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1 **Monoallelic *KCNB2* variants lead to a neurodevelopmental**
2 **disorder caused by altered channel inactivation.**

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51 **Running title:** Pathogenic KCNB2 variants affect K⁺ channel activation and inactivation.

52 **Keywords:** Dysmorphism, global developmental delay, epilepsy, KCNB2, channel inactivation

53 **Abbreviations:** Kv: voltage gated potassium channels, AP: action potential, cRNA:
54 complementary RNA, GV: conductance-voltage, IV: current-voltage, Inac-V: Inactivation-
55 voltage, POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPE: 1-palmitoyl-2-oleoyl-
56 sn-glycero-3-phosphoethanolamine, DPSM: stearyl-sphingomyelin, POPS: 1-palmitoyl-2-
57 oleoyl-sn-glycero-3-phospho-L-serine, POPI: 1-palmitoyl-2-oleoyl-sn-glycero-3-
58 phosphoinositol, WT – wild type.

59 **Abstract**

60 Ion channels mediate voltage fluxes or action potentials that are central to the functioning of
61 excitable cells such as neurons. The *KCNB* family of voltage-gated potassium channels (Kv)
62 consists of two members (*KCNB1* and *KCNB2* genes) that encode KCNB1 and KCNB2 channels,
63 respectively. These channels are major contributors to delayed rectifier potassium currents arising
64 from the neuronal soma which modulate overall excitability of neurons. In this study, we identified
65 several monoallelic pathogenic missense variants in the *KCNB2* gene, in individuals with
66 neurodevelopmental disorders and other neurological conditions such as epilepsy and autism.
67 Recurrent dysmorphisms included a broad forehead, synophrys and digital anomalies.
68 Additionally, we selected three variants where genetic transmission has not been assessed, from
69 two epilepsy studies. We characterized channel properties of these variants by expressing them in
70 oocytes of *Xenopus laevis* and conducting cut-open oocyte voltage clamp electrophysiology. Our
71 datasets indicate no significant change in absolute conductance and conductance-voltage
72 relationships of most disease variants as compared to wild type (WT), when expressed either alone
73 or co-expressed with WT-KCNB2 (except for c.1141A>G, (p.Thr381Ala) and c.641C>T,
74 (p.Thr214Met), which show complete abrogation of currents when expressed alone with the
75 former exhibiting a left shift in activation midpoint when expressed alone or with WT-KCNB2).
76 These variants, however, show collective features of increased inactivation shifted to
77 hyperpolarized potentials. We suggest that the effects of the variants on channel inactivation may
78 contribute to hyper-excitability of neurons, which leads to disease onset.

79 **Introduction**

80 The *shab*-related *KCNB* sub-family of voltage-gated potassium (Kv) channels consists of two
81 genes *KCNB1* [MIM 600397] and *KCNB2* [MIM 608164] that encode KCNB1 (Kv2.1) and
82 KCNB2 (Kv2.2) channels, respectively¹. KCNB1 is well documented as being ubiquitously
83 expressed in several brain regions². Characterization of KCNB2 expression in the brain, in
84 comparison, is less defined due to discrepancies in KCNB2 cloning, and concurrent antibodies
85 used in different studies³. Early studies professed mutual exclusivity of subcellular distribution of
86 KCNB1 and KCNB2 in both principal and inhibitory neurons co-expressing them and by extension
87 their roles in controlling neuronal excitability; KCNB1 is restricted to large clusters in the proximal
88 dendrites and soma of neurons^{4,5}, while KCNB2 is diffusely localized in neuronal dendrites⁶⁻⁸.
89 However, recent reports have detailed KCNB2 expression to localise similarly to KCNB1 in
90 cortical neurons although other neurons may express high levels of KCNB1 or KCNB2 but not
91 both^{3,9,10}. In addition to the cortical expression in the central nervous system, KCNB2 has also
92 been identified in the medial nucleus of the trapezoid body¹¹, the basal forebrain^{12,13} and the spinal
93 cord¹⁴. Single-cell RNAseq data show a high expression in excitatory neurons as well as various
94 types of interneurons (Allen Brain Map, Human MTG 10x SEA-AD dataset).

95 KCNB1 and KCNB2, like other Kvs, are outward rectifiers, i.e., they conduct K⁺ from the cytosol
96 to the extracellular space and provide repolarizing currents that return a depolarized neuron back
97 to the resting state. These channels activate and inactivate slowly compared to the depolarizing
98 sodium currents. Activation is achieved at suprathreshold voltage during an action potential (AP).
99 Such slow activation and inactivation kinetics prolong the duration of KCNB-mediated K⁺
100 conductance; these properties are instrumental to their regulation of repolarization and

101 hyperpolarization phases of an action potential^{15,16}. Consequently, KCNBs help determine
102 interspike interval, AP amplitude and AP firing fidelity during high frequency firing^{11,17-20}.
103 Assigning these effects to neuronal excitability on homotetrameric complexes of either KCNB1 or
104 KCNB2 is a simplified view. KCNB1 and KCNB2 have been shown to form heterotetrameric
105 complexes both *in vitro* and *in vivo*^{3,9}. In addition, KCNB channels co-assemble with the
106 electrically silent KvS channels and auxiliary β -subunits to form heterotetrameric protein
107 complexes that display drastically different biophysical and pharmacological properties as
108 compared to their homotetrameric counterparts^{21,22}. In addition to their role in mediating K⁺
109 conductance, KCNB1 and KCNB2 also exist as non-conducting clusters caused by the formation
110 of ER/plasma membrane junctions that have numerous functions such as inter-organelle
111 communication and calcium signalling, to name a few².

112 Over 29 distinct pathogenic variants in *KCNB1* gene that either truncate or alter the protein
113 sequence of the KCNB1 channels have been identified in individuals suffering from early onset
114 developmental and epileptic encephalopathies [MIM 616056]^{23,24}. The *KCNB1* variants that were
115 functionally characterized were shown in non-native systems to exhibit a multitude of effects on
116 channel activity such as abolished channel function, reduced current density, deficits in voltage
117 sensing, loss in ion selectivity and gain of inward cation conductance²⁴⁻²⁷. Expression of some of
118 these variants in cortical neurons led to reduced repetitive firing properties²⁶. Of note, *KCNB1* KO
119 (*KCNB1*^{-/-}) mice have preserved brain anatomy, and do not exhibit spontaneous epileptic seizures,
120 or visual or motor impairment²⁸. Hippocampal slices of these mice, however, exhibit drug-induced
121 hyperexcitability and stimulation-induced epileptiform activity. Interestingly, homozygous mice
122 expressing a *KCNB1* variant (p.G379R) developed spontaneous seizures as well as proconvulsant-

123 and handling-induced seizures along with being hyperactive, impulsive and having reduced
124 anxiety²⁹.

125 In this study, we identified several variants in the *KCNB2* gene in individuals with
126 neurodevelopmental disorders that has not been associated with a Mendelian genetic disorder in
127 humans in OMIM previously. Most exhibited developmental delays while some also had epilepsy,
128 ADHD, and autism. In addition, we screened the Epi25K dataset and another epilepsy cohort and
129 identified three additional candidate variants. We performed electrophysiological characterization
130 of these variants in oocytes from *Xenopus laevis*. Our data suggests that most *KCNB2* variants
131 show common features of increased channel inactivation with the voltage dependence shifted to
132 hyperpolarized potentials. Based on these observations, we hypothesize that the effects of the
133 variants on channel inactivation may contribute to reduced KCNB2 availability, leading to hyper-
134 excitability of neurons and to disease onset.

135

136 **Materials and Methods**

137 **Clinical and genetic investigations**

138 Variants were identified by trio sequencing of probands. Exome sequencing methods have been
139 described elsewhere (see following references for individuals 1³⁰, 2³¹, 3³², 4³³, 5³⁴, 6³⁵ and 7³⁶. The
140 cohort was assembled with the help of Matchmaker Exchange platform tools³⁷. All clinical
141 information is shared in accordance with local institutional ethical review boards and is in
142 accordance with the ethical standards of the responsible committee on human experimentation
143 (institutional and national) and proper informed consent. A consent for the publication of the
144 photographs included here was obtained from parents or legal guardians.

145 **Molecular Biology and Channel Expression.**

146 Oocytes from *Xenopus laevis* were surgically obtained as described elsewhere³⁸. The human
147 KCNB2 (NM_004770.3) cDNA in pcDNA3.1(+) N-terminus HA tag was purchase at Genescript
148 (Clone ID: OHu25595C). All variants were introduced in KCNB2 construct using the QuikChange
149 Lightning site-directed mutagenesis kit (Agilent Technologies, USA) and were subcloned in
150 pcDNA3.1 containing no tag using EcoR1/XhoI restriction sites. The variants and primers
151 (rev/fwd) are listed in Table S1. To generate high quality and high copy number plasmids, the
152 plasmids were amplified using the CopyCutter EPI400 competent bacteria (Lucigen) to decrease
153 insert toxicity and avoid non-desired additional mutations. cRNA was generated by linearizing the
154 plasmids with a restriction enzyme (*DraIII*) and using the plasmid template for *in vitro*
155 transcription using the mMESSAGING mMACHINE™ T7 ULTRA Transcription Kit (Thermo
156 Fisher Scientific, U.S.A.). For functional expression of the KCNB2 channels, either 1 ng of WT

157 or individual mutant cRNA or 0.5 ng each of WT and one of the mutant cRNA was injected into
158 oocytes and incubated for 12-24 h at 18°C to allow for channel expression.

159 **Electrophysiology**

160 Voltage clamping experiments were performed with a CA-1B amplifier (Dagan Corporation,
161 U.S.A.). Currents were recorded in the cut-open oocyte voltage-clamp configuration³⁹. The
162 external solution used for ionic current recordings contained (in mM): 5 KOH, 110 N-methyl-D-
163 glucamine (NMDG), 10 HEPES, and 2 Ca(OH)₂, pH adjusted to 7.1 with methanesulfonic acid
164 (MES). The internal solution contained (in mM): 115 KOH, 10 HEPES, and 2 EDTA, pH adjusted
165 to 7.1 with MES. The oocytes were placed in a three-part chamber (upper, middle and bottom)
166 containing the external solution. Oocyte membrane exposed in the bottom chamber was
167 permeabilized with 0.2% saponin in internal solution for 30s-1 min for direct current injection into
168 the oocyte. Saponin was washed out and bottom chamber filled with internal solution.
169 Conductance and inactivation of KCNB2 variants were recorded using the protocols illustrated in
170 Fig. 3 and Fig. 5. Both conductance-voltage relation (GV) and Inactivation-voltage relation (Inac-
171 V) was fit to a sum of two Boltzmann relation of the form $G/G_{max} = \text{Minimum} + (\text{Amplitude1} -$
172 $\text{Minimum}) / (1 + \exp((V50(1) - X) / k1)) + (\text{Amplitude2} - \text{Amplitude1}) / (1 + \exp((V50(2) - X) / k2))$ and
173 $I/I_{max} = \text{Maximum} + (\text{Amplitude1} - \text{Maximum}) / (1 + \exp((V50(1) - X) / k1)) + (\text{Amplitude2} -$
174 $\text{Amplitude1}) / (1 + \exp((V50(2) - X) / k2))$, respectively. The decision to use this fit was based on its
175 fidelity and does not necessarily model the underlying processes. Reversal potential for KCNB2
176 variants were determined using the deactivation protocol illustrated in Fig.4. The external and
177 internal solutions used for these recordings are described in Fig.4. The concomitant current-voltage
178 relation was fit to a straight line (linear regression) between -90 and -50 mV; the x-intercept was

179 tabulated as the reversal potential. All data was acquired using the AnalysisX2 software
180 (Département de physique, Université de Montréal, Canada) and analyzed and compiled using
181 MatlabR2022a (The MathWorks, Inc., U.S.A.). Data shown are mean \pm SD with $n \geq 5$ from at
182 least two independent injections.

183 **Molecular dynamics**

184 We created an *in silico* homology model of full-length KCNB2 based on the known structure of
185 the Kv1.2/2.1 chimera⁴⁰. The model was generated using alphafold2⁴¹. The variants discussed here
186 in the manuscript were introduced into the homology structure and the channels set into an *in silico*
187 membrane containing: outer leaflet: POPC:POPE:DPSM in a molar ratio of 59:9:32 and inner
188 leaflet: POPC:POPE:POPS:DPSM:POPI in a molar ratio of 25:38:16:14:7. The system was set in
189 water containing 150mM KCl at a temperature of 300K using charmm-gui⁴²⁻⁴⁴. The different
190 mutant channels as well as the wildtype channel were equilibrated and simulated *in silico* for 100
191 ns using NAMD⁴⁵.

192

193 **Results**

194 **Clinical characteristics**

195 The clinical phenotypes of the six individuals with *de novo* or dominantly transmitted variants in
196 *KCNB2* are outlined in Table 1. Five variants were *de novo*, and one was inherited from a
197 symptomatic father. Proband 7 inherited the variant from an unaffected mosaic father. The age at
198 last evaluation ranged from 21 months of life to 18 years. Individuals were born at term and had
199 normal birth growth parameters. Growth parameters, at last visit, were within the normal range in
200 all individuals.

201 All seven individuals presented with global developmental delay. Six were diagnosed with
202 intellectual disability. Three individuals presented with mild autistic traits while two were too
203 young to make an autistic spectrum disorder (ASD) diagnosis. Two individuals were medicated
204 with Levetiracetam for seizures. Two individuals had hypotonia including one with ataxic cerebral
205 palsy. One individual was diagnosed with attention deficit/hyperactivity disorder (ADHD).

206 Five individuals presented with various facial dysmorphisms (Fig.1). Synophrys was observed in
207 two individuals. A broad forehead was noted in two individuals. Hand anomalies were described
208 in four individuals with one presenting with clinodactyly and another with nail hypoplasia. Mild
209 blepharoptosis, beaked nose, flat mid face, frontal bossing, full lower lip, tongue protrusion and
210 high palate were noted in one individual each.

211

212 Ophthalmological anomalies were found in four individuals. One individual presented with
213 cortical vision impairment, Duane syndrome, hyperopia, astigmatism, and cataracts. Delayed
214 visual maturation, myopia or severe strabismus were found in the other three individuals.

215 Two individuals had heart anomalies, notably one with aortic dilation and one with an abnormal
216 trabeculation of the left ventricular myocardium. Two individuals had genitourinary
217 malformations: one with a neurogenic bladder and the other with a suggestion of a slight shawl
218 scrotum.

219 One individual was diagnosed with diabetes, gingival fibromatosis, low bone density, and
220 oropharyngeal dysphagia. That individual (Proband 1 1, with the c.1141A>G, (p.Thr381Ala)
221 variant) seemed to have more extensive involvement than the others (Supplemental Note: Case
222 Reports), and her phenotype overlapped partially with Zimmerman-Laband (ZLS, [MIMs 135500,
223 616455, 618658]) and DOORS [MIM 220500] syndromes, which are neurodevelopmental
224 disorders with epilepsy (treated with Levetiracetam) and hypoplasia of the terminal phalanges and
225 nails. Of relevance, some of us have previously reported pathogenic variants in potassium channels
226 KCNH1 [MIM 603305] and KCNN3 [MIM 602983] in ZLS [MIMs 135500, 618658]^{46,47}, and
227 vacuolar ATPase subunit ATP6V1B2 [MIM 606939] in ZLS [MIM 616455] and DOORS
228 syndrome^{46,48}.

229 To identify additional candidate variants, we searched epilepsy study data. A missense variant
230 (c.724G>A, (p.Ala242Thr)) was identified in an individual with Sudden Unexpected Death in
231 Epilepsy (SUDEP), from a SUDEP study⁴⁹. Additionally, c.472A>G, (p.Thr158Ala) was
232 identified in two individuals with NAFE (non-acquired focal epilepsy, [MIM 604364, 245570]),
233 and c.1124C>T, (p.Ala375Val) in one individual with GGE (genetic generalized epilepsy, [MIM

234 600669]), all from the Epi25 exome study variant server⁵⁰. The variants were selected as they were
235 absent in gnomAD and affected highly conserved residues. Additional clinical details or parental
236 samples for segregation of the variants were not available to us, thus there is more uncertainty
237 (compared to the 6 first variants identified) regarding the involvement of the variants in the
238 neurological phenotypes of the individuals.

239 Using ACMG criteria⁵¹ through the Varsome Classifier⁵², all variants were predicted to be
240 pathogenic or likely pathogenic (see criteria used in Table S2). The gnomAD missense tolerance
241 score⁵³ for KCNB2 is relatively high with a Z score of 2.25 (range -5 to 5) since there were 511
242 expected missense variants but only 368 were observed. We have assessed the tolerance of each
243 affected amino acid to missense variants using Metadome⁵⁴, and all amino acids are intolerant to
244 missense variants, with scores in table S2 and the tolerance landscape of the proteins in figure S1.
245 Additionally, table S2 shows the pathogenicity prediction and conservation scores obtained from
246 Ensembl's Variant Effect Predictor for various commonly used tools⁵⁵.

247 **Effect of *KCNB2* Variants on Functional Expression and Activation Kinetics**

248 The topology of a KCNB2 monomer, like that of other Kv channels, consists of a N-terminus,
249 hexahelical transmembrane domain including a pore-helix (P-loop ion selectivity filter) and a C-
250 terminus (Fig. 2A). Most of the amino acids mutated in the *KCNB2* variants characterized in this
251 study are highly conserved in homologous proteins across different species (Fig. 2B) and in human
252 KCNB1 (not shown). The exception is amino acid position 646 (hKCNB2), which exists as alanine
253 in some species (including humans) and co-incidentally as valine in others. 3D protein structures
254 of KCNB2 and distribution of the variants characterised in this study are shown in tetrameric (top

255 view, Fig. 2C) and monomeric configuration (side view, Fig. 2D) based on the homology model
256 of the known structure of the Kv1.2/2.1 chimera⁴⁰.

257 To assess biophysical properties and functional expression of the KCNB2 mutants, the cRNAs
258 generated from the plasmids encoding the individual mutants were injected into oocytes of
259 *Xenopus laevis* either alone (1 ng) or in equal amounts with WT-KCNB2 (0.5 ng each). 16-24
260 hours post-injection, the currents were studied using the cut-open oocyte voltage clamp technique.
261 To record absolute amplitudes of K⁺ conductance, the channels were held at -90 mV followed by
262 a depolarising pulse to 100 mV for 200 ms (Fig. 3A). Most variants, when expressed either alone
263 or with WT, did not show a significant change in current amplitudes when compared to WT.
264 Exceptions are the c.641C>T, (p.Thr214Met) and c.1141A>G, (p.Thr381Ala) variants; these
265 mutants showed complete and strong abrogation of K⁺ conductance, respectively (Fig. 3B). K⁺
266 conductance was rescued when the mutants were co-expressed with WT-KCNB2 (Fig. 3D). The
267 c.641C>T, (p.Thr214Met) variant, when co-expressed with WT-KCNB2, and the c.994T>G,
268 (p.Tyr332Asp) variant, when expressed alone, show significant reduction in current amplitudes as
269 compared to WT-KCNB2 (Fig. 3B and 3D, Table 2).

270 We next characterised the activation properties of the *KCNB2* variants by analyzing their
271 conductance-voltage (GV) relationship. Conductance can be inferred from the tabulation of the
272 isochronal current amplitudes at the beginning of the -20 mV step in Fig. 3A. The representative
273 raw traces of the corresponding currents are presented from an oocyte that either expresses the
274 individual variants alone (top) or co-expresses both the individual variant and WT (below). The
275 c.641C>T, (p.Thr214Met) variant was excluded from the GV analyses due to lack of any activation
276 currents by this mutant. The GV datasets (Fig. 3C and 3E) were best fitted by a sum of two

277 Boltzmann distribution that generated two activation midpoints ($V_{50(1)}$ and $V_{50(2)}$) and two
278 corresponding slope factors (k_1 and k_2 , respectively). These parameters are compiled in Table 2.
279 Of all variants, GV profiles of c.1141A>G, (p.Thr381Ala) and c.994T>G, (p.Tyr332Asp) channels
280 showed significant differences when compared to those of WT. However, while c.1141A>G,
281 (p.Thr381Ala) channels showed a ~ 20 mV shift to hyperpolarized potentials in activation $V_{50(1)}$
282 compared to WT, c.994T>G, (p.Tyr332Asp) was shifted by 5 mV to depolarized potentials. The
283 other mutants did not show significant differences compared to WT, although higher k_1 values
284 were observed for the c.911G>A, (p.Arg304Gln) variant. In affected individuals, the variants
285 would be expressed with WT-KCNB2 in a heterozygous manner. We therefore tested the effect
286 on co-expression. While the effects were similar, the voltage dependence and slope of activation
287 was attenuated in the heteromeric as opposed to homomeric expression. This is indicative of
288 difference in activation properties of c.911G>A, (p.Arg304Gln), c.994T>G, (p.Tyr332Asp) and
289 c.1141A>G, (p.Thr381Ala) as compared to WT, but it is notable that the effects were not
290 conserved throughout the mutants nor were the effects consistent among the variants.

291 ***KCNB2* variants show no effect on reversal potential of the channel.**

292 We next investigated if the mutations in *KCNB2* affect the reversal potential of the channel. To
293 do this, we looked at currents evoked by the deactivation protocol illustrated in Fig.4A under
294 conditions of high external $\text{NMDG}^+/\text{Na}^+$ and low K^+ and high internal K^+ and low $\text{NMDG}^+/\text{Na}^+$.
295 The deactivation protocol involves channel opening of the variants at +50 mV followed by the
296 deactivation of the channel at different voltages ranging from +50 mV to -120 mV. The raw traces
297 shown in Fig.4B are representative of an oocyte expressing WT-*KCNB2*. The ensuing current
298 voltage relationship from this protocol leads to the determination of the reversal potential of the

299 channel, representative of the voltage with no net current. As shown in Fig. 4C and D, the reversal
300 potential of all *KCNB2* variants lies between -60 to -80 mV (Table S3), which is closer to the
301 equilibrium potential of K⁺. These results indicate that potassium conductivity by the variants is
302 unaffected in the presence of Na⁺ or NMDG⁺ both extra- and intracellularly.

303 ***KCNB2* variants show greater extent of inactivation when compared to WT-** 304 ***KCNB2***

305 Many Kv channels undergo inactivation either during subthreshold depolarization (referred to as
306 closed state inactivation) or during suprathreshold membrane depolarization (referred to as open-
307 state inactivation)⁵⁶. *Shab*-related KCNB channels are characterised by slow inactivation that
308 strongly influences duration of action potentials during repetitive high frequency firing of different
309 neuronal subsets. To assess voltage dependence and extent of this inactivation, we employed the
310 protocol illustrated in Fig. 5A; the bold lines in the protocol correspond to the raw traces shown in
311 Fig. 5B and C when the variants are expressed either alone or with WT-KCNB2, respectively.
312 Both WT-KCNB2 and the variants exhibit slow inactivation as described previously^{17,57} during
313 sustained depolarizations at +40 mV for up to 20 s.

314 We next assessed and compared voltage-dependence of inactivation of WT-KCNB2 and the
315 variants. All *KCNB2* variants exhibit U-shaped inactivation profiles (Fig. 5D and E) as described
316 previously^{56,58}. Such profiles are indicative of increased inactivation at negative potentials, which
317 is overcome by channel opening at more depolarized potentials because of increased open
318 probability. To determine the mean free energy required to trigger closed state inactivation, we
319 fitted the data between -150 mV to 10 mV with the sum of two Boltzmann's equations. As
320 compared to WT, most-variants, when co-expressed with WT, show a shift to hyperpolarized

321 potentials of the inactivation characteristics (Fig.5E and Table 3). These effects are even more
322 pronounced when the variants are expressed in the absence of WT, indicating these amino acid
323 substitutions contribute to voltage dependency of inactivation (Fig.5D and Table 3). Most variants
324 also showed an increase in the slope factors of the Boltzmann relations k_1 and k_2 (Table 3),
325 especially c.911G>A, (p.Arg304Gln). The slope factors k can be written as ratio of thermal energy
326 and electrical energy per Volt (RT/zF with R: universal gas constant, T: temperature, z the
327 apparent gating charge and F the Faraday constant), indicating that, in these variants, the apparent
328 electrical charge z driving inactivation was reduced. By comparing the extent of inactivation
329 exhibited by the variants with respect to WT (Fig. 5F), most variants showed increased inactivation
330 with the exception of c.1141A>G, (p.Thr381Ala). c.994T>G, (p.Tyr332Asp), when expressed
331 alone, showed the greatest extent of inactivation (~35% more inactivation as WT, Fig.5F). It was
332 remarkable, however, that this effect disappeared in the presence of WT-KCNB2.

333 Similar to the activation kinetics, where c.1141A>G, (p.Thr381Ala) showed a distinct phenotype
334 with fast inactivation at high depolarizations, this variant showed a behaviour distinct to the other
335 variants that we characterized. c.1141A>G, (p.Thr381Ala) opens transiently followed by quick
336 inactivation to a new baseline (Fig. 3A). To check if this variant persists in this inactivated state,
337 we kept the channel open for longer time with our inactivation protocol. As shown in the raw traces
338 represented in Fig. 5G, oocytes expressing WT-KCNB2 (black trace) showed increasing
339 inactivation with time whereas oocytes expressing the c.1141A>G, (p.Thr381Ala) variant did not
340 show inactivation in addition to the rapid inactivation of the open state (blue trace, Fig. 5G, zoomed
341 in Fig.5H) in the 20s post opening of the channel. In contrast, c.1141A>G, (p.Thr381Ala) increases
342 in current indicating that channels are recovered from inactivation (Fig. 5I, *cf.* dashed WT black
343 fit from Fig. 5D and E to c.1141A>G, (p.Thr381Ala) blue data points). The data suggests that

344 there are two types of inactivation in the c.1141A>G, (p.Thr381Ala) mutant, closed state
345 inactivation – typical for KCNB2 – and a very rapid open state inactivation. Because of the rapid
346 open state inactivation, we cannot clearly determine how many channels are expressed, which
347 explains the low currents observed in Fig. 3A. The rapid inactivation is abolished when co-
348 expressed with WT-KCNB2. The heteromers exhibited activation amplitudes (Fig. 3D) and extent
349 of inactivation (Fig. 5F) similar to those expressing WT alone (Tables 2 and 3).

350 In contrast to activation and functional expression, that resulted in variable phenotypes among the
351 variants, an increased inactivation, both in voltage dependence and extent, was common to all
352 variants studied in this work. This communality emphasizes the importance of channel inactivation
353 for the development of the disease.

354 **Discussion**

355 Human homologues of the *Drosophila shab* family are the KCNB family of voltage-gated
356 potassium channels that contain two known members: KCNB1 and KCNB2. Variants in the
357 *KCNB1* gene have been identified in individuals suffering from early onset developmental and
358 epileptic encephalopathies²³. In this study, we identified several variants in the *KCNB2* gene
359 clinically and from the Epi25 and other epilepsy cohorts. This study is the first to identify a
360 channelopathy due to genetic alterations in *KCNB2*. The variants identified clinically are in
361 children and are mostly *de novo* (except for c.1937C>T, (p.Ala646Val) which is inherited).
362 Individuals harboring these variants exhibit a wide array of neurological disorders. Most
363 individuals exhibit delays in either global development, motor milestones or speech/language.
364 These individuals also displayed intellectual disabilities and different dysmorphisms. Dysmorphic
365 facial features were variable across individuals, with synophrys and broad forehead being most

366 common. Functional characterization of the KCNB2 variants revealed a unifying feature: all
367 variants have reduced KCNB2 channel function achieved either by reduced functional expression
368 (c.641C>T, (p.Thr214Met), c.994T>G, (p.Tyr332Asp) and c.1141A>G, (p.Thr381Ala)) and by
369 increased extent of inactivation occurring at more hyperpolarized potentials compared to WT. We,
370 therefore, provide a compelling etiological basis for the onset of the neurological disorders in
371 individuals with mutations in KCNB2.

372 KCNB channels are delayed rectifying channels, i.e., the channels activate and conduct K⁺ under
373 depolarized membrane potentials and undergo slow inactivation. Activation and inactivation of
374 KCNB channels, like most Kv channels, are regulated by two mechanisms governed by structural
375 rearrangements of the protein referred to as “gating”: activation gating and inactivation gating⁵⁹.
376 Activation gating occurs when voltage sensing S1-S4 domains of Kv channels sense membrane
377 depolarization and undergo conformational changes. These structural changes are communicated
378 to the pore domain by electromechanical coupling that leads to pore opening and channel
379 conductance⁶⁰. Inactivation gating involves structural transitions of Kv channels that act as
380 intrinsic negative feedback to inhibit channel conductance and thereby availability, leading to
381 modulation of cellular excitability. Kv2 channels exhibit U-type inactivation arising from pre-open
382 activated but non-conductive channel states⁵⁸. Such inactivation profiles entail a lower degree of
383 inactivation at more depolarized potentials (hence the U-shape of voltage dependence).

384 One of the variants with the most severe disease phenotype is the **c.1141A>G, (p.Thr381Ala)**.
385 The individual (Proband 1, Fig. 1 and Table 1) harboring this variant suffers from global
386 developmental delay with intellectual disability, seizures, and diabetes (for more information,
387 please refer to Supplemental Note: Case Reports). Electrophysiological properties of the variant

388 corroborate with the severity of the disease. When expressed alone, the current amplitude of the
389 c.1141A>G, (p.Thr381Ala) variant is ~10% of that of WT (Fig. 3B and Fig. 5G) and is rescued
390 when this variant is co-expressed with WT (Fig. 3D). Significant changes in activation midpoint
391 of this variant are seen when expressed either alone or with WT (~20 and 5 mV shift in $V_{50(1)}$ to
392 hyperpolarized potentials, respectively) and slope factor (increase in k_1) is observed (Fig. 3C and
393 E and Table 2). As mentioned above, normalised current-voltage relationship of inactivation,
394 displayed in Fig. 5I, shows an increased recovery from inactivation with increasing voltage (cf.
395 blue line for c.1141A>G, (p.Thr381Ala) and dashed black fit for WT). This inactivation profile is
396 very similar to other channels that express the equivalent T→A variant. c.1141A>G,
397 (p.Thr381Ala), present in the channel selectivity filter, is the conserved fourth residue of the
398 TXXTXGYG signature sequence present in all K^+ channels⁶¹. The hydroxyl group of this
399 threonine contributes to one of the four K^+ binding sites in potassium channels⁶². Variants of
400 p.Thr381 equivalent positions in other Kv channels, including the closely related KCNB1, does
401 not produce drastic loss of K^+ conductance as seen with KCNB2^{61,63,64}. When the equivalent
402 threonine in bacterial KcsA channel (T75) was mutated to a glycine, the rate of inactivation was
403 slower by ~2 fold⁶². With a substitution to an alanine (identical to c.1141A>G, (p.Thr381Ala) in
404 KCNB2 in this study), KcsA, Kv1.5 [MIM 176267] and Shaker channels all show a loss in C-type
405 inactivation, indicating the importance of p.Thr381 equivalent threonine in these channels in
406 allosteric coupling of the activation gate and the selectivity filter⁶⁴. When Coonen *et al.* modified
407 the equivalent residue to an alanine in KCNB1 (p.Thr377Ala) and Kv3.1 [MIM 176258]
408 (p.Thr400Ala), the channel variants were resistant to inactivation thereby losing their U-shape
409 profile as shown by their WT counterparts⁶³. We also observe similar reduced inactivation of
410 WT:p.Thr381Ala channels (Fig.5E, cyan trace). Given the involvement of potassium channels in

411 insulin secretion and the presence of diabetes in other potassium channelopathies (ATP-sensitive
412 ones)⁶⁵, it is interesting to note that the individual harboring the c.1141A>G, (p.Thr381Ala)
413 mutation was diagnosed with childhood onset diabetes but was not found to have pancreas islet
414 cell autoantibodies. It remains to be determined if diabetes is strongly associated with KCNB2
415 variants once a larger cohort is established. Previous studies have shown that KCNB2 is expressed
416 in human δ cells of the pancreatic islets⁶⁶. Pharmacological inhibition of KCNB2 currents increase
417 action potential duration and amplitude in these cells⁶⁷ that leads to augmented somatostatin
418 secretion and powerful inhibition of insulin secretion from pancreatic β cells⁶⁸. The lack of
419 functional expression of the KCNB2 c.1141A>G, (p.Thr381Ala) could potentially mimic KCNB2
420 inhibition that leads to somatostatin-induced paracrine blocking of insulin secretion and onset of
421 diabetes in the individual harboring this mutation.

422 The affected individual harboring the **c.641C>T, (p.Thr214Met)** mutation (Proband 4, Fig. 1 and
423 Table 1) exhibited delayed language milestones along with mild autistic traits in infancy, myopia
424 and synophrys. p.Thr214 is present in the S1-S2 linker; this linker (by virtue of its length) has been
425 shown in KCNA2 [MIM 176262] to be essential for N-glycosylation that influences their
426 functioning, proper folding and trafficking to cell surface^{69,70}. However, the S1-S2 linker
427 glycosylation is not conserved in all Kv channels. The threonine present in position 214 in KCNB2
428 is conserved in all human Kv channels⁷¹. The c.641C>T, (p.Thr214Met) variant, when expressed
429 alone, leads to complete abrogation of K^+ conductance (Fig. 3A and B). The lack of functional
430 expression is rescued when the c.641C>T, (p.Thr214Met) variant is co-expressed with WT, albeit
431 significantly lower than WT (Fig. 3D, Table 2). Such observations have also been reported in other
432 Kv channels. Synthetic mutations in KCNA4 [MIM 176266] and KCNC1 [MIM 176258] and
433 disease relevant mutations in KCNB1 of equivalent threonine residues caused intracellular

434 retention of the protein and little to no channel currents⁷¹⁻⁷³. An alanine mutation to the equivalent
435 threonine in KCNQ2 [MIM 602235] and KCNQ3 [MIM 602232] also yielded reduced potassium
436 currents with drastic effects on voltage dependence and kinetics of activation and kinetics of
437 deactivation⁷⁴.

438 The individual harboring the **c.994T>G, (p.Tyr332Asp)** (Proband 5, Fig. 1, Table 1 and
439 Supplemental Notes: Case Reports) mutation in KCNB2 exhibited global development delay with
440 intellectual disabilities and facial dysmorphisms. The c.994T>G, (p.Tyr332Asp) mutation occurs
441 in the S4-S5 linker of KCNB2. The S4-S5 linker provides electromechanical coupling between the
442 voltage sensing and pore domains that leads to voltage gating of Kv channel function. The S4-S5
443 linker is very dynamic; this flexible region present on the intracellular side undergoes
444 conformational rearrangements not only during gating and late gating processes, but also during
445 inactivation of some Kv channels⁷⁵⁻⁷⁸. Interestingly, this tyrosine present at the end of S4-S5 linker
446 is conserved only in KCNB1 and KCNB2. These channels undergo slow inactivation as compared
447 to other inactivating Kv channels. The c.994T>G, (p.Tyr332Asp) variant, when expressed alone
448 or with WT, show minor effects on the coupling between the voltage sensor and pore domains;
449 this is evident by the shift in the voltage dependence of conductance to hyperpolarized potentials
450 in this variant compared to WT (Fig. 3 and Table 2). However, the major effect on the c.994T>G,
451 (p.Tyr332Asp) mutation is the extent of inactivation observed in this variant (Fig.5D and 5F),
452 which is the strongest among the mutants studied here (apart from maybe c.1141A>G,
453 (p.Thr381Ala)). This effect on inactivation, however, is lost on co-expression of this variant with
454 WT (Fig. 5E and 5F). This observation indicates a hitherto unappreciated role of the tyrosine
455 residue at positions 328 and 332 respectively in KCNB1 and KCNB2 on the electromechanical
456 coupling of their activation and in the inactivation of these channels.

457 **c.911G>A, (p.Arg304Gln)**, present in the S4 of KCNB2, is one of many positively charged
458 arginine and lysine residues present in the S4 that mediate sensitivity of the ion channel to voltage
459 fluxes; movement of these positive charges in S4 during membrane depolarization cause
460 conformational changes that lead to the opening of the central channel pore^{79,80}. Variants of
461 arginine residues within the S4 of Kv and several other ion channels have been described
462 associated with different channelopathies^{23,80,81}. Likewise, the individual harboring the c.911G>A,
463 (p.Arg304Gln) mutation in KCNB2 (Proband 6, Fig. 1, Table 1 and Supplemental Notes: Case
464 Reports) exhibited delayed motor, speech and language milestones along with autism spectrum
465 disorder and ADHD. c.911G>A, (p.Arg304Gln) has significant effect on the slope factor k1 of
466 channel activation (Table 2), in the absence or presence of WT, consistent with a reduction of the
467 apparent gating charge in the S4 voltage sensor. This variant, either when expressed alone or with
468 WT, unsurprisingly show significant and drastic effects of the voltage dependence and extent of
469 inactivation (Fig. 5D-F, Table 3).

470 One of the variants identified in this study is the **c.1937C>T, (p.Ala646Val)** mutation in the C-
471 terminus of KCNB2. The individual harboring this mutation in KCNB2 (Proband 3, Fig. 1, Table
472 1 and Supplemental Notes: Case Reports) displayed onset of regression/neurodegenerative disease
473 along with delayed motor, speech and language milestones. The c.1937C>T, (p.Ala646Val)
474 variant, when co-expressed with WT or alone, increased inactivation and altered voltage
475 dependence of inactivation (Fig. 5F, Table 3). The **c.281G>A, (p.Gly94Glu)** mutation was
476 identified in an individual (Proband 2, Fig. 1, Table 1 and Supplemental Notes: Case Reports) with
477 developmental and speech delay along with hypotonia and ataxia cerebral palsy. The other N-
478 terminus variant is the c.472A>G, (p.Thr158Ala) identified in two individuals with non-acquired
479 focal epilepsy from the Epi25k exome study. Both the N-terminus variants show increase in extent

480 of inactivation, especially when expressed with WT (Fig. 5F). There is no precedence on the role
481 of the N-terminus on the inactivation of KCNB2. Interestingly, previous work on exchanging N-
482 terminus of KCNB2 with that of Kv4.2 [KCND2, [MIM 605410]] accelerated inactivation of the
483 KCNB2 chimera⁸². p.Gly94 is a glutamate at equivalent positions in Kv4.2 channels, just as one
484 of our variants (c.281G>A, (p.Gly94Glu)). Detailed investigation on the role of both N and C-
485 termini on KCNB2 channel activity is therefore warranted.

486 **p.Ala375**, like p.Thr381, is present in the pore domain and is located at the pore-helix preceding
487 the selectivity filter of KCNB2. Variant of this residue to Valine (c.1124C>T, p.Ala375Val) in
488 KCNB2, both in the absence or presence of WT, significantly enhances the extent of inactivation
489 exhibited by these channels (Fig. 5D-F). This variant was identified in an individual with genetic
490 generalized epilepsy from the Epi25 exome study. p.Ala375 is conserved in other Kv channels
491 such as KCNB1, Kv1.5 and Shaker at equivalent positions, but not in KcsA, Kv4s and hERG
492 channels. Introducing mutations at p.Ala375 equivalent positions in hERG-1 channel
493 (p.Thr618Ala in Kv11.1/*KCNH2* [MIM 152427]) and Kv1.5 (p.Ala473Thr in *KCNA5*, [MIM
494 176267]) alters voltage dependence of inactivation in these channels⁸³, suggesting the importance
495 of p.Ala375 and analogous regions in the onset of inactivation in numerous Kv channels.

496 In addition, we characterised the **c.724G>A, (p.Ala242Thr)** variant identified in a an individual
497 with Sudden Unexpected Death in Epilepsy (Leu et al., 2015). p.Ala242 in KCNB2 is present in
498 the S2 interspersed between two negatively charged glutamates at position 237 and 247. These
499 glutamates in KCNB2 and equivalent positions in other ion channels are thought to interact with
500 positive charge amino acids in the S4 voltage sensor during resting and activated channel states⁷⁹.
501 We speculate that amino acid substitutions of p.Ala242 ought to affect the voltage sensing of

502 KCNB2. This is evident in the significant changes in slope factors of the voltage-dependence of
503 inactivation in KCNB2-p.Ala242Thr (with or without WT, Table 3) although no changes were
504 observed in activation-voltage relationship of this variant as compared to WT (Fig. 2B-E, Table
505 2). Of note, p.Ala242 is conserved in KCNB1 but is replaced by other non-polar aliphatic amino
506 acids in other Kv channels such as Shaker or Kv1.5 (Isoleucine at equivalent positions) or Kv4s
507 (Leucine/Methionine).

508 Finally, we identified a seventh individual (Proband 7, Fig. 1, Table 1 and Supplemental Notes:
509 Case Reports) harboring a c.827C>T, (p.Pro276Leu) mutation during the revision of this
510 manuscript. It is inherited from an unaffected mosaic father, with the variant present in 10% of the
511 reads in blood DNA.. The patient exhibited global developmental delay with moderate intellectual
512 disability, refractory epilepsy and some behavioral issues. He is being treated with multiple anti-
513 seizure medications (for more information, please refer to Supplemental Note: Case Reports).
514 Brain MRI of this patient showed prominent perivascular spaces.

515 **Conclusions**

516 We report variants in *KCNB2* that are associated with a range of neurological disorders including
517 autism and epilepsy. We show strong evidence that the *de novo* *KCNB2* variants cause
518 neurodevelopmental disorders and that these variants either significantly i) reduce the currents
519 generated by these Kv channels, or ii) shift the voltage dependence of inactivation to
520 hyperpolarized membranes and increase the extent of inactivation as compared to WT.
521 inactivation, in general, is a cumulative effect that is most impactful when trains of stimulations
522 do not allow for recovery from inactivation before the next stimulus⁵⁸. The effect is a reduction of
523 the available functional *KCNB2* channels that shapes the duration and interspike intervals of action

524 potentials, leading to changes in cellular excitability in neurons that express these variants. Further
525 experiments in native systems are warranted to corroborate this hypothesis, which provides the
526 underlying etiological basis of how KCNB2 dysfunction causes disease. Despite variation in the
527 associated diseases, it is remarkable that all variants had a common underlying phenotype on the
528 molecular level.

529 **Declaration of interests**

530 The authors declare no competing interests.

531 **Data and code availability**

532 The published article includes all datasets generated or analyzed during this study, apart from
533 exome sequencing data which are not publicly available due to privacy restrictions. All
534 experimental data will be freely available upon request from the corresponding authors.

535

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550

551 **Author contributions**

552 The study was designed by S.B., J.R., R.B and P.M.C. The individuals were recruited by C.M.L.,
553 J.M.S., R.J.L., K.C., A.L., D.C.K., S.C.R, S.M.S., E.M.M.M.H.V.K., K.K., P.M.V.H., F.D., C.D.,
554 B.R.S., A.A., M.S., E.S.T., S.S.H., I.T. and P.M.C. The disease manifestations were summarized
555 and compared by C.M. and P.M.C. The experiments were performed or supervised by S.B., J.R.,
556 R.B and P.M.C. The data was analyzed by S.B., J.R., R.B and P.M.C. The draft was written by
557 S.B., J.R., C.M., R.B and P.M.C. All authors contributed to revising the manuscript.

558 **Web resources**

559 gnomAD population database, <https://gnomad.broadinstitute.org/>

560 Epi25 exome server: <https://epi25.broadinstitute.org/>

561

563 **Tables**564 **Table 1. Main clinical features of the affected and phenotyped individuals.**

Proband	1	2	3	4	5	6	7
<i>KCNB2</i> variant (NM_004770.3)	c.1141A>G, (p.Thr381Ala)	c.281G>A, (p.Gly94Glu)	c.1937C>T, (p.Ala646Val)	c.641C>T, (p.Thr214Met)	c.994T>G, (p.Tyr332Asp)	c.911G>A, (p.Arg304Gln)	c.827C>T, (p.Pro276Leu)
Inheritance	<i>de novo</i>	<i>de novo</i>	Inherited	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	Mosaic ^a
Sex	Female	Male	Female	Male	Female	Male	Male
Age at last assessment	5 years	2.5 years	3 years	18 years	21 months	9 years	15 years
Medical history							
Brain anomalies (MRI results)	+	N/A	-	N/A	+	N/A	-
Cardiac anomalies	+	+	-	-	N/A	N/A	-
Urogenital anomalies	+	+	-	-	-	-	-
Ophthalmological anomalies	+	+	-	+	+	N/A	-
Growth and Development							
DD	+	+	+	+	+ ^b	+	+
ID	+	N/A	+	+	+	+	+
Autistic features	-	N/A ^c	-	mild	N/A ^c	mild	mild
Epilepsy	+ ^d	-	-	-	-	-	+ ^e
Dysmorphisms							
Facial	+	+	+	-	+	+	-
Hand	-	+	+	-	+	+	-
Synophrys	-	+	-	+	-	-	-
Other features							
Hypotonia	+	+ ^f	-	-	-	-	-
ADHD	-	-	-	N/A	-	+	-
Abbreviations are as follows: N/A: not available, DD: developmental delay, ID: Intellectual disability, ADHD: Attention-Deficit / Hyperactivity Disorder, Mosaic ^a - Individual 7 and his father exhibited genetic mosaicism, + ^b - severe, N/A ^c - patient too young to make an autistic spectrum disorder (ASD) diagnosis, + ^d - seizure effectively treated with Levetiracetam, + ^e - multiple anti-seizure medication (refer to supplemental information), + ^f - Hypotonia with Ataxic cerebral palsy. Please refer to supplemental data for detailed case report for each patient.							

565

566 **Table 2. Activation properties of Kv2.2 variants**

Variants	Without WT						With WT					
	Current Amplitudes (± S.D.) [μ A]	V ₅₀₍₁₎ (mV)	k ₁	V ₅₀₍₂₎ (mV)	k ₂		Current Amplitudes (± S.D.) [μ A]	V ₅₀₍₁₎ (mV)	k ₁	V ₅₀₍₂₎ (mV)	k ₂	
WT	47.7 ± 14.2	-1.9 ± 5.4	6.7 ± 0.9	24.4 ± 5.9	12.3 ± 2.1		47.7 ± 14.2	-1.9 ± 5.4	6.7 ± 0.9	24.4 ± 5.9	12.3 ± 2.1	
p.Gly94 Glu	45.8 ± 13.2	-1.6 ± 5.8	6.7 ± 0.6	24.5 ± 4.4	12.3 ± 1.7		46.5 ± 17.7	-1.4 ± 5.2	6.7 ± 0.8	26.1 ± 10.7	12.3 ± 2.4	
p.Thr15 8Ala	44.8 ± 14.7	-5.6 ± 5.9	6.7 ± 0.8	20.4 ± 6.9	12.1 ± 2.0		39.1 ± 12.6	-2.2 ± 5.7	6.8 ± 0.6	26.3 ± 8.2	13.8 ± 2.3	
p.Thr21 4Met	2.1 ± 2.6***	n.a.					31.8 ± 7.9*	5.0 ± 2.9	7.5 ± 0.6	34.6 ± 4.8*	13.5 ± 1.6	
p.Ala24 2Thr	37.7 ± 10.3	-6.6 ± 3.5	6.7 ± 0.5	18.8 ± 5.6	12.3 ± 1.4		44.5 ± 11.6	-3.9 ± 5.4	6.6 ± 0.5	21.8 ± 7.6	11.8 ± 1.9	
p.Arg30 4Gln	40.6 ± 11.8	-1.2 ± 3.0	8.2 ± 0.9**	26.6 ± 5.0	13.4 ± 2.1		35.8 ± 6.9	0.8 ± 3.9	7.7 ± 0.4*	27.4 ± 5.2	12.9 ± 1.0	
p.Tyr33 2Asp	25.5 ± 5.9**	7.6 ± 3.2**	7.1 ± 0.6	36.4 ± 5.2**	13.9 ± 1.6		48.5 ± 5.7	4.4 ± 2.8	7.0 ± 1.1	30.3 ± 5.3	10.8 ± 1.8	
p.Ala37 5Val	39.9 ± 5.2	-6.4 ± 4.4	7.5 ± 0.9	25.7 ± 6.4	14.0 ± 2.9		43.1 ± 10.8	-3.3 ± 6.2	7.2 ± 0.9	26.5 ± 9.2	13.8 ± 2.2	
p.Thr38 1Ala	7.9 ± 3.7***	-22.7 ± 5.9***	14.3 ± 0.9***	59.1 ± 6.4***	14.0 ± 3.6		44.1 ± 12.4	-7.3 ± 1.6*	10.3 ± 1.3***	26.1 ± 9.1	12.0 ± 4.9	
p.Ala64 6Val	44.2 ± 10.0	-3.3 ± 2.3	6.7 ± 0.6	22.7 ± 3.3	13.3 ± 1.7		42.4 ± 12.9	1.5 ± 5.1	6.2 ± 0.7	27.8 ± 5.5	10.9 ± 1.8	

567 * p<0.05, **p<0.01, *** p<0.001 via Kruskal Wallis one-way ANOVA with post hoc correction
568 for multiple comparisons with Dunn's post-hoc test.

569 n.a. - not analysed; the amplitudes were too low to determine activation properties.

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573

574 **Table 3. Inactivation properties of Kv2.2 variants**

Variants	Without WT				With WT			
	V ₅₀₍₁₎ (mV)	k ₁	V ₅₀₍₂₎ (mV)	k ₂	V ₅₀₍₁₎ (mV)	k ₁	V ₅₀₍₂₎ (mV)	k ₂
WT	-51.3 ± 11.1	11.6 ± 5.0	-19.9 ± 4.0	6.5 ± 1.6	-51.3 ± 11.1	11.6 ± 5.0	-19.9 ± 4.0	6.5 ± 1.6
p.Gly94Glu	-46.9 ± 10.83	10.7 ± 4.9	-23.5 ± 4.7	5.1 ± 0.8	-41.3 ± 13.4	9.8 ± 5.0	-24.0 ± 4.0	4.5 ± 0.9
p.Thr158Ala	-58.7 ± 10.3	11.9 ± 3.4	-29.5 ± 4.4*	5.7 ± 1.7	-59.1 ± 10.9	13.5 ± 3.7	-29.2 ± 9.5	5.5 ± 0.8
p.Thr214Met	n.a.				-34.6 ± 5.0	7.0 ± 2.1	-18.5 ± 0.8	4.7 ± 1.1
p.Ala242Thr	-59.3 ± 5.9	18.1 ± 1.8*	-30.4 ± 2.2*	6.4 ± 0.5	-57.1 ± 16.1	15.4 ± 2.7	-25.3 ± 3.8	6.2 ± 1.4
p.Arg304Gln	-82.9 ± 12.8**	14.6 ± 2.5	-37.8 ± 4.5***	7.1 ± 1.5	-67.4 ± 11.3	18.8 ± 2.1*	-27.6 ± 3.1	6.8 ± 0.5
p.Tyr332Asp	-34.1 ± 11.9	6.5 ± 2.5	-16.8 ± 4.9	4.3 ± 1.0*	-23.0 ± 3.6*	5.3 ± 2.1	-11.0 ± 0.9	4.0 ± 0.9*
p.Ala375Val	-52.1 ± 9.4	9.7 ± 3.2	-34.0 ± 4.2***	3.9 ± 1.0**	-54.3 ± 10.3	15.0 ± 5.2	-29.8 ± 4.3*	6.0 ± 1.6
p.Thr381Ala	n.a.				-60.0 ± 11.5	12.1 ± 3.4	-30.4 ± 7.2*	6.1 ± 1.1
p.Ala646Val	-64.7 ± 14.2	16.2 ± 3.9	-29.8 ± 6.7	6.2 ± 0.8	-61.3 ± 9.0	13.3 ± 2.2	-28.3 ± 3.0	6.0 ± 0.8

575

576 * p<0.05, **p<0.01, *** p<0.001 via Kruskal Wallis one-way ANOVA with post hoc correction
 577 for multiple comparisons with Dunn's post-hoc test.

578

579 n.a. - not analysed; the amplitudes were too low in p.Thr214Met and recovery from inactivation
 580 was seen in p.Thr381Ala that prevented determination of their inactivation properties.

581 References

- 582 1. Misonou, H., and Trimmer, J.S. (2004). Determinants of voltage-gated potassium channel
583 surface expression and localization in Mammalian neurons. *Crit Rev Biochem Mol Biol*
584 *39*, 125-145. 10.1080/10409230490475417.
- 585 2. Johnson, B., Leek, A.N., and Tamkun, M.M. (2019). Kv2 channels create endoplasmic
586 reticulum / plasma membrane junctions: a brief history of Kv2 channel subcellular
587 localization. *Channels (Austin)* *13*, 88-101. 10.1080/19336950.2019.1568824.
- 588 3. Kihira, Y., Hermanstynne, T.O., and Misonou, H. (2010). Formation of heteromeric Kv2
589 channels in mammalian brain neurons. *J Biol Chem* *285*, 15048-15055.
590 10.1074/jbc.M109.074260.
- 591 4. Trimmer, J.S. (1991). Immunological identification and characterization of a delayed
592 rectifier K⁺ channel polypeptide in rat brain. *Proc Natl Acad Sci U S A* *88*, 10764-10768.
593 10.1073/pnas.88.23.10764.
- 594 5. Murakoshi, H., and Trimmer, J.S. (1999). Identification of the Kv2.1 K⁺ channel as a major
595 component of the delayed rectifier K⁺ current in rat hippocampal neurons. *J Neurosci* *19*,
596 1728-1735. 10.1523/JNEUROSCI.19-05-01728.1999.
- 597 6. Hwang, P.M., Fotuhi, M., Bredt, D.S., Cunningham, A.M., and Snyder, S.H. (1993).
598 Contrasting immunohistochemical localizations in rat brain of two novel K⁺ channels of
599 the Shab subfamily. *J Neurosci* *13*, 1569-1576. 10.1523/JNEUROSCI.13-04-01569.1993.
- 600 7. Guan, D., Tkatch, T., Surmeier, D.J., Armstrong, W.E., and Foehring, R.C. (2007). Kv2
601 subunits underlie slowly inactivating potassium current in rat neocortical pyramidal
602 neurons. *J Physiol* *581*, 941-960. 10.1113/jphysiol.2007.128454.
- 603 8. Lim, S.T., Antonucci, D.E., Scannevin, R.H., and Trimmer, J.S. (2000). A novel targeting
604 signal for proximal clustering of the Kv2.1 K⁺ channel in hippocampal neurons. *Neuron*
605 *25*, 385-397. 10.1016/s0896-6273(00)80902-2.
- 606 9. Bishop, H.I., Guan, D., Bocksteins, E., Parajuli, L.K., Murray, K.D., Cobb, M.M.,
607 Misonou, H., Zito, K., Foehring, R.C., and Trimmer, J.S. (2015). Distinct Cell- and Layer-
608 Specific Expression Patterns and Independent Regulation of Kv2 Channel Subtypes in
609 Cortical Pyramidal Neurons. *J Neurosci* *35*, 14922-14942. 10.1523/JNEUROSCI.1897-
610 15.2015.
- 611 10. Trimmer, J.S. (2015). Subcellular localization of K⁺ channels in mammalian brain
612 neurons: remarkable precision in the midst of extraordinary complexity. *Neuron* *85*, 238-
613 256. 10.1016/j.neuron.2014.12.042.
- 614 11. Johnston, J., Griffin, S.J., Baker, C., Skrzypiec, A., Chernova, T., and Forsythe, I.D.
615 (2008). Initial segment Kv2.2 channels mediate a slow delayed rectifier and maintain high
616 frequency action potential firing in medial nucleus of the trapezoid body neurons. *J Physiol*
617 *586*, 3493-3509. 10.1113/jphysiol.2008.153734.
- 618 12. Hermanstynne, T.O., Kihira, Y., Misono, K., Deitchler, A., Yanagawa, Y., and Misonou, H.
619 (2010). Immunolocalization of the voltage-gated potassium channel Kv2.2 in GABAergic
620 neurons in the basal forebrain of rats and mice. *J Comp Neurol* *518*, 4298-4310.
621 10.1002/cne.22457.
- 622 13. Hermanstynne, T.O., Subedi, K., Le, W.W., Hoffman, G.E., Meredith, A.L., Mong, J.A.,
623 and Misonou, H. (2013). Kv2.2: a novel molecular target to study the role of basal forebrain
624 GABAergic neurons in the sleep-wake cycle. *Sleep* *36*, 1839-1848. 10.5665/sleep.3212.

- 625 14. Regnier, G., Bocksteins, E., Van de Vijver, G., Snyders, D.J., and van Bogaert, P.P. (2016).
626 The contribution of Kv2.2-mediated currents decreases during the postnatal development
627 of mouse dorsal root ganglion neurons. *Physiol Rep* 4. 10.14814/phy2.12731.
- 628 15. Johnston, J., Forsythe, I.D., and Kopp-Scheinflug, C. (2010). Going native: voltage-gated
629 potassium channels controlling neuronal excitability. *J Physiol* 588, 3187-3200.
630 10.1113/jphysiol.2010.191973.
- 631 16. Tsantoulas, C., and McMahon, S.B. (2014). Opening paths to novel analgesics: the role of
632 potassium channels in chronic pain. *Trends Neurosci* 37, 146-158.
633 10.1016/j.tins.2013.12.002.
- 634 17. Du, J., Haak, L.L., Phillips-Tansey, E., Russell, J.T., and McBain, C.J. (2000). Frequency-
635 dependent regulation of rat hippocampal somato-dendritic excitability by the K⁺ channel
636 subunit Kv2.1. *J Physiol* 522 Pt 1, 19-31. 10.1111/j.1469-7793.2000.t01-2-00019.xm.
- 637 18. Malin, S.A., and Nerbonne, J.M. (2002). Delayed rectifier K⁺ currents, IK, are encoded by
638 Kv2 alpha-subunits and regulate tonic firing in mammalian sympathetic neurons. *J*
639 *Neurosci* 22, 10094-10105. 10.1523/JNEUROSCI.22-23-10094.2002.
- 640 19. Mohapatra, D.P., Misonou, H., Pan, S.J., Held, J.E., Surmeier, D.J., and Trimmer, J.S.
641 (2009). Regulation of intrinsic excitability in hippocampal neurons by activity-dependent
642 modulation of the KV2.1 potassium channel. *Channels (Austin)* 3, 46-56.
643 10.4161/chan.3.1.7655.
- 644 20. Tsantoulas, C., Zhu, L., Yip, P., Grist, J., Michael, G.J., and McMahon, S.B. (2014). Kv2
645 dysfunction after peripheral axotomy enhances sensory neuron responsiveness to sustained
646 input. *Exp Neurol* 251, 115-126. 10.1016/j.expneurol.2013.11.011.
- 647 21. Bocksteins, E. (2016). Kv5, Kv6, Kv8, and Kv9 subunits: No simple silent bystanders. *J*
648 *Gen Physiol* 147, 105-125. 10.1085/jgp.201511507.
- 649 22. Pongs, O., and Schwarz, J.R. (2010). Ancillary subunits associated with voltage-dependent
650 K⁺ channels. *Physiol Rev* 90, 755-796. 10.1152/physrev.00020.2009.
- 651 23. Bar, C., Barcia, G., Jennesson, M., Le Guyader, G., Schneider, A., Mignot, C., Lesca, G.,
652 Breuillard, D., Montomoli, M., Keren, B., et al. (2020). Expanding the genetic and
653 phenotypic relevance of KCNB1 variants in developmental and epileptic encephalopathies:
654 27 new patients and overview of the literature. *Hum Mutat* 41, 69-80.
655 10.1002/humu.23915.
- 656 24. Torkamani, A., Bersell, K., Jorge, B.S., Bjork, R.L., Jr., Friedman, J.R., Bloss, C.S., Cohen,
657 J., Gupta, S., Naidu, S., Vanoye, C.G., et al. (2014). De novo KCNB1 mutations in epileptic
658 encephalopathy. *Ann Neurol* 76, 529-540. 10.1002/ana.24263.
- 659 25. Calhoun, J.D., Vanoye, C.G., Kok, F., George, A.L., Jr., and Kearney, J.A. (2017).
660 Characterization of a KCNB1 variant associated with autism, intellectual disability, and
661 epilepsy. *Neurol Genet* 3, e198. 10.1212/NXG.0000000000000198.
- 662 26. Saito, H., Akita, T., Tohyama, J., Goldberg-Stern, H., Kobayashi, Y., Cohen, R., Kato,
663 M., Ohba, C., Miyatake, S., Tsurusaki, Y., et al. (2015). De novo KCNB1 mutations in
664 infantile epilepsy inhibit repetitive neuronal firing. *Sci Rep* 5, 15199. 10.1038/srep15199.
- 665 27. Thiffault, I., Specca, D.J., Austin, D.C., Cobb, M.M., Eum, K.S., Safina, N.P., Grote, L.,
666 Farrow, E.G., Miller, N., Soden, S., et al. (2015). A novel epileptic encephalopathy
667 mutation in KCNB1 disrupts Kv2.1 ion selectivity, expression, and localization. *J Gen*
668 *Physiol* 146, 399-410. 10.1085/jgp.201511444.
- 669 28. Specca, D.J., Ogata, G., Mandikian, D., Bishop, H.I., Wiler, S.W., Eum, K., Wenzel, H.J.,
670 Doisy, E.T., Matt, L., Campi, K.L., et al. (2014). Deletion of the Kv2.1 delayed rectifier

- 671 potassium channel leads to neuronal and behavioral hyperexcitability. *Genes Brain Behav*
672 *13*, 394-408. 10.1111/gbb.12120.
- 673 29. Hawkins, N.A., Misra, S.N., Jurado, M., Kang, S.K., Vierra, N.C., Nguyen, K., Wren, L.,
674 George, A.L., Jr., Trimmer, J.S., and Kearney, J.A. (2021). Epilepsy and neurobehavioral
675 abnormalities in mice with a dominant-negative KCNB1 pathogenic variant. *Neurobiol Dis*
676 *147*, 105141. 10.1016/j.nbd.2020.105141.
- 677 30. Campeau, P.M., Kasperaviciute, D., Lu, J.T., Burrage, L.C., Kim, C., Hori, M., Powell,
678 B.R., Stewart, F., Felix, T.M., van den Ende, J., et al. (2014). The genetic basis of DOORS
679 syndrome: an exome-sequencing study. *Lancet Neurol* *13*, 44-58. 10.1016/S1474-
680 4422(13)70265-5.
- 681 31. Hunter, J.M., Massingham, L.J., Manickam, K., Bartholomew, D., Williamson, R.K.,
682 Schwab, J.L., Marhabaie, M., Siemon, A., de Los Reyes, E., Reshmi, S.C., et al. (2022).
683 Inherited and de novo variants extend the etiology of TAOK1-associated
684 neurodevelopmental disorder. *Cold Spring Harb Mol Case Stud* *8*. 10.1101/mcs.a006180.
- 685 32. Cohen, A.S.A., Farrow, E.G., Abdelmoity, A.T., Alaimo, J.T., Amudhavalli, S.M.,
686 Anderson, J.T., Bansal, L., Bartik, L., Baybayan, P., Belden, B., et al. (2022). Genomic
687 answers for children: Dynamic analyses of >1000 pediatric rare disease genomes. *Genet*
688 *Med* *24*, 1336-1348. 10.1016/j.gim.2022.02.007.
- 689 33. Chevarin, M., Duffourd, Y., R, A.B., Moutton, S., Lecoquierre, F., Daoud, F., Kuentz, P.,
690 Cabret, C., Thevenon, J., Gautier, E., et al. (2020). Excess of de novo variants in genes
691 involved in chromatin remodelling in patients with marfanoid habitus and intellectual
692 disability. *J Med Genet* *57*, 466-474. 10.1136/jmedgenet-2019-106425.
- 693 34. Schwantje, M., de Sain-van der Velden, M., Jans, J., van Gassen, K., Dorrepaal, C., Koop,
694 K., and Visser, G. (2019). Genetic defect of the sodium-dependent multivitamin
695 transporter: A treatable disease, mimicking biotinidase deficiency. *JIMD Rep* *48*, 11-14.
696 10.1002/jmd2.12040.
- 697 35. Stolerman, E.S., Francisco, E., Stallworth, J.L., Jones, J.R., Monaghan, K.G., Keller-
698 Ramey, J., Person, R., Wentzensen, I.M., McWalter, K., Keren, B., et al. (2019). Genetic
699 variants in the KDM6B gene are associated with neurodevelopmental delays and
700 dysmorphic features. *Am J Med Genet A* *179*, 1276-1286. 10.1002/ajmg.a.61173.
- 701 36. Hamilton, A., Tetreault, M., Dyment, D.A., Zou, R., Kernohan, K., Geraghty, M.T.,
702 Consortium, F.C., Care4Rare Canada, C., Hartley, T., and Boycott, K.M. (2016).
703 Concordance between whole-exome sequencing and clinical Sanger sequencing:
704 implications for patient care. *Mol Genet Genomic Med* *4*, 504-512. 10.1002/mgg3.223.
- 705 37. Boycott, K.M., Azzariti, D.R., Hamosh, A., and Rehm, H.L. (2022). Seven years since the
706 launch of the Matchmaker Exchange: The evolution of genomic matchmaking. *Hum Mutat*
707 *43*, 659-667. 10.1002/humu.24373.
- 708 38. Bossi, E., Fabbrini, M.S., and Ceriotti, A. (2007). Exogenous protein expression in
709 *Xenopus* oocytes: basic procedures. *Methods Mol Biol* *375*, 107-131. 10.1007/978-1-
710 59745-388-2_6.
- 711 39. Stefani, E., and Bezanilla, F. (1998). Cut-open oocyte voltage-clamp technique. *Methods*
712 *Enzymol* *293*, 300-318. 10.1016/s0076-6879(98)93020-8.
- 713 40. Long, S.B., Tao, X., Campbell, E.B., and MacKinnon, R. (2007). Atomic structure of a
714 voltage-dependent K⁺ channel in a lipid membrane-like environment. *Nature* *450*, 376-
715 382. 10.1038/nature06265.

- 716 41. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O.,
717 Tunyasuvunakool, K., Bates, R., Zidek, A., Potapenko, A., et al. (2021). Highly accurate
718 protein structure prediction with AlphaFold. *Nature* 596, 583-589. 10.1038/s41586-021-
719 03819-2.
- 720 42. Brooks, B.R., Brooks, C.L., 3rd, Mackerell, A.D., Jr., Nilsson, L., Petrella, R.J., Roux, B.,
721 Won, Y., Archontis, G., Bartels, C., Boresch, S., et al. (2009). CHARMM: the
722 biomolecular simulation program. *J Comput Chem* 30, 1545-1614. 10.1002/jcc.21287.
- 723 43. Jo, S., Kim, T., Iyer, V.G., and Im, W. (2008). CHARMM-GUI: a web-based graphical
724 user interface for CHARMM. *J Comput Chem* 29, 1859-1865. 10.1002/jcc.20945.
- 725 44. Lee, J., Cheng, X., Swails, J.M., Yeom, M.S., Eastman, P.K., Lemkul, J.A., Wei, S.,
726 Buckner, J., Jeong, J.C., Qi, Y., et al. (2016). CHARMM-GUI Input Generator for NAMD,
727 GROMACS, AMBER, OpenMM, and CHARMM/OpenMM Simulations Using the
728 CHARMM36 Additive Force Field. *J Chem Theory Comput* 12, 405-413.
729 10.1021/acs.jctc.5b00935.
- 730 45. Phillips, J.C., Hardy, D.J., Maia, J.D.C., Stone, J.E., Ribeiro, J.V., Bernardi, R.C., Buch,
731 R., Fiorin, G., Henin, J., Jiang, W., et al. (2020). Scalable molecular dynamics on CPU and
732 GPU architectures with NAMD. *J Chem Phys* 153, 044130. 10.1063/5.0014475.
- 733 46. Kortum, F., Caputo, V., Bauer, C.K., Stella, L., Ciolfi, A., Alawi, M., Bocchinfuso, G.,
734 Flex, E., Paolacci, S., Dentici, M.L., et al. (2015). Mutations in KCNH1 and ATP6V1B2
735 cause Zimmermann-Laband syndrome. *Nat Genet* 47, 661-667. 10.1038/ng.3282.
- 736 47. Bauer, C.K., Schneeberger, P.E., Kortum, F., Altmüller, J., Santos-Simarro, F., Baker, L.,
737 Keller-Ramey, J., White, S.M., Campeau, P.M., Gripp, K.W., and Kutsche, K. (2019).
738 Gain-of-Function Mutations in KCNN3 Encoding the Small-Conductance Ca(2+)-
739 Activated K(+) Channel SK3 Cause Zimmermann-Laband Syndrome. *Am J Hum Genet*
740 104, 1139-1157. 10.1016/j.ajhg.2019.04.012.
- 741 48. Beauregard-Lacroix, E., Pacheco-Cuellar, G., Ajeawung, N.F., Tardif, J., Dieterich, K.,
742 Dabir, T., Vind-Kezunovic, D., White, S.M., Zadori, D., Castiglioni, C., et al. (2021).
743 DOORS syndrome and a recurrent truncating ATP6V1B2 variant. *Genet Med* 23, 149-154.
744 10.1038/s41436-020-00950-9.
- 745 49. Leu, C., Balestrini, S., Maher, B., Hernandez-Hernandez, L., Gormley, P., Hamalainen, E.,
746 Heggeli, K., Schoeler, N., Novy, J., Willis, J., et al. (2015). Genome-wide Polygenic
747 Burden of Rare Deleterious Variants in Sudden Unexpected Death in Epilepsy.
748 *EBioMedicine* 2, 1063-1070. 10.1016/j.ebiom.2015.07.005.
- 749 50. Epi, C., Chen, S., Neale, B.M., and Berkovic, S.F. (2023). Shared and distinct ultra-rare
750 genetic risk for diverse epilepsies: A whole-exome sequencing study of 54,423 individuals
751 across multiple genetic ancestries. medRxiv. 10.1101/2023.02.22.23286310.
- 752 51. Tavtigian, S.V., Harrison, S.M., Boucher, K.M., and Biesecker, L.G. (2020). Fitting a
753 naturally scaled point system to the ACMG/AMP variant classification guidelines. *Hum*
754 *Mutat* 41, 1734-1737. 10.1002/humu.24088.
- 755 52. Kopanos, C., Tsiolkas, V., Kouris, A., Chapple, C.E., Albarca Aguilera, M., Meyer, R.,
756 and Massouras, A. (2019). VarSome: the human genomic variant search engine.
757 *Bioinformatics* 35, 1978-1980. 10.1093/bioinformatics/bty897.
- 758 53. Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T.,
759 O'Donnell-Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B., et al. (2016). Analysis of
760 protein-coding genetic variation in 60,706 humans. *Nature* 536, 285-291.
761 10.1038/nature19057.

- 762 54. Wiel, L., Baakman, C., Gilissen, D., Veltman, J.A., Vriend, G., and Gilissen, C. (2019).
763 MetaDome: Pathogenicity analysis of genetic variants through aggregation of homologous
764 human protein domains. *Hum Mutat* 40, 1030-1038. 10.1002/humu.23798.
- 765 55. McLaren, W., Gil, L., Hunt, S.E., Riat, H.S., Ritchie, G.R., Thormann, A., Flicek, P., and
766 Cunningham, F. (2016). The Ensembl Variant Effect Predictor. *Genome Biol* 17, 122.
767 10.1186/s13059-016-0974-4.
- 768 56. Bahring, R., Barghaan, J., Westermeier, R., and Wollberg, J. (2012). Voltage sensor
769 inactivation in potassium channels. *Front Pharmacol* 3, 100. 10.3389/fphar.2012.00100.
- 770 57. Schmalz, F., Kinsella, J., Koh, S.D., Vogalis, F., Schneider, A., Flynn, E.R., Kenyon, J.L.,
771 and Horowitz, B. (1998). Molecular identification of a component of delayed rectifier
772 current in gastrointestinal smooth muscles. *Am J Physiol* 274, G901-911.
773 10.1152/ajpgi.1998.274.5.G901.
- 774 58. Klemic, K.G., Shieh, C.C., Kirsch, G.E., and Jones, S.W. (1998). Inactivation of Kv2.1
775 potassium channels. *Biophys J* 74, 1779-1789. 10.1016/S0006-3495(98)77888-9.
- 776 59. Kim, D.M., and Nimigeon, C.M. (2016). Voltage-Gated Potassium Channels: A Structural
777 Examination of Selectivity and Gating. *Cold Spring Harb Perspect Biol* 8.
778 10.1101/cshperspect.a029231.
- 779 60. Blunck, R., and Batulan, Z. (2012). Mechanism of electromechanical coupling in voltage-
780 gated potassium channels. *Front Pharmacol* 3, 166. 10.3389/fphar.2012.00166.
- 781 61. Heginbotham, L., Lu, Z., Abramson, T., and MacKinnon, R. (1994). Mutations in the K⁺
782 channel signature sequence. *Biophys J* 66, 1061-1067. 10.1016/S0006-3495(94)80887-2.
- 783 62. Matulef, K., Annen, A.W., Nix, J.C., and Valiyaveetil, F.I. (2016). Individual Ion Binding
784 Sites in the K(+) Channel Play Distinct Roles in C-type Inactivation and in Recovery from
785 Inactivation. *Structure* 24, 750-761. 10.1016/j.str.2016.02.021.
- 786 63. Coonen, L., Mayeur, E., De Neuter, N., Snyders, D.J., Cuello, L.G., and Labro, A.J. (2020).
787 The Selectivity Filter Is Involved in the U-Type Inactivation Process of Kv2.1 and Kv3.1
788 Channels. *Biophys J* 118, 2612-2620. 10.1016/j.bpj.2020.03.032.
- 789 64. Labro, A.J., Cortes, D.M., Tilegenova, C., and Cuello, L.G. (2018). Inverted allosteric
790 coupling between activation and inactivation gates in K(+) channels. *Proc Natl Acad Sci*
791 *U S A* 115, 5426-5431. 10.1073/pnas.1800559115.
- 792 65. Ashcroft, F.M. (2005). ATP-sensitive potassium channelopathies: focus on insulin
793 secretion. *J Clin Invest* 115, 2047-2058. 10.1172/JCI25495.
- 794 66. Yan, L., Figueroa, D.J., Austin, C.P., Liu, Y., Bugianesi, R.M., Slaughter, R.S.,
795 Kaczorowski, G.J., and Kohler, M.G. (2004). Expression of voltage-gated potassium
796 channels in human and rhesus pancreatic islets. *Diabetes* 53, 597-607.
797 10.2337/diabetes.53.3.597.
- 798 67. Braun, M., Ramracheya, R., Amisten, S., Bengtsson, M., Moritoh, Y., Zhang, Q., Johnson,
799 P.R., and Rorsman, P. (2009). Somatostatin release, electrical activity, membrane currents
800 and exocytosis in human pancreatic delta cells. *Diabetologia* 52, 1566-1578.
801 10.1007/s00125-009-1382-z.
- 802 68. Li, X.N., Herrington, J., Petrov, A., Ge, L., Eiermann, G., Xiong, Y., Jensen, M.V.,
803 Hohmeier, H.E., Newgard, C.B., Garcia, M.L., et al. (2013). The role of voltage-gated
804 potassium channels Kv2.1 and Kv2.2 in the regulation of insulin and somatostatin release
805 from pancreatic islets. *J Pharmacol Exp Ther* 344, 407-416. 10.1124/jpet.112.199083.
- 806 69. Zhu, J., Watanabe, I., Poholek, A., Koss, M., Gomez, B., Yan, C., Recio-Pinto, E., and
807 Thornhill, W.B. (2003). Allowed N-glycosylation sites on the Kv1.2 potassium channel

- 808 S1-S2 linker: implications for linker secondary structure and the glycosylation effect on
809 channel function. *Biochem J* 375, 769-775. 10.1042/BJ20030517.
- 810 70. Watanabe, I., Zhu, J., Sutachan, J.J., Gottschalk, A., Recio-Pinto, E., and Thornhill, W.B.
811 (2007). The glycosylation state of Kv1.2 potassium channels affects trafficking, gating,
812 and simulated action potentials. *Brain Res* 1144, 1-18. 10.1016/j.brainres.2007.01.092.
- 813 71. McKeown, L., Burnham, M.P., Hodson, C., and Jones, O.T. (2008). Identification of an
814 evolutionarily conserved extracellular threonine residue critical for surface expression and
815 its potential coupling of adjacent voltage-sensing and gating domains in voltage-gated
816 potassium channels. *J Biol Chem* 283, 30421-30432. 10.1074/jbc.M708921200.
- 817 72. Marini, C., Romoli, M., Parrini, E., Costa, C., Mei, D., Mari, F., Parmeggiani, L., Procopio,
818 E., Metitieri, T., Cellini, E., et al. (2017). Clinical features and outcome of 6 new patients
819 carrying de novo KCNB1 gene mutations. *Neurol Genet* 3, e206.
820 10.1212/NXG.0000000000000206.
- 821 73. Kang, S.K., Vanoye, C.G., Misra, S.N., Echevarria, D.M., Calhoun, J.D., O'Connor, J.B.,
822 Fabre, K.L., McKnight, D., Demmer, L., Goldenberg, P., et al. (2019). Spectrum of K(V)
823 2.1 Dysfunction in KCNB1-Associated Neurodevelopmental Disorders. *Ann Neurol* 86,
824 899-912. 10.1002/ana.25607.
- 825 74. Full, Y., Seebohm, G., Lerche, H., and Maljevic, S. (2013). A conserved threonine in the
826 S1-S2 loop of KV7.2 and K V7.3 channels regulates voltage-dependent activation. *Pflugers*
827 *Arch* 465, 797-804. 10.1007/s00424-012-1184-x.
- 828 75. Kalstrup, T., and Blunck, R. (2018). S4-S5 linker movement during activation and
829 inactivation in voltage-gated K(+) channels. *Proc Natl Acad Sci U S A* 115, E6751-E6759.
830 10.1073/pnas.1719105115.
- 831 76. Barghaan, J., and Bähring, R. (2009). Dynamic coupling of voltage sensor and gate
832 involved in closed-state inactivation of kv4.2 channels. *J Gen Physiol* 133, 205-224.
833 10.1085/jgp.200810073.
- 834 77. Isacoff, E.Y., Jan, Y.N., and Jan, L.Y. (1991). Putative receptor for the cytoplasmic
835 inactivation gate in the Shaker K+ channel. *Nature* 353, 86-90. 10.1038/353086a0.
- 836 78. Ye, W., Zhao, H., Dai, Y., Wang, Y., Lo, Y.H., Jan, L.Y., and Lee, C.H. (2022). Activation
837 and closed-state inactivation mechanisms of the human voltage-gated K(V)4 channel
838 complexes. *Mol Cell* 82, 2427-2442 e2424. 10.1016/j.molcel.2022.04.032.
- 839 79. Bezanilla, F. (2000). The voltage sensor in voltage-dependent ion channels. *Physiol Rev*
840 80, 555-592. 10.1152/physrev.2000.80.2.555.
- 841 80. Catterall, W.A. (2010). Ion channel voltage sensors: structure, function, and
842 pathophysiology. *Neuron* 67, 915-928. 10.1016/j.neuron.2010.08.021.
- 843 81. Smets, K., Duarri, A., Deconinck, T., Ceulemans, B., van de Warrenburg, B.P., Zuchner,
844 S., Gonzalez, M.A., Schule, R., Synofzik, M., Van der Aa, N., et al. (2015). First de novo
845 KCND3 mutation causes severe Kv4.3 channel dysfunction leading to early onset
846 cerebellar ataxia, intellectual disability, oral apraxia and epilepsy. *BMC Med Genet* 16, 51.
847 10.1186/s12881-015-0200-3.
- 848 82. Gebauer, M., Isbrandt, D., Sauter, K., Callsen, B., Nolting, A., Pongs, O., and Bähring, R.
849 (2004). N-type inactivation features of Kv4.2 channel gating. *Biophys J* 86, 210-223.
850 10.1016/S0006-3495(04)74097-7.
- 851 83. Ferrer, T., Cordero-Morales, J.F., Arias, M., Ficker, E., Medovoy, D., Perozo, E., and
852 Tristani-Firouzi, M. (2011). Molecular coupling in the human ether-a-go-go-related gene-

853 1 (hERG1) K⁺ channel inactivation pathway. J Biol Chem 286, 39091-39099.
854 10.1074/jbc.M111.292060.

855

856 **Figure legends**

857 **Figure 1. Photographs of some of the affected individuals.**

858 Left) Individual 1 throughout the years. Photos and x-rays of her hands and feet, illustrating nail
859 hypoplasia and aplasia, and terminal phalanx hypoplasia (brachytelephalangia). Right) Individual
860 4, illustrating synophrys and nail hypoplasia.

861

862 **Figure 2. Schematic representation of mutations in Kv2.2 protein. A.** Topology of Kv2.2 channel
863 and schematic representation of distribution of *KCNB2* point mutations. The mutations are in the
864 following regions: c.281G>A, (p.Gly94Glu), c.472A>G, (p.Thr158A)la: N-terminus, c.641C>T,
865 (p.Thr214Met): S1-S2 linker, c.724G>A, (p.Ala242Thr): S2, c.827C>T, (p.Pro276Leu): S3,
866 c.911G>A, (p.Arg304Gln): S4, c.994T>G, (p.Tyr332Asp): S4-S5 linker, c.1124C>T,
867 (p.Ala375Val) , c.1141A>G, (p.Thr381Ala): pore helix, c.1937C>T, (p.Ala646Val) – C terminus.
868 **B.** Alignment of the mutated Kv2.2 amino acids across different species. Homology model and
869 mutation distribution of Kv2.2 as a tetramer (Top view, **C**) and as a monomer (**D**) based on the
870 known structure of the Kv1.2/2.1 chimera⁴⁰. The model was generated using alphafold2⁴¹.

871

872 **Figure 3. Activation properties of Kv2.2 variants. A.** Activation currents from oocytes expressing
873 WT-Kv2.2 and variant channels (expressed in the absence (*top*) and presence (*below*), see
874 methods) were evoked by stepping from -90 mV to voltages ranging from -120 to +100 mV in 10
875 mV increments for 100 ms. This was followed by a voltage step to -20 mV for 100 ms and back
876 to -90 mV for 5 s to allow recovery of the channels to deactivated states (protocol illustrated in the
877 left corner). c.641C>T, (p.Thr214Met) does not evoke any currents. c.994T>G, (p.Tyr332Asp)
878 show reduced currents. c.1141A>G, (p.Thr381Ala) shows transient channel opening followed by
879 a rapid inactivation of ionic currents. **B.** Current-voltage (IV) relationship of Kv2.2 variants (*left*)
880 and corresponding box plots of maximal current amplitudes measured at +100 mV (*right*).
881 p.Thr214Met, p.Tyr332Asp and p.Thr381Ala show significant reduction in current amplitudes as
882 compared to WT. **C.** Conductance-Voltage (GV) relationship of *KCNB2* variants when expressed
883 in oocytes alone. IV (**D**) and GV (**E**) relationship of Kv2.2 variants when co-expressed with equal
884 amounts of WT. WT:p.Thr214Met show significant reduction in current amplitudes as compared
885 to WT alone. GV curves were best fitted by a sum of two Boltzmann relations of the form G/G_{max}
886 $= Bottom + (Top_1 - Bottom) / (1 + \exp((V_{50(1)} - X) / k_1)) + (Top_2 - Top_1) / (1 + \exp((V_{50(2)} - X) / k_2))$. The
887 fitting parameters ($V_{50(1)}, V_{50(2)}, k_1, k_2$) for the GV activation relationships have been compiled in
888 **Table 2**. Values are provided as means \pm S.D. from $n > 6$ oocytes per conditions from at least 2
889 independent experiments. Statistical significance was tested by Kruskal Wallis one-way analysis
890 of variance followed by Dunn's post-hoc test comparing amplitudes of the different variants to
891 WT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

892

893 **Figure 4. Reversal potential of Kv2.2 variants. A.** For calculating the reversal potentials of the
894 Kv2.2 variants, currents were evoked by stepping from -120 mV to +50 mV for 100 ms followed

895 by voltages ranging from -120 to +50 mV in 10 mV increments for 100 ms. This was followed by
896 a voltage step to -120 mV for 20 ms and back to the holding potential of -90 mV for 5 s to allow
897 recovery of the channels to deactivated states. The external and internal solutions used in these
898 experiments contained K^+ and either $NMDG^+$ or Na^+ in the concentrations mentioned in the figure.
899 **B.** Representative raw traces of the reversal potential protocol in an oocyte expressing WT-Kv2.2
900 in $NMDG^+$ and K^+ containing external and internal solutions. The resulting IV curves for this
901 protocol of Kv2.2 variants alone (*left*) or co-injected with WT (*right*) in solutions containing either
902 $NMDG^+$ and K^+ (**C**) or Na^+ and K^+ (**D**) are shown. The variants do not seem to alter the K^+
903 selectivity of the channel pore (X-intercept data compiled with 95% C.I. in **Table S3**).

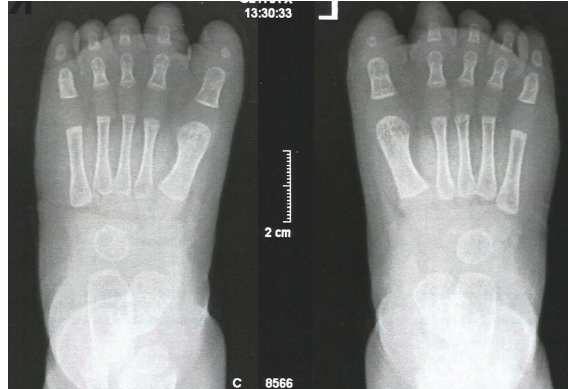
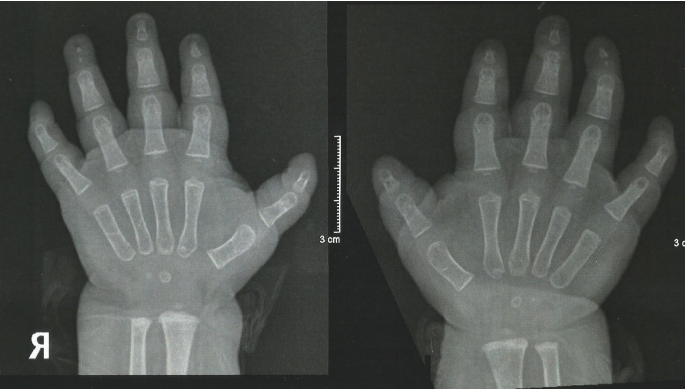
904

905 **Figure 5. Inactivation features of currents by KCNB2 variants.** **A.** Protocol to measure voltage
906 dependent inactivation in Kv2.2 variants. To measure inactivation, currents from oocytes
907 expressing Kv2.2 variant channels were evoked by stepping from -90 mV to voltages ranging from
908 -120 to +40 mV in 10 mV increments for 20 s. This was followed by a voltage step to +60 mV for
909 100 ms and back to -120 mV for 5 s to allow recovery of the channels from inactivation. The raw
910 traces from voltage steps highlighted in thickened line (-90 mV \rightarrow 40 mV \rightarrow 60 mV \rightarrow 120 mV)
911 are highlighted in (**B**) from oocytes expressing the individual variants alone or in from oocytes
912 expressing both WT and a specific variant (**C**). c.641C>T, (p.Thr214Met) was excluded in B
913 because of lack of any currents evoked by this variant. Currents evoked by c.1141A>G,
914 (p.Thr381Ala) is represented and further explained below. The inactivation current-voltage (IV)
915 relationship of these recordings are plotted in (**D**) and (**E**), respectively. The IV relationship was
916 best fitted by a sum of two Boltzmann relations of the form $I/Imax = Top + (Bottom_1 -$
917 $Top)/(1 + \exp((V_{50(1)} - X)/k_1)) + (Bottom_2 - Bottom_1)/(1 + \exp((V_{50(2)} - X)/k_2))$. The fitting parameters
918 ($V_{50(1)}, V_{50(2)}, k_1, k_2$) for the IV relationships have been compiled in **Table 3**. **F.** The fitting
919 function mentioned above also calculates the parameter “Bottom₂”, which describes the extent of
920 inactivation in these variants expressed either alone (top) or with WT (below). The differences in
921 extent of inactivation between the variants and WT (red dashed lines) were tested for significance
922 using the Kruskal Wallis one-way analysis of variance followed by Dunn’s post-hoc test
923 comparing amplitudes of the different variants to WT-KCNB2. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq$
924 0.001 . Values are provided as means \pm S.D. from $n > 6$ oocytes per conditions from at least 2
925 independent experiments. **G.** Representative current trace of an oocyte expressing only the
926 p.Thr381Ala variant (blue trace) or only WT (black trace). p.Thr381Ala expressing oocytes show
927 diminished currents (blue trace) in the inactivation protocol as compared to WT, in a manner like
928 the activation protocol in Fig. 2 and Fig. 3. H represents the raw traces of the inactivation protocol
929 of residual currents of the p.Thr381Ala variant. **I.** The IV relationship of the p.Thr381Ala mutation
930 (blue line) shows recovery of inactivation with increasing voltages as opposed to WT (dashed fit
931 representing the fit to WT IV shown in **D** and **E**).

932

Figure 1

Indiv. 1



Indiv. 4



Figure 2

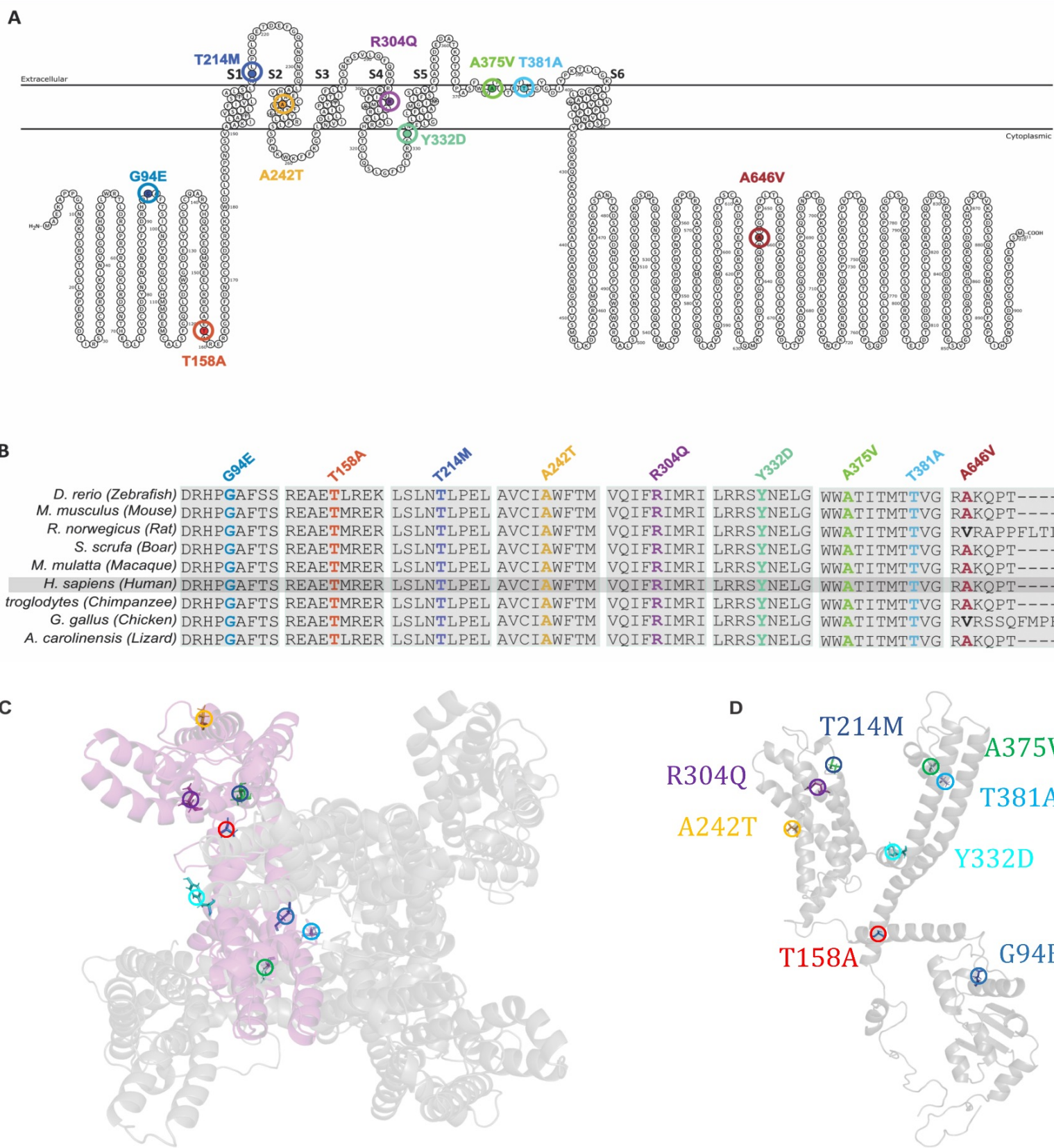


Figure 3

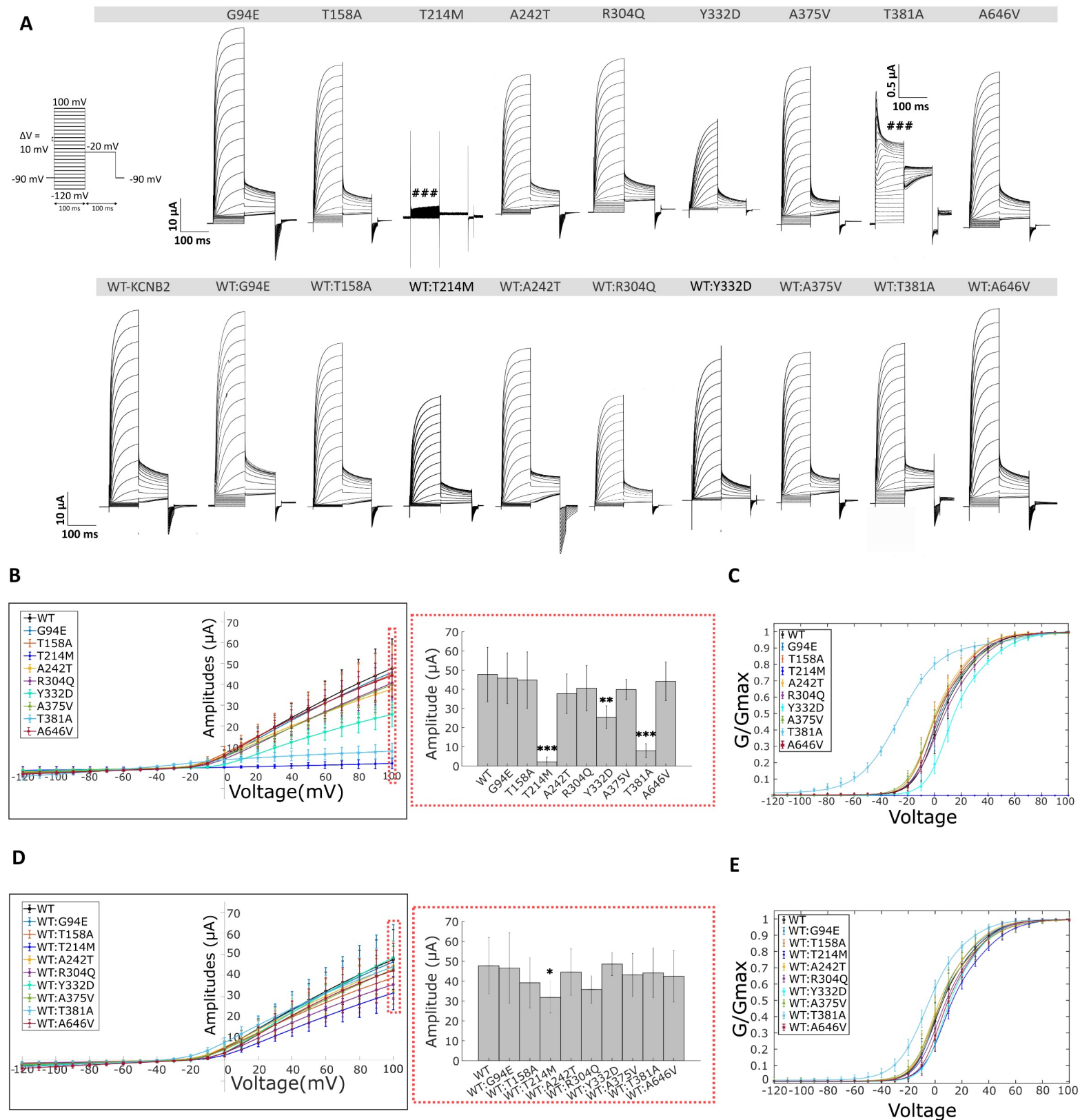
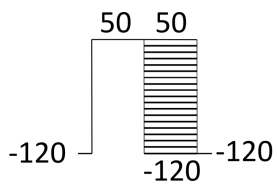
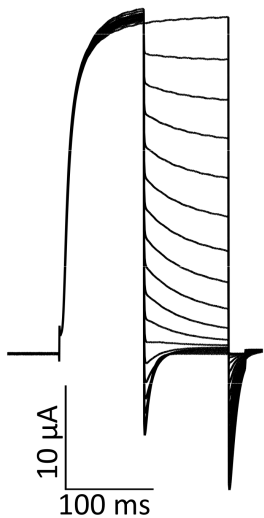


Figure 4

A

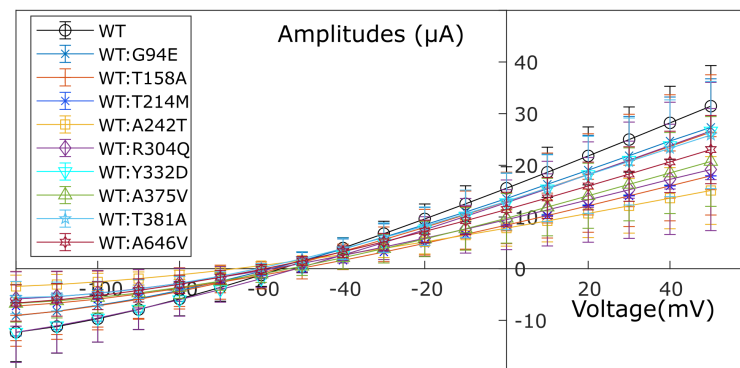
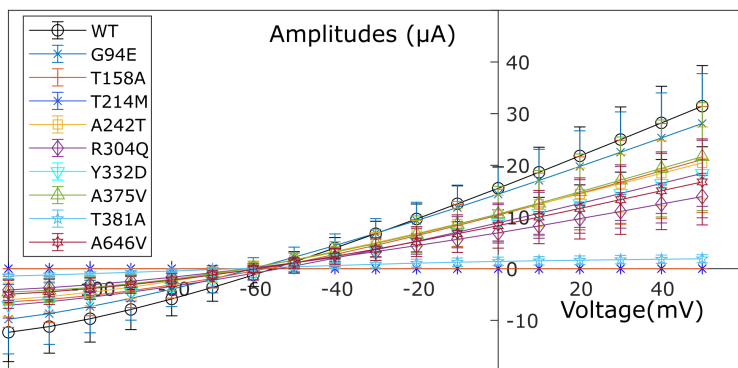


B



C

ES: 110 mM NMDG⁺, 5 mM K⁺
IS: 110 mM K⁺, 5 mM NMDG⁺



D

ES: 110 mM Na⁺, 5 mM K⁺
IS: 110 mM K⁺, 5 mM Na⁺

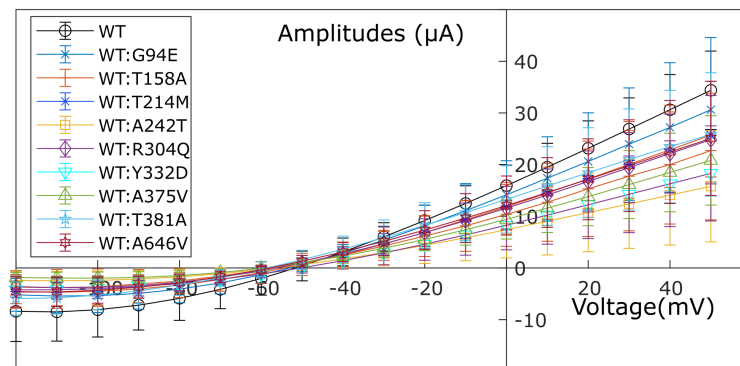
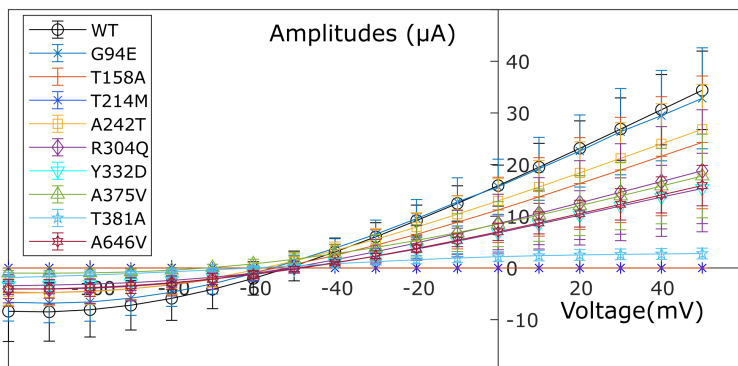
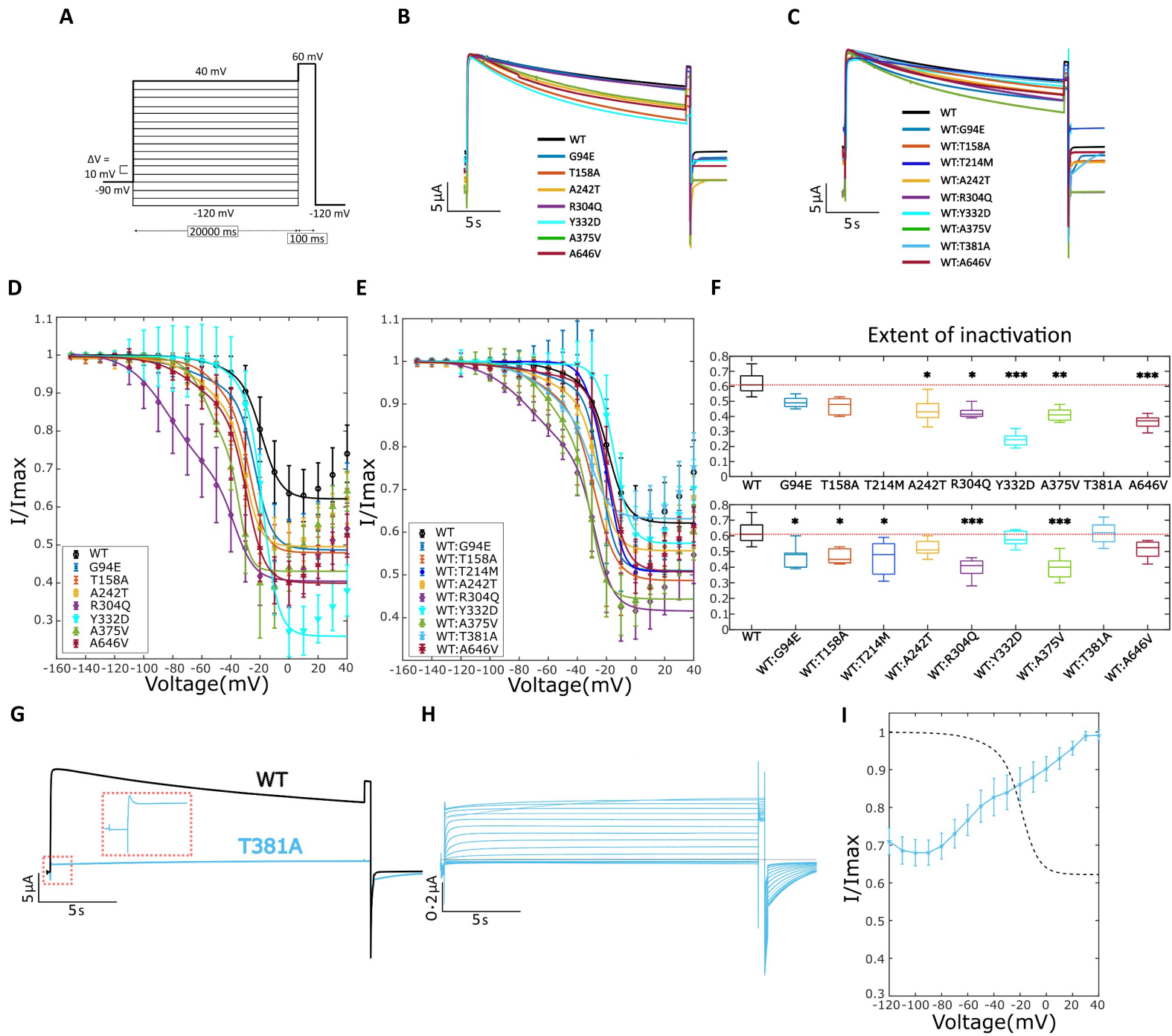


Figure 5



Supplemental Note: Case Reports

Proband 1

KCNB2 variant: c.1141A>G, p.Thr381Ala, *de novo*

Method of Identification: Trio exome and sanger sequencing

Patient information: The patient exhibited global developmental delay, hypotonia and intellectual disability but no autistic traits. The disease phenotype overlapped partially with Zimmerman-Laband and DOORS syndromes, which are neurodevelopmental disorders with epilepsy and hypoplasia of the terminal phalanges and nails. She also exhibited dysmorphisms that included abnormal nails, mild blepharoptosis, beaked nose, flat midface, open mouth, drooling, full lower lip. She has no family history. She was born at term by C-section. Birth weight was 2.79 kg and length was 48 cm. The patient age at last visit was 5 years with a height of 107.5 cm and a weight of 19.7 kg. She exhibited epileptic seizures with effective treatment with Levetiracetam. Her MRI exhibited prominence of the anterior horn of lateral ventricle and 3rd ventricle and decreased bilateral hippocampus. She exhibited aortic dilation and neurogenic bladder. Other clinical features include diabetes, cataract, gingival fibromatosis, cortical vision impairment, Duane syndrome, hyperopia, astigmatism, low bone density, oropharyngeal dysphagia, cataract, and low bone density.

Proband 2

KCNB2 variant: c.281G>A, p.Gly94Glu, *de novo*

Method of Identification: Whole exome and Trio whole genome sequencing

Patient information: The patient, at two years of age, exhibited developmental delay but was making slow progress. He rolled at 6 months, sat at 2 years (propped), and could sit independently (but not stand unsupported) at 2.5 years. He exhibited language delay but made sounds and coos at 2.5 years. At this age, the patient is too young to assess for intellectual disability and autistic traits and no seizures were noted. Neurological assessment revealed Hypotonic/ataxic cerebral palsy per neurology. He also exhibited dysmorphisms that included synophrys and full lashes, single transverse palmar crease on one side, high palate and clinodactyly. The patient had no immediate family history, but there are some still births in extended family (maternal side) and a maternal cousin with possible autism spectrum disorder. The patient was born at 38 5/7 weeks to a G1 P0->1 mother with appropriate prenatal care and no exposures reported. Fetal ultrasound was normal except for concern for small cerebellum. Birth weight was 3.152 kg, length was 50.8 cm, head circumference was 32.3 cm and APGAR score of 7/8. Age at last visit was 2.5 years with weight of 14.8 kg (77th percentile based on CDC boys 2-20 years), height of 98 cm (94th percentile), and head circumference of 48.7 cm (33rd percentile). Other anomalies include abnormal trabeculation of left ventricular myocardium and slight

shawl scrotum. Other clinical features include delayed visual maturation and bilateral sensorineural hearing loss.

Proband 3

KCNB2 variant: c.1937C>T, p.Ala646Val, Inherited

Method of Identification: Trio whole exome and whole genome sequencing

Patient information: The patient exhibited delayed motor milestones, speech and language delay, apraxia, weakness, easily fatigue, irritability, regression, intellectual disability, and other neurodevelopmental disorders but no autistic traits. No seizures were noted. She also exhibited macrocephaly, deep set eyes, broad forehead, horizontal palpebral fissures, thin upper lips, tapered fingers and puffy feet. The patient inherited the mutation from her father in an autosomal dominant manner who was symptomatic with pneumothorax, pain, hypotonia, joint pain, weakness, fatigue and clumsiness. Her brother was also symptomatic who was positive for neurodevelopmental disorder, abnormal EEG, hypotonia, pain, and apraxia. The patient was born at term by C-section. Her birth growth parameters are unavailable. Her age at last visit was 3 years (in 2020) with a head circumference of 52 cm (98% percentile). Other clinical features include microscopic hematuria, episodic edema, and rashes.

Proband 4

KCNB2 variant: c.641C>T, p.Thr214Met, *de novo*

Method of Identification: Trio whole exome sequencing

Patient information: The patient was born at term with a weight of 4.24 kg, length of 53 cm and head circumference of 37 cm. He started walking at 17 months and exhibited language delay, intellectual disability, and mild autistic traits in infancy. No seizures were noted. He also exhibited synophrys, nail hypoplasia and myopia. The patient had no family history, and the age of last visit was 18 years. His growth parameters at this stage included a weight of 64 kg, height of 183 cm and head circumference of 59.5 cm.

Proband 5

KCNB2 variant: c.994T>G, p.Tyr332Asp, *de novo*

Method of Identification: Trio whole exome sequencing

Patient information: The patient exhibited severe global developmental delay and intellectual disability. According to BSID-III, she exhibited motor development p1.8, cognitive development index of 64% (cognitive age of 9 months at age 15.7 months) and language index of 72%. She couldn't walk independently yet with no speech and proximal hypertonia. Slow progress was observed with no regression. . Patient exhibited dysmorphisms in the hands and in the head. The latter included frontal bossing, suggestion of macrocephaly (but not on measurement) and broad forehead. She was born at term by C-section and had normal birth weight (3.335 kg). Her age at last visit

was 21 months with head circumference of 49.5 cm (+1SD). She has a family history of hypertension and obesity. Brain anomalies include delayed myelination, T2 abnormalities in basal ganglia (hyperintensity in globus pallida or hypo-intensity of other ganglia), small volume of thalamus, subtle increased volume of lateral and 3rd ventricles and peripheral CSF, small corpus callosum (especially the posterior part), subtle small volume of adenohypophysis, small cerebellar hemisphere on the left side and cyst in the left temporal side. Other neurological features include tongue protrusion, severe strabismus and retinal 'bear tracks'.

Proband 6

KCNB2 variant: c.911G>A, p.Arg304Gln, *de novo*

Method of Identification: Trio whole exome sequencing

Patient information: The patient exhibited delayed motor milestones, speech and language delay with intellectual disability and autistic traits. Other neurological features include ADHD, mood disorder and aggressive behavior. Facial dysmorphisms include double hair whorl, malar flattening, long philtrum, up-slanting palpebral fissures, epicanthal folds and broad nasal tip. He also had tapered fingers and short toes. He was born at term by vaginal delivery and had a birth weight of 3.28 kg. His age and height at last visit were 9 years and 135.9 cm, respectively. Family history included mental health issues in paternal uncle and paternal grandmother.

Proband 7

KCNB2 variant: c.827C>T, p.Pro276Leu, mosaic in proband (47T, 72C)

Method of Identification: Trio whole exome sequencing

Patient information: The patient exhibited normal motor development with language delay and moderate intellectual disability. He had behavioural issues that were treated with Risperidone, Fluoxetine, Guanfacine and methylphenidate. He suffers from drug-resistant focal and generalized seizures (Age of onset: 3 years and 9 months) that show multifocal abnormalities with frontal predominance on EEG. Previous anti-seizure medications (ASM) include Ethosuximide, VPA, perampanel, topiramate, clobazam, rufinamide, memantine, levetiracetam, IVIG; Current ASM include Lamotrigine, Oxcarbazepine, Sulthiame, Vagal Nerve Stimulator and Diazepam *prn*. Other neurological features include diadochokiensia with monopodal stand more insecure on the left. No brain abnormalities were detected except for non-specific prominent perivascular spaces. He was born at term, Apgar score of 2-6-9 with no perinatal complications. His age at last visit was 15 years with the head circumference of 51.7 cm, height of 150.6 cm and weight at 52 kg. Family history was unremarkable; the father was mosaic with sequencing reads for both T and C nucleotides (17T, 192C).

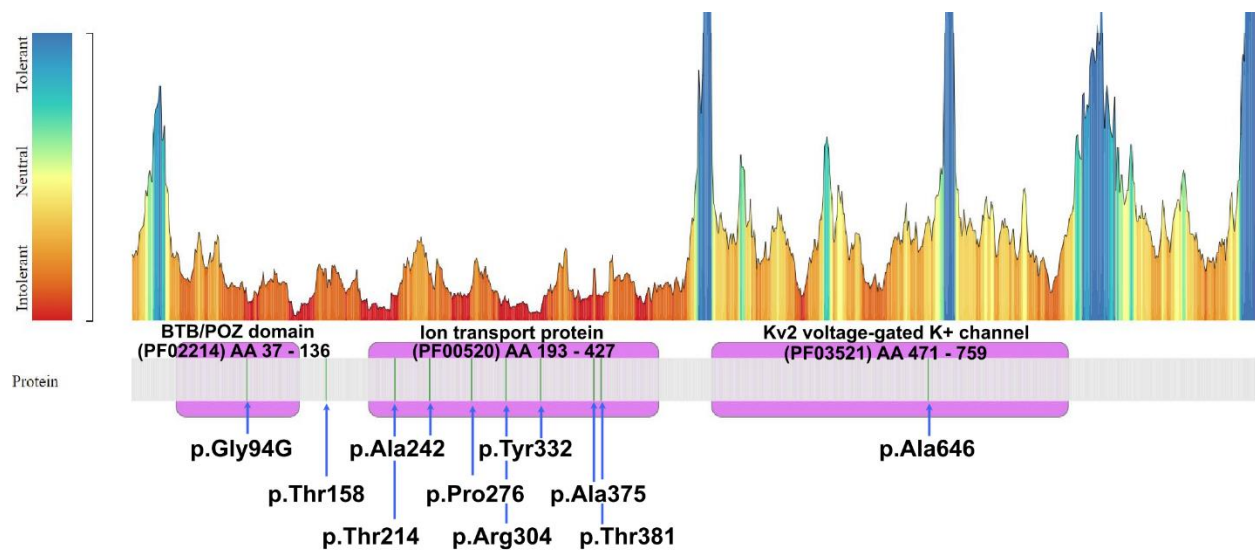


Figure S1. Metadome's missense tolerance landscape for KCNB2 (NM_004770.2). All amino acids are intolerant to missense variants (scores in Table S2).

Table S1. Primers used for mutagenesis.

<i>KCNB2</i> (NM_004770.3) variants	Forward primer	Reverse primer
c.281G>A, p.Gly94Glu	aaatggaagtgaaggctctggatgccgatcaaag	cttgatcgcatccagaagccttcactccattt
c.472A>G, p.Thr158Ala	cgctctcgcatagcctctgcctctcgc	gcgagaggcagaggctatgagagagcg
c.641C>T, p.Thr214Met	ccattgcttctctcaatatgctgccggagc	gctccggcagcatattgagagacaaagcaatgg
c.724G>A, p.Ala242Thr	tccatggtaaaccatgtaatacacacagcctccac	gtggaggctgtgtgtattacatggtttaccatgga
c.911G>A, p.Arg304Gln	gaggatgcgcatgattggaagatctggaccac	gtggtccagatcttccaaatcatgcgcacctc
c994T>G, p.Tyr332Asp	ttcacccttagcgaggatgacaatgaattgggct	agcccaattcattgtcactccgcctaagggtgaaa
c.1141A>G, p.Thr381Ala	tcacatagccaacagcggatcatggtgatggtg	caccatcaccatgaccgctgttggtatggtga
c.1124C>T, p.Ala375Val	ggatcatggtgatggtgaccacaaaatgatgc	gcatcatttgggggtcaccatcaccatgacc
c.1937C>T, p.Ala646Val	ggggggcccctaactcttgggtgctcttct	agaagagcaccaagagtagggggccccc

Table S2. Summary of *in-silico* predictions for all *KCNB2* variants reported in our study

ACMG classification of variants (not taking into account functional data presented here), metadome amino acid missense tolerance scores and pathogenicity prediction and conservation scores obtained using Ensembl's VEP NB. Definition of acronyms and details on the scores with links and references for the tools can be found in the dbNSFP v4 "read me" file at <https://usf.app.box.com/s/6yi6huheisol3bhml8bitcon1oi53pm>

See excel file for data.

Table S3. Reversal potential of Kv2.2 variants

Variants	Without WT	With WT	Without WT	With WT
	ES: 110 NMDG ⁺ , 5 K ⁺ ; IS: 110 K ⁺ , 5 NMDG ⁺		ES: 110 Na ⁺ , 5 K ⁺ ; IS: 110 K ⁺ , 5 Na ⁺	
	V _{rev} in mV (95% C.I.)			
WT	-60.8 (-59.2 to -62.6)		-63.8 (-61.7 to -65.9)	
G94E	-65.3 (-60.8 to -70.1)	-64.3 (-61.1 to -67.6)	-67.6 (-62.8 to -72.8)	-68.1 (-62.1 to -74.8)
T158A	-64.8 (-59.1 to -71.2)	-62.9 (-58.3 to -68.0)	-67.4 (-61.5 to -74.1)	-70.0 (-61.3 to -80.5)
T214M	n.a.	-58.0 (-52.9 to -63.5)	n.a.	-68.3 (-63.6 to -73.4)
A242T	-68.0 (-63.6 to -72.8)	-72.9 (-66.7 to -79.9)	-70.1 (-65.7 to -75.0)	-70.7 (-62.0 to -81.1)
R304Q	-67.6 (-63.5 to -72.0)	-65.0 (-58.6 to -72.1)	-65.0 (-58.0 to -73.0)	-70.7 (-65.5 to -76.4)
Y332D	-60.4 (-56.6 to -64.4)	-56.6 (-53.4 to -60.0)	-64.2 (-58.2 to -70.9)	-64.0 (-57.9 to -70.7)
A375V	-71.2 (-64.9 to -78.3)	-62.0 (-57.6 to -66.7)	-81.6 (-75.5 to -88.6)	-75.2 (-68.9 to -82.4)
T381A	-60.0 (-54.0 to -66.7)	-70.4 (-65.3 to -75.9)	-60.7 (-55.9 to -66.0)	-68.8 (-63.3 to -75.0)
A646V	-66.5 (-60.9 to -72.6)	-66.7 (-62.9 to -70.8)	-61.8 (-57.8 to -66.0)	-69.9 (-64.0 to -76.5)

n.a. - not analysed; the amplitudes were too low for determination of reversal potential.

C.I. - Confidence Intervals