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Targeting $K_{Na}1.1$ channels in *KCNT1*-associated epilepsy

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Abstract

Gain-of-function (GOF) pathogenic variants of *KCNT1,* the gene encoding the largest known potassium channel subunit, $K_{Na}1.1$, are associated with developmental and epileptic encephalopathies accompanied by severe psychomotor and intellectual disabilities. Blocking hyperexcitable $K_{Na}1.1$ channels with quinidine, a class I antiarrhythmic drug, has shown variable success in patients due in part to dose-limiting off-target effects, poor blood-brain-barrier penetration and low potency. In recent years, high-resolution cryo-EM structures of the chicken $K_{Na}1.1$ channel in different activation states have been determined, and animal models of the diseases have been generated. Alongside increasing information about the functional effects of GOF pathogenic variants on $K_{Na}1.1$ channel behaviour and how they lead to hyperexcitability, these tools will facilitate development of more effective treatment strategies. Here, we review the range of *KCNT1* variants and their functional effects, challenges posed by current treatment strategies, and recent advances in finding more potent and selective therapeutic interventions for *KCNT1*-related epilepsies.

KCNT1 mutations are associated with treatment-resistant epilepsies

To date, upwards of 50 distinct missense gain-of-function (GOF) pathogenic variants of *KCNT1* have been associated with severe, refractory, developmental and epileptic encephalopathies (DEE)[1]. *KCNT1* encodes the largest known potassium channel subunit, $K_{Na}1.1$ (Slack, or previously Slo2.2 or $K_{Ca}4.1$), which forms a tetrameric Na^+-activated K^+ channel. $K_{Na}1.1$ and the closely-related $K_{Na}1.2$ (encoded by *KCNT2*) subunits have distinct expression patterns in the central nervous system (CNS) [2,3] but also co-localise in some regions, and can form heteromeric co-assemblies [4,5]. In normal physiology, K_{Na} channels are responsible for generation of the slow **afterhyperpolarisation** (AHP, see glossary) following single action potentials [6,7] or bursts of action potential firing [8]. K_{Na} channels have also been implicated as an important determinant of the resting membrane potential and intrinsic excitability in a number of cell types in the CNS [9,10] and in arterial smooth muscle [11].

Channelopathies of K^+ channels are found in a number of epilepsies, arising from both loss-of-function (LOF) and GOF mutations [12]. *KCNT1* pathogenic variants appear to cause significantly more severe clinical phenotypes, accompanied by intellectual and psychomotor disabilities. The phenotypic spectrum is becoming increasingly broad, and inhibition of overactive $K_{Na}1.1$ by class I antiarrhythmic quinidine as a treatment strategy has had limited success [13-26]. However, there is accumulating information about the mechanisms underlying the GOF effect of DEE-related *KCNT1* pathogenic variants. This, coupled with high-resolution structures of the inactive and active states of $K_{Na}1.1$ generated by cryogenic

electron microscopy (cryo-EM) [27], has provided new opportunities for developing therapeutic interventions.

Range of *KCNT1* disorders

Pathogenic *KCNT1* variants were first identified in two distinct epilepsies; epilepsy of infancy with migrating focal seizures (EIMFS) and autosomal-dominant or sporadic sleep-related hypermotor epilepsy ((AD)SHE) [28,29]. EIMFS is characterised by recurrent migrating, polymorphous seizures, with a typical age of onset before 6 months, after which frequency of seizures increases. The disorder is accompanied by other severe comorbidities such as developmental disorders [30,31] and delayed motor function [32]. Following onset, patients may lose all psychomotor skills previously developed [30]. *KCNT1* pathogenic variants have been identified as the most prevalent variants in patients with EIMFS through whole exome sequencing (WES) studies and result in large increases in $K_{Na}1.1$ current amplitude when mutated channels are expressed *in vitro* [28,32]. Most EIMFS-associated *KCNT1* pathogenic variants are *de novo*, though three separate cases of somatic mosaicism have been identified [17,31,33].The disorder is accompanied by other severe comorbidities such as developmental and psychiatric disorders [30,31] and delayed motor function [32]. Following onset, patients may lose all psychomotor skills previously developed [30].

(AD)SHE, clinically, is a less severe disorder, characterised by motor seizures occurring during sleep, and a mean age of onset of 6 years old [29]. Seizures are, like EIMFS, accompanied by cognitive disabilities. This separates them from (AD)SHE associated with other genes, such as those encoding nicotinic acetylcholine receptor subunits. Furthermore,

(AD)SHE arising specifically from *KCNT1* is defined by more severe seizures and an earlier age of onset than other forms of (AD)SHE [29].

KCNT1 variants have been linked to other hyperexcitability disorders with psychomotor and developmental defects, including Ohtahara syndrome [2,3], Lennox-Gastaut syndrome [26] and Status Dystonicus [34]. There have also been reported cases of West syndrome, leukoencephalopathies and Brugada syndrome [31]. Only one heterozygous LOF variant, causing impaired $K_{Na}1.1$ trafficking, has thus far been reported in a patient that exhibited severe generalised seizures and delayed myelination [35,36]. Cardiac effects have been more recently reported, with pathogenic *KCNT1* variants linked to systemic-to-pulmonary artery collateral-mediated heart disease, or 'collateralopathy' [37-39].

Structure and function of $K_{Na}1.1$

$K_{Na}1.1$ is a member of the SLO subfamily of K^+ channels [40], which exhibit unusually high conductance, and are encoded by four genes in mammals [41]. $K_{Na}1.1$ subunits have structural and functional similarities to other K^+ channels, such as $K_{Ca}1.1$ (BK_{Ca}) and the K_v family of voltage-gated K^+ channels. $K_{Na}1.1$ subunits are, like K_v channels, composed of six transmembrane helices and a loop between the S5 and S6 domains that forms the selectivity filter [27,42]. The channel is weakly voltage-gated and mechanisms underlying its voltage-sensitivity are unknown. Unlike Kv channels and other SLO subfamily subunits $K_{Ca}1.1$ and $K_{Ca}5.1$ (Slo3), $K_{Na}1.1$ does not possess a voltage-sensing domain (VSD) defined by an excess of positively charged residues on its S4 domain [43]. Characteristic of all SLO subfamily members, however, $K_{Na}1.1$ has a large intracellular C-terminal domain containing

two regulation of conductance of K^+ (RCK) domains, and also multiple consensus sites for PKC phosphorylation [27,42]. The C-terminal also contains a nicotinamide adenine dinucleotide (NAD^+) binding domain believed to be involved in potentiating channel activity [44].

$K_{Na}1.1$ is primarily Na^+-activated, despite its weak voltage-sensitivity; in the absence of intracellular Na^+, wildtype (WT) $K_{Na}1.1$ channels show almost no activity in whole-cell patch clamp experiments [45]. Mutational studies have implicated the RCK domains in conferring Na^+ sensitivity of both $K_{Na}1.1$ and $K_{Na}1.2$ [46,47], though this is yet to be corroborated by structural data. The exact mechanisms of channel gating have not been elucidated; there is a narrowing of the intracellular pore vestibule by movement of the S6 helices to the 'closed', Na^+-unbound state, yet there remains sufficient access to the selectivity filter by K^+ ions [27]. This, and other recent functional studies of $K_{Na}1.1$ and closely related $K_{Na}1.2$, point away from a canonical **S6 helix bundle-crossing** as the mechanism of activation gating [48,49]. Rather, it is possible that the channel is gated either by a **hydrophobic gating** mechanism or **selectivity filter gating** mechanism. This is similar to a number of other K^+ channels that lack features of widely-accepted canonical mechanisms of voltage gating, for example an S6 helix bundle-crossing and VSD [50-53].

Pathogenic GOF variants associated with DEEs are clustered in 'hotspots' in the channel structure thought to be involved with gating; particularly around the RCK and NAD^+ binding domains. Pathogenic variants are also found in the pore-forming region on the S5 and S6 helices, and one pathogenic variant has been reported on the S3 helix (Figure 1), which together with those in the intracellular domains may reflect disruption of a range of protein

120 regions that are critical for channel activation. It was earlier suggested that location of
121 mutation on the channel structure could be related to clinical phenotype [54], however
122 G288S located on the S5 helix, and R398Q located on RCK1, have since been reported to
123 cause both (AD)SHE and EIMFS [32,33]. Several mechanisms have been proposed for how
124 pathogenic variants may lead to GOF, such as changes in Na^+ sensitivity [17,55,56] or
125 increased maximum probability of opening (P_o) [56]. Other studies have shown that
126 pathogenic variants increase cooperative gating between channels in the same patch
127 excised from *Xenopus* oocytes [32], or cause $K_{Na}1.1$ to be in a constitutively phosphorylated-
128 like state, as a result of altered interactions with binding proteins such as Phosphatase and
129 Actin Regulator 1 (Phactr1) [28,57,58]. Though pathogenic variants may alter channel
130 activity through different mechanisms, the overall effect is GOF characterised by an increase
131 in outward current amplitude, and a shift in the half-maximal activation voltage in the
132 hyperpolarising direction [14,55].
133
134 Missense *KCNT1* variants are almost always heterozygous, with patients carrying only one
135 mutated allele. Only one patient with Ohtahara syndrome has been reported as
136 homozygous, as a result of uniparental disomy [59]. Due to the severity of phenotypes
137 resulting from heterozygous *KCNT1* pathogenic variants, it is unlikely that homozygous
138 patients would survive. Most efforts to functionally characterise *KCNT1* pathogenic variants
139 involve expression of homomeric mutant channels. Recently, the implications of
140 heterozygous *KCNT1* pathogenic variants [14,55] or *KCNT2* pathogenic variants in co-
141 assembly with *KCNT1* [5] on channel function have been studied *in vitro* using co-
142 expression. These studies show heteromeric variant/WT channels behave with
143 characteristics 'intermediate' between WT and pathogenic variant homomeric channels. In

general, however, there is an absence of information concerning the functional and kinetic effects of heterozygous pathogenic variants.

Treatments and their rationale

KCNT1-related epilepsies are intractable, with conventional therapies only temporarily alleviating symptoms. Antiarrhythmic drugs quinidine and bepridil are efficacious at inhibiting WT and pathogenic variant $K_{Na}1.1$ channels expressed in *Xenopus* oocytes and mammalian cells [14,54,55,60,61]. A third antiarrhythmic drug, clofilium, has also been found to inhibit WT channels *in vitro* [62]. These inhibitors are non-selective and inhibit multiple ion channels, including cardiac cation channels [55,60,62], and only quinidine has been trialled in patients.

Quinidine is a less potent inhibitor than bepridil; the drugs inhibit WT $K_{Na}1.1$ expressed in mammalian cells with IC_{50} values in the order of 100 µM and 1 µM, respectively [55,60,61]. Both have been tested against several pathogenic *KCNT1* variants in whole-cell and single channel patch clamp experiments [14,34,54,55,61]. When applied to two EIMFS-causing variants, M516V and G288S, bepridil inhibited $K_{Na}1.1$ current more potently compared to WT [55]. The same effect was reported when quinidine was tested against (AD)SHE-causing variant Y796H [61]. This raises the possibility that the inhibitors exert an open-channel block and the increase in channel activity with pathogenic $K_{Na}1.1$ variants may potentiate binding by quinidine and bepridil [14,55].

Clinically, quinidine has led to variable results. One study reported only 20% of patients to have more than 50% reduction in seizures, and another found 45% to have more than 25% reduction [15,63]. Furthermore, *in vitro* efficacy does not fully translate to clinical efficacy; clinical and *in vitro* effects of quinidine and other drugs are summarised in Table 1. For example, despite promising inhibition of the variant Y796H expressed in *Xenopus* oocytes and HEK293 cells [54,61], there was no improvement in (AD)SHE symptoms or accompanying developmental symptoms [18]. On the other hand, whilst quinidine was not as effective at reducing K629N $K_{Na}1.1$ currents as other variants, seizure frequency in a patient carrying this EIMFS-causing variant was reduced by 80% [18,54]. In another EIMFS patient, significant reduction of seizure frequency and developmental defects were reported following quinidine therapy [13]. Since heteromeric channels comprising WT and mutated subunits have properties intermediate of channels comprising WT or mutated subunits alone [14,55], efficacy of $K_{Na}1.1$ inhibitors may also be influenced by this.

Quinidine has poor blood-brain-barrier (BBB) penetration and may not reach therapeutically-significant concentration in the CNS [13,18]. A drug that crosses the BBB effectively is desirable, due to the abundance of $K_{Na}1.1$ in the CNS [2,3]. No studies have reported cerebrospinal fluid (CSF) concentration of quinidine in patients with *KCNT1*-related DEEs. Previously, CSF levels of quinidine in humans were measured as between 4-37% of unbound serum concentration [64], suggesting that even at therapeutic circulating concentrations, lower levels reach the CNS. Varying serum quinidine concentrations have been reported in a sample of 7 *KCNT1* patients, ranging 1.23-14.8 µM [65]. The therapeutic quinidine concentration range when used as an antiarrhythmic is 6-15 µM, and toxic effects occur at above 18.5 µM [13]. Considering that quinidine inhibits $K_{Na}1.1$ *in vitro* with an IC_{50}

191 of 100 μM [55,60,61], it is unsurprising that clinical success is limited. Fitzgerald *et al* [15]
192 reported subtherapeutic levels of quinidine in more than half of the patients in their cohort.
193 Increasing the serum concentration to reach therapeutic levels may lead to off-target
194 effects, as was found in a randomised trial in 6 adult patients presenting with *KCNT1*-related
195 (AD)SHE (Australian Therapeutic Goods Administration Clinical Trial Registry, number
196 2015/0151) [19].
197
198 Combination therapy with hepatic cytochrome P450 enzyme-inducers, such as
199 phenobarbital and phenytoin, may hinder quinidine effectiveness by inducing its
200 metabolism and lowering serum concentrations by as much as 50%. This has been
201 evidenced in a patient with EIMFS resulting from two *KCNT1* variants, R356W and
202 P724_L728 dup, though the second is of unknown significance. Serum levels of quinidine
203 were undetectable prior to discontinuation of concurrent phenobarbital administration [21].
204 Similarly, two patients with EIMFS resulting from *KCNT1* pathogenic variants, G288S and
205 A934T, had decreased quinidine serum concentration resulting from combination therapy
206 with phenobarbital [24].
207
208 Bepridil, quinidine, and clofilium all present a problem with selectivity when used
209 therapeutically for epilepsies. Bepridil is primarily an L-type Ca^{2+} channel blocker, but also
210 inhibits fast inward Na^+ current through voltage-gated Na^+ channels in a similar manner to
211 lidocaine [66]. Quinidine inhibits human *ether-a-go-go*-related gene (hERG) K^+ channels
212 expressed in HEK293 cells with an IC_{50} of 0.41 μM [67], which is several orders of magnitude
213 more potent than an IC_{50} in the order of 100 μM required to inhibit $K_{Na}1.1$ expressed in
214 mammalian cells [55,60,61]. Since all three inhibitors are potent inhibitors of hERG

channels, adverse cardiac effects may arise from their clinical use [13]. The hERG channel current is responsible for termination of the cardiac action potential, and inhibition can induce **Torsades de Pointes ventricular arrhythmia**, mimicking the type-2 long-QT (LQT) syndrome phenotype [68]. Though some reports suggest increasing dosage and consequently serum concentration in some patients may lead to LQT-like side effects, Fitzgerald *et al* [15] found there to be no relationship between blood quinidine level and propensity for prolonged **QTc interval**. This raises the possibility that a prolonged QTc interval can present even with subthreshold concentrations of quinidine. There is an unmet need for more potent and selective inhibitors of $K_{Na}1.1$ that supress the effects of GOF pathogenic variants.

Mode of action of known inhibitors

Whilst the mechanism of action of $K_{Na}1.1$ inhibitors was previously unknown, inhibition of hERG channels by quinidine, bepridil and clofilium has been investigated in-depth. These drugs block hERG by interacting with aromatic side chains of residues lining the inner pore vestibule, which is largely hydrophobic, from the intracellular side. It is predicted that inhibitors cross the plasma membrane and block the channel intracellularly [61]. A phenylalanine residue located in the hERG channel pore, F656, was identified as a common determinant of inhibition by the three inhibitors [69-72]. This led to the hypothesis that quinidine and bepridil also bind to the inner pore vestibule of $K_{Na}1.1$. A combination of *in silico* modelling of quinidine and bepridil binding within the pore (Figure 2), utilising structures of the chicken $K_{Na}1.1$ channel and mutational analysis of the human $K_{Na}1.1$ channel, supported this [61]. Indeed, the residue identified as important for quinidine and

bepridil binding to $K_{Na}1.1$, F346, is the equivalent phenylalanine to that determining hERG inhibition by quinidine, clofilium, and bepridil [69-72]. The same residue has also been implicated in an EIMFS-causing *KCNT1* pathogenic variant, F346L and is thus far the only variant found to be completely insensitive to inhibition by quinidine *in vitro*, when expressed in *Xenopus* oocytes [17].

A number of small molecule inhibitors have been identified that inhibit the channel with low- and sub-micromolar potencies. A combination of **structure-based virtual screening** using a $K_{Na}1.1$ cyro-EM structure, and **ligand-based virtual screening** using bepridil as reference, were used to identify inhibitors from a virtual library of 100,000 compounds. It was proposed that the inhibitors had a similar mechanism of action to quinidine and bepridil, blocking the channel via the intracellular vestibule (Figure 2). Furthermore, two of the compounds had limited cytotoxicity and did not inhibit hERG channels in preliminary toxicity screens. It is noteworthy that the two compounds identified that were structurally-similar to bepridil almost completely inhibited hERG channels at 10 µM [61]. As well as identifying potential pharmacophores, starting points for more potent inhibitors of $K_{Na}1.1$, or tools to study the channel further, this work highlights the use of cryo-EM-derived structures of membrane proteins for structure-based drug discovery. Though the structure determined by Hite and MacKinnon [27] was that of chicken rather than human $K_{Na}1.1$, they share 84% sequence homology. The success when testing the compounds against the human $K_{Na}1.1$ channel demonstrates that proteins very close to the human structure can be utilised in the absence of the desired structure.

More recently, a potent small molecule inhibitor, VU0606170, was identified using a high-throughput **thallium flux screen.** This compound inhibited WT channels expressed in HEK293 cells with an IC_{50} of 1.84 µM, and EIMFS-causing pathogenic variant A934T with an IC_{50} of 1.16 µM. Further selectivity experiments found this compound to be inactive against $K_{Na}1.2$, $K_{Ca}1.1$, GIRK1/2, $K_v2.1$, TREK1 potassium channels, and $Ca_v3.2$ and $Na_v1.7$. Though the compound inhibited hERG channels to some degree, the potency was lower than bepridil. Importantly, VU0606170 reduced the firing rate of hyperexcitable cultured cortical rat neurons, providing evidence that selective $K_{Na}1.1$ inhibition may act to diminish hyperexcitability [73]. Similarly, using another well-established high-throughput screen and functional assays, Griffin *et al* [74] identified and characterised a small molecule inhibitor with *in vivo* activity. Compound 31, an optimised derivative of a hit from a high-throughput **rubidium flux screen**, reduced both seizure frequency and interictal spikes in a mouse model of EIMFS-causing pathogenic variant P924L (mP905L). Compound 31 inhibited human WT and mP905L channels *in vitro* with nanomolar and low micromolar potency respectively, and was selective for $K_{Na}1.1$ channels over hERG, $Na_v1.5$, $Ca_v1.2$, I_{Ks}, $K_{Ca}1.1$, and $K_{Na}1.2$ [74]. It is surprising that both VU0606170 and Compound 31 are both selective for $K_{Na}1.1$ over $K_{Na}1.2$ channels, considering their high sequence homology; sharing 78% sequence identity and differing by only one residue in the pore vestibule. Whilst it is possible that these molecules target different and less well-conserved domains of the channel, the reduced potency of Compound 31 with F346L $K_{Na}1.1$ and the importance of trifluoromethyl groups for potency, which in BC12 and BC14 were found to dock between the pore and S5 helices [61] (Figure 2B), suggest that Compound 31 binds the channel pore in a similar manner.

Potential new inhibitor modalities

To date, the literature principally describe $K_{Na}1.1$ pore-blocking drugs, a mechanism linked to non-selectivity due to similarity in structure and pore-lining residues between different K^+ channel subunits. Advances in structure-based drug design and information about the underlying mechanisms of GOF pathogenic variants may give rise to alternative modalities for therapeutics. Targeting other domains, such as those involved in ligand binding and activation by ether Na^+ or NADP, may provide alternative pharmacological opportunities.

Though many K^+ channels have conserved sequences, particularly around the selectivity filter and pore-forming region, RCK domains on the C-terminus are one of the features separating SLO channels from other eukaryotic K^+ channels [41]. Moreover, the RCK domains of $K_{Na}1.1$ and $K_{Na}1.2$ deviate further from those of other members of the SLO family due to the channel being activated by intracellular Na^+. In place of the Ca^{2+}-bowl possessed by $K_{Ca}1.1$ channels, which enables Ca^{2+} activation, the αQ' helix of $K_{Na}1.1$ is extended across the assembly interface between RCK domains of adjacent subunits [42]. Though homomeric pathogenic variant $K_{Na}1.1$ channels are shown to have some activity in the absence of intracellular Na^+ [55,56], the behaviour of heteromeric pathogenic variant and WT channels in the absence of Na^+ has not been widely explored. It is possible that an antagonist acting at the Na^+- binding site of the channel could diminish the effects of GOF pathogenic variants effectively, without residual activity in heteromeric channels.

Previous work using a combination of homology modelling, molecular simulations and mutagenesis has highlighted a conserved aspartate residue as being important for Na^+-sensitivity of rat $K_{Na}1.1$ [46] and $K_{Na}1.2$ [47] channels. In $K_{Na}1.1$, rH823 and rD818 (hH844

and hD839) are part of a proposed Na^+-binding motif, DXRXXH, which is responsible for Na^+ sensitivity in G protein-coupled inwardly- rectifying K^+ (GIRK) channels. Mutation of the aspartate in this motif to neutral or positively-charged residues significantly reduced, but did not abolish, Na^+-sensitivity of the channel expressed in *Xenopus* oocytes [46]. This model of Na^+-activation does not align well with the since determined $K_{Na}1.1$ channel structure, with the conserved loop containing this motif, including the equivalent aspartate side-chain (cD812 in chicken $K_{Na}1.1$), remaining in the same conformation in the active and inactive channel states [27]. Further information is required on the location of the residues involved in Na^+-binding to the RCK domains, and the mechanisms by which Na^+-binding leads to $K_{Na}1.1$ activation. Similarly, whether the NADP binding site can be targeted to prevent the modulatory effects of NADP on Na^+-activation [44] has yet to be explored.

$K_{Na}1.1$ has a number of modulatory cytoplasmic binding partners that could potentially be targeted. For example, Fragile X Mental Retardation Protein (FMRP) increases P_o of $K_{Na}1.1$ [75], and TMEM16C directly interacts with $K_{Na}1.1$ subunits to increase Na^+ sensitivity and increase channel activity [76]. SCYL1 has also been found to increase P_o of $K_{Na}1.1$ when the two proteins are co-expressed in *Xenopus* oocytes, and their overlapping expression patterns in mouse brain regions suggests this binding protein may regulate $K_{Na}1.1$ activity in neurons [77]. Preventing the physical or functional interaction with these proteins may suppress hyperactive $K_{Na}1.1$ channel activity. Conversely, in HEK293T cells, co-expression with Phactr1 suppresses rat $K_{Na}1.1$ current. It is hypothesised that this happens via protein phosphatase 1-mediated (PP1) dephosphorylation of the channel at rS407 (hS426), the critical PKC phosphorylation residue on the C-terminus of $K_{Na}1.1$ [58]. Suppression of $K_{Na}1.1$ by Phactr1 is disrupted by the introduction of two EIMFS-causing *KCNT1* pathogenic variants

334 [57]. The interaction between $K_{Na}1.1$ and Phactr1 may therefore also be a useful therapeutic
335 target.
336
337 $K_{Na}1.1$ channels are activated both by intracellular Na^+ and voltage, whilst lacking the
338 canonical VSD that is found in other voltage-gated K^+ channels [42]. Understanding the
339 interactions of domains that underly the WT $K_{Na}1.1$ gating mechanism would assist in
340 determining the effects of pathogenic variants on channel function, but also in finding new
341 treatment modalities. A negative allosteric modulator that uncouples the interaction
342 between Na^+- binding and channel activation or affects the intrinsic closed-open transition
343 could act to suppress $K_{Na}1.1$, perhaps in a similar manner to modulators of $K_{Ca}1.1$ channels.
344 For example, paxilline allosterically modulates $K_{Ca}1.1$, by preferentially occupying the closed
345 state and decreasing the equilibrium constant *L* described in the Horrigan-Aldrich allosteric
346 model for channel gating, with the overall effect of suppressing current [78]. *L* describes the
347 intrinsic closed to open channel transition in the absence of Ca^{2+}, and no active VSD [79].
348
349 Though it is currently unclear how GOF *KCNT1* pathogenic variants may lead to
350 hyperexcitability, several mechanisms have been suggested. One such hypothesis is that
351 GOF of $K_{Na}1.1$ channels expressed in GABAergic inhibitory interneurons could increase
352 hyperpolarisation [80,81]. This would dampen their inhibitory effect on excitatory
353 interneurons, leading to increased excitation. This has been evidenced by
354 electrophysiological recordings from primary cortical neurons cultured from a mouse model
355 of (AD)SHE-causing mutation Y796H, which resulted in increased $K_{Na}1.1$ current at
356 subthreshold voltages in both non-fast and fast-spiking GABAergic neurons [81]. Another
357 possibility is that pathogenic variant $K_{Na}1.1$ channels activate rapidly in response to action

potentials, enabling more rapid recovery from action potentials. Voltage-gated Na^+ channels, which are responsible for the upstroke of the action potential, may become inactivated and re-primed at a faster rate, increasing the rate of high frequency firing and leading to hyperexcitability. The latter was evidenced by a study of potentially immature iPSC-derived neurons that harbour homozygous variant P924L $K_{Na}1.1$ through genome editing [82]. This genotype, however, does not reflect the usual heterozygous nature of the disorders and the function of homomeric mutant channels may differ from those co-assembled with wild-type subunits.

Considering the potentially neuron-subtype-specific effects of *KCNT1* pathogenic variants, it may be of use to target specific cell types therapeutically. Inhibiting subthreshold $K_{Na}1.1$ current in GABAergic inhibitory interneurons [81], particularly non-fast spiking neurons, could reverse the effects of pathogenic variants on membrane excitability and action potential generation.

Concluding remarks and future perspectives

Following the initial reports of pathogenic *KCNT1* variants [28,29], the broad spectrum of disorders associated with $K_{Na}1.1$ has become apparent. Normal function of $K_{Na}1.1$ is important not only for regulating neuronal excitability [6-10], but also for cardiac and intellectual functions [11,37,38], evidenced by the wide range of phenotypes resulting from pathogenic variants. Understanding the underlying mechanisms of GOF pathogenic variants, particularly in heteromeric assemblies of mutant and WT subunits, will be key in developing more effective and selective treatment strategies. Availability of high resolution cryo-EM

382 structures for the channel in different activation states [27,42], and animal models [83], will
383 aid this in future. As stated in the outstanding questions, determining how Na^+ acts to
384 activate $K_{Na}1.1$, and the sequence of events involved in gating the channel is important in
385 understanding how pathogenic variants exert their effects. This will enable other, more
386 selective, treatment modalities to be explored that suppress $K_{Na}1.1$ current and avoid off-
387 target cardiac effects.

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Glossary

Afterhyperpolarisation: Repolarisation of the membrane potential following an action potential or burst of action potentials. The membrane potential falls lower than resting membrane potential, and this is usually facilitated by K^+ channel activation.

Hydrophobic gating: In K^+ channels gated by a hydrophobic gate, water transitions between liquid and vapour states within the pore as it interacts with hydrophobic pore-lining residues. In the vapour state, the pore cavity becomes 'de-wetted' or collapsed, and this acts as a barrier to ion permeation. De-wetting is dependent on pore diameter.

Ligand-based virtual screening: Computer-aided drug screening technique that identifies structurally similar compounds to a known ligand from a chemical library. These structurally similar compounds can later be docked into the protein of interest and ranked based on predicted binding affinity.

QTc interval: On an ECG, the QTc interval is defined as the time interval between the start of the Q wave, and end of the T wave. This represents the onset of depolarisation, and end of repolarisation of the cardiac action potential.

Rubidium flux screen: A high-throughput technique with the capability to screen large libraries of compounds for K^+ channel modulation, exploiting the ability of Rb^+ to permeate the channels. Cells can be pre-incubated with $^{86}RbCl$ and the degree of efflux during the assay can be determined by liquid scintillation. Alternately, cells can be loaded with cold RbCl and efflux determined by atomic absorption.

S6 helix bundle-crossing: The canonical mechanism of K^+ channel gating. The cytoplasmic end of S6 helices of K^+ channel subunits converge at the bottom of the pore-forming region, forming a 'bundle crossing'. Ion conduction is physically occluded by the S6 helix bundle crossing and widening enables ion conduction.

Selectivity filter gating: A recently identified mechanism of K^+ channel gating. The selectivity filter widens as a result of allosteric coupling with the activation gate, facilitated by pore-lining transmembrane helices, to enable ion conduction.

Structure-based virtual screening: Computer-aided drug screening approach involving docking compounds from a chemical library into a protein structure. Intermolecular interactions can be predicted, and chemicals are ranked based on predicted binding affinities.

Thallium flux screen: Fluorescence-based assay that utilises the permeability of Tl^+ through K^+ channels, to detect modulators of K^+ channels heterologously expressed in mammalian cells. A high-throughput technique with the capability to screen large libraries of compounds.

Torsades de Pointes ventricular arrhythmia: Associated with prolongation of the QTc interval, this acquired or inherited arrhythmia is characterised by 'twisting' of the QRS segment of an ECG. Torsades de Pointes can sometimes lead to ventricular fibrillation and cardiac arrest.

Figure Legends

Figure 1 Structure of the K_{Na}1.1 potassium channel and location of mutated residues. Cryo-EM structure of the chicken K_{Na}1.1 channel in the active conformation (PDB: 5U70 [27]). One subunit of the tetramer is coloured yellow with the residues coloured red indicating those that are altered in the equivalent position in human K_{Na}1.1 by *KCNT1* gain-of-function pathogenic variants. Figure prepared in UCSF Chimera.

Figure 2 Predicted interactions between K_{Na}1.1 and inhibiting compounds. A Structure of the pore domain of the tetrameric chicken K_{Na}1.1 channel [27] comprising the S5, P-loop, and S6 segments. The yellow dashed box indicates the region depicted in the figures in panel B. B Docking poses of quinidine, bepridil, BC13, and BC14 from the study by Cole *et al.* [61] in the K_{Na}1.1 channel pore. Compounds occupy the pore and interact with residues in the S6 segment and also those immediately below the selectivity filter. Potency of each of these inhibitors was reduced by mutation of a phenylalanine in S6 (shown at the bottom of each figure) to serine [61]. Figure prepared in UCSF Chimera.

Table 1: Pathogenic variants in *KCNT1* that have been studied *in vitro* or clinically with inhibitors, their location on the channel structure, and the associated clinical phenotype.

Pathogenic variant	CDS change	Location	Associated disorder	Pharmacological modulation *in vitro*	Clinical response to therapies	References
R209C	c.625C>T	S3 domain	Lennox-Gastaut	N/A	Clinically significant response to quinidine	[26]
A259D	c.776C>A	S4-5 linker	EIMFS	Quinidine significantly decreased current amplitude (300 μM)	No clinical response to quinidine	[20]
Q270E	c.808C>G	S5 domain	EIMFS	N/A	No clinical response to quinidine	[16,63]
					No clinical response to quinidine or ketogenic diet	
V271F	c.811G>T	S5 domain	EIMFS	Quinidine significantly decreased current amplitude (300 μM)	N/A	[17]
L274I	c.820C>A	S5 domain	EIMFS	Quinidine significantly decreased current amplitude (300 μM)	No clinical response to quinidine	[17,63]
					Patient showed minimal response to ketogenic diet and no response to cannabidiol	
G288S	c.862G>A	S5 domain	(AD)SHE EIMFS	Bepridil significantly more potent compared to WT **IC_{50} for bepridil: 0.15±0.05 μM** No significant difference between inhibition of WT and mutant by quinidine **IC_{50} for quinidine: 67±19 μM** Inhibited by test compound 31 **IC_{50}: 221 nM**	Some patients have responded to quinidine, others have not	[15,24,55,63,74]
					No clinical response to quinidine, seizures worsened. Phenobarbital showed slight efficacy but discontinued due to drowsiness.	
					Three patients responded to quinidine, two out of the three had a marked response to ketogenic diet. The third had a marked response to cannabidiol.	
F346L	c.1038C>G	S6 domain	EIMFS	Completely insensitive to quinidine (300 μM) in *Xenopus* oocytes Inhibited by test compound 31 **IC_{50}: 1.77 μM**	N/A	[17,74]
R356W/ P724_L728 dup	c.1066C>T c.2170_2184dup 15	Bottom of S6 domain	EIMFS	N/A	Minor relief with quinidine. Interaction with phenobarbital, resulting in prolonged QTc interval.	[21]
R398Q	c. 1193G>A	RCK1 domain	(AD)SHE EIMFS	Quinidine significantly decreased current amplitude (300 μM)	No clinical response to quinidine	[54,63]

Pathogenic variant	CDS change	Location	Associated disorder	Pharmacological modulation *in vitro*	Clinical response to therapies	References
R428Q	c.1283G>A	RCK1 domain	EIMFS	Quinidine significantly decreased current amplitude (300μM)	Patient responded to phenobarbital and potassium bromide (later died of sudden cardiac arrest). Quinidine exacerbated seizures and was discontinued. Patient responded to quinidine in combination with other antiepileptic medications and ketogenic diet >50% reduction in seizure frequency with quinidine, experienced ventricular tachycardia. Seizures unresponsive to phenobarbital, KBr, clonazepam, clobazam, levetiracetam. Patient showed marked response to ketogenic diet, some response to cannabidiol and no response to quinidine.	[17,24,25,63,84]
L437F	c.1309C>T	RCK1 domain	Epilepsy with status distonicus	**IC_{50} for quinidine: 66 μM**	N/A	[34]
R474C	c.1420C>T	RCK1 domain	Focal epilepsy	N/A	23% reduction in seizure frequency with quinidine; not considered successful. No clinical response to conventional epilepsy therapeutics, methyl prednisolone pulse therapy, and ketogenic diet.	[24]
R474G	c.1420C>G	RCK1 domain	Multifocal seizures	N/A	Clinically significant response to phenobarbital	[84]
R474H	c.1421G>A	RCK1 domain	EIMFS	N/A	No clinical response to quinidine One patient responded to ketogenic diet, another showed no response to quinidine Patient responded to tipepidine and dextromethorphan	[15,23,63]
F502V	c.1504T>G	RCK1 domain	EIMFS	Quinidine significantly decreased current amplitude (300 μM)	Clinically significant response to quinidine	[17]
M516V	c.1546A>G	RCK1 domain	EIMFS	Bepridil significantly more potent compared to WT **IC_{50} for bepridil: 0.3±0.1 μM** No significant difference between inhibition of WT and mutant by quinidine **IC_{50} for quinidine: 46±12 μM**	N/A	[55]
K629N	c.1887G>C	RCK2 domain	EIMFS	Quinidine (300 μM) less effective than when used for Y796H, R428Q and WT	Clinically significant response to quinidine; 80% decrease in seizure frequency	[18,54]

Pathogenic variant	CDS change	Location	Associated disorder	Pharmacological modulation *in vitro*	Clinical response to therapies	References
Y796H	c.2386T>C	NAD^+ binding domain	(AD)SHE	**IC_{50} for quinidine: 38±12.89 µM** **IC_{50} for bepridil: 12.8±2.48 µM** Inhibited by test compounds BC5,6,7,12,13 and 14 with **IC_{50} values ranging from 3.61-17.45 µM**	No clinical response to quinidine	[18,61]
E893K	c.2677G>A	NAD^+ binding domain	EIMFS	More sensitive to quinidine than WT in CHO cells **IC_{50} for quinidine: 9.6±2.5 µM**	Clinically significant response to quinidine; 90% reduction in seizures	[14]
M896K	c.2687T>A	NAD^+ binding domain	EIMFS	Quinidine significantly decreased current amplitude (300 µM)	Severe pulmonary vasculopathy resulting from clinical use of quinidine	[17]
M896I	c.2688 G>C	NAD^+ binding domain	SHE	Quinidine significantly decreased current amplitude (300 µM)	N/A	[54]
P924L	c.2771C>T	C-terminus (next to NAD^+ binding domain)	EIMFS	Quinidine significantly decreased current amplitude (300 µM) Mouse orthologue inhibited by test compound 31 **IC_{50}: 1.012 µM**	N/A	[54,74]
R928C	c.2782C>T	C-terminus (next to NAD^+ binding domain)	(AD)SHE	Quinidine significantly decreased current amplitude (300 µM)	No clinical response to quinidine	[19,54]
A934T	c.2800G>A	C-terminus (next to NAD^+ binding domain)	EIMFS	Quinidine significantly decreased current amplitude (300 µM) Inhibited by test compound VU0606170. **IC50: 1.16 µM**	Clinically significant response to quinidine Seizure frequency initially decreased, but later increased with quinidine therapy. In combination with phenobarbital and KBr, phenobarbital hindered increase in quinidine serum levels One patient showed a marked response to both ketogenic diet and cannabidiol. Another responded to quinidine and low glycemic index diet. A third patient showed no clinical response to quinidine or ketogenic diet	[22,24,54,63,73]
R950Q	c.2849G>A	C-terminus	EIMFS	More sensitive to quinidine than WT in CHO cells **IC_{50} for quinidine: 24±5.7 µM**	No clinical response to quinidine. Significant reduction in seizure frequency in another patient with quinidine therapy. One patient showed some response to cannabidiol therapy, another showed a marked response to quinidine and some response to low glycemic index diet	[14,22,63]
L962P	c.2885T>C	C-terminus	EIMFS	N/A	Some clinical response to ketogenic diet	[63]

Pathogenic variant	CDS change	Location	Associated disorder	Pharmacological modulation *in vitro*	Clinical response to therapies	References
A966T	c.2896G > A	C-terminus	Combination of (AD)SHE and EIMFS phenotype	N/A	Complete and persistent response to phenobarbital in patient	[85]
R1114W/ del 550	c.3340 C>T c.1649-1651delAGC	End of C-terminus	EIMFS	Quinidine significantly decreased current amplitude of R1114W variant (300 µM) but not del 550 variant	No clinical response to quinidine	[20]

Highlights

Gain-of-function pathogenic variants of *KCNT1*, the gene encoding Na^+-activated K^+ channel $K_{Na}1.1$, underlie a broad spectrum of severe and refractory developmental and epileptic encephalopathies accompanied by intellectual disabilities.

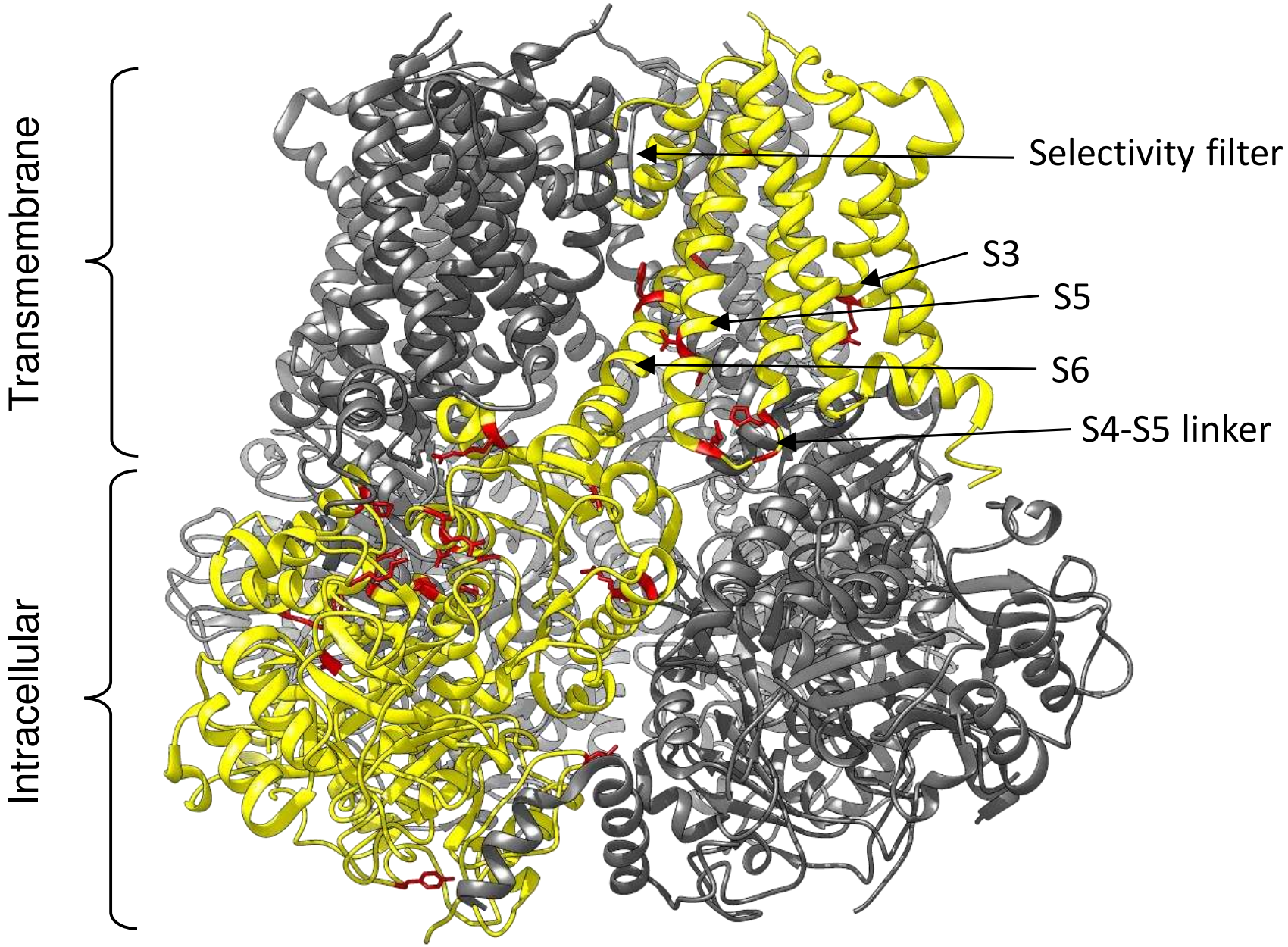
KCNT1 variants likely cause hyperexcitability by impairing GABAergic neuron excitability. Inhibition of mutant $K_{Na}1.1$ channels is the current strategy to suppress hyperexcitability.

Known inhibitors block the inner pore vestibule of $K_{Na}1.1$, similar to how they inhibit cardiac hERG channels. Potent inhibition of hERG is one of the limiting factors for their use, along with low potency.

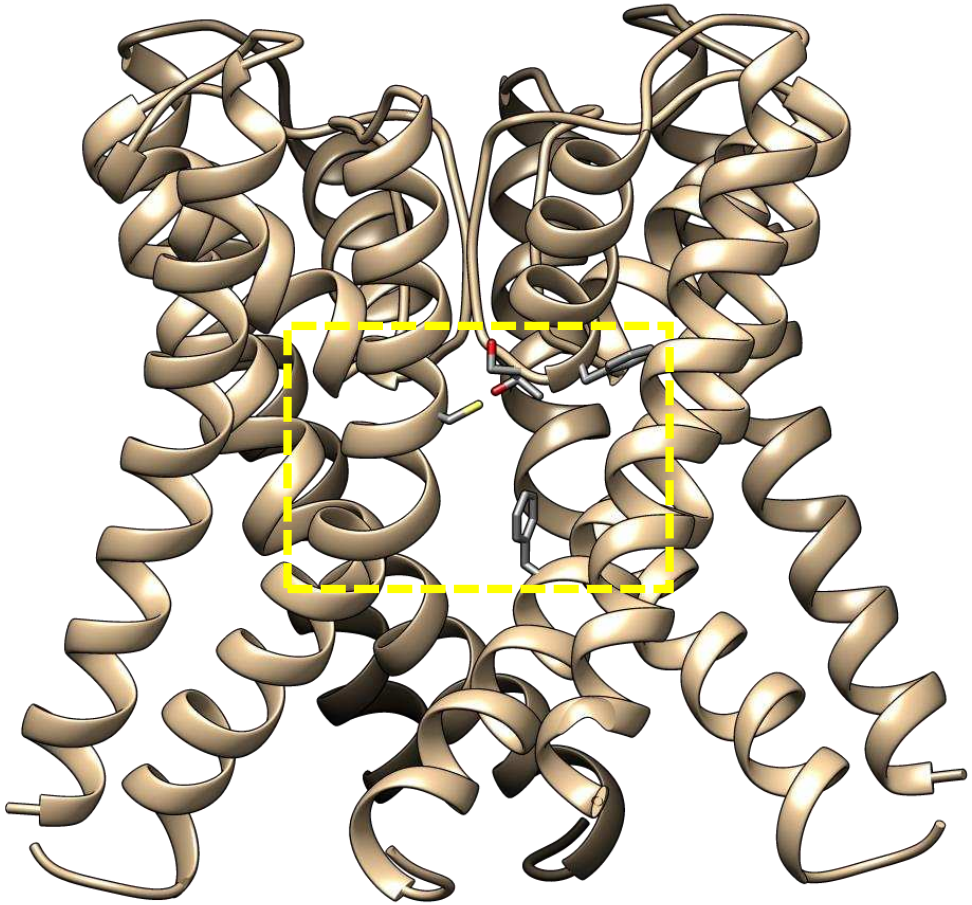
Potent small-molecule inhibitors of the channel have been identified, using both high-throughput screening and *in silico* methods. These inhibitors show promise both in terms of improved selectivity for $K_{Na}1.1$ and efficacy for suppressing hyperexcitable neurons.

Outstanding questions

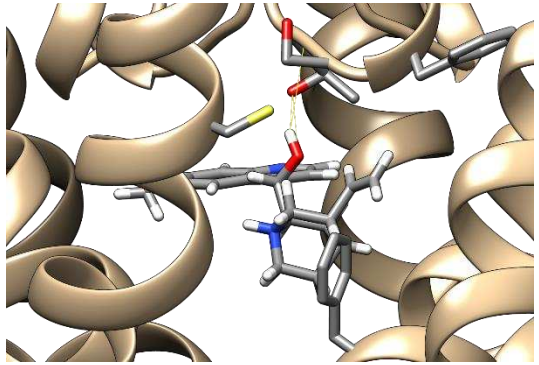
- How is $K_{Na}1.1$ activated by intracellular Na^+?
- How do Na^+ sensing and voltage interact to gate $K_{Na}1.1$, and how is this affected by epilepsy-causing mutants?
- How does heteromeric assembly of mutant and WT $K_{Na}1.1$ subunits affect inhibition and gating of the channel?


Transmembrane
Intracellular
Selectivity filter
S3
S5
S6
S4-S5 linker

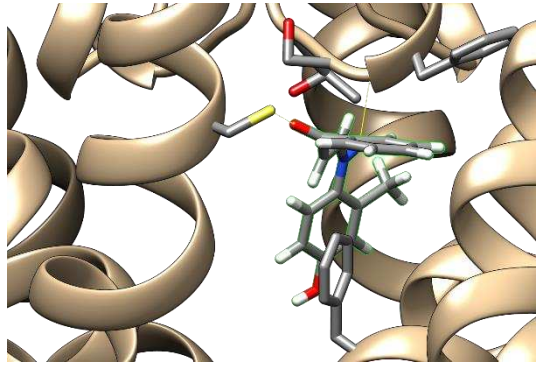
A



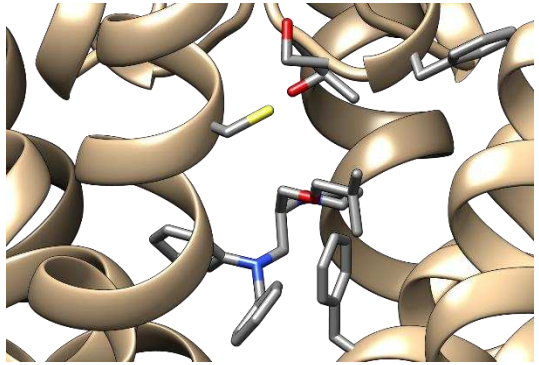
B



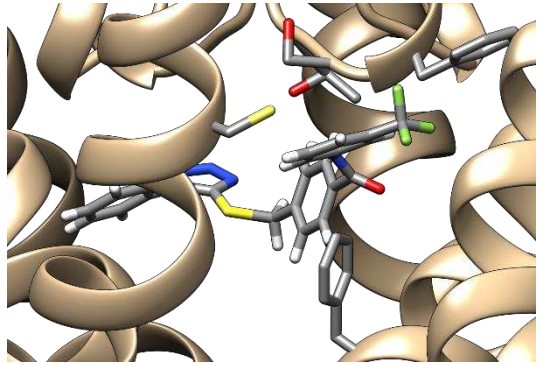
quinidine



BC13



bepridil



BC14