcsA [39] and to the PC binding site in the Kv1.2/Kv2.1 structure [40] (Suppl. Figure S13 B). Furthermore, it strongly resembles the binding pocket determined for a TRPC6 agonist [41] which mimics the action of the native lipid agonist DAG (Figure EV5 D). Analogous small molecule binding sites near the S6/PH intersubunit interface have also been identified for TRPML1 (agonist) [42] and CavAb (antagonist) [43]. This highlights the functional importance of this region in other ion channels and could hint at a druggable pocket near the turret domain. A recent preprint with the structure of Kv3.1a in complex with a positive modulator [31] suggests that the molecule is bound near the S4/S5 linker, close to a second site containing non-protein densities in our structure (Suppl. Figure S4 B and S13 A, Site I in Figure EV1 B). The density in our maps can likely be attributed to a native lipid with its head group in close proximity to R326, corresponding to R6 of the voltage-sensing arginines in S4 helix (Figure EV1 C, top inset). It is also within 5 Å of R332 in S4/S5 linker helix, which we identified as a key residue in controlling activation potential. We carried out coarse-grained MD simulations in a complex lipid bilayer approximating the lipid composition of membranes in eukaryotic organisms, to study which lipids might bind in the sites revealed by cryo-EM. Whereas anionic lipids predominately interact with Kv3.1 residues at site I near the S4/S5 linker, there is no preferential localization at site II (Suppl. Figure S13 C, D) – an interesting result considering the role of anionic lipids at this site within the prokaryotic channel KcsA. CG free-energy perturbation calculations further pointed

towards an absence of preference for anionic lipids in site II: the estimation of the free-energy difference related to the alchemical transformation of zwitterionic PC into anionic PA yielded -1.69 ± 0.67 kJ/mol. On the other hand, the differences in lipid binding characteristics in site I are in line with its similarity to the PIP₂ pocket in Kv7.1 [44] (Figure S13 A) and may be utilized for the development of pharmaceutical drugs designed to specifically target one of the two lipid binding pockets in Kv3.1a.

Discussion

Voltage-gated potassium channels are quintessential regulators for neuronal excitability, and diverse members of the family have evolved to fine-tune the threshold potential for firing, the duration of action potentials and firing rates for specific cell functions. Kv3 channels stand out against the rest of the family due to several kinetic properties which are tailored to sustain high-frequency firing (up to 1 kHz). Here we present structural insights from cryo-EM structures of the human Kv3.1 structure and illuminate several hot spots in the channel architecture that play important roles for: i) the ultrafast activation and deactivation kinetics ii) the large conductance which aids efficient repolarization iii) the positive activation threshold that ensures that Kv3 channels only open during the repolarization phase of the action potential.

We show that the twisted T1 arrangement in Kv3.1 channels enables physical contact to the C-terminal axonal targeting motif and provide evidence that the “second cuff”, created by the tetrameric arrangement of $\alpha 6$ helices in T1, plays a major role in controlling Kv3.1 gating properties through interactions with the S4/S5 linker and the S6 tail. Using alanine scanning mutagenesis, we identify H336 in the S4/S5 linker as major contact to $\alpha 6$ with an essential role for fast activation and deactivation of Kv3.1a. Another important finding from our structures is the role of T1 interactions for open state stability of the pore domain in Kv3 channels. More specifically, we show that interdomain interactions between K449 in S6T and multiple residues in the negatively charged $\alpha 6$ helix of T1 are contributing to stabilisation of the lower gate in the open state, and MD simulations indicate a correlation of these interactions with the activation state of S4. Furthermore, our electrophysiological results also suggest that mutation of residue R332 in the S4/S5 linker profoundly affects the stability of the open state.

These findings are ground-breaking in light of the current model of electromechanical coupling in Kv channels, where the transmembrane region is thought to be primarily responsible for the transmission of signal between the VSD and the pore: the S4/S5 linker is considered an important coupling device transmitting conformational changes in the S4 voltage sensors to the pore domain via interactions with the S6 C-termini [45, 46] and direct interactions between S4 and S5 have been proposed as a non-canonical coupling pathway [47-49]. Due to the remarkable degree of sequence conservation of the S4/S5 linker in Kv3 channels across species, this region was previously suggested to be linked to the specialized Kv3 properties and driving their evolution in vertebrates [2]. Our experimental data indeed confirm that the linker contains unique residues involved in functionally important interactions with the S4 voltage sensor and with an extra control element in Kv3.1a, namely the “cuff” of $\alpha 6$ helices in the T1 domain. According to our structures, the latter is ideally positioned to influence conformational changes

of both S4/S5L linker and S6T and this interpretation is further supported by our MD and electrophysiological data.

Interestingly, Kv3 channels exhibit only a small difference in the $V_{0.5}$ values for Q/V and G/V curves [12], indicating tighter electromechanical coupling compared to other Kv channels in which the curves are further apart [50]. The stronger interlock between S5 and S6 through intersubunit interactions of residues F345 (S5) and M430 (S6) could be one contributor to tighter coupling between VSD and PD in Kv3 channels, since the mechanical pulling force exerted on the pore-flanking S6 helices is increased by this connection. The $\alpha 6$ ring could certainly be a major determinant for promoting this concerted opening of the pore via its clamp-like action to stabilize S6T in the open state. The cooperativity between subunits will likely be enhanced because each $\alpha 6$ helix is in direct contact with two neighbouring pore-flanking S6 helices. It is conceivable that this cross-bridging of the helical S6 bundle could potentially accelerate the final concerted step associated with opening of the pore {Zagotta, 1994, Shaker potassium channel gating. III: Evaluation of kinetic models for activation} and therefore provide a mechanical explanation for the fast activation kinetics of Kv3.1a. In line with this interpretation, we observe sluggish gating for double mutant E116A/D120A in $\alpha 6$. This phenotype suggests that fast activation/deactivation characteristic of Kv3.1 depends on a network of interactions involving the T1 $\alpha 6$ and a disruption of this network might increase the energy barrier of the activation/deactivation process.

Finally, another region of interest for potential enhanced electromechanical coupling is the interface between turret domain and the S1/S2 β -hairpin, which could be key for anchoring the immobile S1/S3 helices in the VSD and therefore allow more efficient S4 movement in response to voltage changes. Additional studies addressing these possibilities are needed to fully understand the intricate gating mechanism of Kv3 channels and its implications for the ability to sustain high frequency firing.

Conclusion And Outlook

The structural findings from the present work allow us to map and understand the underlying molecular mechanisms of disease mutations in the human *KCNC1* gene. Our results may also inspire future studies beyond Kv3 channels because a role of the cytoplasmic T1 domain in modulating gating was demonstrated for other Kv channels [24-26]. Since we identified previously unknown control elements in Kv3 channels, our insights could be transformative for structure-guided drug discovery targeting these novel structural features. Hence, the structures will serve as a blueprint for the rational design of new pharmaceutical drugs against a multitude of channelopathies and other severe CNS disorders linked to malfunction of high-frequency firing.

Methods

Molecular Biology, Virus Production and Protein Expression

Full-length human KCNC1 (isoform A) was cloned from the mammalian gene collection (MGC: 40024733) into LIC-adapted pHTBV C-terminally tagged Strep-II/10-His/GFP vector. Baculoviral DNA from the transformation of DH10Bac with the plasmid was used to transfect Sf9 cells grown in Sf-900™ II media supplemented with 2 % fetal bovine serum (Thermo Fisher Scientific). The resulting virus was further amplified by transducing Sf9 cells followed by incubation on an orbital shaker at 27°C for 70 h, followed by harvesting by centrifugation at 900*g*.

An Expi293F™ cell culture in mid-log phase (2×10^6 cells mL⁻¹) in Freestyle 293™ Expression Medium (Thermo Fisher Scientific) was infected with high-titre baculovirus (3 % v/v) in the presence of 5 mM sodium butyrate. Cells were grown in orbital shaker at 37°C with 8 % CO₂ for 38 h before being harvested by centrifugation at 900*g* for 10 min. The pelleted cells were washed with phosphate-buffered saline, pelleted again, then flash-frozen in liquid nitrogen (LN₂) for storage in -80°C freezer.

Purification of Kv3.1a channels

Whole cell pellet expressing Kv3.1a was resuspended to a total volume of 50 mL per 15 g cell pellet with buffer A (20 mM HEPES pH 7.5, 100 mM NaCl, 50 mM KCl) supplemented with 0.7 % w/v Lauryl Maltoside Neopentyl Glycol (LMNG; Generon) and 0.07 % cholesteryl hemisuccinate (CHS; Generon) for solubilisation. The cells were solubilised at 4°C for 1 h with gentle rotation, then centrifuged at 45,000*g* for 1 h. Washed Strep-Tactin Superflow (IBA) was added to the lysate to a ratio of 0.4 mL resin per 100 mL lysate, and the slurry was gently rotated at 4°C for 1 h. The resin was collected on a gravity flow column and washed with buffer B (buffer A with 0.003 % LMNG and 0.0003 % CHS), then with buffer B supplemented with 2 mM ATP and 5 mM MgCl₂. Kv3.1 was eluted with 6 CV of buffer B supplemented with 5 mM D-desthiobiotin followed by overnight tag-cleavage with TEV protease.

The sample was then concentrated and subjected to a size exclusion chromatography pre-equilibrated with buffer C (buffer A supplemented with 0.04 % digitonin (Apollo Scientific)). Peak fractions were pooled and concentrated to 20 µM.

Cryo-electron microscopy sample preparation, data collection and data processing

For Kv3.1 sample in apo state, 20 µM sample was directly used. For EDTA-incubated sample, 100 mM EDTA was added to the 20 µM Kv3.1 sample for a final concentration of 1 mM EDTA, then it was incubated on ice overnight. For ZnCl₂-incubated sample, 40 mM ZnCl₂ was added to the 20 µM Kv3.1 sample for a final concentration of 400 µM ZnCl₂, then it was incubated on ice overnight.

All samples were frozen on Quantifoil Au R1.2/1.3 200-mesh grids freshly glow-discharged for 30 s, with plunge freezing performed on Vitrobot Mark IV (Thermo Fisher Scientific) set to 100 % humidity, 4°C, 20 s

wait time and 2.5 – 4.5 s blotting time.

The apo-Kv3.1 dataset was collected on a Titan Krios (Thermo Fisher Scientific) operating at 300 keV at Midlands Regional Cryo-EM Facility (MRCEF; Leicester, UK). 4,043 super-resolution dose-fractionated micrographs ($0.42 \text{ \AA pixel}^{-1}$) were collected on a K3 (Gatan) detector at 105,000 x nominal magnification. Micrographs were binned to $0.84 \text{ \AA pixel}^{-1}$ in Relion 3.0.8 during motion correction with 5 by 5 patches using MotionCor2. Motion-corrected images were imported to Cryosparc v2.14.2 [51], then the defocus values were determined by Patch CTF function. 1,539,126 particles were picked with blob picker function, and extracted particles were subjected to three cycles of 2D classification. 58,244 particles from good classes were used to generate an ab-initio model. The particles were then subjected to a heterogeneous refinement with two classes, where the good class contained 49,327 particles. These were then used for non-uniform refinements with C1 and C4 symmetries using the ab-initio model as reference, with final resolutions reaching $3.5 \text{ \AA}$ and $3.2 \text{ \AA}$, respectively.

The EDTA-Kv3.1 dataset was collected on a Titan Krios (Thermo Fisher Scientific) operating at 300 keV at Cambridge Nanoscience Centre (CNC; Cambridge, UK). 7,214 super-resolution dose-fractionated micrographs ($0.42 \text{ \AA pixel}^{-1}$) were collected on a K3 (Gatan) detector at 105,000 x nominal magnification. Micrographs were motion-corrected with Patch Motion Correction on Cryosparc v3.1.0, and defocus values were estimated with Patch CTF function on Cryosparc. 3,177,434 particles were picked with blob picker function, and extracted particles were subjected to two rounds of 2D classification. 217,788 particles from good classes were used to generate an ab-initio model. The particles were subjected to non-uniform refinement with C4 symmetry using the ab-initio model as reference, which resulted in a $3.2 \text{ \AA}$ reconstruction. For the subclass with extended axonal targeting motif, the particles from refinement were symmetry-expanded by C4 symmetry, which were then used for Cryosparc's 3D Variability Analysis with a mask including Kv3.1's cytoplasmic domain. Cluster function was used to isolate 149,365 particles, after which Remove Duplicate Particles function was used to obtain 110,585 unique particles. These particles were used for a non-uniform refinement with C1 symmetry using one of the maps from 3D Variability Analysis as reference, resulting in a $3.6 \text{ \AA}$ reconstruction. For the subclass with interaction between S1/S2 linker and turret domain, the symmetry-expanded particles were subjected to 3D Variability Analysis with a mask including Kv3.1's transmembrane domain. Cluster function was used to isolate 107,532 particles, after which Remove Duplicate Particle function was used to obtain 93,461 unique particles. These particles were used for a local refinement function with the 3D Variability Analysis map as reference and transmembrane domain mask, which resulted in a $3.6 \text{ \AA}$ reconstruction.

The Zn-Kv3.1 dataset was collected on a Titan Krios (Thermo Fisher Scientific) operating at 300 keV at Cambridge Nanoscience Centre (CNC; Cambridge, UK). 5,010 super-resolution dose-fractionated micrographs ($0.42 \text{ \AA pixel}^{-1}$) were collected on a K3 (Gatan) detector at 105,000 x nominal magnification. Micrographs were motion-corrected with Patch Motion Correction on Cryosparc v3.1.0, and defocus values were estimated with Patch CTF function on Cryosparc. 2,285,988 particles were picked with blob picker function, and extracted particles were subjected to two rounds of 2D classification. 224,524 particles from good classes were used to generate an ab-initio model. In order to separate Kv3.1

monomers from dimers, the particles were reextracted to larger box size (900 x 900 pixels downsampled to 450 x 450 pixels) then heterogeneous refinement was performed. 133,488 monomer particles were used for non-uniform refinement with C4 symmetry, resulting in a 3.1 Å reconstruction. For the dimer subclass, 91,036 dimer particles were further cleaned with Remove Duplicate Particles function, leading to 72,764 unique particles. These were used for a non-uniform refinement with C4 symmetry, resulting in a 3.1 Å reconstruction.

Model building and refinement

Transmembrane domain of a monomer model of Kv1.2 (PDB ID: 3LUT) and a tetramerisation domain of a Kv3.1 orthologue from *a. californica* (PDB ID: 3KVT [10]) were fitted to the apo-Kv3.1 map on UCSF Chimera [52], then combined to a single PDB file. The sequence was substituted with Kv3.1's sequence with CHAINSAW, and the model was subsequently manually built in Coot [53]. The model was refined with Phenix real space refine, and its geometry was verified in Phenix [54] with MolProbity. This model was used as reference for EDTA-Kv3.1 and Zn-Kv3.1 maps. Models were manually refined in Coot, which were then refined with Phenix real space refine. The models' geometry were verified in Phenix (MolProbity).

Heterologous expression in *Xenopus* oocytes and electrophysiological recordings

The human Kv3.1a cDNA in pcDNA3.1(+) was kindly provided by Nadia Pilati (Autifony Therapeutics, Ltd.). This cDNA encodes the full-length Kv3.1a plus a C-terminal FLAG tag. The QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) was used to create all mutations except K449A. Kv3.1a-K449A and the corresponding WT without the FLAG tag were generated synthetically in the pcDNA3.1(+) vector (GenScript). The voltage dependence of Kv3.1a with or without the C-terminal FLAG tag is similar (Suppl. Fig. S12 D, E). Plasmid maintenance, mutagenesis, sequence, RNA synthesis and oocyte microinjection were carried out as described before [55]. Care and surgery of *Xenopus laevis* frogs was performed according to a protocol approved by the Thomas Jefferson University IACUC.

Whole-oocyte currents were recorded under two-electrode voltage-clamp conditions using the OC-725C amplifier (Warner Instrument, Hamden, CT, USA) 1-2 days post RNA injection as previously reported [1]. Data acquisition, P/4 on-line leak subtraction and initial analysis were performed using pClamp 10.3 (Molecular Devices, Sunnyvale, CA, USA). The extracellular solution contained (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 2.5 sodium pyruvate, adjusted to pH 7.4 with NaOH. The electrodes were filled with 3 M KCl. All recordings were conducted at room temperature (21–23°C).

Data analysis for electrophysiological recordings

Data analysis, curve plotting, and fitting were performed in OriginPro 9.6 (OriginLab, Northampton, MA, USA). Conductance-voltage relations (G - V) curves were characterized by the best-fit to the 1st-order Boltzmann equation:

$$G(V) = \frac{G_{max}}{1 + e^{\frac{(V_c - V_{0.5})}{k}}}$$

Where, G_{max} is the max conductance and V_c is the command voltage. The best-fit to this equation returned the $V_{0.5}$ and $z = 25.5/k$. To obtain the time constants of current activation at various depolarizing command voltages, we obtained the best-fit to the rising phase of the current (excluding the short initial current lag) assuming a 1st-order exponential function or a sum of exponential terms. Typically, no more than two terms were necessary to obtain a satisfactory fit. A similar approach was used to obtain the time constants of current deactivation at various repolarizing command voltages. The reported time constants are weighted averages of the best-fit time constants. Statistical details and analysis are reported in the figure legends.

Coarse Grain Simulations

Unresolved residues in the cryo-EM structure within the S1/S2, S3/S4 and T1/S1 loops were built using Modeller [1]. The long unresolved loop between the T1 α_6 and S1 helices was modelled as a combination of two unstructured regions $_{121}\text{SFGGAP}_{126}$ and $_{159}\text{DSPDGRPGGF}_{168}$. Coarse-grained simulations were performed using the Martini 2.2 force field [2] with the protein embedded in an asymmetric model membrane [3] using the Martini Bilayer Maker [4] of CHARMM-GUI [5]. After energy minimization and equilibration for 20 ns, three replicates of the system were simulated for 20 μs each using Gromacs 2020 [6] with the protein backbone beads restrained to allow convergence of lipid interactions. The final 7.5 μs of the simulation trajectories were used for analysis using Python MDTraj scripts [7]. A contact was assumed if a residue's bead was within 5.5 Å of a lipid head-group bead and occupancy probability density calculations were performed using a grid of 2 Å resolution.

Free-Energy perturbation calculations following the methodology of Corey et al. [9] were performed to ascertain the identity of lipid site I without preferential interactions in the CG simulations. The PC lipid in CG resolution was alchemically transformed into an anionic PA lipid previously identified to occupy the analogous site in prokaryotic KcSA channels [10, 11]. Coulomb and van-der-Waals interactions were perturbed separately using 10 windows each along the λ chemical space. Each λ window was energy-minimized, equilibrated and then simulated for 300 ns using a leapfrog stochastic dynamics integrator. The *alchemy* library [12] was used to calculate energies from the individual windows through the BAR method [13]. Results were calculated as a mean of three independent perturbation simulations.

Atomistic Simulations

For computational expedience, to ease the translocation of the S4 Arginines [14], the CTC F256A mutant was introduced using PyMOL. The built structure was embedded in a POPC membrane using CHARMM-GUI [5] and solvated in 150 mM KCl solution. Protein, lipids and ions were modelled using the Charmm36M force field [15] and water was described using the TIP3P model. After energy minimization and equilibration, a production simulation was performed by submitting the system to an external electric field yielding a +300 mV transmembrane potential for 5.5 μ s. Long-range electrostatic interactions were calculated using the particle mesh Ewald method [16] and hydrogen-bond lengths were constrained using LINCS [17]. Pressure and temperature were maintained through the use of the Parrinello-Rahman barostat (1 bar) [18] and v-rescale (300 K) [19] thermostat. Hydration profiles were calculated over 30 ns of MD simulations, using the Channel Annotation Package (CHAP)[56].

Data availability

The cryo-EM maps of the human Kv3.1a structures and the corresponding atomic coordinates have been deposited in the Electron Microscopy Data Bank and the Protein Data Bank under the accession codes EMD-13416/7PHH (apo), EMD-13417/7PHI (zinc), EMD-13418/7PHK (dimer in zinc), EMD-13419/7PHL (EDTA), respectively.

Declarations

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Author Contributions

GC, SV, AFC, NAB-B and KLD designed and cloned the constructs. The protein was expressed by GC, NKS, GM and SMMM, and the protein purification was done by GC and NKS. GC, PQ, PC-H and KS collected the EM datasets. GC processed the EM data and built the model. AS and LD performed MD simulations. QL and MC expressed the protein in oocytes and performed electrophysiology experiments. KLD, GC and AS wrote the manuscript. The manuscript was revised by KLD, GC, MC and LD.

Declaration of interests

The authors declare no competing interests.

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Figures

Figure 1

Kv3.1a full-length structure showing alternative T1 arrangement compared to Kv1.2/2.1 and electrostatic ATM/T1 interactions (A) Density maps of the human Kv3.1a channel, coloured by chain. TM region is illustrated by dashed lines. (B) Superposition of a single chain of Kv3.1a (dark blue cartoon) with one protomer of the Kv1.2-2.1 paddle chimera (pdb: 6EBK, shown in yellow cartoon representation), aligning the S4/S5 linker and pore domain. (C) Cytoplasmic view of the T1 domain of Kv3.1a superposed on the Kv1.2/2.1 structure (yellow cartoon) using the same alignment as in B. Angle indicates rotational displacement measured between a vector defined by α atoms of G16/H22 in Kv3.1 and G41/Q47 in Kv1.2-2.1 respectively. (D) Superposition of an individual T1 protomer (residues 8-120, dark blue) from Kv3.1 with the respective region of the Kv1.2-2.1 protomer (yellow). (E) Detailed view of the ATM/T1 contact region. T1 is shown as semi-transparent surface representation/cartoon and the ATM motif C-terminal from S6T is shown as red cartoon. (F) Close-up view of the inset from E, illustrating electrostatic contacts between acidic residues in α 5 of the N-terminal T1 domain and a cluster of lysines (K454-K458) within the ATM of the C-terminus. Red frame indicates location of frameshift variant K457fs associated with EPM7 (ClinVar ID: 692088).

Figure 2

Voltage sensor domain arrangement and pore properties of Kv3.1a (A) Cartoon representation of the VSD S1-S4 (S3 is omitted for clarity) in Kv3.1a, showing the relative positioning of the voltage sensing charges R1-R6 in S4 (shown in yellow) with respect to conserved residues (F256 and E249) of the hydrophobic charge transfer center (CTC), located in S2 (shown in light green). Blue circles highlight voltage-sensing residues located above the position of conserved F256, which is represented by the dotted red line. (B)

Illustration of the VSD arrangement in Kv1.2-2.1 (pdb 6EBK), highlighting the fact that four gating charges (blue circles) are located above F233 compared to only three in Kv3.1a. (C) Plot of the Kv3.1a pore radius, as determined by MOLE [57]. Red dotted line marks the radius of hydrated K⁺. (D) Sequence of S4 residues for Kv3.1a (top) and Kv1.2-2.1 (bottom) with gating charges highlighted in blue. Green and red boxes mark the gating charges which interact with the conserved Glu and Phe residues in S2, respectively. (E) Cross-membrane view of the Kv3.1a pore domain and selectivity filter. For clarity, only two chains are shown. The pore diameter is shown as light grey surface representation. Residues in the PVP hinge region are shown as green sticks, major pore lining residues are highlighted by black frames. Conserved aromatic residues in the lower S6T gate are shown as sticks. F345 in S5 (unique to Kv3 family) is shown as black stick representation. Dashed line indicates *ca* distance between V425 (framed in red), a residue relevant for the gain-of-function mutation V425M associated with epileptic encephalopathy [9]. (F) Cytoplasmic view of the open pore with pore-lining S6 helices from all four subunits shown as cartoon representation. Dashed line indicates *ca* distance between P433 from two diagonal subunits.

Figure 3

The $\alpha 6$ helix of the Kv3.1 T1 domain forms a second cuff below the S4/S5 linker, stabilized by inter- and intrasubunit interactions (A) Cartoon representation of the Kv1.2-2.1 tetramer (light grey) with one

protomer highlighted in dark grey. S4/S5 linker is represented in magenta, S6 is coloured in pale yellow and $\alpha 6$ of the T1 domain is shown in aquamarine blue. (B) Cartoon representation of the Kv3.1a tetramer, illustrating the unique orientation of $\alpha 6$ (teal blue) of the T1 domain. Colour-coding of other elements is the same as in A. (C) Inset from B with an enlarged view of the $\alpha 6$ helix and its interactions with residues in the S4/S5 linker (magenta) and the lower gate formed by S6T (pale yellow). (D) Cytoplasmic view of the tetrameric “ $\alpha 6$ gating cuff” (shown as teal cartoon), located below the gating machinery formed by S4/S5 (magenta) and S6T (yellow). Only helix $\alpha 6$ of T1 is shown for clarity. (E) Inset from D, detailing inter-subunit interactions between $\alpha 6$ helices from adjacent subunits and interactions between $\alpha 6$ and S6T. Zn^{2+} ions are shown as grey spheres.

Figure 4

Mutational and electrophysiological analyses of key residues involved in functional inter-domain interactions between $\alpha 6$, S4/S5 linker and S6T (A) Normalized G–V curves (G/Gmax) of Kv3.1a WT and the indicated mutants. The symbols and error bars represent mean $\pm$ SEM, and the solid lines are the best fits assuming a 1st-order Boltzmann equation (Methods). (B) Scatter graphs comparing of $V_{0.5}$ values from WT and several Ala mutants. Each symbol represents a measurement from an individual oocyte,

and the overlaid horizontal line represents the mean value. In each group, n=9-19 oocytes. To evaluate differences relative to WT, we use the one-way ANOVA test. Significant P values are indicated on the graph. (C) Normalized G-V curves for the indicated $\alpha 6$ mutations as described for panel A. In each group, n=10-11 oocytes. (D) Scatter graph for the indicated $\alpha 6$ mutations as described for panel B. In each group, n=10-11 oocytes. (E) Left and Center: overlay of representative whole-oocyte currents from Kv3.1a WT and the double mutant E116A/D120A. Protocol details as described for panel F. The horizontal and vertical scale bars indicate 100 ms and 1 μ A, respectively. Right: The voltage dependence of the time constants of activation (hollow symbols) and deactivation (filled symbols) from Kv3.1a WT and E116A/D120A. Symbols and error bars in panels D and H represent the mean $\pm$ SEM. (F) Left: Overlay of representative whole-oocyte currents from Kv3.1a WT and the mutant H336A. These currents were evoked by the voltage protocol shown in the inset. Center: Overlay of representative whole-oocyte currents from Kv3.1a WT and the mutant H336A. From a holding voltage of -100 mV, these currents were evoked by a 30-ms step depolarization to +50 mV, which was immediately followed by a 100-ms repolarizing step to 0 mV. To determine the voltage dependence of deactivation, the repolarizing step was changed in 10-mV increments from -50 mV to +30 mV (inset). The horizontal and vertical scale bars indicate 100 ms and 1 μ A, respectively. Right: The voltage dependence of the time constants of activation (hollow symbols) and deactivation (filled symbols) from Kv3.1a WT and H336A.

Figure 5

Architecture of the turret domain of Kv3.1a and its interaction with the S1/S2 loop of the adjacent VSD. (A) Overview of the S5-pore helix linker region ("turret region", shown in shades of blue) and the interface between pore helix PH (green cartoon) and S1/S2 loop of the VSD (surface representation and pink cartoon). (B) Inset I from A: Detailed view of histidines (bold blue sticks) in the turret domain and their interactions with Q409 in S6 (pale yellow) and Y407 (orange sticks) near the selectivity filter of the pore domain. (C) Sequence alignments of the S5-PH linker (left) and the S1/S2 loop region (right) for human Kv3 family members and other human T1-containing Kv channels, highlighting the enlarged turret domain unique to the KV3 subfamily and the location of conserved Kv3-specific Histidines. (D) Inset II from A: detailed view of the PD/VSD interface formed by unique residues at the N-terminal end of the S1/S2 loop of the VSD (pink ribbon) and their interactions with residues in the transition zone between the turret domain (light cyan) and the pore helix (PH in green). Red frame indicates the location of variant C208Y associated with non-progressive myoclonus epilepsy [6].

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