Two epilepsy-associated variants in KCNA2 (K\textsubscript{v}1.2) at position H310 oppositely affect channel functional expression

Teresa Mínguez-Viñas\textsuperscript{1}, Varsha Prakash\textsuperscript{1}, Kaiqian Wang\textsuperscript{1}, Sarah H. Lindström\textsuperscript{1} \textsuperscript{1}, Serena Pozzi\textsuperscript{1}, Stuart A. Scott\textsuperscript{2}, Elizabeth Spiteri\textsuperscript{2}, David A. Stevenson\textsuperscript{3}, Euan A. Ashley\textsuperscript{3}, Cecilia Gunnarsson\textsuperscript{1,4,5} and Antonios Pantazis\textsuperscript{1,6}

\textsuperscript{1}Division of Neurobiology, Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden
\textsuperscript{2}Department of Pathology, Stanford University School of Medicine, Stanford, California, USA
\textsuperscript{3}Division of Medical Genetics, Stanford University, Palo Alto, California, USA
\textsuperscript{4}Department of Clinical Genetics, Linköping University, Linköping, Sweden
\textsuperscript{5}Centre for Rare Diseases in South East Region of Sweden, Linköping University, Linköping, Sweden
\textsuperscript{6}Wallenberg Center for Molecular Medicine, Linköping University, Linköping, Sweden

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Abstract Two KCNA2 variants (p.H310Y and p.H310R) were discovered in paediatric patients with epilepsy and developmental delay. KCNA2 encodes K\textsubscript{v}1.2-channel subunits, which regulate neuronal excitability. Both gain and loss of K\textsubscript{v}1.2 function cause epilepsy, precluding the prediction of variant effects; and while H310 is conserved throughout the K\textsubscript{v}-channel superfamily, it is largely understudied. We investigated both variants in heterologously expressed, human K\textsubscript{v}1.2 channels by immunocytochemistry, electrophysiology and voltage-clamp fluorometry. Despite affecting the same

T. Mínguez-Viñas and V. Prakash contributed equally to this work.
channel, at the same position, and being associated with severe neurological disease, the two variants had diametrically opposite effects on $K_v1.2$ functional expression. The p.H310Y variant produced ‘dual gain of function’, increasing both cell-surface trafficking and activity, delaying channel closure. We found that the latter is due to the formation of a hydrogen bond that stabilizes the active state of the voltage-sensor domain. Additionally, H310Y abolished ‘ball and chain’ inactivation of $K_v1.2$ by $K_v\beta1$ subunits, enhancing gain of function. In contrast, p.H310R caused ‘dual loss of function’, diminishing surface levels by multiple impediments to trafficking and inhibiting voltage-dependent channel opening. We discuss the implications for $K_v$-channel biogenesis and function, an emergent hotspot for disease-associated variants, and mechanisms of epileptogenesis.

Corresponding author
A. Pantazis: Division of Neurobiology, Department of Biomedical and Clinical Sciences, Linköping University, 581 83 Linköping, Sweden. Email: antonios.pantazis@liu.se

Abstract figure legend
The ribbon structure shown is a homology model of the voltage-dependent, $K^+$-selective channel $K_v1.2$ (Pantazis et al., 2020), an important regulator of neuronal excitability. A single $K_v1.2$-subunit is in blue, while the other three subunits in the tetrameric channel are transparent. The arrow origin is at the approximate position of H310: a highly conserved amino-acid in the $K_v$-channel superfamily, at the C-terminus of transmembrane segment S4. In this work, we characterized two variants of $KCNA2$, which encodes $K_v1.2$ channels, from patients with seizures and neurodevelopmental delay. Both result in the substitution of H310. Working on human $K_v1.2$ channels (h$K_v1.2$) and the auxiliary $K_v\beta1$-subunit, we show that, despite occurring at the same site and causing similar disease, the two variants cause opposite effects on $K_v1.2$ trafficking and function.

Key points
- $KCNA2$ encodes the subunits of $K_v1.2$ voltage-activated, $K^+$-selective ion channels, which regulate electrical signalling in neurons. We characterize two $KCNA2$ variants from patients with developmental delay and epilepsy. Both variants affect position H310, highly conserved in $K_v$ channels.
- The p.H310Y variant caused ‘dual gain of function’, increasing both $K_v1.2$-channel activity and the number of $K_v1.2$ subunits on the cell surface.
- H310Y abolished ‘ball and chain’ (N-type) inactivation of $K_v1.2$ by $K_v\beta1$ subunits, enhancing the gain-of-function phenotype.
- The p.H310R variant caused ‘dual loss of function’, diminishing the presence of $K_v1.2$ subunits on the cell surface and inhibiting voltage-dependent channel opening.
- As H310Y stabilizes the voltage-sensor active conformation and abolishes N-type inactivation, it can serve as an investigative tool for functional and pharmacological studies.
Introduction

In this work, we characterized two epilepsy-associated variants of the KCNA2 gene. KCNA2 encodes voltage-gated, potassium-selective, Kv1.2-channel subunits. By themselves, or in association with related Kv1 family subunits (Coleman et al., 1999; Sheng et al., 1993), they form tetrameric, voltage-gated, potassium-selective (Kv) channels. Expressed broadly in both central and peripheral neurons, and more specifically at axon initial segments, juxtaparanodal regions and presynaptic terminals (Trimmer, 2015), Kv1.2 subunits contribute to delayed-rectifier and A-type currents. Thus, they control electrical excitability, regulating action potential generation and ultimately neurotransmitter release (Bean, 2007; Debanne et al., 1997; Guan et al., 2007; Lambe & Aghajanian, 2001). KCNA2 disruption in mice causes severe neurological disease (Brew et al., 2007; Xie et al., 2010) and the first reports of KCNA2 channelopathy in humans emerged in 2015, among children with epilepsy, ataxia and developmental delays (Pena & Coimbra, 2015; Syrbe et al., 2015).

Both KCNA2 variants in this work substitute amino-acid H310. One variant, discovered in a patient with epileptic encephalopathy, is a tyrosine substitution (p.H310Y). The other variant was found in a patient with developmental delay, and it is an arginine substitution (p.H310R). In a previous study on the drk1 channel, the Drosophila homologue of Kv2 channels, alanine substitution of the histidine at homologous position 309 resulted in a ~50 mV depolarizing shift of the activation voltage dependence (Li-Smerin et al., 2000). As the two KCNA2 variants affected the same channel, at the same position, and were associated with severe neurological disease, we originally hypothesized that they impair Kv1.2-channel functional expression in similar ways: likely by loss of function via inhibited voltage-dependent opening.

However, both gain and loss of KCNA2 function have been linked to epilepsy (Doring et al., 2021; Masnada et al., 2017; Syrbe et al., 2015). Mutations can affect several aspects of Kv1.2 functional expression, in terms of voltage-dependent opening and inactivation, and cell-surface trafficking (Nilsson et al., 2022; Pantazis et al., 2020). As such, it was not possible to predict how the two p.H310-variants affected the human Kv1.2 channel. Here, we investigated the effects of p.H310Y and p.H310R on human Kv1.2 functional expression, both to gain insight on how these variants alter Kv1.2 to cause paediatric disease, and to investigate the role of a largely understudied amino acid in the Kv-channel voltage-sensor domain, an emergent hotspot for diseases of electrical excitability.

Materials and methods

Ethical approval

All animal experiments were approved by the Linköping University Animal Care and Use Committee (document number 15839-2018, protocol number 1941). Defolliculated Xenopus laevis oocytes were either purchased from Ecocyte or isolated and prepared from locally kept frogs supplied by Nasco.

Up to five female frogs were kept in an aquarium with dimensions 615 x 435 x 232 (length, breadth, height, in mm) at the Linköping University animal facility. Aquarium water was cleansed continuously using mechanical and biological filters. 10—15% of the water was changed weekly. Water temperature was kept at 17—19°C and conductivity at 400—1000 µS. Frogs were fed twice per week. Artificial mangrove roots and a glass container with stones and large limestones were lowered into the aquaria for environmental enrichment, shelter and as an additional source of lime. Animal facility staff monitored the frogs daily. The facilities are approved by the Swedish Agricultural Agency (Jordbruksverket).

To collect ovaries, frogs were anaesthetized in a water bath containing 1.4 g/l MS-222 Sandoz, 2.4 g/l HEPES, pH adjusted to 7.5 with 10 M NaOH. Anaesthesia was confirmed by foot pinch. The anaesthetized frog was placed on ice and her abdomen was cleaned with 70% ethanol. Lobes of ovaries were removed through a 15 mm abdominal incision and placed into Ca2+-free OR-2 solution (in mM: 82.5 NaCl, 2.5 KCl, 1MgCl2 and 5 HEPES; pH adjusted to 7.4 by NaOH) for further processing. Frogs were treated with analgesics (5 mg/ml Marcin and 2% Xylocain) and the incision was sutured. Frogs were immersed in water with a pillow under the chin, to keep their nose above water, and observed until regaining consciousness (ca. 30 min) prior to being returned to a recovery aquarium for post-surgical monitoring. Frogs were allowed to recover for at least 2 months between surgeries, which alternated between left and right side of the abdomen. Frogs were humanely killed by decapitation under deep anaesthesia if any of these conditions were met: (i) they were sick or not thriving; (ii) they had undergone six surgeries; or (iii) they were housed at the animal facility for over 5 years. The investigators understand the ethical principles under which the journal operates and that their work complies with the Journal of Physiology animal ethics checklist.

Patient clinical genetic testing

Inclusion of the p.H310Y patient in the present study was approved by the Local Ethical Committee (Linköping
University Dnr 2015/129-31) and the family gave informed consent prior to enrolment. The study complies with the ethical guidelines of the 1975 Declaration of Helsinki. DNA extraction from whole blood samples (EDTA) was performed using EZ1 (Qiagen). DNA concentration and quality were determined using a NanoDrop spectrophotometer. Genetic analysis was performed using the Blueprint Genetics Comprehensive Epilepsy Panel (v.6 Feb 22, 2020), including sequence analysis and copy number variation analysis (Table S1). It is a 203 gene panel and includes assessment of non-coding variants. In addition, it also includes the maternally inherited mitochondrial genome. The results showed that our patient was heterozygous for the NM_004974.3 KCNA2 c.928 C>T (p.H310Y) variant. This variant was judged as a variant of unknown significance according to American College of Medical Genetics and Genomics guidelines (Richards et al., 2015) at the time of diagnosis.

The p.H310R variant was detected by clinical exome sequencing. Libraries were prepared for the proband and maternal specimen using Agilent SureSelect Clinical Research Exome kit mixed with an enhanced capture protocol. Exome libraries were run on the Illumina HiSeq2500 (Illumina). Sequencing reads were aligned to the reference genome GRCh37/hg19 and variants were analysed with a filtration algorithm developed in house in Alissa Interpret (Agilent). The KCNA2 variant was confirmed in the proband and was not detected in the maternal specimen by Sanger sequencing. The mean depth of coverage for the exome was 86.7X and the percentage of the exome covered at ≥20× and base phred quality ≥20 was 95.4%.

Molecular biology

All DNA constructs made for this work are available upon reasonable request. Site-directed mutagenesis was performed with a high-fidelity Pfu polymerase (Agilent 600850). Plasmid purification was performed using the ZymoPURE Plasmid Miniprep and DNA Clean & Concentrator kits (Zymo Research). All other molecular biology reagents were provided by New England Biolabs and all synthetic oligonucleotides by Integrated DNA Technologies, unless stated otherwise. All molecular biology operations were confirmed by full sequencing at the Molecular Biology Unit at the Linköping University Core Facility.

Original rat Kv1.2 constructs were converted to the human sequence via three mutations (V9A, G198S and D355E). The pMAX and pCDNA3 vectors were used for expressing Kv1.2 constructs in oocytes (Pantazis et al., 2020) or COS-7 cells (Nilsson et al., 2022), respectively.

The human Kv1.2 subunit (KCNA1; GenBank ID NM172159.2) was acquired from Addgene: plasmid #154084, mOrange-P2A-hKvr1 in the pPAV vector, a gift from Michael Hoppa (Cho et al., 2020); RRID: Addgene_154084. It was subcloned to the pMAX vector for oocyte expression.

Concatenated dimers in pMAX vectors were constructed using previous rat Kv1.2/Kv1.2 or Kv1.2/Kv1.4 dimers as templates (Nilsson et al., 2022). The rat Kv1.2 genes were substituted by human Kv1.2, wild-type or including H310R, by subcloning. All final constructs consisted of an N-terminal partner lacking a stop codon, and the intervening sequence: GCT AGC GAT ACG AAG GAG CGA GGA AAC CTC TTC ACG TCA ACC GGA TCC GCC GCC ACC ATT, corresponding to the amino-acid linker sequence: ASDTK ERGNL FTSTG SAATI.

pMAX plasmids were linearized using PacI and transcribed to cRNA in vitro (messageMAX T7, Cellscript). cRNA was purified using the Monarch RNA Cleanup Kit (New England Biolabs), quantified spectrophotometrically, evaluated by gel electrophoresis, and stored at −80°C.

Immunocytochemistry and flow cytometry

COS-7 cell culture and transfection. COS-7 cells (Merck; ECACC 87021302) were grown in Complete Culture Medium containing: Dulbecco’s modified Eagle’s medium (DMEM)/F 12 Nutrient Mixture (1:1) (Gibco), heat-inactivated fetal bovine serum (FBS) (10%), penicillin (100 units), streptomycin (100 mg/ml) and glutamine (0.5 mm). Cultures were incubated at 37°C with 5% CO2 and passaged twice per week up to p-24. Cells used for flow cytometry were seeded in 12-well plates (approx. 30,000 cells/well). Cells used for immunocytochemistry were seeded in 35 mm glass-bottomed dishes (approximately 15,000 cells/dish). Cells were transiently transected 24 h after seeding using jetOPTIMUS (Polyplus; 1 μl jetOPTIMUS: 1 μg plasmid DNA).

Immunocytochemistry/confocal microscopy. Primary (Rat anti-haemagglutinin (HA); Roche 3F10) and secondary (AlexaFluor 568 conjugated goat anti-rat IgG(H+L); Invitrogen A-11077) antibodies were diluted 1:100 and 1:1000, respectively, in blocking solution. About 48 h post-transfection, COS-7 cells were rinsed with ice-chilled phosphate-buffered saline (PBS) supplemented with 0.9 mM Ca2+ and 0.5 mM Mg2+ (Gibco 14400-133). Cells were fixed with 4% paraformaldehyde in PBS (without Ca2+ or Mg2+, 5 min), and washed with PBS (once quickly, then three times for 5 min each) prior to incubation with blocking solution (2 ml of 5% normal goat serum in PBS) for at least 1 h at room temperature. Blocking solution was replaced with primary antibody solution (0.5 ml) and cells were incubated at room
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Retrieved ovaries were cut into small clusters of 5–7 oocytes, then enzymatically treated with Liberase (7 Units/batch; Roche 05401127001) in 10 ml of OR-2 with agitation using an orbital shaker for 25–40 min. Liberase was removed by washing with OR-2 solution, then manual agitation for 30–60 min was employed to remove follicular layers. Mature (stage V–VI) defolliculated oocytes were selected and stored at 17°C in SOS (in mM: 100 NaCl, 2 KCl, 1.8 CaCl_2, 1 MgCl_2 and 5 HEPES; pH adjusted to 7.0 by NaOH). Oocytes were injected with 50 nl of 0.06 ng/nl hK_v1.2 cRNA 2 days before regular cut-open oocyte Vaseline gap (COVG) experiments. For hK_v1.2 and hK_v1beta1 coexpression, oocytes were injected with 50 nl of 0.06 ng/nl hK_v1.2 cRNA and 0.81 ng/nl hK_v1beta1 cRNA (resulting in a 16-fold molar excess for hK_v1beta1) 2 days before experiments. Finally, for voltage-clamp fluorometry experiments, oocytes were injected with 50 nl of 0.1–0.5 ng/nl hK_v1.2 cRNA 3–5 days before experiments. Injected oocytes were stored at 17°C in SOS.

Flow cytometry. Two days after transfection (see above), COS-7 cells were harvested by trypsinization (0.05%). Thereafter, cells were maintained on ice in the dark. Cells were pelleted at 400 g for 5 min at 4°C and washed twice with Dulbecco’s PBS (DPBS, Gibco). To evaluate K_v1.2(H310Y/R) subunit surface trafficking, cells were stained with 8 μg/ml anti-HA conjugated Brilliant Violet 421 (Brilliant Violet 421 anti-HA.11 Epitope Tag Antibody; BioLegend 682405, RRID: AB_2716037; lot B363302) in 100 μl DPBS/5% FBS for 45 min at 4°C and 200 g. Cells were washed with (i) DPBS/5% FBS and (ii) DPBS. After the final wash, cells were resuspended in 300 μl DPBS. Finally, a single-cell suspension was prepared using pre-separation filters (Miltenyi Biotec) (70 μm) and stored at 4°C in the dark until detection later the same day. Cells were detected using a BD FACSARia III Cell Sorter (FACSDiva 8.0.2 software). Brilliant Violet 421 and enhanced green fluorescent protein (EGFP) were excited at 405 and 488 nm, respectively. The emission filters were 450/40 nm and 550/30 nm, respectively. Cell clumps or fragments were excluded using forward and side scatter gating. All gates were set as in our previous studies using the same experimental paradigm (Nilsson et al., 2022; Pantazis et al., 2020). On average, ~7900 EGFP-positive cells were counted for each experiment (minimum cell count: 3910).

Electrophysiology and voltage-clamp fluorometry

Oocyte preparation. Retrieved ovaries were cut into small clusters of 5–7 oocytes, then enzymatically treated with Liberase (7 Units/batch; Roche 05401127001) in ~10 ml of OR-2 with agitation using an orbital shaker for 25–40 min. Liberase was removed by washing with OR-2 solution, then manual agitation for 30–60 min was employed to remove follicular layers. Mature (stage
were the currents (at 100 mV). In the dark, to label the introduced Cys (A291C) at the S4 extracellular flank (Claydon & Fedida, 2007; Nilsson et al., 2022; Pantazis et al., 2020). The oocytes were then rinsed in dye-free SOS prior to being mounted in the recording chamber. Fluorescence emission and ionic current were simultaneously measured from the same area of membrane isolated by the top chamber. The same electrophysiological apparatus and solutions were used as above. The optical set-up, comprising Thorlabs LED light source, optics and photodiode, Open Ephys LED driver, Semrock light filters, a FEMTO transimpedance amplifier and a Warner Instruments low-pass filter, was as described previously (Nilsson et al., 2022).

Data analysis and modelling

Trafficking assay quantitative analysis. Cell-surface staining was normalized (xnorm) using the following formula:

$$x_{\text{norm}} = \frac{x - \overline{x}_0}{\overline{x}_{\text{WT}} - \overline{x}_0}$$  \hspace{1cm} (1)

Where $x$ is a mean, log10 anti-HA measurement from alive, EGFP-positive cells; $\overline{x}_0$ is the mean of mean, log10 anti-HA measurements from alive, EGFP-positive cells transfected with negative anti-HA control from the same batch; and $\overline{x}_{\text{WT}}$ is the mean of mean, log10 anti-HA measurements from alive, EGFP-positive cells transfected with EGFP-Kv1.2(HA) from the same batch. In this way, the mean of negative-control cells is zero and that of EGFP-Kv1.2(HA) is 1.

Electrophysiological data analysis. All curve-fitting was performed by least squares using Solver in Microsoft Excel, unless stated otherwise.

Steady-state activation was calculated by fitting the macroscopic conductance ($G$) to a Boltzmann distribution:

$$G = \frac{G_{\text{max}}}{1 + \exp\left[\frac{V_0 - V_m}{T}\right]}$$  \hspace{1cm} (2)

where $V_m$ was the membrane potential; $V_0$ was the half-activation potential; $z$ was the effective valence; $F$ and $R$ the Faraday and Gas constants, respectively; $T$ was temperature (294 K). The macroscopic conductance, $G$, was calculated by dividing the current ($I_m$) by the driving force for potassium:

$$G = \frac{I_m}{V_m - E_K}$$  \hspace{1cm} (3)

where $E_K$ was the equilibrium potential for potassium. This formula was used instead of the Goldman–Hodgkin–Katz equation (Clay, 2009) since the current increased linearly at positive potentials and the $G(V)$ curve saturated.

The voltage dependence of slow inactivation was calculated by fitting the fraction of non-inactivated current to a Boltzmann distribution:

$$\frac{I_{\text{post}}}{I_{\text{pre}}} = \frac{1 - \text{ped}}{1 + \exp\left[-\frac{V_0 - V_m}{T}\right]} + \text{ped}$$  \hspace{1cm} (4)

where $I_{\text{pre}}$ and $I_{\text{post}}$ were the currents (at +40 mV) before and after the inactivating pulse, respectively, and ped was the non-inactivating pedestal current.

Channel opening and closing kinetics were determined by fitting traces to an ‘Exponential, standard’ function with $n = 1$ term in Clampfit 11 (Molecular Devices):

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C$$  \hspace{1cm} (5)

To characterize inactivation recovery kinetics, the fraction of recovered current at different intervals at −80 mV after the inactivating pulse (1 min at 0 mV) were fit to the following bi-exponential function:

$$\frac{I_{\text{post}}}{I_{\text{pre}}} = [\alpha_i \exp\left(-\frac{t}{\tau_1}\right) + (1 - \alpha_i) \exp\left(-\frac{t}{\tau_2}\right)] \cdot (1 - b) + b$$  \hspace{1cm} (6)

where $t$ is the time of recovery at −80 mV; $\tau_1$ and $\tau_2$ the time constants of two components, and $\alpha_i$ is the fraction of the first component. The function asymptotes are $b$ (the fraction of non-inactivated current at the end of the inactivation pulse) and 1 (full recovery from inactivation).

The voltage dependence of fluorescence deflections ($\Delta F$; i.e. voltage-sensor domain (VSD) activation) was estimated by fitting $\Delta F$ to a Boltzmann distribution:

$$\Delta F = \frac{\Delta F_{\text{max}} - \Delta F_{\text{min}}}{1 + \exp\left[\frac{V_0 - V_m}{T}\right]}$$  \hspace{1cm} (7)

where $\Delta F_{\text{max}}$ and $\Delta F_{\text{min}}$ were the maximal and minimal $\Delta F$ asymtotically.

Markov model construction and fitting. A 16-state Markov model was constructed in Matlab R2019a (MathWorks), based on previous schemes used for Shaker and Kv1.2 channel gating (Ishida et al., 2015; Zagotta et al., 1994). Each VSD in the model underwent two voltage-dependent activation transitions ($R_1 \leftrightarrow R_2$ and $R_2 \leftrightarrow A$), with forward rates $\alpha_i$ and $\gamma_i$, respectively, and backward rates $\beta_i$ and $\delta_i$, respectively. Activation of all fourteen...
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VSDs allowed a transition to the open state, with forward (opening) rate \( k_o \) and backward (closing) rate \( k_c \), both voltage-independent.

Each voltage-dependent rate constant was expressed according to Eyring theory. E.g.:

\[
\alpha = \alpha_0 \exp \left( \frac{z_e F V_m}{RT} \right)
\]

\[
\beta = \beta_0 \exp \left( -\frac{z_p F V_m}{RT} \right)
\]

for forward and backward rates, respectively, where \( \alpha_0 \) and \( \beta_0 \) were the rate constants, or values of \( \alpha \) and \( \beta \) at \( V_m = 0 \) mV and \( z_e \) and \( z_p \) were the equivalent charge movements up to the transition state for each transition. Calculations for state occupancies and the rate constants were formulated using the \( Q \)-matrix method (Colquhoun & Hawkes, 1977, 1982) as previously (Pantazis et al., 2020). Briefly, \( Q \) is an \( n \times n \)-element matrix, where \( n \) is the number of states (in this case 16) and each element \( q_{ij} \) is the rate constant for the transition from state \( i \) to state \( j \). If there is no connection between states \( i \) and \( j \) then \( q_{ij} = 0 \). Each diagonal element is the negative sum of the off-diagonal elements in its row. The transition rates are then given by:

\[
dp(t) = p(t) Q
\]

where \( p(t) \) was a 1 \times 16 vector of probability (occupancy) for each state such that its sum was 1. It was calculated in Mathworks Matlab using the \textit{ode15s} solver. The voltage steps for the model were simulated with a 43 \( \mu \)s time constant (determined by fitting the voltage-pulse traces to a single-exponential function) to reproduce the cut-open oocyte clamp time constant and reduce stiffness. For initial conditions and background fluorescence calculations, the state occupancies at steady-state were calculated using:

\[
p(\infty) = u^T (SS^T)^{-1}
\]

where \( S \) was \([Q \, u]\) and \( u \) was a 16 \times 1 unit vector.

The macroscopic current was simulated by:

\[
I(t) = p_O(t) G_{max} [V_m(t) - E_K]
\]

where \( p_O(t) \) was the occupancy of the open state, \( G_{max} \) was macroscopic conductance and \( E_K \) the reversal potential for K.

Fluorescence signal (\( \Delta F \)) was derived from model transitions using the following:

\[
\Delta F(t) = \sum_{i=1}^{2} [p(t) - \mathbf{p}_{\infty}] \cdot \mathbf{n}_i \cdot \varphi_i \cdot \Delta F_{total}
\]

where \( \mathbf{p}_{\infty} \) is a vector containing the steady-state occupancies at \(-80\) mV (the holding potential, to subtract background fluorescence); \( \mathbf{n}_i \) were each 16 \times 1 vectors with the number of VSDs in the R\(_2\) or A conformation (\( \mathbf{n}_1 \)) or the A conformation (\( \mathbf{n}_2 \)) for each model state, and divided by 4 (the total number of VSDs); \( \varphi_1 \) and \( \varphi_2 \) were the fractional contributions to the \( \Delta F \) for the R\(_1\) \( \rightarrow \) R\(_2\) and R\(_2\) \( \rightarrow \) A transitions, respectively (\( \varphi_2 \) was constrained to be \( 1 - \varphi_1 \)); and \( \Delta F_{total} \) was the total fluorescence change.

Data (current and \( \Delta F \) traces from activation and deactivation voltage protocols, from exemplar wild-type and H310Y experiments) were fit simultaneously, to allow the constraining of shared parameters. Rate optimization was performed by least squares, using the Bayesian adaptive direct search (BADS) machine-learning, model-fitting algorithm (Acerbi & Ma, 2017).

**Statistics.** All measurements were taken from distinct samples. All comparative statistics (e.g. `relative \( G_{max} `` \) were performed among oocytes or COS-7 cells from the same block of experiments (same oocyte batch or cell passage number, set of RNA injections or DNA transfection, and experiment day). All significance tests were two-tailed Student's \( t \) tests. Errors and error bars are SD, unless otherwise stated.

**Protein structures.** All channel structures are of the K\textsubscript{V}1.2 model made previously (Pantazis et al., 2020) using as templates the K\textsubscript{V}1.2–2.1 atomistic structure in the active/open configuration (Long et al., 2007) and a simulation of its closing transition (Jensen et al., 2012). Structures were analysed using UCSF ChimeraX (Goddard et al., 2018; Petersen et al., 2021), and rendered using UCSF ChimeraX or The Protein Imager (Tomasello et al., 2020).

**Results**

**Clinical manifestation of the two variants**

The KCNA2 c.928C>T (p.H310Y) variant was discovered in a 16-year-old female patient, born at term from non-consanguineous parents. Developmental and growth milestones were normal before disease onset at the age of 18 months, with febrile seizures. At the age of 6.5 years, the patient developed absences, myoclonic and generalized tonic-clonic seizures. The patient received anti-convulsant treatment (Levetiracetam) and the disease was stabilized. EEG showed generalized spikes, spike/poly spike waves with up to 2 s duration and photo paroxysmal response. The patient had mild intellectual disability. The patient's mother and older sister had a history of epilepsy; however, due to family reasons, segregation analysis was not performed.
The KCNA2 c.929A>G (p.H310R) variant was reported in a 9-year-old male patient, born at term from non-consanguineous parents. The patient was initially seen by a medical geneticist at 2 years of age. There was a history of global developmental delay requiring multiple resources including speech therapy, physical therapy and occupational therapy. Since a toddler he had a history of behavioural problems including aggression and lack of impulse control. He was subsequently diagnosed with autism spectrum disorder. Magnetic resonance imaging of the brain was performed at 3 years of age and showed a partially deficient falx with gyral inter-digitation and mild widening of the foramen of Luschka suggestive of mild inferior vermician hypoplasia. Array comparative genomic hybridization and Fragile X testing was previously performed and non-diagnostic. Physical examination at 8 years of age showed macrocephaly (56.5 cm, >98th centile) and borderline macrosomia with weight >99th centile and height at the 93rd centile. He had multiple minor anomalies including prominent cheeks, thin antihelix of the right ear, deep set eyes, and two café au lait macules (one on the wrist and one on the abdomen). At 9 years of age he continued to have global developmental delays. He was speaking just single words with approximately 20 words, able to follow simple commands, and had multiple stereotypes. Prior to 9 years of age he had no seizure episodes and at 5 years of age he had an EEG that was reported as normal. At 9 years of age he presented with a single seizure episode. He had 5 min of generalized tonic-clonic activity with upward gaze consistent with a seizure and was started on valproic acid. The variant was reported to ClinVar in 2018 (#975835, dbSNP: rs1649470988). The patient also harboured a variant in the MED12L gene, which encodes a component of mediator: a macromolecular complex linking gene-specific transcriptional activators with the basal transcription machinery. This rare variant is an in-frame deletion (c.4096_4149del, p.Q1366_P1383del) and was classified as being of uncertain significance.

H310 is a highly conserved site among the Kv channels

Fig. 1A shows the general membrane topology and structure of Kv channels, including Kv1.2. Four Kv-channel subunits are required to form a functional

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**Figure 1. Location and conservation of H310**

A, membrane topology of the Kv1.2 subunit and top view of a homo-tetrameric Kv1.2 channel structure model (Pantazis et al., 2020). B, close-up on the Kv1.2 voltage-sensor domain in the active (top) and resting (bottom) conformations (Pantazis et al., 2020). Positively charged arginine (R) and lysine (K) side-chains on S4 are shown in grey: during activation, they move past a conserved phenylalanine (F) on juxtaposed segment S2, the ‘charge-transfer centre’ (Nilsson et al., 2022). This conformational rearrangement is transduced via the S4–S5 linker, to enable channel opening (Borjesson & Elinder, 2008; Jensen et al., 2012). H310 (orange) is located after the last arginine (‘R6’) of the S4 helix. At the resting state, its side-chain faces towards the cytosol; upon voltage-dependent activation, it faces towards the core of the voltage-sensor domain. C, amino-acid sequence alignment of the S4 helix and the S4–S5 linker of Kv channels, with the conventional numbering of the voltage-sensitive, positively charged residues (R/K). All members of the Kv1 sub-family are shown (Kv1.1–Kv1.8), along with the first members of the other Kv-sub-families (Kv2–Kv12). Shaker, the archetypal Kv1 homologue from Drosophila melanogaster, is also included. All sequences, with the exception of Shaker, are of the human subunits. H310, in orange, is highly conserved. VSD: voltage-sensor domain.
channel. Each subunit is a polypeptide that spans the membrane six times. The first four transmembrane helices (S1–S4) are bundled into a VSD, while the fifth and sixth segments assemble into a central, \(K^+\)-selective pore domain. As such, a \(K_V\) channel consists of four VSDs surrounding and controlling the central pore. H310 is located at the C-terminus of the S4 helix. S4 is a critical element of the VSD, because it can undergo substantial conformational changes depending on the membrane potential (Fig. 1B) (Borjesson & Elinder, 2008). When the membrane is polarized (the inside of the cell is negative compared with the outside), S4, and the VSD overall, are in the ‘resting’ conformation. This keeps the channel pore gate shut, preventing ionic conductance. When the cell becomes more positive (membrane depolarization), positively charged arginine and lysine residues on S4 move outwards. This movement is transduced to the channel gate, favouring channel opening (Jensen et al., 2012). Located just after the final S4 arginine, H310 is carried along this molecular movement, changing its orientation from cytosolic (resting state) to facing the VSD core (active state) (Fig. 1B). Finally, the S4 C-terminal histidine is highly conserved, found in most members of the \(K_V\)-channel superfamily (Fig. 1C).

**H310Y and H310R oppositely affect \(K_V1.2\) trafficking**

To investigate how the H310Y and H310R substitutions affect human \(K_V1.2\) functional expression, we heterologously expressed wild-type and mutant channels in *Xenopus laevis* oocytes and studied them electrophysiologically. Cells expressing \(K_V1.2\)(H310Y) exhibited ~2.8-fold more conductance than those expressing wild-type \(K_V1.2\) (Fig. 2A, B). By contrast, we could not detect any significant current in cells injected with \(K_V1.2\)(H310R) cRNA, compared with uninjected cells (Fig. 2A and B).

The differential interpretations of these experiments are that the mutations affected the channel’s ability to conduct current (single-channel conductance or maximal open probability) or the trafficking of the \(K_V1.2\) subunits to the cell surface. To evaluate the latter, we performed surface-trafficking assays. The mutations were introduced in \(K_V1.2\) constructs for the study of cell-surface trafficking. The constructs possessed an EGFP N-terminally fused to human \(K_V1.2\), to positively report transfection and protein translation. In addition, an HA tag was introduced at the \(K_V1.2\) extracellular loop between S1 and S2: this reported surface expression when labelled with anti-HA fluorescent labels (\(\alpha\)-HA) in unpermeabilized cells (Gu et al., 2003; Nilsson et al., 2022). Figure 2C; Pantazis et al. (2020) shows exemplary confocal micrographs, where a fixed, unpermeabilized, COS-7 cell transfected with the H310Y variant showed more surface labelling (magenta \(\alpha\)-HA signal) than wild-type. On the other hand, the cell transfected with the H310R variant showed no surface staining. Figure 2D shows representative cell-density plots from flow cytometry experiments on COS-7 cells transfected and labelled as above. In these experiments, more H310Y-transfected (EGFP-positive) cells showed positive surface staining than wild-type-transfected cells. A minority of H310R-transfected cells showed surface staining. On average, the extent of surface labelling in H310R-transfected cells was much reduced compared with wild-type, while H310Y was significantly increased (Fig. 2E).

These results suggest that mutation H310Y results in augmented surface trafficking of human \(K_V1.2\) subunits and increased macroscopic conductance. In contrast, H310R resulted in diminished surface trafficking and no detectable channel current. There may be additional effects on channel conducting properties. Our initial hypothesis, that two mutations of the same channel, at the same position, caused neurological disease via the same effect on channel functional expression, could not be supported.

**H310Y facilitates \(K_V1.2\) opening and slows closing**

As H310 is located within the channel VSD, mutations at this site could alter the voltage-sensing properties of \(K_V1.2\) channels. Accordingly, we studied voltage-dependent activation of human \(K_V1.2\) channels with or without H310Y using the COVG voltage-clamp approach (Pantazis & Olcese, 2019; Stefani & Bezanilla, 1998; Taglialatela et al., 1992). We found that H310Y modestly facilitated channel opening, shifting the voltage dependence of activation by ~6 mV to more hyperpolarized potentials (Fig. 3A and B). The strongest effect was on channel deactivation, which was slowed by ~fivefold, whereas channel opening kinetics were not affected (Fig. 3D). These results suggest that H310Y stabilizes the channel in a conducting state, facilitating voltage-dependent opening and delaying closing. Not only did H310Y enhance surface trafficking of human \(K_V1.2\) channels, but it also increased its activity: a ‘dual gain of function’ effect.

**H310Y stabilizes late steps on the channel activation pathway**

The effect of H310Y on \(K_V1.2\)-channel closure, as well as the location of H310 in the voltage-sensor domain, prompted us to investigate the fundamental transitions underlying voltage-dependent function, VSD activation and deactivation. We used voltage-clamp fluorometry to optically track voltage-evoked S4 movements at the same time as pore opening (Cha & Bezanilla, 1997; Claydon &
Fedida, 2007; Gandhi & Olcese, 2008; Mannuzzu et al., 1996; Pantazis & Olcese, 2019). We found that H310Y hardly affected the voltage dependence of VSD activation; but the most prominent effect was the slowing of VSD deactivation (Fig. 4). This finding was consistent with the premise that deactivation of a single VSD is sufficient to close a potassium channel (Gagnon & Bezanilla, 2009; Ishida et al., 2015; Zagotta et al., 1994) and is thus rate-limiting for channel closing.

To mechanistically connect VSD conformational changes and pore opening, we simultaneously fit voltage-clamp fluorometry data (fluorescence and current traces from activation and deactivation protocols, in the absence or presence of the H310Y mutation) to a 16-state, physically relevant model of Kv1.2 voltage-dependent activation (Fig. 5A). The model described a closed channel with four VSDs, each of which could independently undergo two, sequential, voltage-dependent, activation transitions: from a fully resting (R) to an intermediate-resting (R') state; and then from R' to the fully active (A) state. When all four VSDs had achieved the A conformation, the channel could undergo an opening transition. A fit to the model recapitulated the experimentally observed properties of VSD activation and pore opening (Fig. 5B; parameters in Table 1) and showed that the strongest effect of H310Y was on parameter δ0, which was reduced by fourfold compared with wild-type, while other parameters remained largely unchanged (Fig. 5C). δ0 was the rate constant describing deactivation of the VSD from the fully active state to the

Figure 2. H310Y and H310R oppositely affect human Kv1.2 trafficking
A, representative cut-open oocyte Vaseline gap traces from cells injected with cRNA encoding human wild-type Kv1.2 (WT), Kv1.2(H310Y) or Kv1.2(H310R) channels, or un.injected cells. B, relative maximal conductance (gmax, a measure of functional expression) of WT (1.0 (0.51)) and H310Y (2.8 (2.6)). Cells injected with Kv1.2(H310R) cRNA did not exhibit distinguishable current (670 (180) nA at 200 mV) from uninjected cells (550 (94) nA).
C, confocal micrographs and D, flow cytometry (FC) cell-density plots to evaluate Kv1.2 surface trafficking, in unpermeabilized COS-7 cells. The vertical lines separate cells to α-HA-negative (left) and α-HA-positive (right): top: WT (7474 EGFP-positive cells); middle: Kv1.2(H310Y) (8196 EGFP-positive cells); bottom: Kv1.2(H310R) (8804 EGFP-positive cells). Numbers show percentage of α-HA-positive cells among the EGFP-positive cells; n = 9 replicates per condition. E, quantification of the FC results. Mean log(α-HA) signals from EGFP-positive cells were normalized to WT and negative control (α-HA-labelled cells transfected with an EGFP-Kv1.2 construct with a bungarotoxin binding site instead of HA). Left panel: WT: 1.0 (0.017); H310Y: 1.4 (0.046); negative control: 0.0 (0.010). Right panel: WT: 1.0 (0.016); H310R: 2.1 (0.067); negative control: 0.0 (0.038). Errors are SD. EGFP: enhanced green fluorescent protein; HA: haemagglutinin; WT: wild-type.
intermediate-resting state, and its decrease resulted in the increase of the equilibrium between the R2- and A-states (Fig. 5C, \( \gamma_0/\delta_0 \)). Essentially, H310Y specifically stabilized the fully active conformation of the VSD.

To increase confidence in this result, we fit the same dataset to models where different sets of transitions were constrained to be identical between wild-type and H310Y channels, reducing the number of free parameters. A model assuming that H310Y only affected the first

### Table 1. Markov-state model parameters (without WT/H310Y constraints)

<table>
<thead>
<tr>
<th>Transitions or equilibria</th>
<th>Parameter</th>
<th>WT</th>
<th>H310Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha (R_1 \rightarrow R_2) )</td>
<td>( \alpha_0 (s^{-1}) )</td>
<td>380</td>
<td>430</td>
</tr>
<tr>
<td>( \beta (R_1 \leftarrow R_2) )</td>
<td>( \beta_0 (s^{-1}) )</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>( E_{R1 \rightarrow R2} (0 \text{ mV}) )</td>
<td>( \gamma_0 (s^{-1}) )</td>
<td>570</td>
<td>380</td>
</tr>
<tr>
<td>( \delta (R_2 \leftarrow A) )</td>
<td>( \delta_0 (s^{-1}) )</td>
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<td>370</td>
</tr>
<tr>
<td>( E_{R2 \rightarrow A} (0 \text{ mV}) )</td>
<td>( k_0 (s^{-1}) )</td>
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<td>6.2e5</td>
</tr>
<tr>
<td>( \gamma (R_2 \rightarrow A) )</td>
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<td>0.45</td>
</tr>
<tr>
<td>( \delta (R_2 \rightarrow A) )</td>
<td>( z_0 (s^{-1}) )</td>
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<td>0.34</td>
</tr>
<tr>
<td>( E_{A \rightarrow O} )</td>
<td>( k_0 / k_c )</td>
<td>46</td>
<td>50</td>
</tr>
</tbody>
</table>

Parameters for the fitting shown in Fig. 5. ‘WT’ and ‘H310Y’ are used for human K\(_{\text{V}}\)1.2(A291C) or K\(_{\text{V}}\)1.2(A291C,H310Y) channels, respectively, labelled with MTS-TAMRA; ∗: fixed value.
VSD transitions (R₁ ↔ R₂) predicted that deactivation (rate constant β₀) was diminished and the VSDs were stabilized in the intermediate-active state (R₂; Fig. S1, Table S2). However, it provided relatively poor fits to the data (H310Y VSD activation and channel closing kinetics; Fig. S1B). A model assuming that H310Y only affected the second VSD transitions (R₂ ↔ A; Fig. S2, Table S3) recapitulated the fit goodness and the findings of the unconstrained model. Finally, a model that only allowed for a difference in the final, opening/closing transition (C ↔ O; Fig. S3, Table S4) resulted in remarkably good fits, despite the reduction in free parameters. This model predicted that the fully active/open state is stabilized over the fully active/closed state. The differential interpretations of these results are that H310Y stabilized: (i) a final, voltage-independent, step of VSD activation; or (ii) the open state of the channel. Since the H310 side-chain points towards the core of the VSD (Figs 1B and S4A), the H310Y mutation is unlikely to directly perturb pore gating. H310Y could produce an active VSD-conformation distinct from that of wild-type channels, more efficient at stabilizing the open state. The consensus of all fits is that H310Y stabilizes late conformations on the channel activation pathway.

The tyrosine hydroxyl group is responsible for delayed Kv1.2(H310Y) closure

We sought to determine how a tyrosine at position 310 stabilized Kv1.2-channel activation. Stabilization of a protein state occurs by the formation of bonds, which need to break in order for the protein to change conformation. As the tyrosine residue possesses a hydroxyl group capable of forming hydrogen bonds, we effectively ablated it by studying H310F channels (Fig. 6A) – as part of ‘atomic mutagenesis’. Indeed, Kv1.2(H310F) closed with similar kinetics to wild-type – perhaps, even, slightly faster (Fig. 6D–F). Our interpretation of this result is that both histidine and tyrosine can stabilize the active-VSD state by forming hydrogen bonds, but the tyrosine bonds are stronger; substitution by a residue unable to form hydrogen bonds (phenylalanine) resulted in a channel

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**Figure 4.** H310Y modestly affects voltage-sensor domain (VSD) voltage dependence

A, voltage-clamp fluorometry experiments on Kv1.2(A291C) ‘WT’ or Kv1.2(A291C,H310Y) ‘H310Y’ channels. Representative traces show membrane potential (Vₘ), current (Iₘ, black) and simultaneously acquired fluorescence deflections (ΔF, magenta). The protocols probe VSD activation (top) or deactivation (bottom). B, interpretation of the ΔF signal: VSD activation causes fluorophore quenching, reported as negative ΔF. C, WT conductance (G, black filled circles): V₀.5 = 5.4 (9.4) mV, z = 2.3 (0.31) e₀, n = 10 cells; VSD activation (ΔF, magenta filled circles): V₀.5 = −50 (4.8) mV, z = 1.1 (0.13) e₀, n = 10 cells; H310Y G (grey open triangles): V₀.5 = −10 (6.0) mV, z = 2.2 (0.45) e₀, n = 12 cells; ΔF (magenta open triangles): V₀.5 = −45 (5.3) mV, z = 1.4 (0.26) e₀, n = 12 cells. Errors are SD.
that closed faster than both the H310Y mutant and the wild-type. This is consistent with protein biochemistry, as the histidine side-chain forms hydrogen bonds via its nitrogen atoms, which are weaker than those formed by the more electronegative oxygen in tyrosine.

H310F channels exhibited additional perturbations: their voltage-dependent activation was inhibited, shifting by 16 (5.4) mV to more depolarized potentials (Fig. 6C); and their opening was delayed, compared with wild-type (Fig. 6D, F). Both of these findings supported that the phenylalanine at position 310 formed a stabilizing interaction at the resting VSD state, absent or diminished in the histidine or tyrosine variants.

Finally, we sought the putative hydrogen-bond partner of Y310. In a model of wild-type Kv1.2 in the active conformation (Pantazis et al., 2020), H310 can form a hydrogen bond with serine S169, in juxtaposed transmembrane helix S1 (Fig. S4A). Serine has the same side-chain as alanine, plus a hydroxyl group, similar to tyrosine and phenylalanine. We posited that, if Y310 formed a hydrogen bond with S169, this interaction would be abolished by mutation S169A. In turn, this would destabilize the active-VSD conformation, accelerating channel closing (as in the H310F experiments). However, double-mutant Kv1.2(S169A,H310Y) channels showed evidence of increased stabilization of the active state, compared with H310Y: voltage dependence shifted to more hyperpolarized potentials (Fig. S4B, C) and channel closing was slowed down even further (Fig. S4B, D). These results meant that either S169 and Y310 do not interact; or they do interact, and Y310 associates with another hydrogen-bond partner in the absence of S169. While the H310F substitution strongly points to the tyrosine hydroxyl group as the moiety responsible for enhanced channel opening (Fig. 6) – hence hydrogen bonding – the identity of its hydrogen-bond partner is yet unknown.

Figure 5. H310Y stabilizes the active-voltage-sensor-domain (VSD) conformation
A, state-scheme mechanism of voltage-dependent Kv1.2 opening. In the inset, α, β, γ, δ are voltage-dependent rates describing VSD transitions between fully resting (R₁), intermediate-resting (R₂) and fully active (A) states. These are incorporated as closed (C)-state transitions in a channel with four VSDs, in the main scheme. Full activation of all four VSDs leads to the channel open state (O), with voltage-independent rates kₒ, kᶜ. B, fitting of current (pore opening) and fluorescence (VSD activation) datasets (black: data; red: model). Voltage protocols are as in Fig. 4. C, the rate constant determining deactivation of the VSD from the fully active state (δₒ) is substantially (fourfold) decreased in H310Y channels, increasing the equilibrium of the full-activation transition (R₂↔A). That is, H310Y stabilizes the fully active-VSD conformation. Parameters are shown in Table 1.
H310Y delays the recovery of Kv1.2 channels from slow inactivation

Channel opening is linked to the slower process of spontaneous decrease in current, termed slow, or ‘C-type’ inactivation (Suarez-Delgado et al., 2020). Investigating the effects of H310Y on Kv1.2 slow inactivation, we found that the mutation resulted in less complete inactivation, but it did not significantly affect the voltage dependence of this process (Fig. 7A, B). Recovery from slow inactivation, occurring in two phases, was slowed by H310Y, by a factor of ~5 (Fig. 7C, D). While inactivation recovery occurred at much slower timescales than channel closing (c.f. Figs 3D and 7D), it is intriguing that H310Y affected both to a similar extent, by ~fivefold.

Note that the tail currents of Kv1.2(H310Y) channels had a larger amplitude than those from wild-type channels. There was no evidence of altered ionic selectivity in H310Y channels (no differences in the reversal potential, and all depolarization-evoked currents were consistently outward with our solutions); therefore, we believe that the H310Y-channel tail currents appeared larger due to them being slower, thus better resolved, than those from wild-type channels.

H310Y abolished inactivation by Kvβ1 subunits

Kvβ1 subunits, encoded by KCNAB1, are cytosolic proteins that associate with Kv1 channels and impart inactivation (Rettig et al., 1994), i.e. a spontaneous reduction in current following channel opening, in turn shaping neuronal action potentials and modulating synaptic transmission (Cho et al., 2020). Kvβ1 subunits bound to cytosolic N-terminal regions of Kv1 channels mediated channel inactivation by a ‘ball-and-chain’

Figure 6. The tyrosine hydroxyl group is responsible for delayed Kv1.2(H310Y) closure

A, the tyrosine (Y) side-chain includes a hydroxyl group (blue) and a benzene ring (yellow). We investigated phenylalanine (F) substitution of H310, as it lacks the hydroxyl group. B, representative current traces from cells expressing human Kv1.2(H310F). The current from a pulse to −20 mV is shown in blue (c.f. Fig. 3A) C, steady-state voltage dependence of activation. H310F channels had a right-shifted voltage dependence (orange diamonds; $V_{0.5} = 13 \pm 5.4$ mV, $z = 3.2 \pm 0.63$ e, n = 18 cells) compared with WT (black) and H310Y-channels (green; c.f. Fig. 3B). D, super-imposed current traces from cells expressing wild-type (black), H310Y (green) or H310F- channels (orange) during depolarization to 60 mV. Note that H310F channels appeared to open sooner, E, as in B, with a voltage protocol to probe channel closing. The current from a pulse to −120 mV is shown in blue (c.f. Fig. 3C). F, closing and opening kinetics. H310F channels (orange diamonds; $r_{cl} = 1.2 \pm 0.89$ ms, n = 12 cells) closed faster than WT (black line) and H310Y (green line) and opened slower. Data to the left of the dashed line were generated using deactivation voltage-clamp protocols, and data to the right came from activation protocols. Errors are SD.
Disease-associated mutations in the K\textsubscript{v}1.2 voltage-sensor domain

mechanism (Sukomon et al., 2023): a part of K\textsubscript{V}1\beta, the 'ball peptide', is connected to the rest of the protein via a flexible 'chain' region. The ball peptide is thought to bind to the open channel pore, occluding it and preventing ionic flow. It is reminiscent of intramolecular inactivation caused by N-terminal ball-and-chain segments in the Drosophila Shaker K\textsubscript{V} channel (Hoshi et al., 1990) and mammalian K\textsubscript{V}1.4 (Tseng-Crank et al., 1993) – hence it is also known as 'N-type' inactivation. As this inactivation is due to an intramolecular (Shaker, K\textsubscript{V}1.4) or intramolecular-complex (K\textsubscript{V}1/\beta1) binding reaction, it typically occurs with a time-course similar to that of channel opening, so it is also known as 'fast' inactivation, in contrast to the typically much slower, C-type inactivation. Finally, residues proximal to H310, in the S4–S5 linker, have been implicated in fast inactivation (Nunoki et al., 1994). To further characterize the properties of K\textsubscript{V}1.2(H310Y) channels, we evaluated their interaction with K\textsubscript{V}1.2(H310R) subunits have impaired voltage dependence

In our previous study of non-trafficking K\textsubscript{V}1.2-variant p.F233S, we discovered that mutant subunits can be rescued by associating with wild-type K\textsubscript{V}1.2, offering a glimpse of their functional properties as parts of heteromeric 'dimers of dimers' (Nilsson et al., 2022). We did the same for K\textsubscript{V}1.2(H310R) subunits, placing them...
in dimeric concatemers with wild-type Kv1.2 (Fig. 9A). Constructs bearing one H310R subunit (i.e. two mutant subunits in a pseudotetramer; Fig. 9A) had inhibited voltage-dependent activation, shifted by ∼12 mV toward more depolarized potentials, compared with concatenated wild-type subunits (Fig. 9B, C). In cells injected with Kv1.2(H310R)-Kv1.2(H310R) cRNA, no current was observed, compared with that of uninjected cells (Fig. 9B, D), demonstrating that a wild-type partner was necessary for trafficking rescue.

While the relative position of the H310R- subunit (N- or C-terminal) had no effect on the voltage dependence of activation, the steady-state voltage dependence of activation was shifted by ∼12 mV toward more depolarized potentials compared with concatenated wild-type subunits (Fig. 9B, C).

**Figure 8.** H310Y abolished ball-and-chain inactivation by KVβ1

A, representative cut-open oocyte Vaseline gap current traces from human Kv1.2(WT) (left) or Kv1.2(H310Y) (right) channels coexpressed with the human KVβ1 subunit. KVβ1 introduced inactivation in the wild-type channels only. The holding potential was −120 mV to prevent accumulation of inactivation. B, percentage of current at the end of the 50-ms pulse (I50) over peak current (Ipeak), for Kv1.2 channels coexpressed with KVβ1: wild-type (circles, n = 16 cells) or H310Y (triangles, n = 18 cells). 100% (marked by dashed line) means no inactivation. For depolarizations to 80 mV, the percentage was 63 (11)% for wild-type and 99 (0.58)% for H310Y. Errors are SD.

**Figure 9.** Rescued Kv1.2(H310R) subunits are functionally impaired

A, experiments with concatenated dimers of Kv1.2 subunits (expected to assemble as ‘dimers of dimers’) to evaluate the functional properties of trafficking-capped hetero-tetramers containing H310R-subunits. B, representative current traces from combinations of Kv1.2 wild-type (1.2) and H310R (1.2(HR)) subunits. C, steady-state voltage dependence of activation. 1.2–1.2 (filled circles): V0.5 = −1.8 (3.2) mV, z = 2.2 (0.083) e0, n = 8 cells; 1.2–1.2(HR) (open triangles): V0.5 = 12 (4.5) mV, z = 2.8 (0.27) e0, n = 8 cells; 1.2(HR)–1.2 (open diamonds): V0.5 = 11 (4.7) mV, z = 3.2 (0.27) e0, n = 8 cells. D, the 1.2(HR)–1.2(HR) construct did not produce distinguishable current (590 (170) nA at 200 mV) from uninjected cells (520 (93) nA). E, functional expression (shown as maximal conductance, Gmax) relative to 1.2–1.2 (1.0 (0.038)); 1.2–1.2(HR): 0.16 (0.065); 1.2(HR)–1.2: 0.086 (0.059). Errors are SD.
of these constructs (Fig. 9C), it apparently affected the efficiency of trafficking rescue: constructs with a wild-type N-terminal partner, which was translated, folded, and translocated to the endoplasmic reticulum (ER) membrane first, trafficked more efficiently (Fig. 9E). Assuming that, after ER translocation and dimerization, the two constructs would be treated equivalently by the cell secretion apparatus, this result suggests that one aspect of the trafficking impediment involved ‘early’ stages in Kv1.2(H310R) biosynthesis: folding and translocation. In addition, since neither construct achieved full trafficking rescue (maximal conductance levels equal to the WT–WT construct) (Fig. 9D), where it may be compared with the equivalent data from trafficking-deficient Kv1.2 variant F233S, from Nilsson et al. (2022). Briefly, H310R appeared to cause a milder trafficking defect than F233S, as either early or late trafficking aided rescue of Kv1.2(H310R) better than Kv1.2(F233S). This result was consistent with the limited trafficking of Kv1.2(H310R) subunits in COS-7 cells (Fig. 2D, E), compared with no detectable trafficking for Kv1.2(F233S) using the same trafficking assay (Nilsson et al., 2022).

### H310R causes a milder trafficking defect than F233S

Trafficking rescue of Kv1.2(H310R) by wild-type Kv1.2 or Kv1.4 is summarized in Fig. 10D, where p.H310Y causes a dual loss of function: first, it decreases cell-surface subunit abundance (Fig. 2). Second, it increases channel activity (slowed down channel closing via stabilization of the voltage-sensor active state, Figs 3–5) and prevents inactivation by Kvβ1 subunits (Fig. 8). In contrast, p.H310R produces a dual loss of function: first, it diminishes the presence of subunits on the cell surface (Fig. 2), by disrupting both ‘early’ (folding, translocation) and ‘late’ (ER trafficking) events in biosynthesis and secretion (Figs 9 and 10). Second, it inhibits voltage-dependent opening (Figs 9 and 10). Our overall evaluation is that H310, a highly conserved amino-acid (Fig. 1), plays critical roles in channel biosynthesis and trafficking, as well as the regulation of closed and open states, and inactivation by β1. In his book Projections, Karl Deisseroth wrote: ‘Just as when a fabric frays, its hidden structural threads can be revealed (or when a bit of DNA mutates, the original functions of the damaged gene can be inferred), the broken describe the unbroken’ (Deisseroth, 2021). We posit that, by the same token, when an amino acid is substituted, the observed effects can reveal the role of its position along the polypeptide chain. Accordingly, H310, conserved in most Kv1 channels, is critical to all aspects of Kv1.2 functional expression: from early biogenesis, as a nascent string of amino-acids that folds and translocates to the ER; to its journey through the protein secretion pathway, maturing and reaching the

### Discussion

In this work, we investigated two KCNA2 variants associated with encephalopathy. p.H310R produces dual loss of function: first, it decreases cell-surface subunit abundance (Fig. 2). Second, it increases channel activity (slowed down channel closing via stabilization of the voltage-sensor active state, Figs 3–5) and prevents inactivation by Kvβ1 subunits (Fig. 8). In contrast, p.H310R produces dual loss of function: first, it diminishes the presence of subunits on the cell surface (Fig. 2), by disrupting both ‘early’ (folding, translocation) and ‘late’ (ER trafficking) events in biosynthesis and secretion (Figs 9 and 10). Second, it inhibits voltage-dependent opening (Figs 9 and 10). Our overall evaluation is that H310, a highly conserved amino-acid (Fig. 1), plays critical roles in channel biosynthesis and trafficking, as well as the regulation of closed and open states, and inactivation by β1. In his book Projections, Karl Deisseroth wrote: ‘Just as when a fabric frays, its hidden structural threads can be revealed (or when a bit of DNA mutates, the original functions of the damaged gene can be inferred), the broken describe the unbroken’ (Deisseroth, 2021). We posit that, by the same token, when an amino acid is substituted, the observed effects can reveal the role of its position along the polypeptide chain. Accordingly, H310, conserved in most Kv1 channels, is critical to all aspects of Kv1.2 functional expression: from early biogenesis, as a nascent string of amino-acids that folds and translocates to the ER; to its journey through the protein secretion pathway, maturing and reaching the

**Kv1.4 association can fully rescue Kv1.2(H310R) trafficking**

Owing to a shared tetramerization domain (Li et al., 1992), Kv1.2 and Kv1.4 subunits can combine into hetero-tetrameric channels, contributing to neuronal A currents (Bean, 2007; Coleman et al., 1999; Sheng et al., 1993). Kv1.4 subunits are highly trafficking-competent: in contrast to Kv1.2, they lack ER-retention signals and possess a forward-trafficking motif (Li et al., 2000; Manganas et al., 2001; Misonou & Trimmer, 2004). Thus, Kv1.4 subunits increase Kv1.2 trafficking, both wild-type (Manganas & Trimmer, 2000) and a trafficking-impaired variant (Nilsson et al., 2022). Accordingly, we attempted to fully rescue Kv1.2(H310R)-subunit trafficking by combining them with Kv1.4 in dimeric concatamers.

Indeed, the construct where Kv1.4 subunits were N-terminal achieved full trafficking rescue, producing the same maximal conductance as the construct with wild-type Kv1.2 (Fig. 10A, B). This result supported the interpretation that the reduced macroscopic current observed with Kv1.2 concatamers containing H310R subunits (Fig. 9E) was due to diminished trafficking, rather than reduced channel conductance. As previously (Fig. 9E and Nilsson et al., 2022), the construct where the Kv1.2(H310R)-partner was N-terminal achieved less trafficking rescue (Fig. 10A, B). As previously observed in the Kv1.2(F233S) variant (Nilsson et al., 2022), combining mutant Kv1.2 subunits with Kv1.4 did not rescue their impaired voltage dependence (Fig. 10A, C). Finally, when Kv1.4 subunits were N-terminal, the constructs exhibited fast inactivation (Fig. 10A), due to the Kv1.4 N-terminal inactivation particle (Tseng-Crank et al., 1993). Yet, when the Kv1.4 partner was C-terminal, no fast inactivation was observed (Fig. 10A), likely because the inactivation particle was tethered by the dimer linker sequence, as observed previously (Nilsson et al., 2022). These data do not necessarily provide information on whether mutation H310R can affect N-type inactivation, because the Kv1.4 inactivation peptide could act on the wild-type Kv1.4 concatenated subunits.
cell surface; to its function as a delayed rectifier, shaping neuronal excitability in concert with its close and distant KvV-paralogs, and associated β-subunits.

Several lines of inquiry stem from our work about the two variants and KvV channels in general. (1) It is not known whether the mutations affect single-channel conductance and maximal open probability – factors which could also affect macroscopic conductance. This can be tackled by single-channel analysis. (2) The precise mechanism of H310Y stabilization of the channel active/open state is still missing. This can be elucidated from the dwell-time distributions in single-channel recordings. In addition, identification of the Y310 interacting partner can be achieved, for example, by molecular dynamics simulations and subsequent functional studies. (3) The dual aspect of gain- and loss of function produced by H310Y and H310R, respectively, hints at a cellular mechanism that favours the trafficking of readily activatable channels, and disfavours that of channels with inhibited voltage dependence. We posit that this mechanism evaluates the fitness of the voltage-sensor domain, perhaps sampling its conformation. Likely, proteomic studies of ion-channel trafficking can identify chaperone candidates and further elaborate the ‘early’ and ‘late’ checkpoints of KvV-channel biogenesis. (4) VSD activation has been suggested to be involved in both slow (C-type) and fast (N-type, ball-and-chain) inactivation (Olcese et al., 1997; Roux et al., 1998). H310Y, a VSD mutation affecting all three processes, is an excellent tool to further study their relationship. (5) The consequences of the two variants on neuronal excitability remain to be investigated, and are discussed below.

KCNA2 encephalopathy may be caused by highly diverse effects on channel voltage dependence (Doring et al., 2021; Masnada et al., 2017; Pantazis et al., 2020; Syrbe et al., 2015). We previously discussed how loss of Kv1.2 function could produce an imbalance of excitation and inhibition favouring seizure generation (Nilsson et al., 2022; Pantazis et al., 2020). Briefly, loss of Kv1.2 would result in broadened action potentials in the axons of

Figure 10. Kv1.4 can fully rescue Kv1.2(H310R) trafficking
A, representative currents from combinations of human ‘1.2’ or ‘1.2(HR)’ subunits (see Fig. 8) with rat Kv1.4 (‘1.4’), which grant expedited endoplasmic reticulum (ER) trafficking (Li et al., 2000; Manganas et al., 2001; Misonou & Trimmer, 2004; Nilsson et al., 2022). Note that the Kv1.4 inactivation particle (blue filled circles) can only inactivate the heteromers when it is N-terminal. B, N-terminal Kv1.4 completely rescued Kv1.2(H310R), shown as relative maximal conductance (Gmax): 1.4–1.2: 1.0 (0.58); 1.4–1.2(HR): 0.95 (0.59); 1.2–1.4: 1.0 (0.61); 1.2(HR)–1.4: 0.32 (0.22). C, voltage-dependence curves show that H310R-containing heteromers had a ~12 mV V0.5 shift. 1.4–1.2 (filled circles): V0.5 = −2.8 (4.5) mV, z = 1.6 (0.49) e0, n = 8 cells; 1.4–1.2(HR) (open triangles): V0.5 = 11 (2.4) mV, z = 1.5 (0.22) e0, n = 8 cells; 1.2–1.4 (filled circles): V0.5 = −12 (2.7) mV, z = 2.0 (0.15) e0, n = 8 cells; 1.2(HR)–1.4 (open diamonds): V0.5 = 2.7 (4.9) mV, z = 2.1 (0.19) e0, n = 8 cells. Errors are SD. D, summary of studies with concatemers, for trafficking-deficient Kv1.2 variants H310R (Figs 9E and 10B; blue) and F233S (data from Nilsson et al. (2022); orange): (i) for both variants, both ‘early’ (trafficking-competent N-terminal partner) and ‘late’ (ER expedited trafficking) aids are necessary and sufficient for full trafficking rescue; (ii) in the case of H310R, late trafficking impediments are more severe than early; (iii) in the case of H310R, early and late trafficking impediments are additive. Errors are 95% C.I.
Disease-associated mutations in the Kv1.2 voltage-sensor domain

The remarkable conservation of the S4 C-terminal histidine (Fig. 1) suggests that mutations in this position are likely to cause disease. Seven single, missense mutations can occur at the histidine codon, converting it to codons for arginine (R), asparagine (N), aspartate (D), glutamine (Q), leucine (L), proline (P) and tyrosine (Y). To date, virtually all of these substitutions, with the exceptions of L and Q, have been associated with disease, when they occur at the Kv1.2 H310 position (p.H310Y; p.H310R, ClinVar #975835; p.H310P, #1505535; p.H310D, #1801703) and homologous positions in Kv1.2 (KCNA1: p.H313Y, #451654; p.H313R, #384177) and Kv8.2 (KCNV2: p.H393N, #1425846). Several disease-associated variants have also been reported for KvV-channel subunits where the homologous residue is not a histidine: Kv7.2 (KCNQ2: p.R214W (Castaldo et al., 2002), #7385; p.R214Q, #578032; p.R214P, #373698), Kv10.1 (hEAG1, KCNH1: p.K367N, #1493753), Kv11.1 (hERG, KCNH2: p.K538N, #67333; K538R, #200395; p.K538E, #359311). These variants occur in potassium-selective channels expressed in both neurons and cardiac myocytes, so they are likely associated with both neurological and cardiac diseases. Taken together with the multiple roles of this amino acid in channel functional expression, shown here for Kv1.2, it appears that mutations at this position are poorly tolerated. We anticipate that genetic screening of patients with neurological or cardiac diseases of electrical excitability (‘excitopathies’) is bound to reveal more variants at this position.

Ion-channel variants can cause disease by various mechanisms (Cannon, 2007), and their role in epilepsy is increasingly recognized (Macnee et al., 2023). Novel technologies, such as the advent of human induced-pluripotent-stem-cell-derived neurons (Simkin et al., 2022), deep-mutation studies in ion channels (Coyote-Maestas et al., 2022; Macdonald et al., 2023) and genetic tools to tune the expression of pathogenic channel variants (Kanner et al., 2017, 2020) are especially promising for studying and treating currently intractable genetic epilepsies. The mechanistic biophysical evaluation of clinically relevant variants (i) reveals novel insights into ion-channel biogenesis and function; (ii) provides tools for the study of ion-channel properties; and (iii) is essential for the appropriate classification of variants, stratification of patients, and ultimately choice of the most efficacious therapy.

References


### Additional information

#### Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### Competing interests

E.A.A. is a founder and stockholder of Personalis Inc, Deepcell Inc and SVEXA. He is an adviser to Pacific Biosciences, SequenceBio, Nuevocor and Foresite Labs and a non-executive director for AstraZeneca.

#### Author contributions

T.M.-V., V.P., K.W., S.H.L. and S.P. and A.P. performed the experiments and analysed the data. A.P. designed research. K.W., S.H.L. and A.P. contributed experimental materials; S.A.S., E.S., D.A.S. and E.A.A. supervised genetic screening and contributed clinical information for the p.H310R variant. C.G. supervised the genetic screening and contributed clinical information for the p.H310Y variant. T.M.-V. and A.P. wrote the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Supporting information
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