

Channelopathy profiling

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After academic research as a neuroscientist and 20 years of commercial ion channel drug discovery, Marc is now a freelance blogger, advisor and consultant for clients worldwide, where he shares his expertise and enthusiasm for all aspects of ion channel screening. He is particularly interested in automated patch clamp, and exploiting the potential of human iPS stem cell assays to facilitate the successful translation of new drugs into the clinic.

Channelopathies are mutations in ion channel genes that are associated with human disease, and they provide an important validation metric for drug discovery efforts linking specific protein targets to patient populations. Increased clinical use of genome and whole exome sequencing to help diagnose and classify patients has greatly expanded the number of channelopathies in recent years, with over 1300 ion channel mutations now identified in the [OMIM database](#) of human genes and genetic disorders. Information on the functional impact of ion channel mutations is important for two broad reasons:

- Improving structure-function studies of voltage- and ligand-gated ion channels, and
- Understanding the effect of disease-related variants to improve patient treatments

Ion channels lagged behind other protein families when it came to high resolution structures, with many structure-function studies relying on homology models based on a limited number of non-mammalian eukaryotic or prokaryotic x-ray crystallography structures, which limited their predictive power. The recent development of single particle cryo-EM imaging has yielded a large number of high resolution structures of human ion channels from most voltage- and ligand-gated ion channel families, onto which channelopathy mutations can be mapped at atomistic scale and correlated with historical functional data. While biophysical data on channelopathies typically came from low throughput *Xenopus laevis* oocyte and manual patch clamp electrophysiology, the explosion in ion channel structural data has produced a mechanistic 'bottleneck'

which is now being met by high throughput automated patch clamp (APC) platforms, which will be the focus of this article.

Similarly, the proliferation of human genetic sequence data has provided challenges for traditional variant classification techniques, typically based on clinical and functional phenotype categorization and *in silico* protein models. The [ClinVar database](#) is a vital and growing repository of human gene variants classified by disease and drug response, but the clinical and functional annotation of each variant can be extremely variable as it relies on submitted evidence and pathogenicity predictions based on variant location and protein homology models. Most significantly, only 2-5% of human gene variants have reliable classification as either being pathogenic or benign that can be used to inform clinical treatments and patient counseling, leaving the vast majority as 'variants of uncertain significance' (VUS) that require further functional and *in silico* profiling to help diagnosis of rare disease patients and development of novel drugs and clinical interventions (reviewed in ^{1,2}). The first half of this article will provide an overview of the *in silico* tools being used to map and profile the growing list of channelopathies, followed in the second half by case studies using APC to functionally profile and 'decrypt' disease-linked variants in human ion channels.

Channelopathy variant profiling approaches

Researchers have access to two main techniques to characterize channelopathy mutations, namely virtual protein modeling and empirical biophysical measurements. It would be misleading to cast these two approaches to predict and annotate ion channel variants as competing with each other or being mutually exclusive, and more helpful to regard them as complimentary, especially as the *in silico* models require real world data to train their algorithms and test their predictions. Also, the proliferation of high resolution x-ray and cryo-EM ion channel structures in recent years has provided deep insight into the structure-function of ion channels and the mechanisms by which disease- and patient-related variants may affect ion channel protein activity, providing a real-world structural underpinning for both *in silico* and empirical channelopathy datasets.

In silico modeling of ion channel variants

While the main focus of *in silico* models of ion channels has been to understand structure-function relationships of the various transmembrane domains and to undertake virtual screening of ligand interactions with known and new binding sites, these techniques have also been used to predict the pathogenicity and functional effects of channelopathy variants in an effort to stratify patients and offer more accurate diagnosis and therapeutic options. This section will highlight three different *in silico* approaches used to model disease and patient variants in Na_v1.7, Ca_v1.3 and cardiac ion channels.

Atomistic models of Na_v1.7 pain variants

Any review of ion channelopathies can not ignore Na_v1.7, as numerous variants have been identified in patients with painful and painless syndromes (reviewed in ³). However, the vast majority of these variants have been studied individually by various academic groups using manual patch clamp in heterologous cells, native dorsal root ganglion (DRG) and human iPSC sensory neurons, yielding an extensive but disparate biophysical dataset. Academic researchers in the US, EU and UK (part of the PROPANE study group) therefore attempted to incorporate all of this biophysical information on painful Na_v1.7 channelopathy variants into an atomistic structure-function model of the channel (Figure 1) to explain the functional effects of profiled variants, predict the phenotype of novel variants, and provide a deeper understanding of the molecular interactions within and between different Na_v1.7 channel domains. ⁴ As this work predated high resolution cryo-EM structures of the human Na_v1.7 channel, they used a 3D homology model of the bacterial Na_vAb channel and a networked topology algorithm to predict interatomic interactions and the effect of painful gain-of-function variants vs benign mutations and more common population polymorphisms on VSD and pore domain connectivity, complex protein structure and channel function (termed *in silico* mutagenesis). The key parameter for pathogenic variant prediction was a measure called Betweenness centrality, which achieved 76% sensitivity and 83% specificity (ROC of 0.81) across biophysically characterized gain-of-function variants (18 IEM mutations, 6 SFN mutations, 6 PEPP mutations) and 4 benign mutations and 49 SNPs identified among human and mammalian Na_v1.7 channels. Higher pathogenicity was seen for amino acid substitutions within or facing the channel 'core', and those facing the lipid membrane had lower painful pathogenicity. A second paper extended this *in silico* modeling to show that pain-related mutations may perturb a hydrophobic patch near the Na_v1.7 pore and affect hydrophobic interactions near the selectivity filter, predicting pathogenicity with 80.5% sensitivity and 93.7% specificity. ⁵

Caveats include the fact that a closed state conformation of the Na_v1.7 channel was used so other interactions during

the gating cycle could be missed, and all *in silico* models excluded intracellular inter-domain linkers as they are structurally disordered and hard to model (but do contain some pathogenic painful Na_v1.7 variants). Also, there was no discrimination between gain-of-function variant effects on voltage-dependent inactivation, activation or other biophysical parameters contained within existing electrophysiological datasets, suggesting that broader patterns are sufficient to predict pathogenicity, or that more detailed *in silico* models could reveal more subtle patient- and disease-specific alterations in Na_v1.7 channel function that can be used to personalize pharmacological and therapeutic treatments.

Ion channel gene evolution analysis

A basic premise of many *in silico* variant prediction algorithms is that amino acid changes in evolutionarily conserved domains are likely to impact protein function,

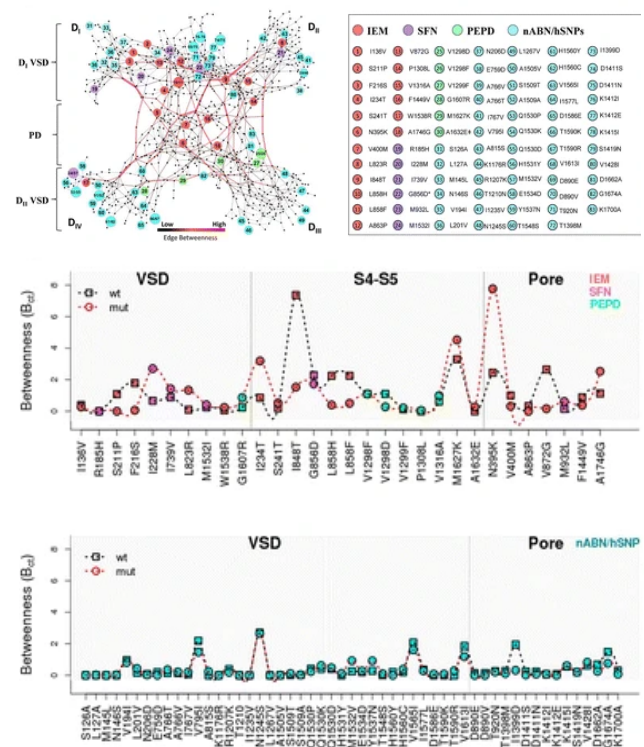


Figure 1 Atomistic model of painful vs benign Na_v1.7 variants (Kapetis et al., 2017 ⁴). Top: View of Nav channel from top showing locations of painful and benign variants in voltage sensor domain (VSD) and pore domains (PD). IEM = inherited erythromelalgia, SFN = small fibre neuropathy, PEPP = paroxysmal extreme pain disorder. Bottom: Betweenness centre scores for painful and benign variants across different Nav channel domains. Figure used under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

whereas variations in non-conserved areas with diverse sequence probably have less significant effects. This technique was recently applied to 80 *de novo* missense variants in the Ca_v1.3 channel, where co-evolutionary analysis of L-type Ca_v1.x channel sequences across the animal kingdom successfully predicted the pathogenicity of known variants in the CACNA1D gene and helped to re-classify several novel variants as either pathogenic or benign, which was confirmed for one subtle variant by patch clamp electrophysiology in transiently transfected tsA-201 cells which showed altered activation. ⁶ This evolutionary model accounts for both single amino acid variants as well as residue interactions within functional domains of the Ca_v1.3 channel independently of clinical, biophysical and structural information, and was able to efficiently and accurately predict pathogenicity of variants which affect Ca_v1.3 gating and are associated with symptoms in patients with autism spectrum disorder and other neurological and endocrine system disease, raising hopes of more effective clinical assessment and interventions.

It is worth noting that the same *in silico* technique may not be an effective predictor of variant pathogenicity for different ion channel proteins and families, and some improvement in model performance can be achieved by combining methods. This was recently shown for analysis of variants in Ca_v1.4 channels linked to stationary night blindness, where a machine learning combination of CACNA1F gene sequence analysis and L-type Ca_v1.1 cryo-EM-based structural homology modeling (which used amino acid properties such as size, charge, hydrophobicity, and position) was shown to accurately predict the molecular and clinical phenotype of over 70 disease-relevant missense and over 300 benign population variants. ⁷ As with other structure-based and homology models of ion channels, most cytoplasmic domains of Ca_v1.4 were omitted due to poor resolution, although it was found that the majority of pathogenic variants were located in conserved residues of transmembrane domains whereas benign variants were less conserved and more likely to be located in regions outside of the constrained homology models.

Those wishing to learn more about these *in silico* approaches may consult a comprehensive comparison of the performance of 16 bioinformatic tools to predict the pathogenicity of hundreds of variants in the Ca_v1.2

channel family. ⁸ This paper is also useful as it analyses 36 VUS mutations, and avoids some of the pitfalls of structure-based models as a significant number of the pathogenic and likely pathogenic Ca_v1.2 variants are found in the cytoplasmic linkers and N-terminus that are often omitted from homology-based algorithms.

- **AlphaMissense protein variant prediction software**

The introduction of Google DeepMind's AlphaFold AI-based 3D protein modeling software has greatly enhanced structure-based studies of large ion channel proteins, aided by the availability of near-atomic resolution cryo-EM structures for most human ion channel family members which have helped constrain and refine 3D protein models based on amino acid sequences. Taking this sequence-based protein structure prediction methodology to the next step, Google DeepMind have now developed a new AI-based machine learning large language algorithm called AlphaMissense that was taught using the 2% of human genetic variants with known clinical classification and patterns in human and primate population genetics to make probabilistic predictions of the pathogenicity of unclassified missense variants. ⁹ Overall, the algorithm classifies 32% of all 71 million human genome missense variants as 'likely pathogenic', 57% as 'likely benign' and significantly reduces the fraction of mutations of unknown significance down to only 11%, with a 90% precision against the ClinVar database (containing ~19,500 annotated variants). Caveats include the fact that the algorithm currently focuses only on single amino acid substitutions, has reduced performance against variants in disordered protein structures, and labels variants as benign if they are frequent in human and primate populations or pathogenic if they are in evolutionarily-conserved domains, although the software is claimed to be able to classify *de novo* variants that it wasn't trained against, which will be an important advantage as genetic testing continues to reveal novel variants in rare and common disease patients.

AlphaMissense marginally out-performs other recent variant classification algorithms, illustrating the significant and exciting progress being made in this field. Specifically looking at ion channels (e.g. included on the list of clinically actionable genes from the American College of Medical Genetics), we see that AlphaMissense can more reliably

"The challenge of profiling the growing number of novel patient- and disease-related channelopathy mutations is being met with high throughput automated patch clamp that can accurately and rapidly characterise their biophysical and pharmacological properties."

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predict known clinical pathogenicity than other variant classification software for ryanodine receptors (RYR1 and RYR2), and $Ca_v1.2$ and $Na_v1.5$ channels but less so for $K_v7.1$, although it also appears to under-estimate the known pathogenicity of a few variants in each protein (false negatives) rather than over-estimate the score of benign variants (false positives). Such approaches will help to bridge the gap between individual gene studies and population genetics, especially in patient and disease groups where genetic risk predictions (e.g. SNPs, GWAS data) and genotype-phenotype associations are still key to trying to understand disease mechanisms, to classify and diagnose new and existing rare disease patients and genetically define cohorts, and ultimately to develop more effective clinical treatments.

AlphaFold and other protein modeling software such as RoseTTAFold and ESMFold are being actively used to better understand ion channel structure-function, and their predictions of 3D structures can now be compared directly to high resolution cryo-EM data (e.g. for $Na_v1.8$, $Ca_v1.1$ and $K_v1.3$ ¹⁰). However, such comparisons are revealing the relative weakness of both computational and structural methods to resolve N- and C-terminus and intracellular domains of voltage- and ligand-gated ion channels, which is a significant shortcoming considering that ion channel trafficking, modulation and biophysics involve these domains. A recent ML-based model of NMDA variant effects on ligand affinity and receptor function also had to omit intracellular domain variants¹⁰ (see below), highlighting the remaining challenges for improved variant effect predictions using structure-based models. Looking forward, predictive protein models of ion channels will be improved by better resolution of disordered intracellular domains, together with access to high quality biophysical and functional data on human genetic variants which is now being provided by academic and industrial groups.

Biophysical assessment of channelopathy variants

The go-to technique for profiling ion channel variants has been electrophysiology as it allows for detailed assessment of changes in functional expression as well as biophysical parameters and pharmacology, which can be synthesized into a classification of each variant as a gain- or loss-of-function and suggest pharmacogenetic treatments for each patient category. Variant profiling has traditionally used labor-intensive techniques such as two-electrode voltage clamp in *Xenopus laevis* oocytes and manual patch clamp of heterologous cell lines, but this work is now being accelerated with access to APC platforms. Another rate-limiting step has been the need to express numerous ion channel variants in host cells, either transiently or with a combination of a stable pore-forming subunit and additional subunits of heteromeric voltage- and ligand-gated ion channels. This process is now also being made more efficient by the use of semi-automated transient transfection techniques (e.g. MaxCyte¹²). Case studies

exemplifying some of these issues and advancements are discussed in the following section, highlighting work on neuronal and cardiac voltage-gated Na_v , K_v and Ca_v channels and ligand-gated NMDA receptors.

APC is facilitating high throughput biophysical profiling of patient channelopathies

One of the main drivers for development of *in silico* variant prediction models has been the large and continually growing number of ion channel mutations and SNPs found after genome sequencing and GWAS studies of patients with undiagnosed, rare and common diseases, many of which have uncertain significance for clinical diagnosis. Generation of biophysical data to validate these ion channel variant models and empirically confirm model predictions, especially for VUS mutations, has until recently been limited by the throughput of labor-intensive electrophysiology screening but this challenge is now being met by the increased use of APC platforms in academia and industry to profile channelopathy variants tied to human disease. This section will highlight several key papers showing how tens or hundreds of Na_v , Ca_v and K_v channel variants associated with neuronal and cardiac syndromes can be reliably characterized by APC (reviewed in¹³). A related paper worth mentioning developed a combined sequence- and structure-based machine learning model that reliably classified over 800 gain- and loss-of-function variants in $Na_v1.x$ and $Ca_v1.x$, $Ca_v2.1$ and $Ca_v3.x$ channels linked to 19 different neurological, cardiac and other diseases, of which 87 were confirmed with biophysical data, revealing shared molecular and pathological mechanisms across this family of closely related ion channel genes.¹⁴

$Na_v1.x$ channel variant profiling

Thousands of $Na_v1.x$ channel variants have been found in patients suffering from both rare and common neuronal and cardiac diseases, some of which have been characterized using manual patch clamp but more recently larger numbers are being profiled by APC. Perhaps not surprisingly due to the highly conserved structure-function of $Na_v1.x$ channels, biophysical studies of specific $Na_v1.x$ variants show that similar amino acid substitutions at the same residue lead to similar excitability phenotypes across different patient populations, exemplified by an early study of I141V/I136V mutations in $Na_v1.4$, $Na_v1.5$ and $Na_v1.7$ channels associated with myotonia, polymorphic ventricular arrhythmias and erythromelalgia, respectively.¹⁵ This section will focus on $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$ channel variants associated with neurological syndromes (reviewed in¹⁶), and then $Na_v1.5$ channelopathies linked to cardiac LQT3 and Brugada syndrome.

- **Neurological Na_v1.x channelopathies**

Mutations in Na_v1.1, Na_v1.2 and Na_v1.6 channel genes are associated with various forms of epilepsy and neurodevelopmental syndromes, but many variants remain uncharacterized (ClinVar VUS designation) and so require detailed functional profiling.¹⁷

There are now around 1500 variants in the SCN2A gene linked to clinical diagnosis in ClinVar, with roughly a third each classified as pathogenic, benign or of unknown significance. A small manual patch clamp study¹⁸ of 6 Na_v1.2 variants showed gain-of-function gating effects linked to infantile epilepsy and DEE, and profound loss-of-function expression deficits in variants from later-onset autism and intellectual delay patients, revealing the wide SCN2A genotype-phenotype spectrum. A more comprehensive APC study¹⁹ largely confirmed this pattern in a larger sample of 28 disease-associated and 4 common variants occurring in all 4 functional domains of Na_v1.2 channels, with gain- and loss-of-function variants producing opposite shifts in voltage-dependent activation and inactivation that either increased or decreased window currents, and many epilepsy and DEE variants also slowing inactivation kinetics. The high throughput assay was also able to compare 21 variant effects in neonatal vs adult Na_v1.2 splice variants, to add greater understanding of subtle developmental effects. This APC data comes from the laboratory of Prof. Al George and collaborators at Northwestern University in the US, who have pioneered a scalable and high throughput workflow for transient and stable cell line transfection and sophisticated channelopathy variant profiling using the SyncroPatch 384 APC platform, which has been applied to a range of Na_v1.x and K_v channelopathies (see below). In a good example of the increased involvement of rare disease foundations and patient and parent charities in channelopathy research, this group subsequently published another APC study of 69 Na_v1.2 channel variants from 81 young patients with DEE or autism in a collaboration with the FamileSCN2A Foundation to provide a more systemic understanding of their biophysical and clinical genotype-phenotype, especially a linkage to non-seizure symptom severity.²⁰ Cluster analysis of clinical and biophysical profiles was able to stratify 5 different groups, with strong statistical correlation of clinical phenotype with Na_v1.2 variant effects such that gain-of-function and mixed function variants predominated in patients with neonatal-onset epilepsy, while moderate loss-of-function variants were seen in infant-onset epilepsy and severe loss-of-function variants were linked to later-onset epilepsy and autism. This combined approach, exploiting high throughput and high fidelity channelopathy variant profiling by APC, can reveal both complex and subtle links between ion channel function and patient phenotypes during development and across different neurological indications, and in turn guide personalized precision medicine and pharmaco-genetic drug discovery approaches.

Around 200 Na_v1.6 mutations are known, including loss-of-function variants in autism spectrum disorder and gain-of-function variants in patients with neonatal epilepsies and DEE. A recent manual patch clamp study of 2 infantile epileptic encephalopathy type 13 variants showed that they are gain-of-function by increasing persistent and window currents, which were selectively inhibited by clinical anti-epileptic drugs Phenytoin, Carbamazepine and Topiramate.²¹ Other manual patch clamp studies of several known and novel SCN8A variants from patients with DEE13 and EIEE also show gain-of-function shifts in voltage-dependent gating, slowed inactivation kinetics and increased window, persistent and resurgent currents, while a novel variant in a patient with autism and intellectual disability was biophysically confirmed to be loss-of-function.^{22,23} A recent preprint²⁴ from Al George and collaborators profiled 13 DEE variants by APC, revealing a complex spectrum of gain- and loss-of-function biophysical effects on Na_v1.6 current density, voltage-dependence of activation and inactivation and inactivation kinetics, and confirmed the overriding effect to enhance persistent and window currents. The opposing functional properties seen for specific variants confounds the assignment of a simple binary classification, so the complex data was summarized and analyzed using heat map tables and 'radar' or spider plots, a growing trend for APC channelopathy profiling studies (see Figure 3). Significantly, the authors improved on previous approaches by comparing Na_v1.6 variant effects in neonatal and adult exon 5 splice variants (detecting some significant differences which may impact pharmacogenetic treatments for young vs adult patients), and expressing Na_v1.6 channels in a TTX-S current depleted 'LoNa_v' ND7/23 neuroblastoma cell line to avoid the need for using TTX-binding site mutant TTX-R channels (which exhibited significant functional differences compared to the wild-type channels).

- **Cardiac Na_v1.5 channelopathies**

There are several different types of cardiac Na_v1.5 channelopathies, including LQT3 typified by increased persistent or 'late' current that prolongs the cardiac action potential plateau, and Brugada Syndrome where loss-of-function variants decrease current density and alter the PR interval of the cardiac ECG rather than produce long QT (reviewed by^{25,26}). Up to 80% of the ~1400 missense variants in Na_v1.5 are of unknown significance, complicating diagnosis of patients with potential cardiac risk based purely on genomic data.

APC profiling is now being used to help characterize cardiac channelopathies in Na_v1.5, hERG and K_v7.1 channels,^{13,27,28} and researchers at Vanderbilt University in the US provide a good case study of such efforts. Their first Na_v1.5 APC study using the SP384 platform characterized 83 SCN5A gene variants: 10 previously studied LQT variants with a known spectrum of Na_v1.5 channel function, 10 suspected benign variants from unaffected individuals or general population

polymorphisms, and 63 variants suspected of being linked to Brugada syndrome.²⁹ An efficient mutagenesis and Na_v1.5 gene expression workflow was developed by introducing multiple restriction sites across different 'zones' of the SCN5A gene and stable transfection into an inducible cell line that selected against non-integrated cells. For APC profiling each 384 well plate contained 5 variants and one block of wild-type cells (N=64 single hole wells per cell type), and used standard voltage protocols to measure biophysical properties. The validation set of 10 mixed variants showed good concordance across current densities and other properties between APC and published MPC data, and their biophysical parameters were used to set a matrix of cut-offs for experimental classification of variants as benign or pathogenic. Accordingly, all 10 predicted benign Na_v1.5 variants were confirmed to have normal peak current density and half-activation voltage, while most Brugada-linked variants had significantly reduced current expression and a mixture of gain- or loss-of-function effects on activation; no significant effects were seen upon inactivation (voltage-dependence, current decay or recovery kinetics) or late current (a feature of LQT3 variants). Overall, 2/2 predicted benign and 14/61 VUS variants were confirmed by APC to be or likely benign, 9/10 likely pathogenic were confirmed to be deleterious, and significantly 35/61 VUS were reclassified as pathogenic, with 12/61 remaining undiagnosed and 49/61 VUS being functionally reclassified. The majority of Brugada variants exhibiting partial or complete loss of current expression were found in or close to the pore-forming S5 and S6 helices and S5-S6 linker, whereas benign variants were in other transmembrane domains, and distance from the pore was a good predictor of functional phenotype (similar to the study of Ca_v3.3 schizophrenia loss-of-function mutants;³⁰ see below). There was a trend for more common Brugada variants to more consistently reduce Na_v1.5 current density, confirming the main clinical phenotype of Brugada syndrome to reduce the activity of Na_v1.5 channels during the peak of the cardiac action potential, rather than induce LQT. Also, the clinical anti-arrhythmic Mexiletine (and low temperature cell incubation) was able to rescue some I-o-f Brugada mutants, suggesting that trafficking and/or protein folding deficits contribute to Na_v1.5 current reduction.

A follow-up study from the same group showed that heterozygous co-expression of wild-type Na_v1.5 channels with many Brugada loss-of-function variants (38/50) produced a dominant negative effect to markedly reduce current expression measured in HEK cells by APC, evidence that Na_v1.5 channels can form dimers.³¹ Whilst there was evidence for coupled gating in heterozygous Na_v1.5 dimers as most variants affected activation, the lack of effect on inactivation parameters or late current amplitude were similar to homozygous Brugada variant channels. However, heterozygous co-expression of wild-type Na_v1.5 and Brugada variant channels revealed more detail on the spectrum of current density deficits not seen previously in homozygous cell lines where most of these variants failed

to express at all, offering a deeper insight for structure-function studies of Na_v1.5 channels and patient stratification and diagnosis. Significantly, patients with these dominant negative variants had an increased burden of clinical cardiac risk compared to those with haplo-insufficient variants (e.g. frameshift, splice, or nonsense), directly and reliably linking Na_v1.5 channel genotype to APC biophysical profile and clinical cardiac risk phenotype.

Finally, the Vanderbilt group has collaborated with leading cardiac ion channel researchers in Australia to replicate their Na_v1.5 variant SP384 profiling assay at a 2nd site, demonstrating the ability of APC to produce consistent and reproducible screening assays if robust workflow and QC techniques are employed. For example, there was a very strong correlation between Na_v1.5 current densities recorded at Vanderbilt University and the Victor Chang Cardiac Research Institute for 49 Brugada variants, and using the 'control' biophysical parameters recorded from a training set of 25 benign variants the APC assay could accurately distinguish between benign and 23/24 pathogenic variants.³² The 'calibrated' APC Na_v1.5 assay was then used to reclassify 3/4 unknown variants in new patients from several Brugada families as pathogenic, thereby providing clinical-grade functional evidence for patient classification.

K_v channels

As approximately 90% of genotyped patients with long QT syndromes (LQTS) carry variants in Na_v1.5, hERG and K_v7.1 channels these have garnered most attention from clinicians, as well as researchers using APC to profile cardiac channelopathies (reviewed by^{13,27}, also see²⁸). Accordingly, APC case studies on hERG and K_vLQT1 variant profiling will feature in the next section. The 2nd section will focus on neurological indications for which a broad range of K_v channel variants have been linked with diverse human diseases, with detailed APC case studies on K_v7.2 and K_v3.1 channels.

- **APC profiling of cardiac channelopathies in hERG and K_v7.1**

Carrying on with the cardiac theme from above, we start with an overview of several recent papers from Australian researchers at the Victor Chang Cardiac Research Institute who are using SP384 APC assays to biophysically profile large numbers of LQT2 patient-derived hERG variants.³³⁻³⁶ While most LQT2 mutations in the KCNH2 gene encoding K_v11.1 channels are loss-of-function and reduce trafficking and hERG current activity and prolong the cardiac action potential to cause arrhythmia, several questions remain over the mechanism of this effect which were addressed in these studies. Firstly, comparison of APC hERG current dysfunction after homozygous vs heterozygous expression with wild-type channels revealed that most of the 30 variants exerted a dominant negative trafficking effect to

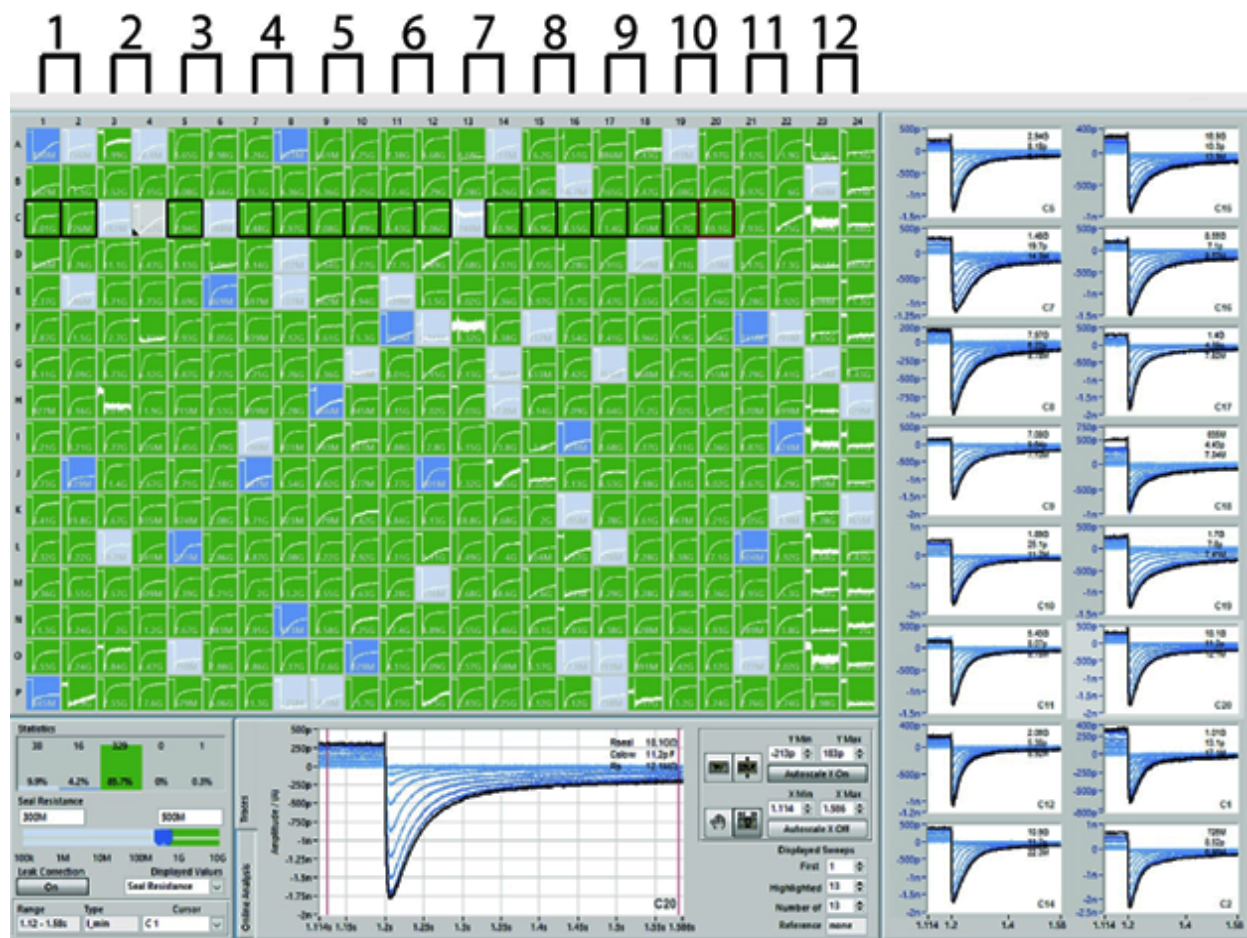


Figure 2 High throughput APC profiling of hERG LQT2 variants across a SP384 plate (Ng et al., 2021). Left, each doublet of columns contains a single variant or control cell line, with those in green passing stringent QC filters. Right, exemplar hERG tail currents from wells containing hERG variants (small block boxes in left panel). Figure used under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

reduce current expression, which was largely reflected in results from a cell surface expression assay.³³ This is similar to the dominant negative effect of Brugada variants in $N_{av}1.5$ channels (described above) and for $K_{v}7.1$ cardiac and $K_{v}7.2$ neuronal channelopathies (described below), and reflects the autosomal dominance of most LQT syndromes. Secondly, detailed APC biophysical data (activation voltage, ion selectivity, current kinetics) revealed that 7 hERG variants altered channel gating to reduce function by a haploinsufficiency mechanism. Overall, combination of the cell surface trafficking and APC functional biophysical assay in the 1st study was able to confirm ClinVar predictions of pathogenic or benign phenotype in 17/17 variants and reclassify 3 VUS variants as likely benign, and notably indicated that 10/10 variants lacking a ClinVar category were likely pathogenic. Also, these assays revealed that 11/13 pore domain and most proximal C-terminal variants exerted a dominant negative trafficking and expression deficit, whilst 2 variants exhibited deactivation gating effects that reduced the function of

hERG channels correctly trafficked to the cell surface. The same APC current density assay (Figure 2) was then used to functionally profile an additional 31 hERG variants with ClinVar categorizations, which confirmed 17/17 pathogenic missense variants and 13/14 benign variants (indicating 100% sensitivity and 93% specificity of the APC assay), with 3 variants also showing gating changes.³⁶ This calibrated APC assay was then used to classify another 44 variants with VUS ClinVar categorization, confirming 6 were likely pathogenic and 31 with moderate loss-of-function. Finally, additional KCNH2 missense mutations were profiled by APC to create a statistically powered dataset of 30 benign and 30 pathogenic variant hERG current density values which were analyzed to give a Z score indicating significant loss-of-function when current density was <55% of wild-type, indicative of haploinsufficiency.³⁷ Using this cutoff the authors then profiled 18 KCNH2 missense variants found in a cohort of childhood onset LQTS patients, revealing that 14 were likely pathogenic loss-of-function and the remainder largely normal. The authors claimed that the level of hERG

channel function in the calibrated and validated APC hERG current density assay could be correlated with clinical LQTS scoring regimes and the QT interval measured in affected and related patients, confirming that a validated hERG APC assay could be used to provide 'functional evidence' for variant genetic classification.

A massive follow-up study³⁵ used the high throughput hERG trafficking assay to profile ~1450 missense variants in the KCNH2 exon 2 'hotspot' as well as 77 synonymous and nonsense variants of which nearly half significantly reduced surface expression. A subset of ~450 of these missense trafficking variants were then profiled by APC to confirm heterozygous dominant negative effects on current density in around half and gating deficits in ~10%, requiring 92 384 well plates to collect over 22,000 successful QC'd patch clamp recordings (mean of N=39 for each variant). Most common polymorphisms were functionally normal whereas most of the 74 variants lacking ClinVar categorization were found to be likely pathogenic, while the 42 hERG VUS were distributed widely from normal wild-type current density to pathogenic loss-of-function.

This combined functional hERG variant profiling workflow is designed to accelerate and enhance the ability of clinicians to diagnose new cardiac arrhythmia patients, as many of the >1000 LQTS variants are unique to a family or small number of individuals and there is little information on the pathogenicity of many existing or novel variants discovered during genome sequencing. The use of an inducible, stable FLP-In transfection cell line construct is well suited to heterozygous variant expression and high success APC recordings, with the authors claiming that a new hERG variant can be expressed and profiled within 2 months. Also, generating reliable functional data to categorize the cardiac risk of hERG variants will aid the predictive abilities of bioinformatic software tools.

Mutations in the KCNQ1 gene encoding cardiac $K_{v7.1}$ channels were the first to be associated with long QT syndrome (LQT1), with ~500 different amino acid sequence variants detected after patient genome and exome sequencing. Characterizing such a large number of channelopathy mutants is very challenging, and prone to variation when using manual patch clamp across different laboratories and experimental conditions. In contrast, APC offers an opportunity for efficient and consistent profiling of multiple variants under identical conditions, exemplified by a study of 78 KCNQ1 channelopathies.¹² A total of eight 384 well APC plates were sufficient to confirm the functional effects of 30 training set variants split between LQT1 disease-linked mutations previously profiled by MPC and non-disease population polymorphisms, with a mean of N=56 replicates obtained for each variant, and capacity for testing 200-300 wells of control cell data in parallel to enable reliable statistical analysis of changes in current density, kinetics and voltage-dependence. Most KvLQT1 variants, studied in CHO cells co-transfected with KCNE1 and either wild-type

or variant KCNQ1 sequences, exhibited dominant negative loss-of-function effects, alongside some large negative shifts in activation that were not seen in non-disease polymorphisms. In addition, 23/35 variants with uncertain ClinVar categorization were functionally 'decrypted' as either benign or pathogenic using high content APC data, offering important insights for clinicians treating these cardiac patients and their families.

- **Neuronal K_v channelopathy case studies**

Mutations in various K_v channel genes are associated with different types of epilepsy (KCNQ2, KCNA2, KCNT1, KCNB1, KCNH1), pain (KCNQ2), neurodevelopmental syndromes (KCNQ5, KCNB2, KCNN3) and ataxia (KCNA1, KCNC3, KCND3); reviewed by³⁸; also see³⁹ for an APC study of KCNB1 $K_{v2.1}$ variants. This section will focus on recent APC case studies of KCNQ2 and KCNC1 genes encoding $K_{v7.2}$ and $K_{v3.1}$ channels.

As highlighted above, Prof. Al George and collaborators at Northwestern University in the US have developed an efficient workflow using high throughput cell transfection and 384 well APC to profile large numbers of variants in Na_v and K_v channels. For KCNQ2 the challenge involves ~500 different amino acid sequence variants in $K_{v7.2}$ channels detected in epilepsy patients, many of which are still of uncertain pathogenicity. Their recent study profiled 79 missense variants and 2 deletions associated with developmental and epileptic encephalopathies (DEE) and present in the general population, comparing effects on current density and biophysical measures of voltage-dependence and current kinetics. Recordings were made from 9,480 cells in parallel under identical APC conditions to create a large dataset of high content, high quality data suitable for identification of subtle changes in $K_{v7.2}$ channel function.⁴⁰ As the neuronal M-current comprises $K_{v7.2/7.3}$ heteromers, and most patients are heterozygous for KCNQ mutations, a stable CHO $K_{v7.3}$ cell line was created into which wild-type and variant $K_{v7.2}$ genes were co-expressed; control recordings on manual and APC showed very similar biophysical and pharmacological properties, and robust QC filters were applied to ensure high quality APC data. As expected the majority of 24 rare population variants had little effect upon heteromeric $K_{v7.2/7.3}$ current activity, while 16 of 18 variants with known (e.g. MPC data) or predicted pathogenicity were confirmed to affect current properties on APC; gain-of-function variants shifted activation to the left, whilst loss-of-function variants shifted the activation curve in a depolarizing direction. Most epilepsy-associated KCNQ2 mutations were loss-of-function, reducing current density and exhibiting dominant negative effects in $K_{v7.2/7.3}$ heteromers, and importantly these deficits were reversed by the canonical $K_{v7.2}$ channel opener Retigabine, validating current drug discovery efforts to develop selective $K_{v7.2/7.3}$ channel activators as novel anti-convulsants. Most significantly, many of the 39 mutations with previous VUS designation were shown by APC to functionally alter KCNQ2 current amplitude and produce shifts in activation

voltage-dependence, and only a few were 'rescued' after heterozygous expression with the KCNQ3 subunit, confirming the dominant negative effects of most KCNQ2 epilepsy mutations. Similar conclusions were made from an independent study of 11 KCNQ2 variants from Chinese developmental and epileptic encephalopathy patients which were profiled by manual patch clamp, with 9 of these loss-of-function variants affecting residues outside of the previous APC publication.⁴¹

An earlier study of the *de novo* missense variant R320H in the KCNC1 gene linked to patients with myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK) revealed another interesting use of APC profiling to correlate K_v channelopathy biophysics to clinical phenotypes.⁴² This variant produces an overall loss-of-function effect by reducing current expression which is expected to increase CNS neuron excitability, although there was also a small hyperpolarising shift in activation that could promote $K_v3.1$ channel activity at sub-threshold membrane potentials. Noting that fever could improve clinical symptoms in many patients, the authors used the temperature control capabilities of the Patchliner APC platform to repeat their room temperature experiments at 36 °C and 39 °C, revealing a larger effect of the R320H variant to shift activation to the left as well as reduce the temperature-dependent increase in inactivation compared to wild-type $K_v3.1$ currents, effects that could 'correct' the loss-of-function deficit in variant $K_v3.1$ channel activity.

- **Ca_v channel variant analysis can be more complicated**

As well as voltage-dependent biophysics, studies of Ca_v channels also need to look at current kinetics and charge integrals as the magnitude and kinetics of Ca^{2+} influx into muscle, neuroendocrine and neuronal cells is important for signaling, plasticity and toxicity. Similarly, analysis of disease-related variants in Ca_v channels also need to sample an expanded palette of functional parameters in order to correctly classify gain or loss-of-function changes and their predicted pathogenicity.

An excellent example of this complex biophysical analysis of Ca_v channelopathies was published in 2022 where US academics used APC to profile 57 rare and common missense variants in $Ca_v3.3$ T-type channels (CACNA11 gene) found in a cohort of 10,000 Swedish schizophrenia patients and compared their properties to common population polymorphisms and wild-type channels.³⁰ A total of 3,300 mutant-expressing and 924 wild-type cells were subject to a series of voltage protocols to assay changes in expression (current density) and biophysical properties, with the large sample size enabling detection of large as well as subtle changes in voltage-dependent activation and inactivation. Rare $Ca_v3.3$ variants from the general population typically reduced current amplitude, whilst common schizophrenia variants affected channel activation and rare schizophrenia variants affected the

voltage-dependence of inactivation, but common $Ca_v3.3$ variants did not adversely influence voltage-dependent activation or inactivation. $Ca_v3.3$ variants affecting current density were mainly found near the channel pore and selectivity filter, while those associated with altered voltage-dependence were unsurprisingly found within VSD helices and linkers. Overall, this dataset suggests that rare $Ca_v3.3$ variants are gain-of-function, and support GWAS studies that a loss-of-function in $Ca_v3.3$ is a molecular phenotype that may protect against schizophrenia. This group is now extending their Ca_v channelopathy profiling work to also measure current inactivation and deactivation kinetics, and are using a cloud-based software suite which creates 'spider' or 'radar' plots of key biophysical parameters that reveal how the overall function of Ca_v channels are changed by each gene variant (Figure 3). This information is also being integrated into cryo-EM-based homology models to map individual and similar variants to specific ion channel domains, increasing the understanding of structure-function in Ca_v channels and revealing shared patterns of variant effect which could inform pharmacogenetic treatments and patient stratification.

Finally, an academic group in the US has developed a fascinating story around $Ca_v1.2$ variants found in patients with LQT8 Timothy Syndrome, which predominantly affect cardiomyocytes but also manifest neurological symptoms such as autism and developmental delay.⁴³ Manual patch clamp profiling of 7 variants showed that DIII S6 mutations were gain-of-function as they notably shifted activation to the left and slowed deactivation, similar to that seen for DI S6 mutations. LQT8 mutations appear to reduce Ca^{2+} - and voltage-dependent inactivation of $Ca_v1.2$ channels independently, but both effects are expected to increase Ca^{2+} entry into cardiomyocytes and neurons. Significantly, the authors conclude that variants associated with neurological symptoms also shift $Ca_v1.2$ activation, an effect seen as a shift to mode 2 gating in single channel recordings. The functional effect of these complex changes in $Ca_v1.2$ activity are being translated to phenotypic assays in human LQT8 iPSC cardiomyocytes and neurons (see below).

- **Ligand-gated receptor channel profiling**

A major challenge in studies of ionotropic receptors is to deliver agonist rapidly and consistently to avoid receptor desensitization and allow for reliable EC_{50} determinations which define ligand-gated ion channel function. It is somewhat surprising that some groups still use *Xenopus laevis* oocytes for ionotropic receptor experiments given that this preparation suffers from a large membrane area, invaginations and vitelline membranes that can all affect agonist access to receptors in plasma membrane and promote receptor desensitization and ligand potency shifts, which is weighed against historical precedent and datasets and the relative ease of transiently expressing ionotropic receptor proteins in this system. Some groups are now using micro-perfusion techniques to rapidly apply

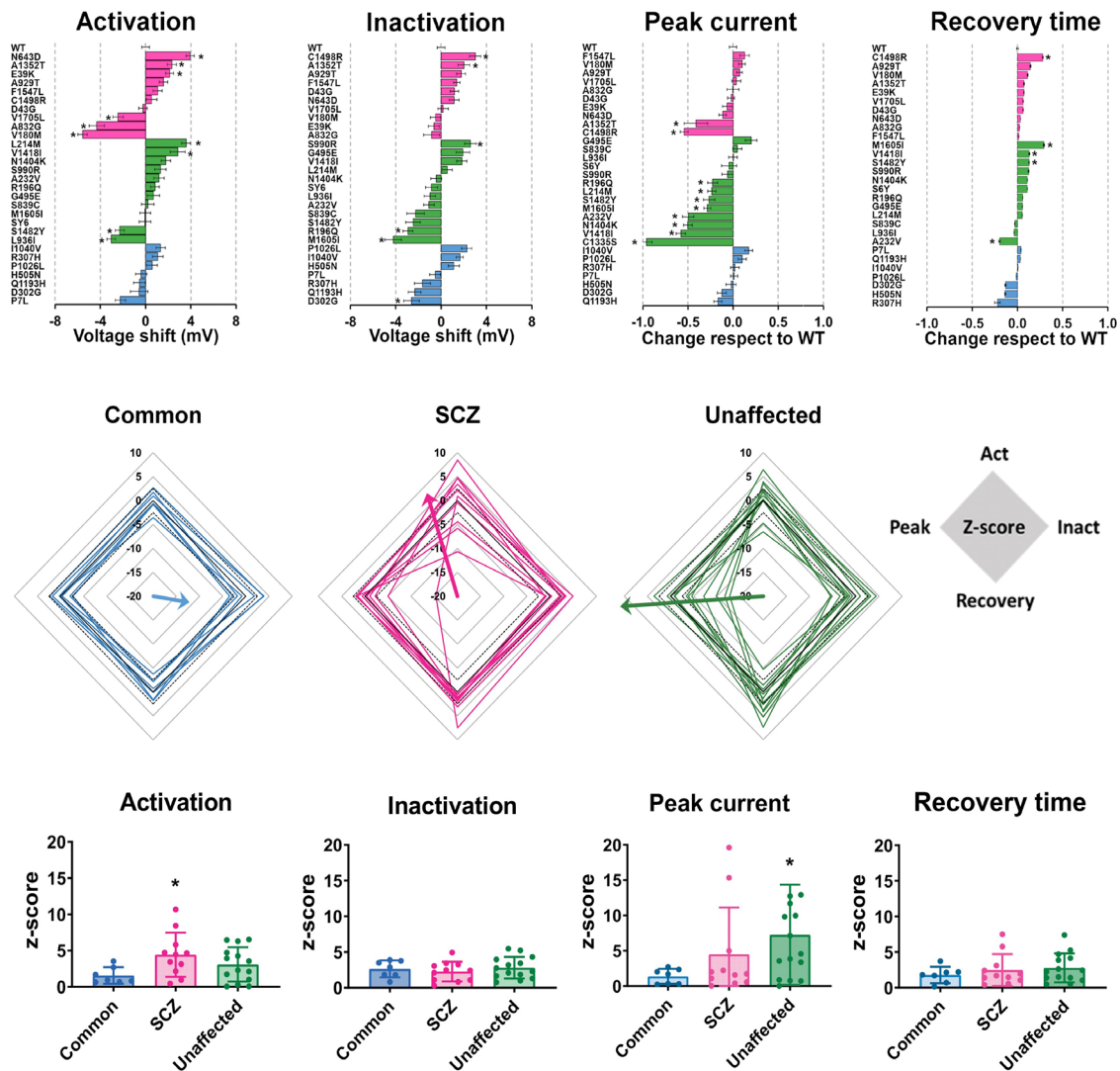


Figure 3 Biophysical profiling of common and rare schizophrenia $Ca_v3.3$ variants (Baez-Nieto et al., 2022). Upper row: Graphical summary of altered biophysical properties and expression for $Ca_v3.3$ variants from common (blue), schizophrenia cohort (pink) and unaffected samples (green). Statistical significance denoted by asterisks (*). Middle row: Radar or spider plots of the data from the top row for each $Ca_v3.3$ variant, arranged according to the schematic at the right and plotted as statistical Z-score. Lower row: Mean statistical Z-scores for each biophysical parameter broken down by genetic cohort. Figure used under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

ligands onto heterologous cells, but as with *Xenopus laevis* oocytes this is a slow, technical and labor intensive method which can struggle to efficiently profile the growing number human patient and disease channelopathy variants being discovered in ligand-gated receptor channels. In contrast, while the 1st generation of APC platforms were not designed or optimized for rapid liquid additions necessary for studies of ligand-gated ion channels, current APC devices are now well-suited to such studies as they offer flexible and rapid liquid addition options, such as stacked tips or microfluidic flow.

It is not uncommon that a single major ion channel exhibits

a variety of genetic changes, and that a single protein target can be linked to different disease phenotypes. This makes the deconvolution of variants and their effect on ion channel function and possible linkage to specific etiologies critical to developing a better understanding of disease processes, genetic risks, and genotype-phenotype relationships that could help diagnose and treat individual patients. APC can help to effectively collect and analyze detailed, high fidelity information on a large collection of variants within a single ion channel under identical conditions, and this includes studies of ligand-gated NMDA receptors which are the focus of this section.

An early paper used manual patch clamp to profile 9 mutations in the NMDA receptor GluN2B subunit found in patients with autism, intellectual disability and Lennox Gastaut and West syndromes.⁴⁴ The small sample size for each patient group made it difficult to detect specific trends in receptor properties for each clinical category, with the authors finding a mixture of loss- and gain-of-function effects including reductions in glutamate potency (C461F), increased desensitization (P553L) and decreased current density (C461F), alongside disrupted divalent cation permeation, Mg²⁺ block and current rectification (N615I and V618G), respectively. The changes in Mg²⁺ block were recapitulated in EPSCs recorded from rodent hippocampal neurons transiently transfected with the latter two variants, both linked to West syndrome.

A later paper used APC to characterize over 20 mutations in the NMDA receptor GRIN2A gene associated with schizophrenia or epilepsy and developmental delay and intellectual disability for their biophysics and pharmacology properties.⁴⁵ Loss-of-function protein truncating and missense mutations in GRIN2A were associated with schizophrenia symptoms, whereas a mixture of gain- and loss-of-function effects were seen in variants from developmental delay and intellectual disability patients. Gain-of-function GluN2A mutations increased glutamate potency, while loss-of-function phenotypes included rightward shifts in glutamate potency, reduced maximal current activation, or complete loss of expression. Also, the loss-of-function mutant M653I associated with developmental delay acted in a dominant-negative manner, whereas the schizophrenia loss-of-function variant Y698C did not. The APC profiling of these GluR channelopathy mutations serves to functionally link their location within the protein to predicted clinical severity, and highlight differential treatment options for each patient population.

A recent preprint extended this work to combine biophysical profiling and ML algorithms to classify and predict the pathogenicity of 991 missense variants across GRIN1, GRIN2A and 2B subunits of NMDA receptors associated with neurodevelopmental disorders and epilepsy.⁴⁶ The variants included 201 patient-derived and 631 from the general population, of which 127 already had published functional data (gain- or loss-of-function) and a further 47 which were characterized by electrophysiology in this study using *Xenopus laevis* oocytes and HEK293 cell patch clamp to generate biophysical data to train their ML models. Structure-function modeling of the new biophysical data indicated that the spatial proximity of variants to the pore and agonist (Glutamate, glycine) and antagonist (Zn²⁺, Mg²⁺) binding sites was a major predictor of their pathogenicity and effects on receptor function. Taking these new insights, their new ML models were then able to predict pathogenicity and receptor function changes with good accuracy, and reclassify numerous variants in ClinVar previously of uncertain significance.

However, it should be noted that this study, like others based on computational and cryo-EM protein structure models,¹⁰ struggles to model variants in poorly resolved protein domains such as the N- and C-terminus and intracellular loops, regions important for ion channel activity and modulation and where disease-linked variants can also be found. The NMDA variant ML model also excluded data on protein surface expression and trafficking, although this was collected in a HEK cell β -lactamase enzyme reporter assay. Also, a ML model based solely on biophysical effects of amino acid substitutions was far less predictive of pathogenicity, possibly as it did not include expression data and lacked some information on deactivation kinetics, although it did confirm that loss-of-function variants were significantly closer to glutamate binding residues, whereas gain-of-function variants cluster closer to the pore and inhibitory Mg²⁺ binding sites. Finally, the authors claim that their NMDA receptor pathogenicity and functional predictor ML models out-performed AlphaMissense, similar to a soon-to-be published study from the Pan laboratory of Ca_v2.1 variants associated with intellectual disability and developmental delay, suggesting that AlphaMissense may struggle to model the functional effect of single amino acid substitutions in large multi-domain transmembrane ion channel proteins.

Emerging issues and challenges

The vast majority of ion channelopathy profiling publications have focused on measuring changes in current expression and biophysical parameters, greatly aided by use of medium and high throughput APC platforms. Surprisingly, although such APC platforms are commonly used in industry for drug screening, there has been far less work looking at the impact of ion channel variants on other important functional measures such as changes in channel modulation and pharmacology, but this is beginning to change.

Channelopathy variants can alter channel modulation

While transmembrane ion channel VSD and pore regions have garnered most of the attention of *in silico* and biophysical channelopathy variant analysis, there is growing awareness that cytoplasmic domains also harbor disease-associated mutations and that these may affect intracellular signaling and modulation.

For example, several studies have looked at the impact of cardiac and neuronal channelopathies on phosphorylation of voltage- and ligand-gated ion channels. A paper on Na_v1.5 Brugada Syndrome found two variants in the domain I-II linker that reduced cell surface expression and current density, but they also disrupted protein kinase A phosphorylation and up-regulation of Na_v1.5 channels by adrenergic receptor stimulation, in both heterologous HEK cells and rodent ventricular cardiomyocytes.⁴⁷ Also, an exome sequencing study of schizophrenia patients found

a minority with variants GRIN1 (NR1 subunit) and GRIN2A (NR2A subunit) NMDA receptor genes.⁴⁸ Whilst many were predicted to directly disrupt NMDA receptor function, other patient mutations were predicted to have indirect effects to disrupt Fyn tyrosine kinase (but not serine-threonine or Src kinase) phosphorylation sites which normally up-regulate NMDA receptor activity and modulate trafficking. In contrast, a gain-of-function IEM mutation in Na_v1.7 channels introduced a novel threonine site to the DII S4-S5 linker and enhanced protein kinase C but not kinase A protein phosphorylation, which might contribute to gating changes that increase pain sensitivity.⁴⁹

More recent data from a large collaboration of UK, EU and US researchers looking at KCNK9 imprinting syndrome showed that as well as gain- and loss-of-function effects on current density of 15 TASK3 K2P channel variants profiled by HEK cell manual patch clamp, there were notable decreases in channel inhibition by acidic pH and Gαq GPCR signaling.⁵⁰ Structure-based homology models and molecular dynamic studies showed variant effects on the pore, selectivity filter and gating helix modules which correlate with effects on permeability and gating of TASK3 channels. Significantly, most of the patient-derived variants clustered towards the intracellular side of transmembrane helices and within cytoplasmic linkers (Figure 4), where they were projected to affect channel trafficking, gating, and intracellular modulation. This new *in vitro* functional and modulation data re-classified several VUS and likely pathogenic variants as pathogenic, but also revealed nuanced patterns in functional effects of novel variants that may help inform clinical interventions.

A similar phenomena of dual gating and modulation effects

has been described for disease-related variants in the CLC-7 organelle Cl⁻/H⁺ transporter channel, which is linked to osteopetrosis, neurodegeneration, and rare lysosomal storage disease.⁵¹ The gain-of-function mutation Y715C leads to increased Cl⁻ transport (measured by manual patch clamp), hyper-acidified lysosomes and hypopigmentation, with structural homology modeling suggests that the mutation disrupts the linkage between cytosolic and transmembrane domains of the CLC-7 channel. In addition, inhibition of CLC-7 channels by the lysosome-specific phosphoinositide PI(3,5)P2 is reduced by the Y715C variant, adding another mechanism for its gain-of-function effect.

Thus, it seems likely that channelopathy variants in other ion channels may exert dual effects on their biophysics and physiological modulation (e.g. Na_v and Ca_v phosphorylation, K_v7.x phosphoinositide regulation), revealing further complexity in genetic disease processes and potential therapeutic treatments.

Channelopathy variants can also affect pharmacology

Channelopathy variant effects on pharmacology can impact many aspects of drug discovery and clinical treatment, such as benchmarking and validation of screening assays and driving pharmacogenetic approaches to identify best drug treatments for individual patients. This section will include case studies on Na_v1.7, K_v3.x, K_v7.2 and Ca_v1.2 channels covering a range of neuronal and cardiac diseases.

Gene sequencing has revealed numerous gain-of-function variants in Na_v1.7 channels associated with painful syndromes (reviewed in³), and the subtle differences

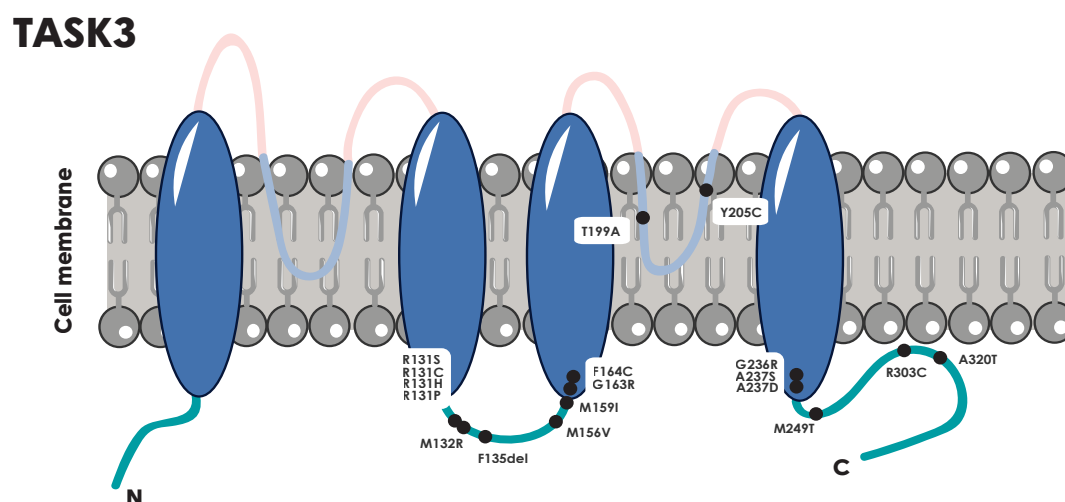


Figure 4 Location of KCNK9 imprinting syndrome variants in TASK3 channel (Cousin et al., 2022). Figure modified and used under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

in their biophysical effects and patient phenotypes has driven interest in personalized medicine pharmacogenetic testing. For example, Na_v1.7 variants linked to PEPD show different pharmacology to wild-type channels, with increased block of β 4 subunit-induced resurgent current but reduced use-dependent inhibition by Carbamazepine, Riluzole and Anandamide.⁵² Similarly, several IEM variants from Taiwanese pain patients are less sensitive to block by Lidocaine, as is the N395K variant that disrupts the local anaesthetic binding site.^{53,54} In contrast, Mexiletine is a more potent inhibitor of IEM variants I848T and L858F,^{53,55} while Carbamazepine selectively normalizes the gain-of-function shift in Na_v1.7 activation in IEM variants V400M and S241T,^{56,57} mirroring the efficacy of low doses of these drugs in certain IEM patients. Similarly, Na_v1.7 variants from patients with SFN expressed in heterologous cells had variable sensitivity to voltage- and use-dependent block by Lacosamide that mirrored the analgesic effect of this drug *in vivo*, with enhancement of fast inactivation being a key discriminator between responders and non-responders.⁵⁸

A recent manual patch clamp study of variants in the KCNC2 gene associated with developmental and epileptic encephalopathies (DEE) showed that V469L reduced the amplitude of K_v3.2 currents by occluding the pore, while gain-of-function V471L increased current amplitude and shifted activation by stabilizing the open state.⁵⁹ Intriguingly, the broad-spectrum K_v channel pore blocker 4-AP had weaker effect on the gain-of-function K_v3.2 variant but was equipotent against wild-type channels and the V469L variant predicted to have an altered pore structure. Similarly, profiling of a rare *de novo* variant in the KCNC1 gene encoding inhibitory neuron K_v3.1 channels confirmed it was functionally a gain-of-function, but had strikingly altered pharmacology compared to wild-type channels in a drug repurposing screen.⁶⁰ Notably, inhibition of V434L channels by 4-AP (clinically approved as Fampridine to treat Muscular Sclerosis) was reduced, and the K_v3.1 positive allosteric modulator AUT1 (developed by Autifony Therapeutics for tinnitus and schizophrenia) became a moderately active antagonist. These KCNC1 and KCNC2 K_v3.x channel pharmacology case studies clearly show that pharmacological data needs to be collected alongside variant biophysical profiling, to both classify the pathogenicity and 'sign' of novel rare disease-related variants, and also to inform decisions about drug repurposing and personalized medicine diagnosis and treatment across diverse DEE patients.

While the KCNQ2 APC profiling paper discussed above showed that current density reductions in epilepsy-associated loss-of-function K_v7.2 channel variants were restored by the K_v7.x opener Retigabine, the extensive pharmacology dataset also revealed some variation in drug sensitivity across 57 variants.⁴⁰ Notably, variants affecting residues within the ion selectivity filter with strong dominant-negative effects were least sensitive, whereas gain-of-function variants within the VSD and in some other

areas of K_v7.2 channels were most responsive to Retigabine. Significantly, this data revealed genotype-dependent differences in Retigabine efficacy which could be linked to symptom type and severity, suggesting that personalized pharmacogenetic approaches using APC profiling may yield precision therapies for specific populations of developmental and epileptic encephalopathy patients.

Finally, LQT8 variants in Ca_v1.2 channels that inhibit inactivation were also found to reduce use- and frequency-dependent inhibition by state dependent blockers such as Verapamil, Nimodipine and Amlodipine, mirroring their lack of efficacy in the clinic.⁶¹ Biophysical data and computational modeling indicated that the altered pharmacological sensitivity was tied to changes in Ca_v1.2 inactivation rather than variants effects on the drug binding sites.

Integration and translation of channelopathy variant effects

There remains a clear challenge in studies of human disease channelopathies to integrate all of the diverse, and sometimes conflicting, effects of each variant on ion channel expression, biophysics and modulation into a reliable prediction of a net functional change and its potential clinical pathogenicity. Current methodologies are complementary and based on comparisons of sequence (e.g. ClinVar) or structural homology (e.g. *in silico* algorithms), and these are being improved by the proliferation of patient-derived genome and biophysical data, but some information is still missing or not being incorporated into existing models. For example, few studies test effects of ion channel variants on protein trafficking (e.g. with use of western blots or tagged proteins or immunohistochemistry^{35,41,62}), and some of these assays suffer from poorly specific reagents. Also, biophysical experiments require a minimum current density to enable reliable patch clamp recordings, and many variants manifest complex and contrasting positive and negative effects to alter current density vs voltage-dependent and inactivation properties which makes assignment of a gain- or loss-of-function effect very difficult; for example, how do you classify a variant that moderately reduces current expression but exhibits gain-of-function biophysical effects? Although it is possible to summarize complex variant effects in 'radar plots' of biophysical and current density changes, and in the future also include data on altered modulation, what still seems to be lacking is a comprehensive method to integrate and reduce all of this information into a single functional 'call' for each ion channel variant. It seems likely that AI-ML algorithms will be able to accomplish this task, and may be able to segment and classify diverse effects of ion channel variants into different categories of functional effects relevant to each tissue, organ and disease.

Translational assays are gaining traction to predict the functional effects of channelopathies, which range from biophysical to phenotypic.

- **Biophysical assays using current and action potential waveform clamp**

Both manual and APC amplifiers are capable of current clamp recordings of passive membrane potential properties in recordings from heterologous cell lines, and it is also possible to apply 'action potential waveform' voltage clamp protocols to heterologous cells that emulate cardiac or neuronal action potentials, which can be used to detect changes in variant channel current amplitude, kinetics and stimulus-dependent modulation. For example, $\text{Ca}_v1.2$ LQT8 Timothy Syndrome mutations were profiled using a train of neuronal action potential waveforms, with only single domain I and III S6 variants associated with neurological symptoms producing broader 'action currents' and an increase in stimulus-dependent inactivation which were linked to their effects on $\text{Ca}_v1.2$ deactivation kinetics and activation gating.⁴³ In contrast, cardiac-selective variants do not change brief, high frequency neuronal action potential responses but their effects on Ca^{2+} and voltage-dependent inactivation broaden cardiac action potentials. Similarly, $\text{Ca}_v3.3$ gain-of-function variants associated with intellectual disability, developmental delay and seizures, which hyperpolarised voltage-dependent properties and increased window currents close to RMP and also slowed current inactivation, were profiled in HEK cells using action potential waveforms modeled on thalamic relay neurons.⁶³ Ca^{2+} influx (i.e. $\text{Ca}_v3.3$ current charge integral) evoked by each virtual action potential was notably increased (via increased persistent pacemaker currents), and complex changes were seen during 20 Hz action potential waveform trains with profound early facilitation followed by later attenuation attributed to accumulation of increased variant $\text{Ca}_v3.3$ channel inactivation. Finally, an elegant *in silico* approach used published MPC biophysical data from sick sinus syndrome HCN4 channel loss-of-function variants to drive a virtual action potential waveform clamp in a model of the human sino-atrial node, reliably predicting changes in If 'funny' pacemaker current and clinically observed minimum or resting heart rates in bradycardia patients.⁶⁴

Several groups have used *in silico* models of neurons to confirm the functional effects of channelopathy variants, using the biophysical parameters extracted from manual and APC recordings of human wild-type and mutant channels to seed Hodgkin-Huxley equations and passive membrane properties in models of action potential firing and neuronal excitability. Most significant has been the work from Australian and US researchers linked to Praxis Precision Medicines who have used *in silico* models of cortical neurons to characterize excitability effects of patient-derived $\text{Na}_v1.1$ and $\text{Na}_v1.2$ epilepsy and DEE variants and test the effects of drug candidates 'live' using dynamic action potential clamp methods, with this data informing the approval and patient stratification of subsequent clinical trials.⁶⁵⁻⁶⁷ Several academic studies of $\text{Ca}_v3.x$ channelopathies have also added their biophysical data into *in silico* models of thalamic relay neurons, finding that

gain-of-function variants in $\text{Ca}_v3.3$ channels (associated with intellectual disability, developmental delay and seizures) and $\text{Ca}_v3.2$ variants found in trigeminal neuralgia patients that all left-shifted Ca_v channel voltage-dependence and potentiated window currents also increased neuronal excitability by lowering the threshold and increasing the duration and frequency of action potential firing.^{63,68} In contrast, loss-of-function variants in $\text{Ca}_v3.2$ channels found in ALS patients that shift activation and inactivation decreased *in silico* thalamic relay neuron excitability, an effect that was less severe in heterozygous variant/wt virtual neurons that mirrored reduced symptom severity in ALS cohorts.⁶²

- **Translational assays using channelopathy iPSCs**

There are now many publications on human iPSCs as translational disease models, including numerous studies taking advantage of patient-derived and genetically engineered channelopathy variants associated with neurological, cardiac and other syndromes. For example, a recent review outlined how iPSC cortical neurons expressing epilepsy channelopathy variants in *SCN1A*, *SCN2A*, *SCN8A*, *KCNQ2*, *KCNT1* and *CACNA1A* genes can be used in electrophysiological and phenotypic assays to better understand specific patient cohorts and epilepsy subtypes, and screen for appropriate seizure treatments,¹⁷ and another reviewed NMDA receptor physiology and pharmacological screening.⁶⁹ This section will focus on iPSC case studies related to the ion channel targets described above, notably $\text{Na}_v1.7$, $\text{Ca}_v1.2$, *hERG*, $\text{K}_v7.1$ and $\text{Na}_v1.5$ channels.

For $\text{Na}_v1.7$ the focus is on pain, and numerous iPSC sensory neuron reagents have been created over the last decade expressing specific channelopathy variants linked to various rare diseases such as IEM, SFN and PEPD (reviewed in⁷⁰⁻⁷²). Manual patch clamp and plate-based electrophysiology readouts are commonly used to assess changes in $\text{Na}_v1.7$ channel biophysics, sensory neuron excitability and test analgesic drugs, and it was found that the simplified architecture of iPSC sensory neurons are well suited to APC voltage- and current-clamp recordings.⁷³ Another interesting and important finding has been that while modeling of single gain-of-function $\text{Na}_v1.7$ channelopathy variants in iPSC sensory neurons can help explain the biophysics and role of this channel in regulating excitability in a native human background, there is additional complexity in some pain patients. For example, two papers from Steve Waxman's laboratory took advantage of clinical access to newly diagnosed and exome sequenced patients with inherited erythromelalgia to show that patient-derived iPSC sensory neurons display significant differences in action potential firing and spontaneous activity that parallel the different pain sensitivities of the subjects and their family members.^{74,75} In both cases, cryptic gain-of-function variants in *KCNQ2* and *KCNQ3* genes that encode the neuronal $\text{K}_v7.2/7.3$ M-current and dampen sensory neuron excitability were identified in less affected individuals, highlighting the

unique advantages of patient-derived iPSCs to unbiasedly model human diseases.

iPSC neurons have also been created to model the biophysical and phenotypic effects of $\text{Ca}_v1.2$ variants linked to autism, revealing the effects of channel gating deficits on native L-type Ca_v currents as well as their effect on action potential shape and neuronal excitability.⁷⁶ The authors also showed changes in Ca^{2+} dynamics and gene expression that demonstrate the power of iPSC neurons to model the neurological pathogenesis of $\text{Ca}_v1.2$ variants in Timothy Syndrome.

The same US laboratory has used stem cell cardiomyocytes (iPSC-CMs) to bridge the gap between their biophysical studies of LQT8 $\text{Ca}_v1.2$ variants in HEK cells and clinical pharmacology, recapitulating the loss of efficacy of the state-dependent blocker Verapamil to inhibit native cardiac $\text{Ca}_v1.2$ currents in LQT8 compared to wild-type iPSC-CMs.⁶¹ Various LQT8 variants prolong the iPSC-CM action potential as expected, and shortening of the action potential by Verapamil was significantly reduced in iPSC-CMs derived from a LQT8 patient compared to wild-type, mirroring the poor clinical efficacy of this drug in LQT8 patients.

iPSC-CM models of LQT1, LQT2, LQT3 and Brugada Syndrome cardiac arrhythmia have also been created by introduction of specific channelopathy variants in *KCNQ1*, *KCNH2* and *SCN5A* genes encoding $\text{K}_v7.1$, hERG and $\text{Na}_v1.5$ channels into wild-type iPSCs or derivation of iPSC-CMs from LQT patients (reviewed in²⁵). As well as generating biophysical data on cardiac channelopathies effects on ionic currents and action potentials in native human cardiomyocytes using manual patch, these reagents are amenable to high throughput APC for efficient LQT disease modeling and drug screening.⁷⁷ As with iPSC neuron studies described above, the native (albeit immature) phenotype of differentiated iPSC-CMs can reveal hidden heterogeneity and complexity of cardiac channelopathies that are missing from reductionist studies of single ion channel variants in heterologous cells. For example, LQT patient-derived iPSC-CMs can model more complex genetics where several different ion channel and other gene variants are present and specific ion channel variants have subtly different effects, and these phenotypic assays can be used for drug screening to find more effective and novel anti-arrhythmics.⁷⁸⁻⁸¹

An excellent recent review on the use of APC and iPSC-CMs to synergistically decode Brugada Syndrome variants in $\text{Na}_v1.5$ and other channels discusses many examples of this phenotypic approach, including several patient-specific pharmaco-genetic drug repurposing studies.²⁶ So, it is worthwhile to end on a study that brings together the major threads of this blog by demonstrating how a combination of functional and translational biophysical and pharmacological experiments revealed important complexity in the phenotype of cardiac arrhythmia patients.

iPSC-CMs from two Brugada patients revealed the expected reduction in $\text{Na}_v1.5$ current density largely produced by a rightward shift in activation, but also showed that changes in $\text{K}_v4.3$ -mediated *I*_{to} current and *KCND* gene expression and $\text{Ca}_v1.2$ window currents contributed to changes in action potential shape and arrhythmias.⁸² Significantly, manual patch and Patchliner APC recordings from iPSC-CMs showed that two phosphodiesterase inhibitors previously shown to suppress hypothermia-induced arrhythmia could selectively normalize *I*_{to} current amplitude and reverse the Brugada $\text{Na}_v1.5$ variant-induced repolarization abnormalities, providing a comprehensive case study of how APC can facilitate channelopathy variant profiling, disease modeling and drug repurposing to offer new treatments to patients.

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