

## Invited review

**Molecular basis of inherited calcium channelopathies: role of mutations in pore-forming subunits<sup>1</sup>**Lynn MCKEOWN, Philip ROBINSON, Owen T JONES<sup>2</sup>*Faculty of Life Sciences, the University of Manchester, Manchester, UK***Key words**

calcium channels; channelopathies; mutation

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<sup>2</sup> Correspondence to Dr Owen T JONES.  
Phn 44-0-161-275-5604.  
Fax 44-0-161-275-5600.  
E-mail owen.t.jones@manchester.ac.uk

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**Abstract**

The pore-forming alpha subunits of voltage-gated calcium channels contain the essential biophysical machinery that underlies calcium influx in response to cell depolarization. In combination with requisite auxiliary subunits, these pore subunits form calcium channel complexes that are pivotal to the physiology and pharmacology of diverse cells ranging from sperm to neurons. Not surprisingly, mutations in the pore subunits generate diverse pathologies, termed channelopathies, that range from failures in excitation-contraction coupling to night blindness. Over the last decade, major insights into the mechanisms of pathogenesis have been derived from animals showing spontaneous or induced mutations. In parallel, there has been considerable growth in our understanding of the workings of voltage-gated ion channels from a structure-function, regulation and cell biology perspective. Here we document our current understanding of the mutations underlying channelopathies involving the voltage-gated calcium channel alpha subunits in humans and other species.

**Introduction**

Voltage-gated calcium channels (VGCCs) are critical determinants of physiological function in both excitable and non-excitable cells<sup>[1,2]</sup>. The ability of VGCCs to couple changes in membrane potential to the influx of the pivotal “second messenger” calcium (Ca<sup>2+</sup>) bestow VGCCs with a unique and privileged position among ion channels in the coupling of electrical signaling to intracellular biochemical events. In tissues such as muscle and heart, VGCCs are used for specific functions such as neurotransmitter release and excitation-contraction coupling, respectively. More generally, VGCCs orchestrate cell excitability<sup>[3]</sup>, second messenger signaling<sup>[4]</sup> and gene expression<sup>[5]</sup>.

Owing to such diverse roles, it is perhaps not too surprising that disruption in VGCC function has been implicated and, in many cases, demonstrated to underlie diverse inherited pathologies ranging from cardiac failure to epilepsy. The goal of this article is to review disparate data on such calcium “channelopathies” from a molecular perspective, focusing on the key pore-forming alpha subunits.

**Structure and function of VGCCs**

Although the existence of VGCCs had been known for many decades, their emergence as functionally discrete subtypes was not revealed until the pioneering electrophysiological studies of Tsien<sup>[6]</sup>, Lux<sup>[7]</sup> and many others during the 1980s. Originally, VGCCs were classified into two groups, according to their functional and pharmacological characteristics: T VGCCs [low voltage-activated (LVA) subtype]; and N, L, P/Q, and R VGCCs [high voltage-activated (HVA) subtypes]<sup>[8]</sup> (Table 1). However, molecular cloning, expression and biochemical studies revealed inadequacies in the above classification notation and a more rigorous structure-based nomenclature (Table 1) was subsequently introduced<sup>[9]</sup>.

All VGCCs are large (>400 kDa) heteromers comprised minimally of 3 core subunits  $\alpha_1$ ,  $\alpha_2/\delta$ , and  $\beta$  found in a 1:1:1 stoichiometry<sup>[8]</sup>. Expression studies in *Xenopus* oocytes<sup>[10,11]</sup> and transfected mammalian cells<sup>[12–14]</sup> have revealed that the  $\alpha_1$  subunits contain the gating, channel pore and inactivation machinery required for function. However, interactions between  $\alpha_1$  and the auxiliary  $\alpha_2/\delta$  and  $\beta$  subunits are required for optimal cell surface expression and channel kinetics.

**Table 1.** Classification and nomenclature of VGCCs. Each channel type is defined by a specific core  $\alpha 1$  protein subunit and current yielded, either high voltage-activated (HVA) or low voltage-activated (LVA). Adapted from Catterall *et al*<sup>[8]</sup> and Ertel EA *et al*<sup>[9]</sup>.

Name	Class	Current type	Alpha subunit	Protein size (kDa)	Ligands
CaV1.1	L	HVA	$\alpha 1_S$	212	Dihydropyridines
CaV1.2	L	HVA	$\alpha 1_C$	240	Dihydropyridines
CaV1.3	L	HVA	$\alpha 1_D$	187	Dihydropyridines
CaV1.4	L	HVA	$\alpha 1_F$	222	Dihydropyridines
CaV2.1	P/Q	HVA	$\alpha 1_A$	257	$\omega$ -Agatoxin IVA
CaV2.2	N	HVA	$\alpha 1_B$	262	$\omega$ -Conotoxin-GVIA
CaV2.3	R	HVA	$\alpha 1_E$	252	SNX-482
CaV3.1	T	LVA	$\alpha 1_G$	250	Kurtoxin <sup>a</sup>
CaV3.2	T	LVA	$\alpha 1_H$	261	Kurtoxin <sup>a</sup>
CaV3.3	T	LVA	$\alpha 1_I$	224	None

<sup>a</sup> Ligand is low affinity and most probably unreliable.

Additional protein interactions have been identified that appear necessary for trafficking and regulation (Table 2).

A cardinal feature of VGCCs is their extraordinary propensity for diversity. In mammals, 10  $\alpha_1$ , 3  $\alpha_2/\delta$  and 4  $\beta$  subunit genes have been identified. Moreover, most of the RNA transcripts have been shown to undergo alternative splicing, and the number of reported variants is growing rapidly<sup>[8]</sup>. The precise nature of the  $\alpha_1$ ,  $\alpha_2/\delta$  and  $\beta$  gene products in the VGCC complexes define their biophysical characteristics, therefore such diversity has significant functional implications<sup>[8]</sup>. However, the contribution of any ion channels to integrative physiology also depends on their distribution. Thus, it is notable that specific VGCCs have unique, but often overlapping, patterns of expression in discrete regions of brain and other tissues. Even the distribution over the cell surface can differ<sup>[15–22]</sup>, suggesting that diversity is used to hone voltage-dependent  $\text{Ca}^{2+}$  influx to the demands of discrete functional compartments<sup>[23]</sup>.

An emerging paradox concerns a subunit, termed  $\gamma$ , first identified as a constituent of the skeletal muscle VGCC but now known to have relatives in heart and brain<sup>[24]</sup>. Precisely what function these  $\gamma$  subunits serve is unresolved. Expression studies suggest an ability to normalize calcium currents to those resembling endogenous VGCCs<sup>[25]</sup>. However, not all  $\gamma$  subunits show this effect and one subunit,  $\gamma_2$ , has been shown to interact with the AMPA-subtype of glutamate receptors<sup>[26]</sup>.

## Ca<sup>2+</sup> channelopathies

Inherited defects in VGCCs give rise to some of the most interesting and widely studied channelopathies<sup>[27]</sup>. Here we

summarize, specifically, current information on those defects arising from mutations in the pore-forming alpha subunits in human and other model systems (Table 1).

**CaV1.1 ( $\alpha 1_S$ )** The pore-forming subunit of CaV1.1, encoded in humans by the *CACNA1S* (formerly *CACNL1A3*) gene on chromosome 1q 31–32<sup>[28]</sup>, is expressed mainly in skeletal muscle traverse tubules where it mediates excitation-contraction coupling and calcium homeostasis<sup>[29,30]</sup>.

Muscular dysgenesis in mice is a lethal mutation derived from a frameshift at nucleotide 4010. The resulting deletion of the C-terminus leads to a loss in muscle contraction and was the first mutation reported to affect calcium currents *in vivo*<sup>[31,32]</sup>. Missense mutations in *CACNA1S* have been identified in human cases of hypokalemic periodic paralysis (hypoPP) and malignant hyperthermia susceptibility (MHS)<sup>[33–38]</sup>. The Arg-His or glycine substitutions found in hypoPP (Table 1) are located in the voltage-sensing segments (S4) of domains II and IV, leading to a loss of myotube function<sup>[33,39,40]</sup>. Arg-His mutations have also been found in patients suffering from MHS. However, although both allelic, MHS and hypoPP appear to be distinct non-overlapping diseases<sup>[41]</sup>. Genetic studies of MHS have mainly linked associated mutations to the ryanodine receptor (RyR). However, as the RyR comes under the control of CaV1.1 and the R1086H mutation is located in the cytoplasmic loop between transmembrane spanning segments 3 and 4, such mutations might disrupt the functional link between the CaV1.1 and RyR<sup>[36]</sup>.

**CaV1.2 ( $\alpha 1_C$ )** CaV1.2 is primarily localized to cardiac or smooth muscle but is also found in endocrine cells and neurones. Functionally, CaV1.2 mediates excitation-contraction coupling in smooth and cardiac muscle, hormone secretion and action potential propagation in sino-atrial and atrio-

**Table 2.** Summary of major cytoplasmic proteins interacting with VGCC  $\alpha 1$  subunits, their binding sites and roles.

Protein	VGCC 1 binding site	Role/effect	Reference
Syntaxin 1	Synprint motif (II–III linker)	SNARE member, vesicle exocytosis	149
SNAP-25	Synprint motif (II–III linker)	SNARE member, vesicle exocytosis	150
Cysteine string protein	Synprint motif (II–III linker)	Vesicle exocytosis	151
Synaptotagmin	Synprint motif (II–III linker)	Vesicle exocytosis	152
CASK	C-terminus	VGCC–vesicle coupling	153
MINT-1	C-terminus	VGCC–vesicle coupling	153
Calmodulin	C-terminus (IQ domain)	Post $\text{Ca}^{2+}$ -influx inactivation	154
G-protein $\beta\gamma$ subunit	Domain I–II linker, C-terminus	GPCR-coupled inactivation	155
Protein kinase C, protein kinase A	Domain I–II linker, Synprint motif, C- and N-termini	Dependent on channel type and binding site	1
AKAP-79	Domain II–III linker	Postsynaptic L-type channel trafficking	156
Calbindin	C-terminus	Decrease L-type channel activity in pancreatic beta cells	157
CaMKII ( $\text{Ca}^{2+}$ /calmodulin-dependent kinase II)	C-terminus	Facilitation of $\text{Ca}^{2+}$ current	158

ventricular nodes<sup>[42]</sup>.

The CaV1.2 subunit is encoded by the *CACNA1C* (formerly *CACNL1A1*) gene on chromosome 12p13.3<sup>[43]</sup>. In mice, knockout of the CaV1.2 gene is lethal due to cardiac dysfunction (Seisenberger *et al* 2000). However, two *de novo* missense mutations in CaV1.2 in humans result in Timothy syndrome, a multi-system disorder including syndactyly, immune deficiency, long QT syndrome and ventricular arrhythmias during infancy<sup>[44]</sup>. These gain of function phenotypes arise from highly conserved glycine substitutions for either Arg at 406 or Ser at 402<sup>[44]</sup>. Mutation G406 is located in alternatively spliced exon 8A, at the cytoplasmic face of the transmembrane segment S6 of domain I, whereas mutation G402 is located within the transmembrane region. As glycine can act as a hinge-point in  $\alpha$  helices, such mutations have been suggested to disrupt the activation gate<sup>[44]</sup>. Further support for the role of CaV1.2 in cardiac development and dysfunction comes from studies on zebrafish whose embryos can survive without blood flow for several days. In their study, Rottbauer *et al*<sup>[45]</sup> mapped the genetic mutations responsible for the zebrafish *isl* lethal mutant. Mutants expressing two *isl* nonsense mutations at M379 or M458 (which both caused premature truncation of CaV1.2) present with abnormal heart growth during development.

**CaV1.3 ( $\alpha 1_D$ )** The pore-forming subunit of the CaV1.1 channel is mainly expressed in endocrine cells of the pituitary and adrenal chromaffin cells, but is also found in sensory cells and in low densities in atrial muscle, heart and neurons. Originally classed as the neuroendocrine L-type channel, CaV1.3 plays a role in hormone secretion, mood

behaviour, and control of cardiac rhythm at rest<sup>[46]</sup>.

The CaV1.3 subunit is encoded by the *CACNA1D* (formerly *CACNL1A2*) gene on chromosome 3p14.3<sup>[47]</sup>. Insights into the role of Cav1.3 in cardiac tissue have largely evolved due to electrophysiology studies on cells from knockout mice<sup>[48]</sup> and cardiac cells from human patients<sup>[49]</sup>. Although no human gene defect has been reported, animal models have provided useful insights into potential deficits. In one study, Wappl *et al* created a mouse model in which the high dihydropyridine sensitivity of CaV1.2 subunits was eliminated by replacement of Thr1066 in helix IIIS5 with a tyrosine residue<sup>[50]</sup>. As the distribution of CaV1.2 and 1.3 often overlap, and they both mediate L-type currents, the creation of this mouse model allowed Sinnegger-Brauns *et al*, to isolate the function of CaV1.3 in brain, pancreatic beta cells and the cardiovascular system<sup>[51]</sup>. These studies ruled out a direct role for CaV1.3 in insulin secretion, cardiac inotropy, and arterial smooth muscle contractility but suggested it might play a role in depression. Although no linkage of CaV1.3 channel mutations has been reported for human inherited diseases, mice carrying a targeted null allele display profound congenital deafness, thus providing insight into the molecular basis of CaV1.3 function in auditory processing<sup>[52,53]</sup>. Interestingly, the *ise*, mutant form of zebrafish larvae displays a deafness-imbalance phenotype, arising through two mutations (R1250X and R284C) in a gene encoding for the CaV1.3 channel<sup>[54]</sup>. The first mutation involves exchange of an Arg codon for a stop codon at position 1250 and results in a nonsense mutation in domain IVS4 that disrupts the transmembrane region and removes the

**Table 3.** The position of inherited and *de novo* mutations in  $\alpha 1$  genes and their resulting phenotypes. Mutation refers to amino acid unless followed by (n) for nucleotide. Ic, intracellular loops; Ec, extracellular loops.

Channel	Gene	Mutation	Type	Location	Inherited disease	Reference		
CaV1.1	<i>CACNA1S</i>	R528H	Missense	IIS4	HypoPP	33–35		
		R528G	Missense	IIS4	HypoPP	38		
		R1239G	Missense	IVS4	HypoPP	42		
		R1239H	Missense	IVS4	HypoPP	133		
		R1086H	Missense	Linker III–IV	MHS	36,37		
CaV1.2	<i>CACNA1C</i>	G406R	Missense	IS6	Timothy syndrome	44,50		
		G402S	Missense		Timothy syndrome	44,50		
CaV1.4	<i>CACNA1D</i>	S229P	Missense	Ic loop IS4–S5	XLCSNB	133		
		341delC	Frameshift	Linker I II	XLCSNB	55		
		G369D	Missense	IS6	XLCSNB	56,133		
		R508Q	Missense	Linker I II	XLCSNB	56,134		
		I745T	Missense	IIS6	XLCSNB	136		
		R680X	Nonsense	Pore IIS5–S6	XLCSNB	57		
		R830X	Nonsense	Ec loop IIIS1–S2	XLCSNB	55		
		R958X	Nonsense	IIS4	XLCSNB	56		
		991insC	Frameshift	Pore IIIS5–S6	XLCSNB	55		
		R1049W	Missense	Pore IIIS5–S6	XLCSNB	56		
		L1068P	Missense	Pore IIIS5–S6	XLCSNB	133		
		1159delC	Frameshift	Ec loop IVS1–S2	XLCSNB	55		
		R1234X	Nonsense	IVS4	XLCSNB	55		
		S1254I	Missense	IVS4	XLCSNB	57		
		R1285S	Missense	IVS4	XLCSNB	57		
		Q1348X	Nonsense	Pore IVS5–S6	XLCSNB	56		
		L1364H	Missense	Pore IVS5–S6	XLCSNB	56,134		
		W1386X	Nonsense	C terminal	XLCSNB	55		
		W1440X	Nonsense	C terminal	XLCSNB	133		
		1516delC	Frameshift	C-terminal	XLCSNB	135		
		K1591X	Nonsense	C-terminal	XLCSNB	56		
		R1919H	Missense	C-terminal	XLCSNB	57		
		3133insC (n)	Frameshift	Pore IIIS5–S6	XLCSNB	56		
		3658–3669(n)	Deletion	IVS2	XLCSNB	56		
		CaV2.1	<i>CACNA1A</i>	R192Q	Missense	IS4	FHM	85
				R195K	Missense	IS4	FHM	95
S218L	Missense			Ic loop IS4–S5	FHM	137		
R583Q	Missense			IIS4	FHM	138		
T666M	Missense			Pore IIS5–S6	FHM	85		
V714A	Missense			IIS6	FHM	85		
D715E	Missense			IIS6	FHM	94		
K1336E	Missense			Ec loop III S3–S4	FHM	94		
Y1385C	Missense			IIIS5	FHM	139		
V1457L	Missense			Pore IIIS5–S6	FHM	140		
R1668W	Missense			IVS4	FHM	95		
L1682P	Missense			IVS4	FHM	141		
W1684R	Missense			IVS4	FHM	95		
V1696I	Missense			IVS5	FHM	95		
H253Y	Missense			Pore IS5–S6	EA-2	92		

(continue)

Channel	Gene	Mutation	Type	Location	Inherited disease	Reference
		SS	Missplice	IIS2	EA-2	142
		2145del 8(n)	Frameshift	IIS5	EA-2	92
		2317del8(n)	Frameshift	Pore IIS5-S6	EA-2	142,92
		FS1067X	Frameshift	Linker II-III	EA-2	143
		FS1144X	Frameshift	Linker II-III	EA-2	144
		FS1294X	Frameshift	IIS1	EA-2	85,142
		R1279X	Nonsense	IIS2	EA-2	145
		SS	Missplice	IIS3	EA-2	85
		4451delC(n)	Frameshift	IIS5	EA-2	92
		SS	Missplice	Pore IIS5-S6	EA-2	142
		Y1444X	Nonsense	Pore IIS5-S6	EA-2	142
		R1547X	Nonsense	Linker III-IV	EA-2	142
		5056del8 (n)	Frameshift	IVS1-S2	EA-2	142
		5123del	Frameshift	IVS2	EA-2	143
		R1662H	Missense	IVS4	EA-2	146
		H1736L	Missense	IVS5-S6	EA-2	147
		E1757K	Missense	Pore IVS5-S6	EA-2	148
		SS	Missplice	IVS3	EA-2	87
		7213 GAG (n)	Expansion	C-terminal	Spinocerebellar ataxia type 6	96
		R1664Q	Missense	IVS4		149
CaV3.2	CACNA1B	F161L	Missense	IS2-IS3	Childhood absence epilepsy	126
		E282K	Missense	Pore IS5-S6	Childhood absence epilepsy	126
		C456S	Missense	Linker I-II	Childhood absence epilepsy	126
		G499S	Missense	Linker I-II	Childhood absence epilepsy	126
		P648L	Missense	Linker I-II	Childhood absence epilepsy	126
		R744Q	Missense	Linker I-II	Childhood absence epilepsy	126
		A748V	Missense	Linker I-II	Childhood absence epilepsy	126
		G773D	Missense	Linker I-II	Childhood absence epilepsy	126
		G784S	Missense	Linker I-II	Childhood absence epilepsy	126
		V831M	Missense	IIS2	Childhood absence epilepsy	126
		G848S	Missense	IIS3	Childhood absence epilepsy	126
		D1463N	Missense	Pore IIS5-S6	Childhood absence epilepsy	126
		A480T	Missense	Linker I-II	IGE	128
		P618L	Missense	Linker I-II	IGE	128
		G775D	Missense	Linker I-II	IGE	128

carboxy-terminal tail. The second mutation substitutes a highly conserved Arg for a Cys residue at position 284 within the extracellular region of the pore loop between S5 and S6 of domain I.

**CaV1.4 (α<sub>1F</sub>)** The CaV1.4 subunit, encoded in humans by the gene *CACNA1F*, maps to chromosome Xp11.4<sup>[55]</sup> and is mainly expressed in retinal rods and bipolar cells, spinal cord, adrenal gland and mast cells. As its main cellular function is thought to be neurotransmitter release from photoreceptors, it is no surprise that mutations in this channel are involved with inherited diseases of the eye.

The locus for X-linked congenital stationary night blindness type 2 (XLCSNB-2) was mapped to the *CACNA1F* gene<sup>[55,56]</sup> and several mutations have since been identified.

In case studies of patients with XLCSNB, over 73 *CACNA1F* mutations have been detected, of which 51% are nonsense mutations, 32% missense mutations and 8% frameshifts<sup>[57]</sup>. A list of several of the mutations identified and their positions within the channel is given in Table 2. Very recently, Wei and Hemmings detected a genetic association between schizophrenic patients and the *CACNA1F* locus, although the exact mutations are unknown<sup>[58]</sup>. The prevalence of visual abnormalities in schizophrenia makes an association with *CACNA1F* especially interesting.

**CaV2.1 (α<sub>1A</sub>)** The P/Q-type calcium channel CaV2.1 (α<sub>1A</sub>) represents one of the most important channels both from a physiological perspective and its role in channelopathy. Found throughout the nervous system,

CaV2.1 is considered to be the primary VGCC controlling fast neurotransmitter release, especially at excitatory synapses. Not surprisingly, CaV2.1 is found in high concentrations in the presynaptic nerve terminus, where it exists in discrete release sites that are more efficiently coupled to the vesicle release machinery than other VGCCs<sup>[59-62]</sup>. However, CaV2.1 is also found throughout the dendritic arbour, especially in cerebellar Purkinje cells, where it contributes to integrative dendritic physiology<sup>[63]</sup>.

Historically, one of the first lines of evidence linking CaV2.1 to neurological disorders came from studies of the tottering (*tg*) mouse<sup>[64]</sup>. The *tg* mouse is a neurological mutant displaying ataxia, and involuntary spasms indicative of tonic-clonic seizures as well as neurophysiological signs of absence epilepsy<sup>[65]</sup>. The underlying defect in *tg* mice has been identified, through positional cloning, as a point mutation (P601L) in *cacna1a*, the mouse CaV2.1 gene<sup>[64]</sup>. Using whole cell patch clamp methods, Wakamori *et al* found significant (40%) decreases in P-type (Ba<sup>2+</sup>) currents in dissociated Purkinje cells obtained from *tg/tg* versus wild-type (*wt*) mice that could be replicated in a simple heterologous expression system<sup>[66]</sup>. Surprisingly, they found no change in the voltage-dependence of activation or inactivation, single channel conductance or reversal potential, suggesting that the decreased current density is not due to impaired ion conductance or activation/inactivation mechanisms.

Three other mouse mutations showing varying degrees of seizure activity have been identified that map to the *cacna1a* locus rocker (*rkr*), tottering leaner (*tg<sup>la</sup>*) and rolling Nagoya (*tg<sup>rol</sup>*)<sup>[64,67,68]</sup>. Remarkably, each mutant mouse shows considerable differences in the extent and times of onset of seizure, cerebellar atrophy and ataxia. Thus *tg*, *tg<sup>la</sup>* and *rkr* but not *tg<sup>rol</sup>* mice show seizure activity, whereas *tg<sup>la</sup>*, *rkr* and *tg<sup>rol</sup>* but not *tg* mice show marked ataxia<sup>[65,67-69]</sup>.

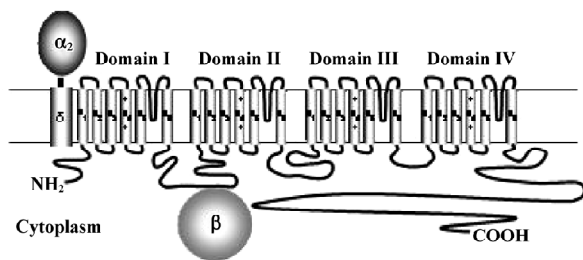
Rocker arises through a point mutation within the extracellular S<sub>5</sub>-S<sub>6</sub> region (T1310K) of domain III<sup>[67]</sup>. Surprisingly, the precise effect of the *rkr* mutation on P/Q currents has not been forthcoming, but is likely to resemble those in *tg* mice. A distinct mutation in domain III, (R1262G), within the S<sub>4</sub> voltage sensor, occurs in *tg<sup>rol</sup>* mice<sup>[68]</sup>. This mutation displays a marked reduction in the voltage sensitivity of channel activation. The *tg<sup>la</sup>* mutant has absence seizures, severe ataxia and cerebellar damage<sup>[70]</sup>. In *tg<sup>la</sup>*, a point mutation at a splice/donor consensus sequence leads to aberrant RNA splicing in the region encoding the carboxy terminus of the  $\alpha_{1A}$  subunit. As a result, translation yields two primary protein products corresponding to truncated CaV2.1 subunits bearing a novel and distinct C-termini. Electrophysiological studies on Purkinje cells show that the major deficit caused

by the *tg<sup>la</sup>* mutation is a 60% reduction in P/Q-type currents or current densities compared to the wild-type mouse<sup>[66,71,72]</sup>. Single channel recordings suggest the decrease in current densities is not due to effects on either the channel conductance or lifetimes<sup>[72]</sup>, but due to effects on channel opening probability (P<sub>o</sub>) or, more likely, a decrease in channel densities at the cell surface, perhaps due to a trafficking defect. Interestingly, in transfected cells, only the *tg<sup>la short</sup>* form shows a significant reduction in current density<sup>[66]</sup>.

Just how the phenotypes of these spontaneous mouse mutants arise is unclear but might give insight into the analogous human conditions<sup>[73]</sup>. In general, aberrant activity of CaV2.1 channels cannot be functionally compensated for at many central synapses. Apart from the 'gain of function' FHM mutations (below), impairment of neurotransmission appears to be the rule for CaV2.1 knockout<sup>[74-76]</sup>, *tg/tg*<sup>[77]</sup> and *tg/tg<sup>rol</sup>*<sup>[78,79]</sup>. However, at the climbing fiber-Purkinje cell synapse, evoked glutamate release is similar between wild-type and CaV2.1<sup>-/-</sup>, *tg<sup>rol</sup>/tg<sup>rol</sup>* or *tg/tg* mice<sup>[76,78]</sup>. Inhibitory transmission does not appear to be affected<sup>[80]</sup>. An obvious complication is the degree to which alternate VGCCs can stand in for the aberrant CaV2.1 channels and to what extent this might contribute to the neurological phenotype. Based on their similar trafficking and biophysical properties, the most likely replacements are expected to be the CaV2.2 VGCCs. Indeed, compensation by CaV2.2 has been documented at the calyx of Held synapse in CaV, CaV2.1<sup>-/-</sup> knockout mice and other mutants<sup>[74,77,78,81]</sup>. However, there is also evidence for upregulation of both CaV2.2 and CaV2.3 at the neuromuscular junction of CaV2.1<sup>-/-</sup> mice<sup>[82]</sup>.

In humans, the gene encoding CaV2.1 is designated as *CACNA1A* (formerly known as *CACNLA4*) and is localized to a large 300 kb region containing 47 exons at chromosome position 19p13<sup>[83]</sup>. Whereas gene expression yields a primary transcript of 9.8 kb, several splice variants have been identified<sup>[84]</sup>, most notably an isoform that differs in exon 37 by 97 nucleotides.

In humans, the cardinal mutation associated with CaV2.1 is a rare disorder termed episodic ataxia type 2 (EA-2) that causes paroxysmal attacks of cerebellar ataxia that can last for several days. In 1996, Ophoff *et al* identified 2 mutations in unrelated patients displaying EA-2 that mapped to the *CACNA1A* gene<sup>[85]</sup>. One mutation involves a base deletion and the other occurs at a splice junction site, but both are predicted to lead to a frameshift such that the CaV2.1 protein is truncated prematurely after the S1 region of domain III (Figure 1). The partially complete channel is, thus, predicted to be non-functional or to be incorrectly folded and trafficked. More recently, additional familial EA-2 mutations have been



**Figure 1.** The predicted structure of a prototypical VGCC consists of an  $\alpha 1$  pore-forming subunit and at least two auxiliary/regulatory subunits,  $\beta$  and  $\alpha 2d$ . The  $\alpha 1$  subunit is a transmembrane protein organized into 4 repeating domains (I–IV), each containing 6 hydrophobic transmembrane regions (S1–S6). Segments S5 and S6 of each domain line the channel pore (P loop) and S4 is designated as the voltage sensor.

identified that induce mis-splicing either through a G–A substitution at an intron–exon boundary or, especially interesting, through a 4 bp ACGT deletion within an intron (the first described for *CACNA1A*), which might unmask a cryptic splice downstream<sup>[86]</sup>. Typically, clinical signs of EA-2 are evident prior to adulthood. However, just recently, a case of late-onset (aged 61 years) EA-2 has been identified involving a 9 bp insertion in the cytoplasmic domain II–III linker. To date this is the largest insertion reported for the human gene<sup>[87]</sup>. Quite what the mutation does is unclear. Although it lies within the II–III linker, it is just downstream of the synprint region for SNAP-25/syntaxin binding. Expression studies indicate a significant (approximately 82%) reduction in current and a 20 mV depolarization shift in activation threshold with some change in activation and inactivation kinetics. Together, these data might be indicative of an effect on gating. Why the clinical symptoms appeared so late in this patient is unclear. An examination of compensatory expression of other VGCCs in this patient would certainly be interesting. A novel *CACNA1A* mutation, IVS36-2A>G, at the 3' acceptor splice site of intron 36 was identified by sequencing<sup>[88]</sup>. It is the first described *CACNA1A* acceptor splice site mutation and the most C-terminal EA-2-causing mutation reported to date.

Another highly debilitating *CACNA1A* channelopathy is the rare autosomal dominant disorder familial hemiplegic migraine (FHM). Characterized by intense attacks of migraine with aura, often lasting for several days, FHM has often been misdiagnosed as epilepsy or stroke<sup>[89]</sup>. Cerebellar dysfunction has also been noted in some families. Following the initial work by Ophoff *et al*<sup>[85]</sup>, several missense mutations have been found within *CACNA1A* that lead to increased calcium influx through the expressed channels<sup>[90]</sup>.

However, the precise biophysical characteristics conferred by the mutations on the CaV2.1 channel are not identical. Thus, single channel recordings on expressed channels show changes in gating with mutants T666M, V714A, and I1819L, but not R192Q. Recovery from inactivation can be slower (T666M) or faster (V714A and I1819L) compared to wild-type CaV2.1<sup>[91]</sup>. Subsequent studies have found an increased propensity for activation at weakly depolarizing potentials for three additional FHM-associated mutants<sup>[92]</sup>. Powerful insights into the neuropathology of FHM have come from a recent study by Van den Maagdenberg *et al*<sup>[93]</sup> who generated a transgenic mouse model bearing the human *CACNA1A* mutation R192Q. Recordings from cerebellar granule cells showed increased CaV2.1 channel current densities, which activated at more negative voltages in R192Q mice than wild-type channels. Significantly, the R192Q mice showed enhanced neurotransmission and susceptibility to cortical spreading depression. Taken together the above suggests that FHM is a *CACNA1A* channelopathy that arises through a gain of function that enhances neurotransmitter release.

As FHM and EA-2 both involve *CACNA1A*, an interesting question concerns the extent to which they overlap. In this respect it is notable that approximately 20% of FHM cases show signs of mild cerebellar ataxia<sup>[94]</sup>. Although cross-correlational prediction is not straightforward, 83% of patients with six missense mutations associated with migraine also showed ataxia and or nystagmus<sup>[95]</sup>.

A third clinical disorder associated with mutations in CaV2.1 has been identified. Termed Spinocerebellar ataxia type 6 (SCA6), this disorder appears in early middle age (30–40 years) and is characterized by a mild progressive (over a subsequent 25 years) cerebellar atrophy causing dysarthria, nystagmus, ataxia, loss of gait and sometimes death<sup>[96]</sup>. Unlike EA-2 and FHM, SCA6 appears to arise through a shift in the reading frame and triplet (CAG) repeat expansion at the distal carboxy terminus<sup>[97]</sup>. Based on a study by Ishikawa *et al*<sup>[98]</sup> the critical size of the CAG encoded polyglutamine stretch appears to be 19 repeats, with cases showing longer stretches having poorer neurological outcomes and earlier disease onset. Precisely what functional effects the SCA6 remains unclear. In a model of SCA6, elongation of the polyglutamine tract in SCA6 CaV2.1 caused a concomitant hyperpolarizing shift of voltage-dependent inactivation to more negative potentials, suggesting an overall reduction of calcium influx might contribute to SCA6 symptomology. However, just recently, compelling evidence has been presented that a portion of the CaV2.1 carboxy-terminus is cleaved *in vivo* and can enter the nucleus by virtue of nuclear localization signals<sup>[99]</sup>. Although the wild-type carboxy ter-

minal fragment is weakly toxic, a fragment containing an expanded polyglutamine tract (Q33) corresponding to SCA6 is highly toxic to Purkinje neurons and other cells. Thus, SCA6 might share a similar mechanism of action with some other expansion disorders where pathogenesis requires entry of a polyglutamine-containing fragment into the nucleus.

Given the above, one would anticipate that mutations in the human CaV2.1 gene are associated with epilepsy<sup>[100]</sup>. Nevertheless, the evidence has been slow to emerge. One early study found no statistically significant evidence that genetic variants of the *CACNA1A* gene might play a causative role in common forms of idiopathic generalized epilepsy (IGE)<sup>[101]</sup>. Moreover, reports of an allelic association of a silent single nucleotide polymorphism (SNP8) with IGE<sup>[102,103]</sup> have been refuted<sup>[104]</sup>. Nevertheless, there is growing recognition that in some cases patients with EA-2 also show an epilepsy phenotype most usually of a primary generalized nature<sup>[105,106]</sup>.

The first human EA-2-epilepsy case was described in 2001 by Jouvenceau *et al* in an 11-year-old who showed frequent episodes of ataxia and poorly controlled absence seizures, and generalized tonic-clonic seizures<sup>[105]</sup>. The underlying mutation was found to lie in exon 36 (C5733T) giving rise to a premature truncation behind the domain IV S6, and, thus, complete loss of the C-terminus. In an expression system, the primary effect of this mutation is a massive loss of functional channels at the cell surface. However, it is interesting to note that the mutation appears to have a dominant-negative effect when co-expressed with wild-type CaV2.1 (as anticipated for the heterozygous state). It is our contention that it is only a matter of time before further epilepsy-associated *CANAI1A* mutations are documented.

**CaV2.2 ( $\alpha_{1B}$ )** Given its established role alongside CaV2.1 in neurotransmitter release, it is remarkable that mutations in the *CACNA1B* gene (locus 9q34) have not been identified in the human population. Based on studies in CaV2.2<sup>-/-</sup> knockout mice one would anticipate problems in nociception<sup>[107]</sup>, decreases in sympathetic nervous system function<sup>[108]</sup> and alterations in response to ethanol<sup>[109]</sup> and anaesthetics<sup>[110]</sup>.

**CaV2.3 ( $\alpha_{1E}$ )** The CaV2.3 channel (*CACNA1E*) (locus 1q25-q31)<sup>[83]</sup> is primarily localized to the somata and dendrites of central neurones. However, such channels are also found in the nerve terminals of central synapses<sup>[111,112]</sup> where they might participate in transmitter release<sup>[113]</sup>. Although no human mutations have been identified in CaV2.3, observations in knockout mice by Jing *et al* predict that mutations impairing this VGCC are likely to affect glucose-stimulated insulin release from pancreatic beta cells by facilitating the global entry of calcium needed for granule replenishment<sup>[114]</sup>.

**CaV3.1 ( $\alpha_{1C}$ )** The CaV3.1 subunit gene *CACNA1G*, located on chromosome 17q22<sup>[115]</sup> is thought to encode a T-type (ie low threshold) VGCC. This channel is highly expressed in brain, especially on dendrites, and it is considered to be the primary T-channel in the thalamus<sup>[116]</sup>. However, CaV3.1 is also found in the ovary, placenta and heart<sup>[117]</sup>. To date no mutations have been identified in inherited human diseases. However, studies on knockout mice indicate reduced sleep patterns<sup>[118,119]</sup>, bradycardia and delayed atrioventricular conduction<sup>[5,120]</sup>.

**CaV3.2 ( $\alpha_{1H}$ )** In humans, the CaV3.2 gene (*CACNA1H*) has been mapped to chromosomal locus 16p13.3<sup>[6,121]</sup>. This subunit appears to be widely expressed in brain (especially the neocortex), kidney, smooth muscle, liver and heart. Targeted knockout studies in the rat nociceptive root ganglion suggest CaV3.2 plays a role in nociception<sup>[7,122]</sup>. Knockout mice show constitutively constricted coronary arterioles and focal myocardial fibrosis<sup>[123]</sup>, and CaV3.1 knockouts in human spermatazoa demonstrate that CaV3.2 is a key player in the T-type current accompanying the acrosome reaction<sup>[124]</sup>. Mutations in *CACNA1H* are now thought to underlie diverse epilepsies. Thus, in 2003, Chen *et al* found 12 missense mutations in 14 patients with childhood absence epilepsy<sup>[125]</sup>, and several of the 12 appeared to promote calcium influx during activation<sup>[126]</sup>. Even more recently, a study by Heron *et al*<sup>[127]</sup> identified 3 missense mutations and a single nonsense mutation in *CACNA1H* in a subset of patients with IGE. On expression, these latter mutations yield statistically significant changes in the kinetics of activation and inactivation of the CaV3.2 channel. Interestingly, many of these mutations lie in the same domain I-II linker region. Even more significant is the possibility that mutations in this region might block the selective inhibition of this VGCC by G-protein  $\beta_2\alpha_2$  subunits<sup>[128]</sup>. Changes in T-type channels have long been implicated in epilepsy<sup>[100]</sup>.

**CaV3.3 ( $\alpha_{1I}$ )** In humans, the *CACNA1I* gene maps to chromosome position 22q12.3<sup>[129]</sup>. In common with the other T-type VGCCs it is expressed highly in brain<sup>[130]</sup> where it is thought to play a role in thalamic oscillation<sup>[131]</sup>. Little is known about possible CaV3.3 gene defects, either in the human population or inferred from knockout animals.

## Summary

With just a few exceptions, pathological mutations have now been identified in every VGCC pore-forming alpha subunit in humans. In many cases the pathology can be predicted on the basis of the tissue patterns of gene expression. Studies on spontaneous mouse mutants provide important



clues to the human condition and, in the case of knockout or knockin mutants, might predict disease phenotype and outcome. What is especially striking is the extent to which small differences in function can have major and very distinct effects on behavior. Such phenotypic pleiotropy does not simply reflect gain or loss of VGCC function, but more subtle effects on biophysical parameters such as pore conduction, gating and inactivation kinetics. In some cases, effects could be attributed to sites of interaction with proteins involved in channel regulation. However, a largely unexplored area is the extent to which VGCC mutations affect channel trafficking to and from the cell surface. Just recently, Papazian's group<sup>[132]</sup> identified mutations in *tg/tg* mice that appear to disrupt trafficking of CaV2.1 to the cell surface. Preliminary work in our laboratory suggests that similar effects might occur in other VGCC mutants. Whatever their origin, it is clear that the list of VGCC mutations identified in the human population will continue to expand. It is our contention that a detailed understanding of the structural and functional basis of such mutations is essential for treating the disorders they manifest.

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